Innate immune response in neonatal hypoxic-ischemic brain injury

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2. List of Papers

This thesis is based on three publications named by roman numbers throughout the text:


3. Abbreviations

ASC  Apoptosis-Associated Speck-like Protein Containing a Caspase-recruitment Domain
AIM2  Absent in Melanoma 2
ALR   Absent in Melanoma like Receptor
AMPA  Alfa-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid
AP    Activator Protein
ATP   Adenosine Triphosphate
BBB   Blood Brain Barrier
BCSFB  Blood Cerebrospinal Fluid Barrier
CLR   C-type lectin Receptors
CNS   Central Nervous System
CSF   Cerebrospinal Fluid
CT    Cycle Threshold
CTG   Cardiotocography
DAMPs Damage Associated Molecular Patterns
DEGs  Differentially Expressed Genes
DNA   Deoxyribonucleic Acid
EDTA  Ethylenediaminetetraacetic Acid
ELISA Enzyme-linked Immunosorbent Assay
EMT   Epithelial mesenchymal transition
FELASA Federation of European Laboratory Animal Science Association
GABA  Gamma-Aminobutyric Acid
HIE   Hypoxic Ischemic Encephalopathy
HI    Hypoxia Ischemia
HMGB1 High Mobility Group Box 1
HT    Therapeutic Hypothermia
IL    Interleukin
ILCOR International Liaison Committee on Resuscitation
INF   Interferon
IPA   Ingenuity Pathway Analysis
LPS   Lipopolysaccharide
MBB   Meningeal Brain Barrier
MCAO  Middle Cerebral Artery Occlusion
MET   Mesenchymal Epithelial Transition
MHC   Major Histocompatibility Complex
MRI   Magnetic Resonance Imaging
MRS   Magnetic Resonance Spectroscopy
MyD88 Myeloid Differentiation Primary Response Gene 88
NAC   N-acetylcysteine
NACA  N-acetylcysteine Amide
NE    Neonatal Encephalopathy
NF-kB Nuclear factor-kB
NLR   Nucleotide-binding Oligomerization Domain-like Receptors
NLRC4 NLR Family CARD Domain Containing Protein 4
NLRP1 Nucleotide-binding Oligomerization Domain and Leucine Rich Repeat Pyrin 1 Domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NLRP3</td>
<td>Nucleotide-binding Oligomerization Domain and Leucine Rich Repeat Pyrin 3 Domain</td>
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<tr>
<td>NMDA</td>
<td>N-Methyl D-Aspartate</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen Associated Molecular Patterns</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>RCT</td>
<td>Randomized Clinical Trial</td>
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<td>RLR</td>
<td>Retinoic Acid Inducible Gene I like Receptor</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>TLR</td>
<td>Toll like Receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing Adapter-inducing Interferon-β</td>
</tr>
<tr>
<td>TTC</td>
<td>Triphenyltetrazolium Chloride</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deocynucleotidyl Transferase dUTP Nick End Labeling</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
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4. Introduction

4.1 Hypoxic Ischemic Encephalopathy

Hypoxic ischemic encephalopathy (HIE) is the most common form of neonatal encephalopathy (NE) [1]. NE is defined as a state of neurological depression within the first days of life in an infant born at or beyond 35 weeks of gestation [2;3]. It can be caused by a variety of events such as neonatal stroke, intraventricular hemorrhage, but also by non-neurological diseases such as hyperbilirubinemia, hypoglycemia, and neonatal sepsis [1]. When induced by a hypoxic-ischemic event, it is defined as HIE. Sarnat and Sarnat introduced a grading system for HIE in 1976 [4], which in the 1980s was modified by Levene et al [5]. Based on clinical examination, HIE is graded as mild, moderate or severe (Table 1). Mild if the child is irritable and hypotonic with sucking problems. Moderate if lethargic with moderate hypotonia, seizures and need for gastric tube feeding. Severe if the neonate is comatose, suffers sustained seizures and failure of spontaneous respiration.

<table>
<thead>
<tr>
<th>MILD</th>
<th>MODERATE</th>
<th>SEVERE</th>
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<tbody>
<tr>
<td>Irritability/Hyperalert</td>
<td>Lethargic</td>
<td>Comatose</td>
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<tr>
<td>Mild hypotonia</td>
<td>Moderate hypotonia</td>
<td>Severe hypotonia</td>
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<tr>
<td>Poor sucking</td>
<td>Requires tube feeding</td>
<td>Failure to maintain spontaneous respiration</td>
</tr>
<tr>
<td></td>
<td>Seizures</td>
<td>Prolonged seizures</td>
</tr>
</tbody>
</table>

Table 1. Hypoxic Ischemic Encephalopathy as categorized by Sarnat and Sarnat and modified by Levene et al. (The incidence and severity of post-asphyxial encephalopathy in full-term infants. Early Hum Dev. 1985. Levene et al)
4.1.1 Incidence of HIE
HIE is a major contributor to neonatal death and disability affecting 2-6/1000 term newborns in high income countries [5], with moderate to severe HIE in 0.5-1/1000 [6]. Children suffering from HIE may develop long term complications ranging from learning disabilities, to reduced cognitive function, sensory defects, epilepsy and cerebral palsy [7-9]. There is a strong correlation between grade of HIE and outcome [10]. Few children with mild HIE show neurological impairment at preschool age. In contrast, rate of death or disability ranges from 44-55% in children with moderate and severe HIE [11]. With a lifelong need for medical treatment and follow-up, the clinical and socioeconomic burden is significant [12]. Worldwide, the low and middle-income countries carry the biggest burden, with an incidence of HIE up to 10-20/1000 live births [13].

