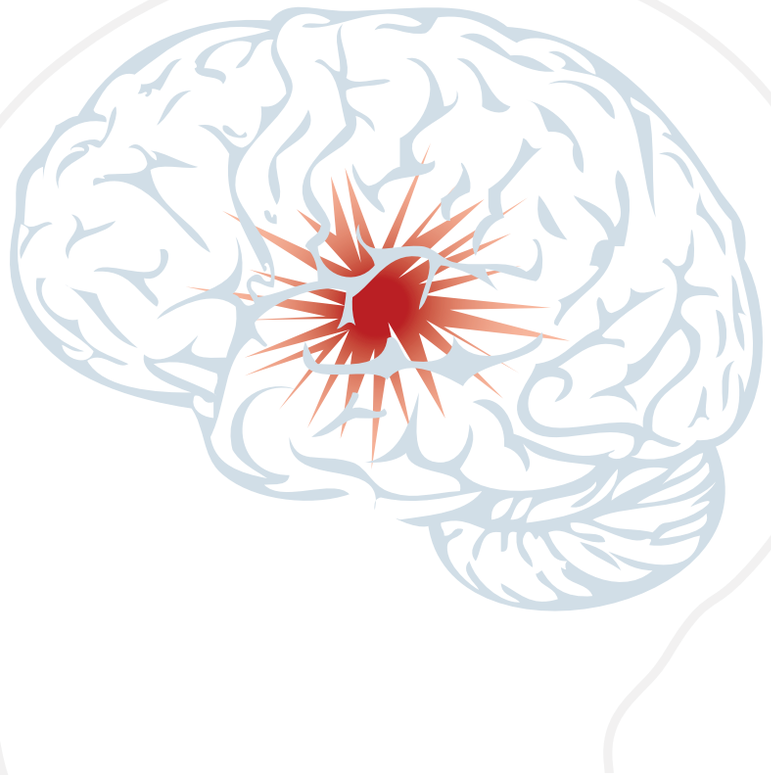
Inflammation in the immature brain

The role of Toll-like receptors



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Linnea Stridh Inflammation in the immature brain - The role of Toll-like receptors

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” The beginning of knowledge is the discovery of something we do not understand.”

Frank Herbert (1920-1986)

Inflammation in the immature brain

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ABSTRACT

Infection/inflammation and/or hypoxia-ischemia (HI) are major causes of perinatal brain injury. Toll-like receptors (TLRs), important components of innate immunity, have been shown to be involved in brain injury, both after infectious and endogenous, non-infectious, stimuli. The **overall aim** of this thesis was to study the expression of TLRs in the immature brain, choroid plexus and endothelial cells after inflammatory stimuli and/or HI, and to investigate the role of TLRs, their adaptor proteins MyD88 and TRIF in brain damaging processes after HI. TLR stimuli, HI or a combination of them both was performed on mice at postnatal day 9. Brain injury and inflammatory responses were evaluated with immunohistochemistry, RT-qPCR and cytokine analyses. All investigated TLRs were expressed under basal conditions in the neonatal brain and several of the receptors were regulated in the brain, choroid plexus and blood brain barrier after inflammatory stimuli and/or HI. Additionally, systemic stimulation of TLR 1/2 and TLR 4 decreased the expression of occludin, a tight junction protein, in the choroid plexus. TLR 2 was constitutively expressed in astrocytes in white matter and in neurons in the paraventricular nucleus and contributed to brain damage following HI. In contrast, MyD88 and TRIF did not appear to play a role in the injury process after HI alone. Both lipopolysaccharide (LPS), a TLR 4 ligand, and Poly I:C, a TLR 3 ligand, sensitized the brain to HI in wild type mice. This effect was blocked in MyD88 and TRIF deficient mice. Both Poly I:C and LPS increased the proinflammatory cytokine levels in the brain and this increase was blocked/reduced in the TRIF and MyD88 deficient animals. **To conclude**, TLRs are expressed under basal conditions and regulated during inflammation in the brain as well as in choroid plexus and blood brain barrier. In particular, we found that TLR 2 contributes to injury following HI, indicating that it has a function in sterile inflammation in the neonatal brain. Further, both MyD88 and TRIF play essential roles in LPS/Poly I:C-sensitized HI neonatal brain injury. These findings suggest that TLRs are important in both physiological and pathological processes in the immature brain and may provide novel targets for neuroprotective therapies in the future.

Keywords: hypoxia-ischemia, immature brain, inflammation, innate immunity, Toll-like receptors

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SAMMANFATTNING PÅ SVENSKA

Ungefär 2 av 1000 födda fullgångna barn riskerar att utveckla hjärnskada från händelser som sker före, under eller strax efter födseln. Dessutom är förekomsten av hjärnskada betydligt högre hos barn som föds för tidigt. Nedsatt syre- och blodtillförsel (hypoxisk ischemi; HI) och/eller infektion är bidragande orsaker till att hjärnan skadas och kan leda till neurologiska handikapp så som cerebral pares, inlärningssvårigheter och epilepsi. Identifiering av mekanismerna bakom skadans uppkomst och på så vis förbättrade behandlingsmöjligheter skulle innebära ett rikare liv för många barn.

Infektioner i blodet eller HI kan leda till inflammation i hjärnan. Inflammationen uppkommer genom att kroppens immunförsvar aktiveras. Immunsystemet har två försvarslinjer, medfödd och förvärvad immunitet. Det medfödda immunförsvaret upptäcker angripande mikroorganismer i kroppen genom speciella mottagare, så kallade receptorer, på kroppens celler. När dessa receptorer upptäcker en mikroorganism, aktiveras det medfödda immunförsvaret och en inflammation uppstår för att på så sätt förstöra mikroorganismen. Toll-lika receptorer (TLR) är en viktig del av det medfödda immunförsvaret och de är specialiserade på att upptäcka specifika molekyler (antigen) från främst bakterier och virus. Man har även upptäckt att TLR kan reagera på kroppsegna molekyler som kommer från skadade celler och vävnad. Man har idag identifierat ett tiotal TLRs hos människa och mus. När TLR upptäcker antigen aktiveras en signaleringskaskad via två olika adaptorproteiner, MyD88 och TRIF. Detta leder till att inflammatoriska signalmolekyler bildas.

Syftet med den här avhandlingen är att undersöka TLRs roll vid skada orsakad av infektion, HI eller en kombination av de båda i den omogna hjärnan. I försöken har en modell i neonatal mus använts för att framkalla en hjärnskada liknande den man ser hos nyfödda barn.

Vi fann att TLR finns uttryckta i den omogna hjärnan och i hjärnans barriärer, och att deras genuttryck regleras vid en infektion eller efter HI. Efter infektion minskade dessutom genuttrycket för occludin, ett protein som har som uppgift att ”limma ihop” cellerna i barriären, vilket kan leda till en öppning av barriären. En öppen barriär kan leda till att inflammatoriska molekyler och celler i blodet lättare tar sig in i hjärnan och därmed orsakar inflammation.

Vi fann också att TLR 2 bidrar till hjärnskada efter HI medan adaptorproteinerna MyD88 och TRIF inte påverkade skadestorleken efter HI. Vi upptäckte att aktivering av TLR 3 och TLR 4 ökade hjärnans känslighet för HI och mössen fick större hjärnskador. Möss som saknade genen för MyD88 eller TRIF var skyddade mot denna ökade känslighet. Vid stimulering av TLR 3 och TLR 4 ökade också nivåerna av inflammatoriska molekyler i hjärnan. Denna effekt uppstod inte i mössen som saknade MyD88 eller TRIF.

Dessa fynd visar att TLRs finns i den omogna hjärnan och att de kan vara viktiga både vid friska och sjuka tillstånd. Dessutom visar vi att TLRs kan spela roll i hjärnans barriärer. Genom att förstå TLRs roll i den inflammatoriska processen som följer efter en hjärnskada kan vi på sikt komma närmare målet att hitta bättre behandlingsstrategier än de som finns idag.

LIST OF PAPERS

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

I. **Regulation of Toll-like receptor 1 and -2 in neonatal mouse brain after hypoxia-ischemia**
Linnea Stridh, Peter L.P. Smith, Andrew S Naylor, Xiaoyang Wang and Carina Mallard
J. Neuroinflammation 2011, 8:45

II. **Lipopolysaccharide Sensitizes Neonatal Hypoxic-Ischemic Brain Injury in a MyD88-Dependent Manner**
Xiaoyang Wang, Linnea Stridh, Wenli Li, Justin Dean, Anders Elmgren, Liming Gan, Kristina Eriksson, Henrik Hagberg and Carina Mallard
J. Immunol. 2009;183;7471-7477

III. **TLR3 activation increases the vulnerability of the neonatal brain to hypoxia-ischemia**
Linnea Stridh, Xiaoyang Wang and Carina Mallard
In manuscript

IV. **Regulation of Toll like receptors in choroid plexus and endothelial cells in the immature brain after inflammatory stimulation**
Linnea Stridh, Xiaoyang Wang, Holger Nilsson and Carina Mallard
In manuscript

CONTENT

ABBREVIATIONS

AIF	apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP 1	activator protein 1
ATP	adenosine triphosphate
BBB	blood-brain-barrier
BCSFB	blood-cerebrospinal fluid barrier
cDNA	complementary DNA
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
CVO	circumventricular organ
dsRNA	double stranded RNA
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FADD	Fas associated protein with death domain
G-CSF	granulocyte colony-stimulating factor
HBSS	Hank's balanced salt solution
HI	hypoxia-ischemia
i.p.	intraperitoneally
IFN	interferon
IHC	immunohistochemistry
IKK	IκB kinase
IL	interleukin
IP-10	IFN-γ induced protein 10
IRF	interferon regulatory factor
JNK	c-jun N-terminal kinase
KO	knock out
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MyD88	myeloid differentiation primary response protein 88
NF-κB	nuclear factor κB
NMDA	N-metyl-D-aspartate
NO	nitric oxide
Pam	Pam3CSK4
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PND	postnatal day
Poly I:C	polyinosinic-polycytidylic acid
PVN	paraventricular nucleus
RIP 1	receptor-interacting protein 1
ROS	reactive oxygen species

RT-qPCR	reverse transcription-quantitative PCR
SARM	sterile alpha and TIR motif containing protein
TBS	tris-buffered saline
TIR	toll-IL-1 receptor resistance
TLR	toll-like receptor
TNFRSF	tumor necrosis factor receptor superfamily
TNF-α	tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing interferon beta
WT	wild type

1 INTRODUCTION

The developing brain is vulnerable to many different factors before, during and after birth. Low levels of oxygen or nutrients and/or the presence of infections/inflammation, can result in fetal brain injury. The risk of developing perinatal brain injury is about 2 in 1000 live births in term infants and even higher in preterm infants [1-4].

1.1 Perinatal brain injury

Perinatal hypoxia-ischemia (HI) is a major cause of brain injury in the newborn and can result in a range of motor and neurodevelopmental disabilities, such as cerebral palsy, mental retardation, visual motor or visual perceptive dysfunction, and epilepsy [5-7]. HI can occur due to different reasons such as occlusion/compression of the umbilical cord or impairment of blood flow and gas exchange of the placenta before or during birth. The preterm infant, due to immaturity of cardiovascular and lung function, might also suffer from circulatory and respiratory problems after birth, which can lead to HI.