4.1.2 Etiology
Often, the underlying cause of HIE remains unknown and this represents a huge challenge for clinicians. The timing and intensity of hypoxic-ischemic events are of uttermost importance as antenatal events are probably out of reach for treatment, while peripartum and postpartum events might be treated with hypothermia (see 4.1.4). In children presenting with NE, clinicians search for evidence of sentinel hypoxic-ischemic events in the peripartum period that may direct the diagnosis towards HIE. The ”Task force on neonatal encephalopathy” defined umbilical cord prolapse, uterus rupture, severe abruption placenta, maternal cardiovascular collapse and fetal exsanguinations as acute peripartum or intrapartum sentinel events that may cause HIE [3]. Postnatal causes of NE can be respiratory failure due to obstructed airways seen with meconium aspiration, congenital abnormalities, pneumothorax or sepsis. Recently, a large retrospective cohort from Sweden, which has a similar health
care system as in Norway, was published [14]. When including 46749 women from southeast Sweden with singleton pregnancies from 2009-2013, they found an incidence of HIE of 1.7/1000 compared to the national incidence in the same period of 1.3/1000. Both maternal and obstetrical risk factors were analyzed. An acute obstetrical event during labor was the strongest risk factor with an odds ratio of 23.3. Nulliparity and abnormal admission cardiotocography (CTG) were also identified as risk factors for HIE [14].

4.1.3 Diagnostics
Given the diverse etiology of NE, clinicians are challenged to treat the neonates that are born with HIE and not those showing neurological depression due to other diseases. Clinical diagnosis of HIE in newborns with NE is based on evidence of an acute peri- or intrapartum hypoxic-ischemic event that is sufficiently strong to cause brain injury, often indicated by pathological fetal heart monitor patterns. The Apgar score was introduced by Dr. Virginia Apgar in 1952 to quickly assess children at 1, 5 and 10 min after birth and is still used worldwide. Based on skin circulation, heart rhythm, respiration, tonus and response to stimulus, health care personnel score neonates from 0-10. Although a score below 5 at 5 or 10 min clearly confers a risk for cerebral palsy, most children with low Apgar score will not develop cerebral palsy. With an Apgar score above 7, it is unlikely that a peripartum hypoxic-ischemic event caused NE. Since the Apgar score is affected by many factors, it cannot be used alone to predict if NE is caused by a hypoxic-ischemic event [15]. In 2014, the “Task force on neonatal encephalopathy” published renewed guidelines to retrospectively identify such events [3]. Neonatal signs consistent with an acute intra- or peripartum event were: a) an Apgar score at 5 and 10 minutes of less than 5, b) fetal umbilical artery
acidemia with pH less than 7 and/or base excess less than -12mmol/L, c) Magnetic Resonance Imaging (MRI)/Magnetic Resonance Spectroscopy (MRS) findings consistent with HI brain injury and d) presence of multisystem organ failure consistent with HIE. The likelihood of HIE increases with an increasing number of elements found in the patient. Although MRI/MRS findings are useful, in clinical practice they are rarely available before treatment needs to be started. In addition, investigations to search for alterations in cytokines in newborns that need resuscitation can give future help in guiding treatment of HIE [16].

4.1.4 Treatment
Skilled personnel attending birth is vital as 15% of term newborns will not breathe spontaneously without assistance and 2% will require intubation [17]. Their initial management of the newborn and knowledge is therefore important for survival in the transition from intra to extra uterine life. Based on acceptance of toxicity of 100% oxygen delivered during resuscitation [18], the International Liaison Committee on Resuscitation (ILCOR) guidelines were in 2010 changed to use room air during resuscitation of term and near term infants [17]. However, when HIE is first diagnosed, the only available treatment option for HIE is therapeutic hypothermia (HT). Cooling for 72 h to 33-34 °C should be started within 6 h in children with moderate or severe HIE. According to the latest Cochrane review from 2013 comparing HT with normothermia, HT reduces the risk of death or major disability by 15% (61% vs. 46%) with a number needed to treat of seven [6]. This implies that approximately half of babies treated with HT still suffer major disability or death [19], hence improvement in outcome is modest [20]. Furthermore, children with mild HIE stand without specific treatment, and there are evidence that these children have an
increased likelihood of behavioral problems in childhood [21]. In addition, HT for HIE in low to middle income countries does not improve mortality [22], meaning it is out of reach for the largest proportions of babies born with HIE. Altogether, this prompts for further research to obtain possible additional treatment options for neonates suffering HIE, and a short summary of some of the most promising strategies will be given in chapter 4.2.4.

4.2 Mechanisms of injury in HIE
The severity and duration of a hypoxic-ischemic insult, the metabolic status including temperature of the child and the gestational age, are crucial factors determining the outcome of hypoxic-ischemic brain injury [23]. The development of brain injury can be divided into several phases [23]. Following a hypoxic-ischemic event, the brain suffers reduced oxygen and nutrient delivery. Depending on the strength and duration of the hypoxic-ischemic event, this leads to a primary energy failure or primary phase of injury. With restoration of blood flow and oxygenation, glucose metabolism and levels of Adenosine Triphosphate (ATP) normalize; this is called the latent phase, and represents a possible window of opportunity for treatment. The secondary energy failure or phase of injury ensues after approximately 6h. It is characterized by a new decrease in ATP levels and cell death, due to impaired mitochondrial functions, despite normalization of oxygen levels [24]. Hyperoxia used during resuscitation has finally been recognized to further aggravate damage in this phase via oxidative stress, resulting in the implementation of room air for resuscitation of newborns from 2010 [25].

Most research has focused on possible interventions in the latent phase, but increasing evidence shows that brain injury and repair continue into a third phase probably
extending the therapeutic window [26]. In cerebral palsy, the role of chronic inflammation is exemplified by increased levels of the inflammatory cytokine tumor necrosis factor (TNF) in plasma of 7 year old children [27].

![Figure 1. The different phases of brain injury following neonatal HI.](image)

Depending on strength and duration of the HI insult, cell death occurs in the immediate post reperfusion phase. This acute phase is however followed by a latent phase with restoration of energy levels that approximately last for 6 hours. After the latent phase, a secondary phase of injury follows with decreasing energy levels and cell death, called the secondary energy failure or brain injury. Finally a third phase of injury ensues that is largely affected by scar formation and inflammatory processes.


### 4.2.1 Excitotoxicity

The reduced oxygen and glucose delivery during HI events result in reduced energy production of ATP as the metabolism in mitochondria switch from aerobic to anaerobic ATP production. ATP is required in cell membrane Na⁺/K⁺ pump. Loss of this essential pump function results in increased levels of intracellular Na⁺, Ca²⁺ and Cl⁻ and causes the cell membrane to depolarize. The influx of Na⁺ causes cytotoxic edema. Depolarization releases glutamate from nerve terminals. Stimulation of postsynaptic glutamate receptors such as N-methyl-D-Aspartate (NMDA) receptors and α-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors,
result in further depolarization and Ca\(^{2+}\) influx [28]. Increased susceptibility to HI brain injury in neonates is linked to increased expression of glutamate receptors in the developing brain [29;30]. Intracellular Ca\(^{2+}\) can bind and activate lipases, proteases and endonucleases destroying proteins, lipids and RNA/DNA and ultimately lead to cell death. This process in which glutamate neurotransmission leads to over activation of postsynaptic neurons and cell death is referred to as excitotoxicity [31].

4.2.2 Oxidative stress
During the conversion of oxygen to water in the mitochondrial respiratory chain, the continuous production of reactive oxygen species (ROS) needs to be neutralized by antioxidants. This balance is destroyed during hypoxia, where an increased amount of ROS production occur leading to oxidative stress. Reoxygenation with 100% O\(_2\) can further aggravate this imbalance [32]. If ROS combine with NO, they form reactive nitrogen species (RNS) [33]. Together, different ROS and RNS, highly reactive species, cause lipid peroxidation, the oxidative destruction of free fatty acids, causing damage to cell membranes and eventually cell lysis. In addition lipid peroxides interact with Ribonucleic Acid (RNA)/Deoxyribonucleic Acid (DNA) and cause RNA/DNA fragmentation. Due to its high content of free fatty acids and low antioxidant defense, the newborn, especially premature brain is particularly susceptible to oxidative stress [25]. Major antioxidant systems are superoxide dismutases (SOD), catalases and the tripeptide glutathione [34]. Glutathione is perhaps the most abundant antioxidant, but its availability is limited in newborns, especially in preterm [35] and under oxidative stress the physiological production of glutathione becomes insufficient to counteract the increased ROS production [36]. Antioxidants, such as N-acetylcysteine amide (NACA), which can replenish the glutathione level can therefore serve an interesting neuroprotective potential.
4.2.3 Inflammation

The third main contributor to neonatal hypoxic-ischemic brain damage is inflammation. Many cell types are involved in the inflammatory response as shown in Figure 2. Microglia, which is the resident brain macrophage, is a central executor cell in the inflammatory process, but astrocytes, the scar forming cells in the central nervous system (CNS), are also capable of inflammatory cytokine production and gain increasing focus in mediating inflammation. In addition, mast cells are known as early activators of the inflammatory system. Although initially essential for resolution of damaged tissue, the inflammatory response can overshoot its target, further enhancing damage, as discussed below (Chapter 4.3).

Figure 2. Different cell types such as microglia, macrophages, mast cells and astrocytes are involved in the inflammatory response to hypoxic-ischemic brain injury. Reprinted by permission from Springer Nature: *Nat Rev Neurol.*, 2015 Apr;11(4):192-208. doi: 10.1038/nrneurol.2015.13. Epub 2015 Feb 17. Hagberg H¹, Mallard C², Ferriero DM³, Vannucci SJ⁴, Levison SW⁵, Vexler ZS⁶, Gressens P⁶. Copyright 2018.
4.2.4 Neuroprotective strategies

In the following section a short description of promising neuroprotective strategies are summarized:

*Melatonin*

Melatonin, an endogenous hormone that regulates circadian rhythm has anti-inflammatory, antioxidant and antiapoptotic effects in high doses. A systematic review and meta-analysis of adult focal cerebral ischemia showed a 43% reduction in infarction size [37]. In the piglet model melatonin augments hypothermic neuroprotection [38]. In a Randomized Clinical Trial (RCT), early administration was feasible and may ameliorate brain injury [39].

*Erythropoietin*

Erythropoietin is secreted by the kidneys in response to cellular hypoxia. In high doses in conjunction with HT it improved outcome in a nonhuman primate model of perinatal asphyxia [40]. Both phase 1 and phase 2 trials have been conducted and showed that a high dose of erythropoietin with HT result in better short term outcome. A phase 3 trial is now conducted.