Maternal or intrauterine infection/inflammation is thought to be another major contributing factor underlying perinatal brain damage [8]. Intrauterine infection/inflammation can manifest either as clinical chorioamnionitis or fetal inflammatory response syndrome, both of which can be life-threatening to the fetus, or as histological chorioamnionitis that may be clinically silent (reviewed in [9]). There is clear evidence that chorioamnionitis is a major risk factor for preterm birth [10] and intrauterine infections have been identified as a risk factor for developing cerebral palsy in both term and preterm infants [11-13]. Furthermore, maternal infection has been associated with the development of perinatal brain injury not only directly, but also indirectly by increasing the vulnerability of the brain to a secondary insult, such as hypoxia, hyperoxia, mechanical ventilation, or other infections [14-17]. In term infants, birth asphyxia may be preceded by antenatal infections and the combined exposure to infection and asphyxia creates an additive effect and increases the risk of developing spastic cerebral palsy [18].

The pattern and distribution of perinatal brain injury is dependent on the severity of the insult and the age at which it occurs [19]. The recognition that different regions of the brain have different susceptibility to injury at different maturational stages has led to the knowledge that particular cell types within the central nervous system (CNS) are selectively vulnerable to brain insults. If an ischemic insult occurs early in gestation and the baby is born prematurely, cerebral white matter injury is most frequently observed [5, 20, 21]. Further, at term age equivalent, several imaging studies have reported reduction in grey matter volume associated with white matter injury in infants born prematurely [22, 23]. In the term infant with ischemic brain injury, damage is often characterized by injury to deep grey matter and cerebral cortex [24].

1.2 Mechanisms of brain injury after hypoxia-ischemia

The development of brain injury after HI is not a single "event" but is rather a process that proceeds over time. Magnetic resonance imaging (MRI) studies show progression of lesion size over the first few days after injury [25] and animal studies show that after the HI insult, many neurons die over a period of days to weeks [26]. The injury process after HI can be divided into two parts consisting of the primary phase that starts during and/or immediately after the insult and secondary phase that starts hours after the primary insult and can continue for several days [27, 28].

HI induces a reduction in the supply of oxygen and nutrients to the brain, which results in a shift from aerobic to anaerobic metabolism [29, 30]. Anaerobic metabolism is an energy-inefficient state resulting in: rapid depletion of high-energy phosphate reserves, including adenosine triphosphate (ATP), accumulation of lactic acid, and the inability to maintain cellular functions. This disrupts active transport processes, resulting in intracellular accumulation of sodium, calcium, and water and a membrane depolarization. The membrane depolarization then results in a release of excitatory neurotransmitters, specifically glutamate, from axon terminals. Glutamate binds to N-metyl-D-aspartate (NMDA)-, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-, and kainate receptors resulting in an influx of calcium and triggering a cascade of cellular events that

mediate cell death, including generation of reactive oxygen species (ROS) and nitric oxide (NO), and lipidperoxidation (reviewed in [27, 31-34]).

If the primary insult is transient, cerebral perfusion is restored and oxygen and glucose levels, and intracellular pH is normalized [28, 35]. However in many cases, a secondary energy failure will occur within hours after the initial insult, leading to another wave of glutamate release, formation of ROS and NO, inflammatory reactions and apoptosis.

1.2.1 Cell death

The severity of the insult may determine the mode of cell death, with severe injury resulting in necrosis, and a milder insult resulting in apoptosis [36, 37], although there is a continuum between these modes of cell death [38]. Necrosis is a passive process of cell swelling, disrupted cytoplasmic organelles, loss of membrane integrity, eventually lysis of the cell, and activation of an inflammatory process. In contrast, apoptosis is an active process distinguished from necrosis by the presence of cell shrinkage, nuclear pyknosis, chromatin condensation, and genomic fragmentation. Apoptosis is also the mechanism for refining cell connections and pathways during brain development [39]. Studies have shown that apoptosis may play a prominent role in the evolution of HI injury in the neonatal brain and may be more important than necrosis after injury [40].

The two main apoptotic pathways are the intrinsic and the extrinsic pathway. Exitotoxicity (glutamate), oxidative stress, and other factors lead to injury of the mitochondrial membrane. The intrinsic pathway starts with permeabilisation of the mitochondrial membrane which leads to the release of several proapoptotic factors into the cytoplasm including cytochrome c, apoptosis-inducing factor (AIF), caspase-9, and endonuclease G [41]. Release of cytochrome c leads to the activation of caspase-9 and is followed by the conversion of procaspase-3 to active caspase-3 [26, 42]. Caspase-3 activation results in proteolysis of essential cellular proteins, including cytoskeletal proteins and kinases and can commit the cell to the morphological changes characteristic of apoptosis [43]. Activated caspase-3 has been shown in human postmortem brain tissue of full-term neonates with severe perinatal asphyxia [44].

The extrinsic pathway is initiated by the activation of cell surface receptors responsive to inflammatory stimuli such as the tumor necrosis factor receptor superfamily (TNFRSF), where the Fas death receptor is one of the most studied TNFRSF members [45-47]. Activation of Fas involves caspase-8 and subsequently caspase-3 activation [47, 48]. HI has been found to activate Fas death receptor signaling in the neonatal brain [49].

1.3 Inflammation

Inflammation is part of the biological response of vascular tissues to harmful stimuli, such as pathogens. Inflammation is characterized by the cardinal signs redness, heat, swelling, and pain and is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. However, extensive, prolonged, or unregulated inflammation is highly detrimental and can cause more damage to the tissue than the initial inflammatory stimuli. The inflammatory process involves invasion of inflammatory cells, such as neutrophils and monocytes/macrophages, and the production of inflammatory mediators, such as cytokines, chemokines, and ROS. In addition to infection, trauma and HI can trigger an inflammatory response, a so-called "sterile inflammation". This process is not fully understood but it has been shown that endogenous molecules from damaged cells and tissues can trigger an inflammatory response through interacting with receptors that normally detect microbial signals [50-52].

1.3.1 Inflammation in the brain

For a long time the CNS was considered to be "immunologically privileged"; i.e. protected from the immune system due to the blood-brain-barrier (BBB), that restricts the access of inflammatory cells and molecules into the brain. However, it is becoming evident that leukocytes as well as cytokines and chemokines can cross the intact BBB. It is also known that the CNS can exert its own immune response, mainly through microglia and also to a lesser extent through astrocytes [53].

The inflammatory response, in conjunction with excitotoxic (glutamate) and oxidative responses, is the major contributor to ischemic injury in the immature brain (Reviewed in [54]. After a HI injury, the generation of ROS and the accumulation of intracellular calcium in neurons and other brain

cells, leading to inflammatory cell activation and infiltration, trigger the immune response. Ischemic brain cells secrete inflammatory cytokines and chemokines causing upregulation of adhesion molecules in the cerebral vasculature and recruitment of peripheral leukocytes into the ischemic lesion. Once activated, inflammatory cells can release a variety of cytotoxic reagents such as cytokines, matrix metalloproteinases (MMPs), NO, and more ROS. This can all contribute to more cell damage as well as disruption of the BBB and extracellular matrix. Whether inflammation is good or bad in the brain after injury is still under debate. For example, inhibition of microglia have been found to both worsen [55] HI injury and protect [56] the brain against HI damages.

1.3.2 Inflammatory mediators

Neutrophils

Neutrophils are one of the most important cell types of the innate immune system and their main function is to eliminate pathogens, which have invaded the tissue. Neutrophils are normally residing in the blood but in response to inflammatory mediators released from sites of injury/infection, activated neutrophils migrate into the tissue towards the site of injury. In the immature brain, accumulation of neutrophils have been demonstrated within the blood vessels and in the injured tissue at early time points (up to 24 h) after HI [57, 58], and they can contribute to injury after HI as depletion of neutrophils was found protective [58, 59]. Neutrophils can enhance injury via several different mechanisms, including ROS production [60] and release of MMP-9 [61], acting both from within the tissue and from the peripheral circulation.

Microglia/macrophages

Macrophages are another important cell type in innate immunity and are specialized for removal of foreign particles/bacteria, phagocytosis of apoptotic/damaged cells, pathogen recognition and clearance, and immune regulation. Macrophages are derived from monocytes in the blood, which then migrate out into different tissues of the body and differentiate into macrophages. Microglia are the resident macrophages in the brain and act as the first and main form of active immune defense in the CNS (reviewed in [62]). Activated microglia are found abundant in the brain following HI [57, 63, 64], producing several mediators known to be injurious to the brain such

as proinflammatory cytokines [65, 66], NO [67], and MMPs [68], and they remain in the injured brain for weeks [69, 70]. While microglia contribute to the ischemic injury by secreting inflammatory mediators, they may also be involved in repair and neurogenesis [55, 71, 72].

Astrocytes

Astrocytes play an important role in the brain where they perform many functions, including provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, structure and maintenance of the BBB, and a role in the repair and scarring process of the brain after injury (reviewed in [73, 74]). Astrocytes can also play an immune modulating role in the brain [75]. In response to brain injury, astrocytes in the affected area will become activated. This activation process is called reactive gliosis and is triggered by cell death, inflammatory mediators and plasma proteins. Activated astrocytes migrate to the injured area and contribute to glial scars, which act as a barrier between the injured and the healthy tissue. In addition to restricting injury, activated astrocytes may also be involved in the inflammatory response after HI, since they have been shown to produce various inflammatory cytokines and chemokines in response to ischemia [76-79].

Cytokines

Cytokines are small proteins (~25kDa) that are released by various cells in the body, usually in response to an activating stimulus, and induce responses through binding to specific receptors. They usually act in an autocrine or paracrine manner, thus affecting both the behavior of the releasing cells and cell-to-cell communication. Cytokines are upregulated in the brain after a variety of insults, including ischemic insults and inflammation. They are expressed in immune cells, but also produced endogenously in resident brain cells, including glia and neurons [66, 80], reviewed in [81].

Cytokines are often divided into proinflammatory and anti-inflammatory cytokines, where interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), IL-6, and IL-10 are among the most studied ones in relation to inflammation in the brain. Both IL-1β and TNF-α can be released from activated macrophages or other glial cells at sites of infection [82-84]. TNF-α is upregulated in the adult brain after ischemia [85, 86] and in vitro experiments indicates that TNF-α induces apoptosis of oligodendrocytes [87, 88]. In vivo

intracerebral injection of TNF-α or IL-1β in newborn rats induced microglial activation, hemorrhage, and myelin damage [89].

Recently, repeated systemic administration of IL-1β was shown to induce white matter damage in neonatal mice [90]. Following neonatal HI, IL-1β is increased and can be even further amplified by a simultaneous infection [66, 91-93]. Genetic deletion of IL-1β was not protective against HI [94], whereas administration of IL-1ra, an inhibitor of IL-1β, protected the neonatal brain after HI [92, 93]. These differences may be due to the fact that IL-1β has different functions at various time points, which are all abrogated in the KO mice, while the inhibitor is operating only during the early phase of injury.