*Xenon*

The noble gas xenon inhibits NMDA signaling and has antiapoptotic properties. In pigs exposed to hypoxia, xenon augmented neuroprotection with HT [41], but in a RCT, xenon did not show any short term benefit above cooling alone [42].

*Argon*

The noble gas argon is a Gamma-Aminobutyric Acid (GABA) agonist and has antiapoptotic properties. Combined with HT it showed improved MR findings compared to HT alone and phase 2 studies are under approval.
**Allopurinol**

Allopurinol reduces free radical production, and in high doses it serves as a free radical scavenger. In piglets treated with allopurinol, reduced brain damage was seen on MRI [43] and there is an ongoing RCT to assess the effect of allopurinol and HT in neonates with HIE.

**Stem Cells**

Autologous stem cell transfusion is postulated to decrease brain damage through paracrine signaling and not direct integration or proliferation. Various animal models have shown neuroprotection with different modes of stem cell transfusion [44]. In a phase 1 study, autologous umbilical cord stem cell infusion was shown feasible [45].

**Magnesium**

Magnesium works by stabilizing membrane potential and blocking excitatory amino acids such as glutamate. Outcome of different animal experiments was found to be highly inconsistent in a metaanalysis [46].

**N-Acetylcysteine (NAC) and N-Acetylcysteine Amide (NACA)**

N-acetylcysteine (NAC) is a thiol antioxidant used in the clinical setting of paracetamol intoxicity and as a mucolytic agent [47]. NAC serves to replenish the glutathione status and advantage over other antioxidants such as vitamins C, D, K and lipoic acid has been addressed to efficiency of NAC to synthesize and replenish glutathione [48;49]. NAC has also anti-inflammatory effect through inhibition of Nuclear factor (NF)-κB activity [47]. In postnatal day 7 rats, combination of NAC and HT ameliorated brain damage [50]. When applied to the piglet model of neonatal hypoxic-ischemic brain injury, NAC treatment resulted in significantly lower levels of inflammatory cytokines [51]. However, a disadvantage of NAC is its low bioavailability [47]. The amide from of NAC, NACA, has been synthesized with enhanced lipophilic property, membrane permeability and antioxidant capacity
compared to NAC [52], NACA can thus potentially cross the blood brain barrier (BBB) to a greater extent and serves as a neuroprotectant. Neuroprotective effect of NACA has been shown in mouse models of multiple sclerosis [53], Parkinson disease [54] and traumatic brain injury [55]. So far, it has however not been evaluated in animal models of neonatal HIE.

4.2.5 Treatment Research at Department of Pediatric Research

At the Department of Pediatric Research, we have a long history of exploring basic mechanisms of perinatal hypoxic-ischemic brain damage by addressing different neuroprotective strategies using models ranging from cell culture studies to animal models with mice, rat and pigs. Effects of different oxygen concentrations during resuscitation have been a central research field at our institute and recently among several both PhD Embjørg Wollen and PhD Anne Gro W Rognlien addressed this in their theses. PhD Jannicke Andresen explored possible neuroprotective effects of nicotine in perinatal asphyxia using newborn piglets. PhD Yngve Sejersted investigated effects of DNA glycosylases in maintenance and DNA repair in hypoxic-ischemic brain damage in neonatal mice. The institute has also searched for neuroprotection with inhaled hydrogen (PhD Rønnaug Solberg) and NAC (PhD Helene Østerholt). In collaboration with PhD student Torkil Benterud, we have studied potential neuroprotective effects of NACA. Finally PhD Håvard Garberg has examined the effect of cannabinoids and PhD student Marianne Huun has examined the effect of fish oil in the piglet model of neonatal hypoxic-ischemic brain injury.
4.3 Inflammation in HIE

4.3.1 The inflammatory response

Inflammation is a double edge sword. As a living organism, we are dependent on a well-balanced inflammatory response that is both capable of reacting to invading organisms and initiate healing of damaged tissue and at the same time respect undamaged self. On the other side, an inflammatory response that is too strong or that does not resolve, can lead to chronic inflammation and increased damage.

While the adaptive immune system is specific and creates an immunological memory to subsequent infections, the innate immune system is constructed to react immediately to a vast diversity of molecules based on recognition of molecular patterns. Compared to adults, neonates have reduced major histocompatibility complex (MHC) II expression capability [56], thereby reducing their capacity to activate adaptive immune responses, rendering them more dependent on their innate immune responses. The adaptive immune responses in neonatal HIE are seen in the second and third phase of injury, with infiltration of antigen presenting cells and T-cells [57]. The choroid plexus has been suggested to be a site for leukocyte entry and important for resolution of brain damage [58], but the role of the adaptive immune cells in neonatal HIE still remains poorly elucidated [57].

A classical inflammatory response, consists of four components; inducers, sensors, mediators and target cells (Figure 3)[59]:

**Figure 3: The inflammatory response**
**Inducers**

Stimulators of innate immune activation are named Damage Associated Molecular Patterns (DAMPs) or Pathogen Associated Molecular Patterns (PAMPs) meaning that both endogenous sterile injury and exogenous infections are able to stimulate these responses [60]. Well known DAMPs are High Mobility Group Box 1 (HMGB1), nuclear and mitochondrial DNA[61].

**Sensors**

So far, five main types of pattern recognition receptors (PRRs) have been characterized; Toll like receptors (TLRs), Nucleotide binding oligomerization domain like receptors (NLRs), C-type lectin receptors (CLRs), Retinoic acid inducible gene I like receptor (RLRs), Absent in melanoma like receptors (ALRs) [62]. These receptors are expressed by classical innate immune cells such as dendritic cells and macrophages, but also by non professional cells such as endothelium, epithelium and fibroblasts [61]. In the brain not only microglia, but astrocytes, neurons and oligodendrocytes express PRRs [63].

Humans express ten different TLRs [64], and mice twelve [61]. TLR4 was the first TLR to be discovered in humans[65]. A mouse strain resistant to lipopolysaccharide (LPS) induced shock was hypothesized to be caused by a mutation in a LPS receptor gene. This gene was identified in 1998 as TLR4 [66]. LPS activation of TLR4 leading activation of NF-κB is now known as a cornerstone in the innate immune response to gram negative bacteria, and is also a necessary step in the activation of inflammasomes, such as the Nucleotide-binding oligomerization domain and leucine rich repeat pyrin 3 domain (NLRP3) as described later [67].
**Mediators**

Cytokines are the messengers of the immune system. They are secreted by immune cells such as macrophages, microglia, neutrophils, B and T-cells, but also by stromal cells such as astrocytes and endothelial cells in the brain[68]. Examples of cytokines are chemokines, lymphokines, TNF, interferons (INF) and interleukins (IL). They serve to regulate immune response by pro or anti-inflammatory capacity. Examples of anti-inflammatory cytokines are e.g. IL-1 receptor antagonist, IL-4, IL-10, IL-11, IL-13 and Transforming growth factor (TGF)β [69]. Typical pro inflammatory cytokines are IL-1, IL-8, IL-12 IL-18, TNF and INFγ [68]. The IL-1 family consists of 11 cytokines, where IL-1β and IL-1α are the most studied and possess strong pro inflammatory capacity [70]. They both bind to the same IL-1 receptor complex and induce fever, vasodilatation, hypotension and increase expression of adhesion molecules on endothelial cells mediating transmigration of cells [71;72].

**Target**

Cells and tissues, i.e., the inflamed tissue that is targeted by the inflammatory response and cells that are activated and or attracted during an inflammatory response (i.e. neutrophils, endothelial cells or hepatocytes)

**4.3.2 Innate Immune Activation in HIE.**

Within minutes after ischemia, inflammatory cytokines are released, but at the same time, the inflammatory process can continue for several weeks [57]. Thus, the inflammatory process encompasses the entire injury phase, making it an attractable target for intervention. In the setting of neonatal HI brain injury, where the latent
phase only last for approximately 6 hours, innate immune responses are therefore potential early targets for intervention.

However, inflammation can also sensitize the neonatal brain to HI, as administration of LPS, the gram negative bacterial endotoxin, prior to HI, increases brain damage in neonatal rats [73]. In clinical practice, it has been documented that sepsis of the newborn is associated with increased brain damage and poor outcome [74]. Cerebrospinal fluid (CSF) of asphyxiated term infants show increased levels of pro inflammatory cytokines, such as IL-6 and IL-8, and their levels correspond to the degree of HIE [75]. Furthermore, from experiments with neonatal rats, the complex time dependent role of inflammation in regard to HI brain injury has been demonstrated by Eklind et al.[76]. LPS worsened brain injury when administered 72 h before or 6 h after, while administration 24 h prior to HI decreased brain injury. Timing is therefore of uttermost importance when addressing the innate immune response.

From animal experiments, several interventions that dampen the early inflammatory response have proven neuroprotective [57]. Following a hypoxic-ischemic event, injured tissue is thought to release DAMPs that bind to PRR on various cells in the CNS as shown in Figure 3. The TLR group is the most extensively investigated PRR group in neonatal HI models [57]. Neonatal mice constitutively express TLR1-9 in the forebrain [77]. Moreover, TLRs 1-4 are expressed in the choroid plexus, which, as previously mentioned, has been suggested as an important entry site for inflammatory cells in neonatal HI [78]. Following activation, all TLRs, except TLR3, signal through a myeloid differentiation primary response gene 88 (MyD88) dependent pathway that through a cascade of events, leads to activation of transcription factors such as NF-κB
and activator protein 1 (AP-1) [61;79]. These transcription factors initiate transcription of hallmark inflammatory cytokines such as IL-1β, IL-6, IL-18 and TNF [80]. TLR3 signals through MyD88 independent/ TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway [61]. Animal models have shown that TLR3 and TLR4 stimulation increase susceptibility to neonatal HI [73;81]. Deficiency of TLR2 confers neuroprotection to neonatal HI brain damage [77], however deficiency of two important downstream TLR adaptor proteins MyD88 and TRIF do not [81;82].

The role of innate immune responses in neurological diseases is well recognized such as in the etiology of Alzheimer’s and Parkinson’s disease, stroke and epilepsy [83]. However, accurate mechanisms of involvement is still poorly understood, and the contribution of the different cell populations such as microglia, astrocytes, oligodendrocytes and endothelial cells needs further investigation to develop effective therapeutic approaches [83]. In addition, the inflammatory response has shown huge difference dependent on age, in regard to BBB, leukocyte migration and microglial activation as discussed in more detail in the next chapter.