IL-6 is mostly considered as a proinflammatory cytokine but the influence of IL-6 on the development of brain injury is more unclear. Although studies have consistently demonstrated elevated IL-6 levels in asphyxiated infants [95] and mice overexpressing IL-6 develop severe neurologic syndromes [96], others find that mice deficient in IL-6 develop more severe brain injuries [97].

IL-10 is an anti-inflammatory cytokine which acts by inhibiting IL-1, TNF-α, and IL-6 [98-100]. Studies in adult animals and in cell cultures have shown that IL-10 has neuroprotective effects against glutamate-induced [101] or HI-induced [102, 103] neuronal death and against lipopolysaccharide (LPS)- or interferon (IFN)-induced oligodendrocyte cell death [104]. Furthermore, IL-10 counteracts acute effects of endotoxin on cerebral metabolism, microcirculation and oxygen tension during HI in the perinatal brain [105, 106].

Chemokines

Chemokines are a family of small (8-14kDa) cytokines that have chemoattractant properties, inducing cells to migrate towards the source of the chemokine. Chemokines control cell migration, proliferation, differentiation and angiogenesis. In an inflammatory situation, chemokines act mainly as chemoattractants for leukocytes, recruiting inflammatory cells, such as monocytes and neutrophils from the blood to the site of injury. Chemokines are upregulated in the immature brain after HI [57].

In the adult brain, inhibition or deficiency of the chemokine monocyte chemoattractant protein-1 (MCP-1) is associated with reduced ischemic brain injury [107, 108], whereas overexpression is associated with increased injury [109]. In neonatal rodents, MCP-1 expression is increased following HI [110] and transient focal ischemia [70]. MCP-1 has also been found to be upregulated in the brain after inflammatory stimuli, such as LPS and virus associated double stranded RNA (dsRNA) [111, 112], and MCP-1 deficiency decreased brain inflammation after LPS [113]. IFN-γ induced protein 10 (IP-10) has been found to be expressed by astrocytes, both in vivo and in vitro, after virus associated dsRNA stimuli [112, 114].

1.3.3 Intracellular inflammatory signaling pathways

Both cerebral ischemia and systemic infection results in regulation of gene expression in the brain, including rapid transcriptional activation of proinflammatory factors [115, 116].

Nuclear factor κB

Nuclear factor κB (NF-κB) is a protein complex that controls the transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, and bacterial or viral antigens (reviewed in [117]). NF-κB is normally located in the cytoplasm as a heterodimer composed of p65 and p50 subunits, bound to the endogenous inhibitor protein IκB. Phosphorylation of IκB by an upstream IκB kinase (IKK) releases NF-κB, allowing it to translocate into the nucleus and bind to functional κB-sites. NF-κB induces several important genes involved in inflammation, such as TNF-α, IL-6, IL-1β and MCP-1 [118-122].

Mitogen-activated protein kinases

The mitogen-activated protein kinase (MAPK) pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis (reviewed in [123, 124]). The MAPK family is comprised of three subfamilies, extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK). MAPKs are activated within the protein kinase cascades called "MAPK cascade". Each one consists of three enzymes, MAPK, MAP kinase kinase (MKK) and MAP kinase kinase kinase (MKKK) that are activated in

series. Firstly, MKKK is activated by extracellular stimuli and phosphorylates MKK on its serine and threonine residues, and this MKK then activates a MAPK. Upon activation, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated, leading to expression of target genes resulting in a biological response. ERK has been found to be generally protective in both adult and neonatal brain injury whereas p38 is best known as a transducer of stress-related signals, regulation of inflammatory genes production [123] and NF-κB recruitment to selected targets [125].

1.4 Barriers of the brain

The CNS is protected from the changeable milieu (e.g. ions, solutes, pathogens and proinflammatory cytokines) of the blood stream through the BBB and the blood-cerebrospinal fluid (CSF) barrier (BCSFB, see review [126-128]). The BBB and BCSFB act both as barrier systems to maintain CNS homeostasis, a necessity for proper neuronal function, as well as transport systems providing the brain with essential nutrients.

The BBB is composed of endothelial cells, pericytes, basal lamina, and astrocytes (figure 1A) [129]. The BBB results from specialized properties of the endothelial cells, their intercellular junctions, and a relative lack of vesicular transport. The BCSFB consists of a single layer of epithelial cells in the choroid plexus that overlay an extensive network of fenestrated capillaries (figure 1B). Besides its barrier function, epithelial cells in choroid plexus produce and secrete CSF.

Both BBB and BCSFB form physical barriers by a network of tight junctions between adjacent barrier forming cells (figure 1C, reviewed in [130]). The tight junctions are the key functional components of the CNS barriers and they limit the intercellular diffusion of substances such as hydrophilic molecules. Under pathological circumstances, such as inflammation or HI, tight junctions in the BBB seem to be readily modified and dysfunction of the BBB is typically followed by an increased permeability of the barrier allowing diffusion of water, proteins and solutes, leading to perivascular edema (reviewed in [131]).

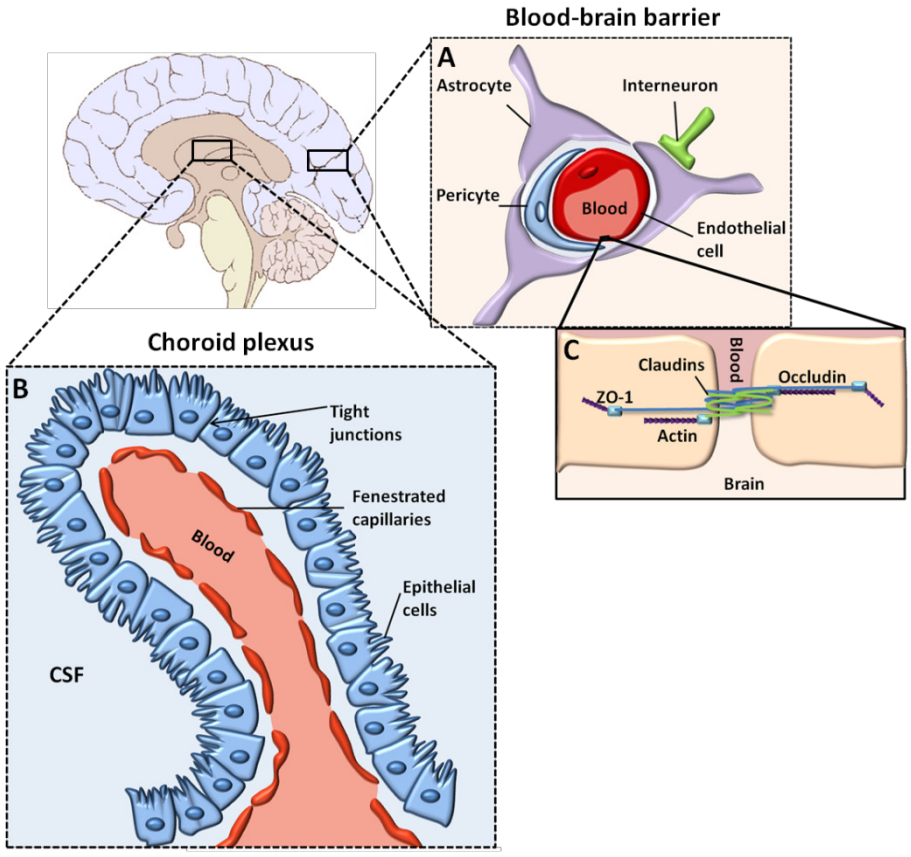

Figure 1. Barriers of the brain. Schematic drawing of the blood-brain barrier(BBB, A) and choroid plexus (B). The BBB is composed of endothelial cells, pericytes and astrocytes. Barrier properties are formed by tight junctions between the endothelial cells (C). Choroid plexus consists of a single layer of epithelial cells overlaying an extensive network of fenestrated capillaries. The blood-CSF barrier is formed by tight junctions between the epithelial cells. Besides its barrier function, epithelial cells in choroid plexus produce and secrete CSF.

1.4.1 Brain injury and barriers of the brain

Disturbance of the brain barrier systems due to inflammation has been implicated as one of the leading causes in the pathology of several neurological diseases both in the young and the ageing brain [132-134]. Studies have shown that at the time of myelination, systemic inflammation results in increased BBB permeability to plasma proteins, specifically in white matter tracts, with a reduced amount of myelin as a result [134, 135].

In connection to inflammatory stimuli, LPS treated mice exhibited a broken basal lamina and pericyte detachment from the basal lamina at 6-24 h after LPS injection. This was correlated with increased microglial activation and increased cerebrovascular permeability [136]. In the choroid plexus, LPS induced an acute phase response with upregulation of genes involved in immune-mediated cascades and down-regulation of genes involved in maintenance of the barrier function [137-139].

HI also affects the CNS barriers [68, 140]. After HI, opening of the BBB occurs shortly after the insult and MMP-9 contributes to this change [68]. In a rodent model, choroid plexus shows a selective vulnerability to ischemia and BCSFB disruption seems to occur before damage to the BBB [141].

1.5 Toll-like receptors

The immune defense of the body can be divided into innate and adaptive immunity, where the innate immune response is the first line of defense against microbial infections. The targets of innate immune recognition are the conserved molecular patterns of microorganisms, also called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) play a key role in the innate immune system by recognizing a wide variety of PAMPs, such as peptidoglycan, LPS, bacterial DNA and dsRNA (for reviews see [142-144]). In addition to their role in pathogen detection and defense, TLRs act as sentinels of tissue damage and mediate inflammatory responses to aseptic tissue injury. Host-endogenous molecules associated with damaged cells and tissues, such as heat shock proteins, high mobility group box 1 protein, and RNA, have been shown to activate TLRs [50-52].

1.5.1 Toll-like receptor signaling

The TLR family consists of 13 members and TLR 1-9 are expressed in both mice and humans. They can be classified into two groups based on subcellular localization. The first group includes TLR 1, 2, 4, 5, and 6, which are all present at the plasma membrane [145]. The second group includes TLR 3, 7, 8, and 9, which localize to intracellular compartments such as endosomes and they sense in particular viral and bacterial nucleic acids. Viral particles are endocytosed and degraded in late endosomes or lysosomes, and

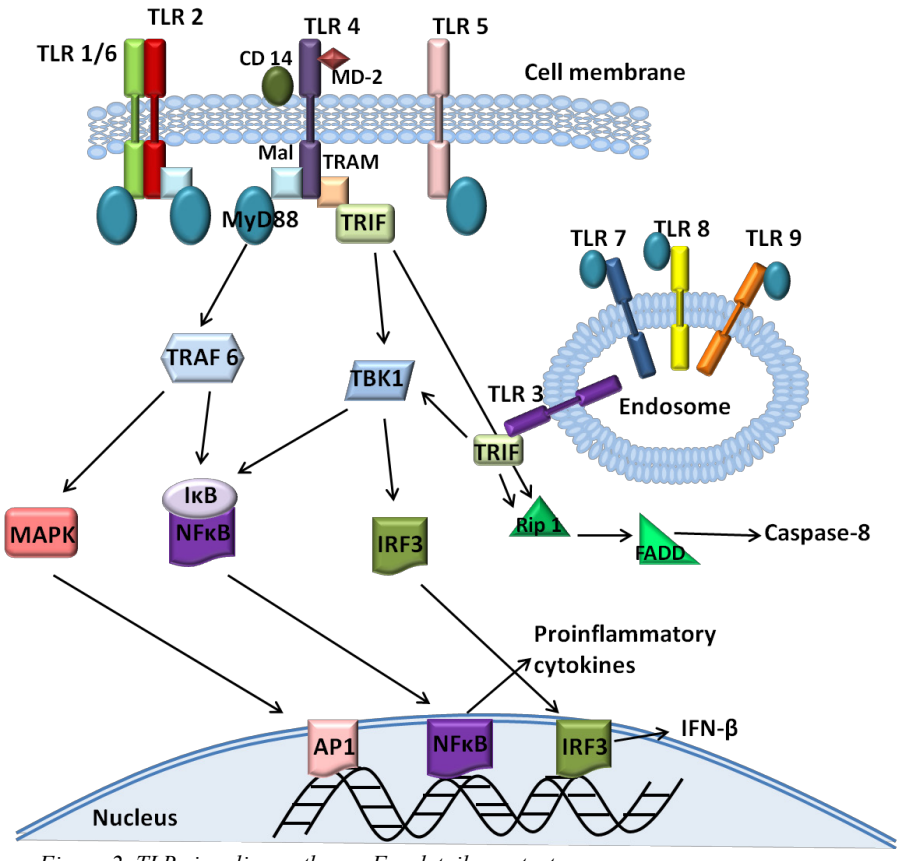

Figure 2. TLR signaling pathway. For details see text.

this degradation causes the release of viral DNA and RNA, which then can come in contact with the TLRs.

The key signaling domain involved in TLR signaling is the Toll-IL-1 receptor resistance (TIR) domain (figure 2). All TLRs have this TIR domain and they signal via the recruitment of various TIR domain-containing adaptor proteins [143]. Upon activation, each TLR family member, except TLR 3, signals through the myeloid differentiation primary response protein 88 (MyD88)- dependent pathway (reviewed in [146-148]).

MyD88 is an adaptor protein, which upon recruitment to the activated receptor initiates a signaling cascade leading to activation of different transcription factors, e.g. NF-κB and activator protein 1 (AP1), and the generation of proinflammatory cytokines such as IL-6 and TNF-α. TLR 3

signals through the MyD88-independent pathway, initiated by the TIR domain-containing adaptor inducing interferon beta (TRIF) adaptor molecule. Recruitment of TRIF leads to the activation of the transcription factor interferon regulatory factor (IRF)-3 and -7 and the generation of antiviral molecules such as IFN-β. However, reports show that TRIF signaling also induces NF-κB-dependent transcription, partially through interaction with receptor-interacting protein 1 (RIP 1) kinase [149, 150]. RIP 1 also links TRIF to the apoptotic cascade, via Fas associated protein with death domain (FADD) and caspase-8 [151]. TLR 4 uses both MyD88 and TRIF.

MyDD adapter-like (MAL) protein is a bridging protein, used by TLR 4 (and to a lesser extent by TLR 2), and is involved in recruitment of MyD88. This provides an extra control on TLR 4 signaling, because MAL is subject to multiple regulatory mechanisms. The fourth adaptor protein, TRIF-related adaptor molecule (TRAM), is used only by TLR 4 and is needed to recruit TRIF. Again, this provides an extra control of TLR 4 signaling, because TRAM is regulated by phosphorylation of protein kinase C ε [152]. The fifth and final adaptor is sterile alpha and TIR motif containing protein (SARM), which has been shown to inhibit signaling by TRIF and thereby limiting the IRF 3 pathway that is activated by TLR 3 and 4 [153].

1.5.2 TLRs and brain injury

TLRs are constitutively expressed in the adult brain [154, 155], where they are widely expressed in different cell types (reviewed in [156]). Not much is known about the direct role of TLRs in neonatal brain injury in humans but emerging data suggest a role for TLRs in preterm birth. Fetal membranes in the human placenta express TLRs, and the expression of TLR 2 and TLR 4 is increased in preterm delivery with histological chorionamnionitis [157-159]. Several studies have also shown an association between TLR polymorphisms and preterm birth [160, 161].

In animal models, it has been shown that LPS, a TLR 4 ligand, injected directly into the brain induces white matter injury [162, 163]. Also systemic exposure to LPS can cause cerebral white matter injury in a variety of animal models [164-168]. Additionally, systemic administration of LPS sensitizes both the immature and the adult brain to a subsequent HI insult [169-173]. Stimulation of TLR 2 has been found to impair neonatal mouse brain

development [174] and to be involved in neurodegeneration induced by group B streptococci [175].

As mentioned above, host-endogenous molecules associated with injury, e.g. heat shock proteins and RNA from necrotic cells can act as ligands to TLRs. This suggests that TLRs may be initiating some of the damaging inflammatory response to ischemic injury and there is increasing evidence that TLRs do play a role in ischemic damage. In adult studies, TLR 4 is found to be upregulated after ischemia reperfusion [176] and mice lacking TLR 2 or TLR 4 are less susceptible to transient focal cerebral ischemia/reperfusion damage [177, 178]. While reports suggest that TLR 2 and 4 are important for stroke-like injury, much less is known about the role of the TLR adaptor proteins in brain injury. In the adult, neither disruption of MyD88 nor TRIF signaling protects against cerebral ischemia alone [179, 180]. On the other hand, contradictive reports show that stimulation of the TRIF pathway either is neuroprotective by reprogramming the response of the adult brain to stroke [181] or exacerbates chronic neurodegeneration [182]. In the immature brain, neither TLR 4 nor MyD88 contribute to neonatal HI brain damage, without LPS [156]. The role of TRIF in neonatal brain injury is unknown.

2 AIM

TLRs have been shown to be involved in brain injury, both after infectious and endogenous, non-infectious, stimuli. Most previous studies have been performed in the adult brain and knowledge about the role of TLRs in the immature brain is lacking. **The overall aim** of this thesis was to study the expression of TLRs in the immature CNS, its downstream intracellular signaling pathways, and brain injury outcomes after inflammatory stimuli with different TLR ligands and/or HI.

Specific aims were:

- To investigate the expression and regulation of TLRs in the brain, choroid plexus and microvessel endothelial cells after HI and inflammatory stimuli.

- To investigate the role of the adaptor protein MyD88 in LPS-sensitized neonatal HI brain injury.

- To investigate the role of TRIF-dependent mechanisms in neonatal HI brain injury.

3 MATERIALS AND METHODS

The material and methods used in this thesis are thoroughly described in the individual papers. A general description of material and methods with comments are presented below.

3.1 Animals

All animals were housed at Experimental Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden. Mice were kept in a 12 h light-dark cycle with free access to food and water. All animal experiments were approved by the Animal Ethical Committee of Gothenburg (No. 314-05, 277-07, and 374-09). Several different genetically modified mouse strains were used: C57BL/6J wild type (WT) mice were bought from Charles River (Germany) (I, IV), TLR 1 knock out (KO) mice were purchased from Oriental BioService, Inc (Tokyo, Japan) (I), TLR 2 KO mice (B6.129-Tlr2tm1Kir/J) (I) and TRIF KO mice (C57BL/6J-Ticam1Lps2/J) (III) were bought from the Jackson Laboratory (USA), MyD88 KO mice were a kind gift from Kristina Eriksson (Department of Rheumatology and Inflammation Research, University of Gothenburg, Sweden). The MyD88 KO mice were originally from Dr Kawai, Department of Biochemistry, Hyogo College of Medicine, Japan (II).

3.1.1 Breeding and genotyping (paper I-III)

In general mice were bred to obtain WT, KO and heterozygote littermates (paper I-III). Mice were genotyped with different protocols depending on genotype. The genotype of MyD88 KO mice was determined by PCR of genomic DNA obtained from mouse tails, as previously described [183]. The WT allele was detected using the forward primer (5'-TGGCATGCCTCCATCATAGTTAACC-3') and the reverse primer (5'-GTCAGAAACAACCACCACCATGC-3'), and the mutant allele detected using the forward primer (5'-TGGCATGCCTCCATCATAGTTAACC-3') and the reverse primer (5'-ATCGCCTTCTATCGCCTTCTTGACG-3'). The PCR products were run on an agarose gel and WT mice were identified by a

single 550-bp band, MyD88 KO mice by the presence of a single 650-bp DNA band, and HET mice were identified by the presence of both bands.

The genotype of TRIF KO mice was determined by reverse transcription-quantitative PCR (RT-qPCR) of genomic DNA. Genotypes were detected through melting curve based genotyping. Primers used for the amplification step were: forward (5'-CCAATCCTTTCCATCAGCCT-3') and reverse (5'-CACTCTGGAGTCTAAGAAG-3') (Tib Molbiol, Germany). For melting curve analysis following probes were used: (5'-CACATGTGGGGCCACACAGGGG-FL) and (5'-LC640-CCAGTCATCTGATGACAAGACTGAG-PH) (Tib Molbiol). The amplification protocol comprised an initial 5 min denaturation at 95°C, followed by 40 cycles of denaturation for 30 sec at 95°C and annealing/extension for 30 sec at 55°C followed by 1 min at 72°C and thereafter a melting curve analysis consisted of 1 min denaturation at 95°C followed by 3 min at 45°C and an increase of temperature to 95°C with a rate of 0.11°C/s on a LightCycler 480 (Roche). WT mice were identified with a melting temperature of 65-66°C, TRIF KO mice with a melting temperature of 59-60°C and heterozygotes were identified by the presence of both melting temperatures.

Comment: In an attempt to minimize the number of animals used, TLR 1, 2 and TRIF KO mice were bred to obtain WT or KO litters. TLR 1 and TLR 2 mice were bred to obtain KO litters all through the experiment and they were therefore not genotyped for each experiment. To minimize variations due to external factors, the different genotypes (WT and KO) were run in the HI chamber at the same time when possible. MyD88 KO animals were crossed with WT to produce heterozygotes, which were bred further to produce littermate animals with mixed genotypes including MyD88 homozygous (KO), heterozygote, and WT mice.

3.2 Hypoxic-ischemic brain injury model (paper I-III)

At postnatal day (PND) 9, mice were anesthetized with isoflurane (3.0% for induction and 1.0-1.5% for maintenance) in a mixture of nitrous oxide and

oxygen (1:1). Through a small incision, the left common carotid artery was permanently ligated with prolene sutures and the incision was then closed again, (the whole procedure was less than 5 min). Mice were returned to the cage and allowed to recover for 1 h and then placed in an incubator circulated with a humidified gas mixture (10.00 ± 0.01% oxygen in nitrogen) at 36°C for 50 min. After hypoxia, the pups were returned to their dam until sacrifice.