4.3.3 Cellular mediators of inflammatory processes in HI

Microglia
In the brain, microglia (the resident brain macrophage) and mast cells are the first immune cells to be stimulated following HI [84;85;85]. Microglia constitute 5-15% of the total brain cell population [86] surveilling the brain parenchyma with thin ramified processes. During neurodevelopment they serve to maintain the neural cell pool,
balanced between elimination and survival of neurons [87]. Upon activation, they rapidly transform into round and amoeboid morphologies, secrete inflammatory cytokines, migrate and possess phagocytic capacity [88]. Resident microglia, rather than infiltrating macrophages contribute to the early inflammatory phase [89] and activated microglia peaks approximately 24 h after neonatal HI. Following ischemic brain injury, resident microglia secrete cytokines such as the potent inflammatory cytokines IL-1β, IL-18 and TNF [90]. Microglia also secrete proteases, that are enzymes cleaving other proteins, complement factors, which is a set of proteins that can be serially activated resulting in cell lysis, as well as activate NMDA mediated toxicity, thereby contributing to secondary energy failure [90-92]. IL-1β is known as a major mediator of acute and chronic inflammation and the most potent endogenous pyrogen [93]. IL-1β modulates the BBB and stimulates astrocytes and microglia to produce chemokines attracting inflammatory cells. A second peak of microglial activity occurs after 1 week and consists mainly of infiltrating macrophages [94]. Thus, addressing persistent microglial activation leading to chronic inflammatory injury could be a target for intervention in the tertiary phase of injury.

The dual role of innate immune responses in neonatal HI is well exemplified by animal experiments with minocycline treatment [95]. Minocycline, a broad spectrum antibiotic, also function as a microglial inhibitor. In both adult [96] and neonatal rats [97] it was first shown to ameliorate brain injury. Furthermore, levels of inflammatory cytokines were reduced. However, when tested in neonatal mice, minocycline treatment worsened HI brain injury [98]. When the observation period was lengthened from 24 h to 7 days following HI, minocycline had lost its neuroprotective effects, even in rats [99]. Finally, an age dependent effect was reported, as post natal day 9 (P9) mice, corresponding to human neonate, were only transiently neuroprotected.
while postnatal day 30 mice, corresponding to human juvenile stage, showed sustained improvements in neuronal injury [100].

Furthermore, pharmacological depletion of microglia has suggested an endogenous protective role of microglia. Intracerebral injection of liposome-encapsulated clodronate before transient middle cerebral artery occlusion (MCAO) in P7 rats resulted in increased brain damage and levels of several cytokines and chemokines [101].

Experiments inhibiting NF-κB, a central transcription factor in the cascade of innate immune activation, further exemplifies the importance of accurate timing when inhibiting the inflammatory system. NF-κB (see chapter 4.3.2) is a central transcriptional regulator of many inflammatory cytokines and levels of NF-κB follows a biphasic activation pattern with a top at 3-6 h and then at 24 h after neonatal HI [102]. With the administration of a decoy oligonucleotide that can bind and inhibit NF-κB effects, inflammatory cytokines are reduced [103] as well as brain injury following neonatal HI [104]. Others have proposed that the effect of NF-κB inhibition is through the apoptotic pathway, rather than cytokine response [105]. Once again, timing was shown crucial in innate immune responses as early inhibition of NF-κB resulted in neuroprotection, while late NF-κB activity provided endogenous neuroprotection [102].

Inflammation research in brain injury has mainly focused on microglia, since these cells not only respond rapidly, but also because they are essential inducers of IL-1β and IL-18 release [106]. However, there is also substantial evidence that astrocytes, mast cells and endothelial cells are central in the inflammatory cascade [83].
**Mast cells**
Mast cells are cells of hematopoietic origin, typically located in close relation to outer layers and barriers such as the ependymal lining and BBB, and are well known from the allergic and anaphylactic reactions. They represent together with microglia the first cells to initiate a defense against exotoxins, allergens, invading pathogens and sterile injury such as HI [107]. Following HI, mast cells mediate excitotoxic injury through increasing TGF-β1 toxicity [108]. When treating neonatal rats submitted to HI brain damage, with a mast cell stabilizer, long lasting neuroprotection was reported [85].

**BBB damage and immune cell invasion**
The brain has traditionally been considered an immune privileged site [109]. It is protected from the systemic blood circulation by the BBB, the meningeal brain barrier (MBB) and the blood cerebrospinal fluid barrier (BCSFB) [110]. These barriers are composed of cells tightly linked through tight junctions making them selectively permeable to specific molecules by membrane transport [110]. The BBB is composed of a thin endothelial cell layer, basement membrane, astrocytic end feet and pericytes. The MBB is the most complex and consists of a three layered membrane; the dura mater, arachnoideal membrane and pia mater. The BCSFB is composed of a thin layer of epithelium over fenestrated capillaries, called the choroid plexus, responsible for the cerebrospinal fluid production. In the BBB, tight junctions are present between endothelial cells, in the MBB, they are present between arachnoideal cells and in the BCSFB, barrier forming cells are epithelial cells with tight junctions [111]. However, this immune privilege is severely challenged once inflammation is established, and both systemic and central immune stimulators can induce inflammation within the CNS [57].
The thin endothelial cell layer of the BBB expresses a variety of different connexin hemichannels [112]. Connexin hemichannels allow intercell communication when opposed to form gap junctions [113], in the healthy state, while undocked hemichannels remain closed. Following brain damage, undocked hemichannels can open, resulting in Ca\(^{2+}\) influx and propagation of damage [112]. At birth, the BBB is functional with no fenestrations and evidence suggest that it is not as permeable as once thought [114;115]. It is however clear that the BBB is damaged after neonatal HI with increased permeability to larger endogenous molecules such as albumin and IgG [116;117] and smaller exogenous molecules [118]. Vulnerability to BBB damage also seems to be developmentally regulated as P7 rats show more albumin extravasation than P21 rats following HI. Restoration of BBB function, at least in regard to passive penetrance has been shown to occur between 3 [118] and 7 days [119]. Little is however known about BBB function in regard to active metabolism and protein transport following neonatal HI [120].