A combined model of infection and HI was used in paper II and III, where LPS (0.3 mg/kg, i.p. or polyinosinic-polycytidylic acid (Poly I:C, 10 mg/kg, i.p.) was administered to the animal 14 h prior to the HI insult.

Comment: The Rice-Vannucci HI model used in this thesis is one of the most commonly used experimental models used to study perinatal HI brain injury [30]. Following HI, damage is restricted to the cerebral hemisphere ipsilateral to the common carotid artery occlusion. Injury is associated with infarction in the middle cerebral artery territory, including subcortical and periventricular white matter, striatum/thalamus, hippocampus, and cerebral cortex [30, 184] and the degree of damage is dependent on the duration of the systemic hypoxia [68]. The peak in brain growth occurs at around P7 in the rat, which occurs around term in humans [185]. However, with respect to cortical maturity [186], the presence of a periventricular germinal matrix, development of BBB, synapse formation (reviewed in [187]), and maturity of oligodendrocytes [188], the PND 7-9 rodent is slightly more immature than the term human fetus. Thus brain maturation in PND 8-PND 9 mice, used in the present thesis, is mostly characteristic of near term human brain development.

3.3 Drug administration (paper II-IV)

Pam3CSK4 (Pam, InvivoGen) 5mg/kg (IV), Poly I:C (InvivoGen) 5 mg/kg (III) and 10 mg/kg (III, IV), or LPS (O55:B5; Sigma-Aldrich or ultrapure, #423, List biological laboratories, Inc.) 0.3 mg/kg (II, IV) was administered intraperitoneally (i.p.) to mice on PND 8.

Comment: Pam is a synthetic lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins and induces NF-κB activation through TLR 1/2 activation. The dose given was chosen from previous studies where we

have shown that TLR 1/2 signaling pathway stimulation/activation impairs immature brain development [174]. Poly I:C is a synthetic analog of dsRNA, a molecular pattern associated with viral infection. Both natural and synthetic dsRNA is known to induce type I IFNs through activation of TLR 3. The Poly I:C dose given was chosen through a dose-response experiment where the maximum IFN-β response was measured by RT-qPCR (III). LPS is a constituent of the cell wall of gram negative bacteria and works as a ligand to TLR 4. LPS is commonly used experimentally to simulate infection and the dose given in this thesis has previously been shown to affect the inflammatory response in the brain and sensitize the immature brain to HI injury [116, 169].

3.4 Tissue preparation

For immunohistochemical staining, animals were deeply anesthetized and intracardially perfused with saline and 5% buffered formaldehyde (Histofix; Histolab). Brains were rapidly removed and immersion fixed in 5% formaldehyde for 24h. Brains were kept in a 30% sucrose solution until they were cut or put through dehydration with graded ethanol and xylene/ X-tra solv® (Medite,Germany) and embedded in paraffin. Coronal sectioning (25 µm/section) was performed on a sliding microtome (Leica SM2000R, Leica Microsystems, Sweden), and sections were stored in tissue cryoprotectant solution (25% ethylene glycol, 25% glycerol and 0.1 M phosphate buffer) at -20°C (I). Paraffin embedded brains were cut coronally (10 µm/section) on a rotating microtome (Leica RM2165, Leica Microsystems, Sweden) (I,II,III).

For messenger RNA (mRNA) analysis, animals were deeply anesthetized and intracardially perfused with saline. Brains were rapidly dissected out; snap frozen and stored at -80°C until analysis. Brain tissue was homogenized with Qiasol lysis reagent homogenizer (Qiagen, Sweden) and total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Sweden) according to the manufacturer's instructions. RNA was measured in a spectrophotometer at 260-nm absorbance. (I,III,IV)

For enzyme-linked immunosorbent assay (ELISA), brains were rapidly dissected on ice, homogenized by sonication in ice-cold isolation buffer consisting of protease inhibitor mixture (Complete Mini) and 10% FCS

(Perbio; HyClone) in phosphate buffered saline (PBS), and centrifuged twice at 5,000 x *g* for 5 min and twice at 10,000 x *g* for 10 min; the supernatant was collected and stored at -80°C until use (II).

3.5 Immunohistochemistry

For paraffin embedded sections (I-III), antigen recovery was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% horse or goat serum (depending on the species used to raise the secondary antibody) in PBS. Sections were incubated in primary antibody at 4°C overnight, followed by the appropriate secondary antibody (biotinylated or fluorescent) for 60 min at room temperature. For biotinylated antibodies, visualization was performed using Vectastain ABC Elite (Vector Laboratories) with 0.5 mg/ml 3,3-diaminobenzidine enhanced with 15 mg/ml ammonium nickel sulfate, 2 mg/ml β-D glucose, 0.4 mg/ml ammonium chloride, and 0.01 mg/ml β-glucose oxidase (all from Sigma-Aldrich). Sections were analyzed on a Nikon Optiphot-2 microscope equipped with an AVT dolphin F145B camera (Allied Vision Technologies).

Free floating sections (I) were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS; 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase activity. Nonspecific binding was blocked for 30 min in blocking solution (3% goat serum and 0.1% Triton-X 100 in TBS) and the sections were incubated with primary antibody in blocking solution at 4°C for 48 h, followed by the appropriate secondary antibody for 60 min at room temperature. Visualization was performed as described above. Sections were analyzed under an Olympus BX60 fluorescence microscope equipped with an Olympus DP50 cooled digital camera. Fluorescent staining was captured with a Leica TCS SP2 confocal system (Leica, Heidelberg, Germany) with channel settings appropriate to the fluorophores present. Sequentially scanned, grey scale Z-stacks were pseudocolored and processed in ImageJ (version 1.42u; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij) before final processing in Adobe Photoshop (version 11.0.2; Adobe Systems Inc., San Jose, CA).

Table 1. Antibodies used in immunohistochemistry

Antibody	Dilution	Company, product number	Paper
HuC/D	1:500	Molecular Probes, A21271	I
GFAP	1:1000	Covance, PCK-591P	I
Iba-1	1:1000	Abcam, ab5076	I
MAP-2	1:1000	Sigma-Aldrich, clone HM-2	I-III
MBP	1:10 000	Covance, SMI-94R	II-III
Neu N	1:1000	Chemicon International, MAB377	I
NeuN- Alexa 488	1:1000	Chemicon International, MAB377X	I
Olig2	1:1000	R&Dsystems, AF2418	I
TLR 1	1:500	Imgenex, IMG-5012	I
TLR 2	1:100	Imgenex, IMG-526	I

Comment: Immunohistochemistry (IHC) is a sensitive method for the localization of antigens in tissue sections. There are a few things to consider when performing IHC. Antigen retrieval is important since many antigens can become ”hidden” when processing the tissue. The specificity of the antibodies is crucial, as non-specific binding leads to false positive results. One way to check for non-specific binding of secondary antibodies is to incubate sections with secondary antibody without the addition of primary antibody. Incubation of the primary antibody with its recombinant antigen prior to staining can also be used to detect non-specific staining. In this thesis the specificity was tested by incubating sections with secondary antibody only.

3.6 Neuropathological analysis

For brain injury evaluation, brains were embedded in paraffin and cut into 10 µm frontal sections. For evaluation of grey matter injury, every 40^{th} (I) or 50^{th} (II,III) section throughout the brains was stained for microtubule-associated protein-2 (MAP-2) and the MAP-2 area outlined and measured (Micro Image

version 4.0 Olympus). Infarct area was assessed as the MAP-2 negative area in the ipsilateral hemisphere, tissue loss was calculated by subtracting the MAP-2 positive volume of the ipsilateral hemisphere from the contralateral hemisphere and atrophy was calculated by subtracting the MAP-2 positive volume and infarct volume of the ipsilateral hemisphere from the contralateral hemisphere. Total infarct, atrophy and tissue loss volume, was calculated according to the Cavalieri Principle using the following formula: $V = \Sigma A \times P \times T$, where V = total volume, ΣA = the sum of areas measured, P = the inverse of the sections sampling fraction, and T = the section thickness.

Regional neuropathology was evaluated in MAP-2-stained sections using a semiquantitative neuropathological scoring system (II). Briefly, cortical injury was graded from 0 to 4, 0 being no observable injury and 4 being confluent infarction encompassing most of the hemisphere. Damage in the hippocampus, striatum, and thalamus was assessed both with respect to hypotrophy (shrinkage; grades 0–3) and observable cell injury/infarction (grades 0–3) resulting in a neuropathological score for each brain region (grades 0–6). The total score (0 –22) was the sum for all four regions.

Subcortical white matter injury was analyzed by measuring the area (Micro Image version 4.0, Olympus) of positive staining for myelin basic protein (MBP) in both hemispheres at striatum and hippocampal levels (II,III). One section for each level was analyzed per animal. The MBP area in the ipsilateral hemisphere was compared with the contralateral hemisphere to calculate the proportion (%) of white matter damage.

Comment: MAP-2 is a commonly used marker for evaluation of tissue loss after brain injury [189]. MAP-2 is expressed in neurons and dendrites, and the loss of MAP-2 staining indicates neuronal death. MBP is a protein, which is a constituent of the myelin sheath of oligodendrocytes and is also believed to be important in the process of myelination of neurons. Loss of MBP can be an indicator of disruption in the myelination process or reduced myelin [190, 191].

3.7 Reverse transcription-quantitative PCR

Analyses of mRNA expression were performed with RT-qPCR. QuantiTect Reverse Transcription Kit (Qiagen, Sweden)(III, IV) or Superscript RNase H-reverse transcriptase kit (Invitrogen, CA, USA) random hexamer primers and dNTP (Roche Molecular Biochemicals, USA) (I) was used to synthesize first strand complementary DNA (cDNA) according to the manufacturer's instructions. Each PCR (20 µl) contained 2 µl cDNA diluted 1:4, 10 µl Quanti Fast SYBR Green PCR Master Mix (Qiagen, Sweden), 2 µl PCR primer, and 6 µl H20. The amplification protocol comprised an initial 5 min denaturation at 95°C, followed by 40 cycles of denaturation for 10 sec at 95°C and annealing/extension for 30 sec at 60°C on a LightCycler 480 (Roche, Sweden). Melting curve analysis was performed to ensure that only one PCR product was obtained. For quantification and for estimating amplification efficiency, a standard curve was generated using increasing concentrations of cDNA. The amplification transcripts were quantified with the relative standard curve and normalized against reference genes.

Table 2. Primers used with RT-qPCR. All from Qiagen, Sweden

Gene	Product number	Paper
CD31	QT01052044	IV
Claudin-1	QT00159278	IV
Claudin-5	QT00254905	IV
Fas	QT00095333	III
GAPDH	QT01658692	III
Hprt-1	QT00166768	I
IFN-β	QT00249662	III
IL-1β	QT01048355	III
IL-6	QT00098875	III
IL-10	QT00106169	III
IP-10	QT00093436	III
MCP-1	QT00167832	III
Occludin	QT00111055	IV
TLR 1	QT00157430	I
TLR 2	QT00129752	I
TLR 3	QT00122983	IV
TLR 4	QT00259042	IV
TNF-α	QT00104006	III
YWHAZ	QT00105350	III
ZO-1	QT00493899	IV

In paper I, we also analyzed gene expression with the RT^2-PCR-Profiler PCR Array for TLR signaling pathways. cDNA-synthesis was performed by using the RT^2 First Strand Kit (SABiosciences, USA) following the manufacturer's instructions. The mouse TLR signaling pathway RT^2-PCR-Profiler PCR Array (SABiosciences, USA) was carried out according to manufacturer's instructions using the LightCycler 480 system (Roche, Sweden). The raw data obtained from the Lightcycler 480 software was uploaded into GEarray Analyzer software (SABiosciences, USA) for analysis.

Comment: RT-qPCR is a good method to use for quantification of specific mRNA transcripts within a sample. RT-qPCR has an advantage over traditional PCR in that the kinetics of the reaction for every cycle can be monitored and data collection in the exponential phase of the PCR is possible. The choice of reference genes is important when you do a relative quantification. A reference gene should be constitutively expressed and not change due to treatments or time points within an experiment. The reference genes provide an internal control for variability in RNA loading and the efficiency of the PCR. The reference genes in this thesis were selected either from reference genes used in the RT^2-PCR-Profiler PCR Array (I) or from genes suggested after evaluation with the Mouse Endogenous Control Gene Panel (TATAA Biocenter) (III, IV).

3.8 Cytokine assay (II)

Cytokine-chemokine expression in brain homogenate supernatants from WT and MyD88 KO mice were performed using a Linco Research mouse 22-Plex kit (No. MCYTO-70K-PMX22; Millipore) containing premixed beads capable of detecting a variety of cytokines, according to the manufacturer's instructions. This microbead array allowed for the simultaneous detection of multiple inflammatory molecules in a single 75 µl brain homogenate sample, as previously described [192]. Results were analyzed on a Bio-Plex workstation (Bio-Rad) and normalized to the amount of protein per well. The level of sensitivity for each microbead cytokine standard curve ranged from 1 to 35 pg/ml [192]. Protein concentrations were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad).

3.9 Isolation of the blood and CSF brain barrier (IV)

Choroid plexus

Animals were deeply anesthetized and decapitated. Brains were rapidly dissected out and placed in a petri dish with PBS. Choroid plexus from the lateral ventricles were dissected out under a microscope, snap frozen and stored at -80°C until analysis.

Microvessel endothelial cells

Endothelial cells and small vessels were isolated by microvessel isolation. 25 µl of sheep anti rabbit magnetic beads (Dynabeads® M-280 Sheep anti-Rabbit IgG, 112.03D, Invitrogen), resuspended in Hank's balanced salt solution (HBSS, 14025-050, Invitrogen), were incubated with 5 µl of rabbit anti-human CD31 antibody (ab32456, Abcam) at 4°C over night.

Animals were deeply anesthetized and decapitated. Brains were rapidly dissected out and placed in ice cold HBSS for ~10 min. Brains were diced into ~1 mm^3 cubes and placed in 1 ml HBSS with 10µl collagenase/dispase (10269638001, Roche) at 37°C for 30-45 min. Every 15 min, the cell suspension was drawn through needles with decreasing bore size (21G and 23G). The cell suspension was centrifuged for 7 min at 1000 g and washed three times in HBSS and then resuspended in the antibody-magnetic bead solution prepared from the day before and incubated for 30 min at 4°C. After incubation, the tubes containing cell suspension and magnetic beads were placed in a magnetic tube rack where beads and bound cells adhere to the side of the tube and remaining cell suspension could be removed. The bead and cell mix were washed 5 times in cold HBSS and then retained as the endothelial cell enriched fraction until analysis.

Comment: The purity of the isolation product was controlled by examining the mRNA expression of CD31 in the endothelial cell enriched fraction compared to brain homogenate sample. The efficiency of the isolation method has previously been examined by FACS analyses with a high yield of CD 31 positive cells and high purity (97%) as a result (M Evans, H Stolp-Personal communication).

3.10 Statistics

In paper I, Brain infarct data were analyzed with one way ANOVA followed by Dunnett's Multiple Comparison Test to compare total infarct volume between genotypes or infarct area at each brain level between groups (WT, TLR 1 KO and TLR 2 KO). Data are presented as mean ± SD. Gene array data were analyzed with GEarray Analyzer software (SABiosciences, USA).

In paper II, data were analyzed by two-way ANOVA with genotype (WT or MyD88 KO) and treatment (vehicle or LPS) as factors followed by the Bonferroni post hoc test with a 95% confidence interval.

In paper III, brain injury and IFN-β expression data were analyzed with two-way ANOVA with genotype and treatment or treatment and time point as factors followed by Bonferroni post hoc test with a 95% confidence interval. The other gene expression data were analyzed with one-way ANOVA followed by Bonferroni post hoc test with a 95% confidence interval. WT and TRIF KO data were analyzed separately.

In paper IV, gene expression data were analyzed with Students t-test or one way ANOVA followed by Dunnett's Multiple Comparison Test.

Data are presented as mean ± SEM and significance was set at $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software).

4 SUMMARY OF RESULTS

4.1 TLR are expressed under normal conditions and after HI in the immature brain (I)

Gene expression

PND 9 mice were subjected to HI and sacrificed at 30 min, 6 h and 24 h post-HI and gene expression analysis for TLR 1-9 was performed with the RT^2-PCR-Profiler array. mRNA for TLR 1-9 was found expressed both in normal brain and after HI. Additionally, we found that HI affects the separate TLRs differently (paper I, Table 1). TLR 1 mRNA expression showed both down-regulation (30 min, $p = 0.004$) and up-regulation (6 h and 24 h, $p = 0.00001$). TLR 2 was up-regulated at both 6 and 24 h ($p = 0.005$, $p = 0.006$, respectively) and mRNA expression of TLR 7 was up-regulated at 24 h ($p = 0.005$). Down-regulation only was found with TLR 5 at 24 h ($p = 0.004$) and TLR 8 at 30 min ($p = 0.006$). The gene expression of TLR 3, 4, 6, and 9 did not change after HI.

Protein expression and distribution in the brain

To further study TLR regulation following injury, we performed immunohistochemical studies of the TLRs that displayed significant up-regulation of mRNA in response to HI, i.e. for TLR 1 and 2. We did also perform IHC with antibodies against TLR 3, 4, 5, 7, 8, and 9 but the resulting staining was negative or unspecific and not further analyzed. There was no TLR 1 positive cells detected in normal control brains or in the contralateral (non-damaged) hemisphere following HI. At 24 h after HI, TLR 1 positive cells were found expressed in the ipsilateral (damaged) hemisphere, mainly in hippocampus. These cells were later identified as neurons (paper I, figure 2 and 3). TLR 2 positive astrocytes were observed mainly in the hippocampus, subcortical white matter and stria terminalis. TLR 2 was also shown to be expressed in a specific population of neurons in the paraventricular nucleus (PVN) of hypothalamus (paper I, figure 2 and 4). The immunohistochemical expression of TLR 2 did not change after HI.

4.2 TLRs are expressed and regulated in the BBB and BCSFB of the immature brain (IV)

The BBB and the BCSFB protect the CNS from the changeable environment of the blood stream and control the transport of molecules into the CNS. Inflammatory conditions can lead to disturbed barrier function and therefore, we wanted to examine the expression of TLRs in choroid plexus and endothelial cells and how they are regulated after inflammatory stimuli with different TLR ligands.

WT mice were given ligands for TLR 1/2 (Pam), TLR 3 (Poly I:C) or TLR 4 (LPS) at PND 8 and were then sacrificed 14 h later. Choroid plexus and brain endothelial cells were isolated and the gene expression for TLR 1-4 was examined with RT-qPCR. All TLRs investigated were expressed both in choroid plexus and endothelial cells under normal (saline) conditions (paper IV, figure 2). In choroid plexus, stimuli with Pam resulted in an increased mRNA expression of TLR 1 and 2 and a decreased expression of TLR4. Poly I:C increased specifically the expression of TLR 3. Interestingly, LPS did not affect TLR 4 mRNA expression but increased the expression of TLR 1 and TLR 3.

In endothelial cells, a similar expression pattern was observed with an increased expression of TLR 1 and TLR 2 after Pam exposure and increased expression of TLR 3 after Poly I:C (paper IV, figure 3). Similar to that observed in the choroid plexus, LPS did not affect TLR 4 expression in endothelial cells. In contrast to the choroid plexus, LPS induced an increased TLR 2 expression in the endothelial cells.

To evaluate barrier properties after stimuli with TLR ligands, we examined mRNA expression for the tight junction proteins claudin-1, claudin-5, occludin and ZO-1. In choroid plexus, occludin was down regulated after both Pam and LPS stimuli. No differences were observed in the expression of the other tight junction proteins after administration of the different TLR ligands. In brain endothelial cells, there were no differences in expression in any of the tight junction proteins investigated.

4.3 TLR signaling pathways are involved in HI injury (I-III)

To further evaluate the role of TLRs in neonatal HI, we performed studies with transgenic mice lacking functional TLR 1, TLR 2, MyD88 or TRIF. MyD88 and TRIF are the two main adaptor proteins downstream of the TLRs. After HI, TLR 2 KO mice had a significantly smaller infarct volume ($p = 0.005$), whereas no protection was observed in the TLR 1, MyD88 or TRIF KO mice compared to WT mice (paper I-III). These data confirm reports in adult animals [179, 180].

LPS sensitizes the brain to HI via signaling through TLR 4 [169, 173] and this leads to bigger injuries than with HI alone. In paper II, we demonstrate an increased tissue loss in LPS pretreated WT mice after HI compared to HI only ($p \leq 0.001$) (paper II, figure 1). Similarly, evaluation of regional neuropathology showed that brain injury was significantly increased in LPS-pretreated WT mice by 112% in the cortex, 76% in the thalamus, 62% in the striatum, and 51% in the hippocampus. There was also a significant increase in loss of subcortical white matter after HI in LPS treated WT mice compared to HI alone ($p \leq 0.05$) (paper II, figure 2). In the MyD88 KO mice, no differences were seen between LPS pretreated mice and HI alone, neither in grey matter nor in white matter injury. Two-way ANOVA revealed a significant interaction between genotype and treatment for both grey matter ($p < 0.006$) and white matter ($p = 0.0077$). Our results are in agreement with previous findings that LPS pre-exposure sensitizes the neonatal brain to HI injury [169] and we show here that LPS sensitization of the neonatal brain to HI is regulated in a MyD88-dependent manner.