In adult mice, stroke generated by MCAO results in early neutrophil invasion. Neutrophils produce inflammatory cytokines, chemokines and ROS and contribute to the disruption of the BBB in adult stroke [121]. In contrast, following HI in neonatal rats, neutrophil invasion occurs in a lesser degree as it was shown only in a transient manner at 12 h after HI [92]. Neutrophil depletion prior to neonatal HI however resulted in less brain swelling [122], but whether the effect was due to reduced neutrophils in the peripheral blood or invading the brain was not examined.

The significance of lymphocyte invasion in neonatal HI remains elusive. Lymphocyte invasion has been reported at different time points, up to 35 days [92] to several months after HI [123]. However, experiments inhibiting lymphocyte egress from
lymph nodes showed no difference in brain injury. This indicates little involvement of these cells in the early injury phase [124].

**Astrocytes**

Astrocytes respond to various CNS stimuli with hypertrophy and hyperplasia in a process called astrogliosis. They are central in the glutamate metabolism and their end feet constitute a part of the BBB to form a neurovascular unit. Following brain injury, they are key constituents of the astrocytic scar and express MHC II and PRRs [125]. Notably, astrocytes are capable of IL-1β production [126-128]. Although astrocytes are classically believed to function mainly as supportive cells for neurons, the astroglial scar can exert both pro and anti-inflammatory effects [129]. Again, a balanced astrogliosis and scar formation is crucial in confining inflammation to the epicenter and restoring brain homeostasis [130]. Their role as supportive cells for neurons, have also been pointed out as a potential target for intervention. In a recent report by Morken et al, this cell population’s mitochondrial capability to assist neurons was particularly affected in a neonatal rat model of HI [131]. Finally, there is emerging interest on the inter cell communication between astrocytes, neurons and microglia. Following brain injury microglia activate hemichannels and gap junctions. These are important in the normal cell-cell communication, but in a pathological setting, play a role in the propagation of injury between glia such as astrocytes and microglia [132;133]. Unopposed connexin hemichannels are in the resting state closed, but following injurious stimuli such as hypoxia ischemia, they can open, disrupting resting potential, cause Ca influx, glutamate and ATP release [112]. There are 21 known connexin hemichannels, and astrocytes are the predominant cell type expressing connexin 43 [112]. Connexin 43 is a key mediator of brain damage and is one of the main targets for intervention in cardiac and brain ischemic reperfusion.
injury [134]. ATP release through opened hemichannels, such as connexin 43 is furthermore implemented in NLRP3 inflammasome activation [135], as described later.

4.3.4 Inflammasomes, platforms for activation of IL-1β and IL-18
With the first discovery of an inflammasome in 2002, Martinon et al [136] paved the way for a new understanding of innate immune responses, leading to more detailed characterization of immune diseases and development of pharmaceutics to treat them. Inflammasomes are large multi molecular complexes composed of a sensing unit (PRR), the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and the effector protease, caspase-1 [137]. They regulate the processing of IL-1β and IL-18 from their proforms through the action of caspase-1 [138]. Inflammasomes are named based on their PRR, and although many have been reported to mediate inflammasome activity in vitro, few have well described physiological functions in vivo. The best characterized are Nucleotide-binding oligomerization domain and leucine rich repeat pyrin 1 domain (NLRP1), NLRP3, NLR family CARD domain containing protein 4 (NLRC4) and Absent in melanoma 2 (AIM2) [138].

4.3.5 NLRP3 in neonatal HIE
The most extensively studied inflammasome is the NLRP3 inflammasome [139]. NLRP3 is a cytosolic macromolecule that reacts to a wide spectrum of both PAMPs and DAMPs. The canonical activation of NLRP3 inflammasome is a two step process as illustrated in Figure 4. First, NLRP3, as well as pro-IL-1β and pro-IL-18 are upregulated, typically through activation of TLR4 [140]. A second stimulus is needed for NLRP3 inflammasome assembly such as ATP, pore forming toxins, crystalline
substances or amyloid-β fibrils [137;141]. This diversity probably rules out the direct interaction with any of the potential stimulators and the exact mechanism for NLRP3 activation is still not clear. So far, three models of NLRP3 activation have been proposed; (1) Lysosomal rupture, (2) cytosolic ROS production and (3) reduced cytosolic K⁺ concentration.

**Figure 4.** Activation of NLRP3 inflammasome results in release of IL-1β and IL-18. See text for detail. Courtesy Arne Yndestad.

Lysosymal rupture is the consequence of frustrated phagocytosis that occurs when cells try to engulf crystals or other non-degradable molecules. From ruptured lysosomes, release of proteases such as cathepsin B occurs, and cathepsin B has been reported to activate NLRP3 [139]. Lysosymal rupture also releases NADPH oxidases, which can activate the NLRP3 inflammasome, but most NLRP3 activating ROS probably origins from mitochondrial failure. Blocking the respiratory chain has been shown to induce a robust NLRP3 dependent IL-1β release. Finally K⁺ efflux can be mediated by extracellular ATP binding to the P2X7 receptors in the plasma cell membrane and is also essential in NLRP1 and AIM2 inflammasome formation [139;142;143].
Upon activation of the NLRP3 inflammasome, NLRP3 associate with ASC and pro-caspase-1 is subsequently recruited and autocleaved into active caspase-1 [137]. Active caspase-1 cleaves pro-IL-1β and pro-IL-18 into their active forms and can also initiate pyroptosis, a programmed inflammatory cell death mode characterized by cell swelling and early cell membrane rupture [59]. Release of IL-1β and IL-18 occurs rapidly after active caspase-1 release, and is regulated through cleavage of a cytosolic protein; gasdermin D. Gasdermin D itself is also cleaved by caspase-1 and results in a destabilization of the plasma membrane that allows for cytokine release.