TRIF is the adaptor protein downstream of TLR 3 and TLR 4. Stimulation of the TRIF pathway via activation of TLR 4 has been shown to protect the adult brain from subsequent ischemic injury via TRIF-induced IFN-β [181]. In paper III, we examined whether or not activation of the TRIF pathway affects neonatal HI brain injury. The combination of Poly I:C administration and HI, with a 14 h interval between the two events, were performed in WT and TRIF KO neonatal mice and neuropathology was examined 5 days after HI. In WT mice, Poly I:C pretreated mice showed an increased infarct volume ($p \leq 0.001$), tissue loss ($p \leq 0.001$), and atrophy ($p \leq 0.01$) compared

to control mice (saline/HI) (paper III, figure 2). Poly I:C pretreatment did also increase subcortical white matter loss both at hippocampal level ($p \leq 0.001$) as well as striatum level ($p \leq 0.001$) (paper III, figure 3). The sensitizing effect of Poly I:C was blocked in TRIF KO mice both in grey matter and white matter. These results show that pre-activation of the TRIF pathway, mediated by Poly I:C, sensitizes the immature brain to a subsequent ischemic injury.

4.4 Inflammation responses after TLR stimuli (II and III)

As mentioned above, cytokines and chemokines are upregulated in the brain after a variety of insults, including stroke and inflammation. In an attempt to understand the sensitizing effect LPS and Poly I:C have on the brain we examined the role of MyD88 and TRIF in mediating inflammatory mediators after systemic administration of LPS or Poly I:C.

Inflammatory response after LPS (II)

LPS was administered to WT or MyD88 KO mice and the animals were sacrificed at 2, 6, and 14 h after LPS. The production of inflammatory mediators in the brain was examined using multiplex ELISA and the results were broadly divided into proinflammatory and anti-inflammatory cytokines, and chemokines-chemotaxic molecules. In WT mice, significant changes were seen in the proinflammatory cytokines IL-1α, IL-5, IL-6, IL-7, and TNF-α (paper II, figure 4). The LPS induced increases in these cytokines were largely inhibited in the MyD88 KO animals. No significant interaction or genotype-dependent differences in response to LPS was seen for the proinflammatory cytokines IL-15 and IL-17 and the anti-inflammatory cytokines IL-9, IL-10, and IL-13 (paper II, figure 4 and 5). The chemokines-chemotaxic molecules IP-10, macrophage inflammatory protein (MIP)-1α, granulocyte colony-stimulating factor (G-CSF), KC (also called chemokine (C-X-C motif) ligand 1), and MCP-1 showed similar expression patterns as the proinflammatory cytokines with a significant interaction with genotype and time (paper II, figure 6). Also here, the response was inhibited in the MyD88 KO mice.

Inflammatory response after Poly I:C (III)

Poly I:C or saline was administered to WT and TRIF KO mice and the animals were sacrificed at 6 and 14 h after Poly I:C. Genes associated with proinflammatory and anti-inflammatory immune responses and chemotaxis were analyzed with RT-qPCR. All proinflammatory (IL-1β, IL-6, TNF-α), and chemotaxic genes (MCP-1, IP-10) investigated, as well as the anti-viral molecule IFN-β were upregulated in the WT mice at 6 h and/or 14 h after Poly I:C (paper III, figure 1 and 5). The gene expression of the anti-inflammatory cytokine IL-10 was below detection limit in both WT and TRIF KO mice. No changes were seen between treatment groups in the TRIF KO mice.

5 DISCUSSION

Infection/inflammation and/or HI are major causes of perinatal brain injury. TLRs are important components of the innate immunity and have been shown to be involved in brain injury in the adult, both after infectious and endogenous, non-infectious, stimuli. In this thesis we have studied the expression of TLRs in the immature brain and their role following neonatal inflammation and/or HI.

5.1 TLR expression in the immature brain

We demonstrated the presence of TLR 1-9 mRNA in the normal neonatal brain under physiological conditions. Additionally, the presence of TLR 1-4 mRNA was shown in brain endothelial cells and choroid plexus. Similarly, studies in human cerebral tissue have indicated that most of the TLRs are expressed on the mRNA level in numerous cell types [155]. In vitro studies also indicate a broad expression of TLR mRNA in several neural cell types [154, 193-195], although the expression of neuronal TLR mRNA is more controversial [196]. The presence of TLRs has also been demonstrated on rat cerebral endothelial cells [197]. To our knowledge, this is the first report showing TLR mRNA expression in the choroid plexus of the perinatal brain. Further it is the first general characterization of TLR gene response in the neonatal brain after HI.

After inflammatory stimuli or HI, the gene expression of several TLRs was changed. The regulation of TLR genes after inflammatory stimuli or neonatal HI is consistent with several studies in the adult brain. Previous studies have shown a changed TLR expression in rat cerebral endothelial cells after inflammatory stimuli with TNF-α and by oxidative stress [197] and an increased expression of TLR 2 and TLR 3 has been demonstrated on endothelial cells after stimuli with Pam and Poly I:C [198, 199]. After ischemia, studies have shown up-regulation of TLR 2 mRNA expression after middle cerebral artery occlusion (MCAO) [178], and induction of TLR 2, 4, and 9 mRNA after focal ischemia in mice [200]. Also, in a model of spinal cord injury, there was an increased mRNA expression of TLR 1, 2, 4,

5, and 7 [201]. This is to some extent similar to our results indicating an increase of TLR 1, 2, and 7 after HI. However, in contrast to studies in adult rodents, we found reduced mRNA expression of TLR 5 (24 h after HI) and TLR 8 (30 min after HI). We did not find changes in TLR 3, 4, 6 and 9 after neonatal HI, which suggests that the TLR response to cerebral ischemia differs in the adult compared to the developing brain.

We found a specific up-regulation of TLR 1, 2 and 3 in both choroid plexus and endothelial cells after stimulation with each of the respective ligands (Pam and Poly I:C). The TLR 4 mRNA expression however, did not change after LPS administration. TLR 4 mRNA has been found to be expressed in the CNS, particularly in choroid plexus [196]. Our results confirm the presence of TLR 4 mRNA in these CNS structures, but may indicate that LPS-induced TLR 4 activation does not necessarily involve TLR 4 regulation on a gene expression level. Similarly, we have previously noted a lack of TLR 4 gene expression regulation in the CNS in response to LPS in neonatal rats [169]. Interestingly, TLR 4 mRNA was reduced in choroid plexus following Pam administration. This suggests that there may be interactions between the TLR 2 and TLR 4 receptors, which have also previously been reported in the adult brain [202]. We also found an up-regulation of TLR 1 and TLR 3 in the choroid plexus after LPS. TLR 3 was found up-regulated after LPS exposure in human peripheral blood monocytes and this up-regulation appears to be positively regulated by a TLR 4-MyD88-NF-κB dependent signaling pathway [203]. Whether this could be true also for the LPS regulated TLR 1 and 3 mRNA expressions in choroid plexus needs to be further investigated.

In addition to TLR gene expression, we also investigated protein expression of the TLRs that was most regulated following HI, i.e. TLR 1 and TLR 2. We found an increase of TLR 1 positive neurons in the injured hemisphere after HI. To our knowledge, there are no previous studies of TLR 1 expression after ischemic injury, either in the adult or developing brain. In an adult model of neurocysticercosis, TLR 1 expression has been demonstrated on infiltrating cells such as microglia/macrophages[154]. These differences in expression may be due to age-and/or injury- dependent factors.

TLR 2 was constitutively expressed in the neonatal brain and the expression did not change with HI. Thus, the pattern of protein expression after HI differed to some extent to the mRNA expression. This kind of differences between gene expression and protein expression have previously been reported [154] suggesting that TLRs may be posttranscriptionally regulated.

TLR 2 was expressed in white matter astrocytes as well as in a specific population of neurons in PVN. Our results goes well with previous studies where TLR 2 expression has been detected in microglia, neurons, ependymal cells and astrocytes, [154] and a constitutive expression of TLR 2 has been demonstrated by in situ hybridization specifically in stria terminalis, PVN, and in the supraoptic nucleus [204]. The function of the TLR 2 positive neurons in PVN is to our knowledge unknown. PVN consists of several subpopulations of neurons involved in neuroendocrine function, such as homeostatic control, by releasing corticotropin-releasing hormone (CRH) and vasopressin. In close proximity to the PVN, there are specific structures, usually referred to as circumventricular organs (CVOs), which are lacking BBB (reviewed in [205]). CVOs are localized at strategic points close to the midline of the brain within the ependymal walls lining the 3rd and the 4th ventricle. Because of their fenestrated endothelial cells, they lie within the blood milieu and thus form a blood-CSF barrier. CVOs play a role in neurochemical and neurosecretory functions, in that the neurons located in the CVOs can directly sense hormonal stimuli and other substances in the blood or secrete hormones into the blood. Previous studies have shown that CVOs are the site of entry for immune cells into the CNS and CSF during inflammatory circumstances [206], and inflammatory stimuli, such as LPS and IL-1β, are known to activate secretory neurons in PVN [207-209]. Based on these results, we speculate that the TLR 2 positive neurons in the PVN that we found in paper I may participate in the neuroendocrine response of the hypothalamus and may act as sensors for incoming inflammatory signals.

5.2 TLRs and brain injury

In paper I, we examined the role of TLR 1 and TLR 2 in neonatal hypoxic-ischemic brain damage. Even though there was an increased TLR 1 expression in injured areas of the brain after HI, lack of TLR 1, in TLR 1 deficient mice, did not protect from HI injury. As far as we are aware, this is

the first study that has investigated the role of TLR 1 in HI brain damage. In contrast, TLR 2 expression did not change after HI, but we found that TLR 2 deficiency protected the immature brain from HI damage. With respect to TLR 2, our results correspond well with previous reports in adult animals. The majority of existing studies have shown a damaging [195, 200, 210] role for TLR 2 in the ischemic injured brain, although one study has reported that TLR 2 deficient mice develop larger infarcts following acute cerebral ischemia/reperfusion injury [204]. Furthermore, intracerebral administration of the TLR 1/2 agonist Pam caused brain damage, which was prevented in TLR2 KO mice [211]. In support we have previously shown that repeated stimulation of the TLR 2 receptor during early neonatal development results in brain injury and this effect is blocked in TLR 2 deficient mice [174].

Several TLR 2-dependent functions in CNS have been described, both in the normal healthy situation and after injury. Cerebral cortical neurons up-regulate TLR 2 in response to ischemia/reperfusion injury and an increased number of apoptotic neurons are found in the murine dentate gyrus 24 h after stimulation with a TLR 2 agonist [195, 211]. TLR 2 has also been found to be involved in adult hippocampal neurogenesis [212] and after a spinal cord injury, TLR 2 is important for the regulation of inflammation and gliosis as well as the repair and functional recovery [201]. Thus, it appears that TLR 2 signaling is emerging as a critical component of CNS function, both in health and disease.

All TLRs, except TLR 3, use the adaptor protein MyD88 for intracellular signaling. TLR 3 signaling is dependent on the adaptor protein TRIF, while both MyD88 and TRIF are associated with TLR 4 signaling. In paper II and III, we investigated the role of the adaptor proteins MyD88 and TRIF in HI injury. Neither MyD88 KO, nor TRIF KO mice were protected following HI alone. This coincides well with previous findings in adult mice [179, 180]. MyD88 is believed to be the sole adaptor protein downstream of TLR 2. The lack of protection in MyD88 KO mice (Paper II), while TLR 2 KO mice demonstrated reduced infarct area following HI (Paper I), may seem contradictory but suggest that TLR 2 may play a role in neonatal ischemic injury, independent of MyD88. A differential effect in MyD88 KO and TLR 2/4 KO mice following brain damage has also been shown in the adult [213]. How MyD88 is involved in other TLR 2-dependent functions in CNS, or

whether TLR 2 may also have additional/alternative downstream signaling pathways, is still not known.

We further investigated the role of the adaptor proteins MyD88 and TRIF by combining specific stimulation of either TLR 3 (Poly I:C) or TLR 4 (LPS) with a HI insult. We show that MyD88 plays an essential role in LPS-sensitized HI brain injury since MyD88 KO mice were protected against the combined LPS and HI effect (Paper II). These results support our previous findings identifying LPS as an important factor in altering the vulnerability to HI injury in the neonate [169-172] and provide novel evidence that the sensitizing effect of LPS is mediated via MyD88. This is the first report identifying a role for MyD88 regulation in LPS-induced HI brain injury.

We also show that pretreatment with the TLR 3 agonist Poly I:C sensitizes the neonatal brain to subsequent HI through a TRIF-dependent pathway (Paper III). In contrast, activation of the TRIF pathway has been shown to be protective against ischemic injury in the adult [181]. This effect was mediated via TRIF/IRF3-induced IFN-β. We could not detect such a relationship in our studies, despite showing a marked increase in IFN-β following Poly I:C administration. However, in support of our studies, it has also been shown that Poly I:C exacerbates the inflammatory response and accelerates neurodegeneration in the adult brain [182] and increases the susceptibility of midbrain dopaminergic neurons to subsequent neurotoxicity [214]. Furthermore, maternal immune challenges with Poly I:C precipitates neurodevelopmental diseases later in life in rodents [215]. Taken together, our data suggests that activation of TLR 3/TRIF by Poly I:C is detrimental to the neonatal brain.

5.3 Inflammatory response after TLR stimuli and/or HI

We have previously shown that peripheral LPS exposure regulates the expression of a large number of genes in the neonatal brain [116], including a significant number associated with inflammatory processes, such as cytokines and chemokines. We report in paper II and III that exposure to LPS or Poly I:C increase mainly proinflammatory cytokine and chemokine levels in the brain whereas there were no significant changes in anti-inflammatory

cytokines. The proinflammatory response to LPS and Poly I:C was largely abolished in MyD88 KO mice and TRIF KO mice respectively. These results are supported by previous studies, both in vitro[192, 216, 217] and in vivo [111, 182, 218, 219].

It is known that systemic inflammation can damage the brain through induction of inflammatory mediators, such as cytokines (reviewed in [220]). Additionally, proinflammatory cytokines cause damage if injected directly into the brain [89] and have the capacity to exacerbate brain damage [221], whereas blocking proinflammatory cytokine production by gene deletion reduces brain damage [66, 222, 223].

We do not know whether the cytokines in the brain after LPS or Poly I:C are produced locally by resident microglia in the brain or produced by infiltrating cells. LPS, when systemically administered, induce a systemic production of cytokines in blood borne cells, which can increase the permeability of the BBB and thereby permit inflammatory mediators to access the brain (reviewed in [224]). LPS itself does not appear to cross the BBB but instead bind to endothelial cells of the brain [225] and to areas of the brain lacking BBB [226] and thereby inducing a CNS response, mainly through activation of microglia. Resident microglia, and not infiltrating cells, have been found to be the main macrophage type activated in the brain after ischemic injury [227] and this suggests that resident microglia may be the main source of cytokine production in the brain also after systemic inflammation. In support, in studies in adult animals, it was shown that non-hematopoietic cells are critical for the CNS inflammation following systemic LPS exposure. This effect was not dependent on cytokine production in the blood [196]. Further studies are needed to investigate if Poly I:C works in the same way.

Both MCP-1 and IL-1β are known to be able to exacerbate neonatal excitotoxic lesions [221, 228]) and deletion of the entire TNF gene cluster abolishes LPS-mediated increase in neonatal HI injury [229], suggesting that these cytokines may play a role in inflammatory-induced neonatal brain damage. The direct influence of IL-6 on the development of brain injury is more unclear. Although studies have consistently demonstrated elevated IL-6 levels in asphyxiated infants [95] and mice overexpressing IL-6 develop severe neurologic syndromes [96], others find that mice deficient in IL-6 develop more severe brain injuries [97].

Although there was an early increase in proinflammatory cytokines and chemokines in WT mice after LPS exposure, the inflammatory response was more or less resolved at 14 h, the time point when HI was induced. A similar response was seen for IL-6 and IP-10 after Poly I:C exposure, whereas MCP-1 expression was still high, and IL-1β, TNF-α expression was even further increased at 14 h. It is unclear to what extent the inflammatory response contributed to the increased vulnerability of the brain to HI. It was previously shown that cell medium from LPS stimulated microglia cells can kill both neurons [230] and astrocytes [231] and at least neuronal death was dependent on MyD88.

Both MAPK and NF-κB play important roles in the proinflammatory response. IL-6, IL-1β, TNF-α, and MCP-1 are all regulated by the transcription factor NF-κB [118-122] after inflammatory stimuli. It has also been shown that Poly I:C gives rise to an induction of IFN-β, TNF-α and IP-10 in vivo and that the activation of NF-κB and the MAPKs JNK and ERK are required for the TLR 3 induced IP-10 gene expression [114]. Studies have also shown that NF-κB activation of IL-6 occurs through MAPKs [232]. Additionally, we, and others have previously shown that MAPKs are involved in neonatal HI [233-235] and inhibition of NF-κB activity attenuates HI brain injury in neonatal mice, independent of cytokine production [236, 237]. Recently, we also demonstrated that inhibition of p38 MAPK blocks cytotoxic effects of LPS-stimulated microglia on astrocytes [231]. MAPKs and NF-κB seems to be closely related and therefore, it could be useful to not only focus on the end product of the inflammatory response, but rather investigate the molecules upstream of the proinflammatory cytokines to explore future treatment strategies.

5.4 TLRs and barriers of the brain

The BBB and the choroid plexus have been suggested to be the site of entry of inflammatory mediators into the CNS during systemic inflammation. Furthermore, in response to LPS, the barrier permeability and genes regulating barrier maintenance and inflammatory cascades are affected both in BBB and in the blood-CSF barrier [136-139]. It is shown in this thesis that activation of TLR 3 with Poly I:C (paper III) or TLR 4 with LPS (paper II) sensitizes the immature brain to HI injury and we have previously shown

that systemic stimulation of TLR 2 impairs neonatal mouse brain development [174].

In order to test whether stimulation of TLRs leads to changes in brain barrier properties in the immature brain, we investigated the gene expression of several tight junction proteins in brain endothelial cells and choroid plexus (paper IV). Tight-junctions are made up of a range of proteins where occludin and claudins are transmembrane components and ZO-1 is one of the principal anchoring proteins of occludin inside the cell membrane. The claudins differ somewhat between tight junctions throughout the body and claudin-5 is believed to be the principal claudin in brain endothelial cells. Claudin-1 is found together with claudin-2 and claudin-11 in the epithelial cells of choroid plexus [238].

In BBB endothelial cells, we found that there was little regulation of tight junction proteins by any of the TLR ligands. However, in epithelial cells of the choroid plexus there was a significant down-regulation of occludin both after Pam and LPS exposure. Others have shown that activation of the TLR 2/6 complex lead to increased BBB permeability through downregulation of claudin-5 and occludin [197]. Furthermore, it has been shown that specific stimulation of TLR 2 or TLR 3 impair endothelial function by increased endothelial permeability, increased production of inflammatory cytokines and reactive oxygen species, and increased apoptosis of ECs [198, 199]. The exact molecular function of the different tight junction proteins and how they interact is still under debate. Studies of claudin-5 knock-out animals have shown that it can have a direct effect on blood-brain barrier permeability [239]. There are also several studies showing that changes to barrier permeability often is associated with alterations of both occludin and ZO-1 [240-242]. Our data suggest a possible mechanism where systemic stimulation of TLRs in the neonate may lead to disturbances in choroid plexus barrier function that may affect CNS injury at later stages. Future studies should investigate the functional barrier properties after TLR stimulation in neonatal animals to better understand these relationships.

6 CONCLUSIONS OF THE MAIN FINDINGS IN THE THESIS

- TLRs are expressed under basal conditions in the immature brain in numerous cell types in the brain parenchyma as well as in choroid plexus and blood brain barrier, suggesting that they may play essential roles in normal neurophysiological processes. Several of the TLRs are also regulated in the brain, choroid plexus and blood brain barrier after HI. Stimulation of TLR 1/2 (Pam) or TLR 4 (LPS) decreased the mRNA expression of the tight junction protein occludin in choroid plexus. These results suggest a role for TLRs in neuropathology and that TLRs may not only be involved in parenchymal inflammation, but may also play a role in transport across the blood and cerebral spinal fluid barriers.

- TLR 2 contributes to brain damage after HI alone, indicating that it has a role in sterile inflammation in the neonatal brain. In contrast, the adaptor proteins MyD88 and TRIF do not appear to play a role in the injury process after HI alone. These somewhat contradictory results of the neuroprotective effect in MyD88 (adaptor molecule downstream of TLR 2) and TLR 2 deficient mice suggest that there may be alternative TLR 2 downstream pathways in the CNS, apart from MyD88. Future studies should investigate this possibility by studying downstream TLR 2 associated pathways in detail following neonatal HI.

- MyD88 plays an essential role in LPS-sensitized HI brain injury; this support our previous findings identifying LPS as an important factor in altering the vulnerability to HI injury in the neonate and provide novel evidence that the sensitizing effect of LPS is mediated via MyD88. In recent years the importance of bacterial infections, resulting in inflammation in both preterm and term infants have been emphasized. This study indicates that the adaptor protein MyD88 may be a critical component in these events.

- Activation of the TRIF pathway, with Poly I:C, prior to HI markedly increased brain injury. The increased vulnerability to HI was associated with TRIF-dependent induction of both type I interferons, proinflammatory cytokines, and chemokines, suggesting that several pathways, including different transcription factors and MAPKs, are involved in the cerebral response to TRIF stimulation. Poly I:C is a synthetic mimic of double stranded RNA viral products. Therefore, the Poly I:C-induced increase in vulnerability to HI may indicate that viral infections could impact on HI brain injury in the newborn.

It is nowadays well established that infection/inflammation contributes to the development of perinatal brain damage in both preterm infants and full term children subjected to birth asphyxia (HI). These injuries can cause severe neurological and cognitive dysfunction, but also more subtle disturbances in behavior, learning and perception. The brain can probably be affected by both a systemic as well as local inflammation, which can influence brain maturation and development or modulate the brain's vulnerability to other insults. However, the inflammatory response is complex and may also have neuroprotective properties. Today there is a lack of treatment for perinatal brain damage and of satisfactory knowledge of underlying mechanisms and how therapies may affect the immune system. The current thesis has demonstrated that TLRs are involved in immature brain injury, both after infection- and HI-induced inflammation, or a combination of them both. Thus TLRs may provide novel targets for neuroprotective therapies in the future.

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