In addition, a noncanonical NLRP3 inflammasome activation pathway has been described through caspase-11 in mice and caspase-4 and -5 in humans [137]. Caspase-11 is able to bind directly to cytosolic DNA and induce NLRP3 assembly of a primed NLRP3 [137].

A substantial amount of research involving neuroinflammatory effects of IL-1β and IL-18 have been performed, mainly in adult rodent models of stroke, intracerebral hemorrhage, traumatic brain injury, multiple sclerosis and Alzheimer disease [144]. IL-1β and IL-18 mRNA and protein are upregulated after cerebral ischemia in adult rodent models [145-147] as well as in neonatal HI rodent models [148;149]. Both microglia and astrocytes have been proposed as sources of IL-1β and IL-18 [145]. A neuroprotective effect of IL-1Ra injection in rodents has been shown in both adult ischemia and neonatal HI models [148;150]. Similarly, mice deficient in IL-1α and IL-1β were neuroprotected after cerebral ischemia [151]. In neonatal HI, these knockout mice were not neuroprotected, however mice with combined deficiency in IL-1β and IL-18 showed smaller infarct volumes indicating a particular role for IL-18.
in neonatal HI [152]. Moreover, caspase-1 deficient mice also show diminished infarct volumes following both adult ischemia and neonatal HI [153;154].

The NLRP3 inflammasome has been implicated in the disease progression of Alzheimer [155] and multiple sclerosis [156], prion disease and amyotrophic lateral sclerosis [157], but its involvement in other CNS diseases such as Parkinson’s disease, stroke or neonatal HIE still remain to be investigated. Neither has the role of the ASC component of the NLRP3 inflammasome, which also constitute an essential part of other inflammasomes, been identified. In summary, it is still not known whether neonatal HI brain injury induces expression of NLRP3 and ASC, and if it does, in which cell types. And most importantly, how NLRP3 and ASC affect the outcome of a hypoxic-ischemic event is not known.
5. Aims of the Study

In this thesis, we aimed to identify basic mechanisms in neonatal HI brain injury that might serve as novel therapeutic targets. We hypothesized that deficiency of NLRP3 inflammasome components would dampen the inflammatory response and result in neuroprotection in neonatal mice and that the antioxidant/anti-inflammatory agent NACA would ameliorate brain damage in the piglet model of neonatal asphyxia.

Specific aims were to:

Paper I:
- Examine if NLRP3 expression could be found in the neonatal brain following HI.
- Evaluate the effect of NLRP3 deficiency in the early (24 h) phase of brain injury.

Paper II:
- Since damage following HI propagates beyond the early phase, we aimed to examine if NLRP3 deficiency was neuroprotective at a later stage, namely 7 days after HI.
- As ASC is an essential component of both NLRP3 and other inflammasomes, we aimed to investigate if ASC deficiency would ameliorate brain damage 7 days after HI.

Paper III:
- We exploited another animal model, the piglet model, to further explore the inflammatory effects of hypoxia and examine if there was a neuroprotective effect of the potential anti-inflammatory drug NACA.
6. Materials and Methods

6.1 Modified Vanucci model for newborn mice
In paper I and II we used the well established Vanucci model of HI with modifications for use in neonatal mice. This is a widely accepted model for neonatal HI. We based our research on the C57BL/6 J background and both wildtype (WT) and knockout mice were generated from this line. In our first report we used heterozygous breeding. In paper II, with three genotypes, animals were bred in a homozygous pattern. The animals were given food and water ad libitum and were housed and bred at 24 °C on a 12:12 hour light:dark cycle. All experiments were approved by the regional veterinary institute and followed Federation of European Laboratory Animal Science Association (FELASA) C recommendations.

At P9, pups were randomized to HI or control. Animals were analgesized with buprenorphine (0.1mg/kg) and sedated with 4% isoflurane. A small skin incision was made and the left common carotid artery was carefully dissected out. Ligation of the left common carotid artery was performed by a cautherisator and skin incision closed by absorbable suture. All surgery was performed within 5 minutes. Pups were then left for recovery for 1 h before hypoxia with 10% O2 for 1 h. Sham operated animals were injected with s.c. ibuprofene, sedated with isoflurane, before a skin incision was made and closed, but did not undergo common carotid artery ligation, nor hypoxia. In the experiments from our first report, hypoxia was performed in a custom maid chamber with control of oxygen level and temperature. In experiments from the second report, a hypoxia chamber Invivo500 (Thermo Scientific) was used (Figure 5).
Animals were sacrificed at different time points, 3 h, 24 h, 72 h and 7 days following HI. We harvested brains for RNA/Protein analysis, Immunohistochemistry and Triphenyltetrazolium Chloride (TTC). We also developed a method to sample enough blood for protein analysis when decapitating mice as described later.

6.2 Piglet model
Newborn piglets, all from the same farmer, in healthy condition with age of 12-36 h were used. The piglets were gently handled and weighted to minimize stress. They were anesthetized with a bolus injection of fentanyl (25 microg/kg), midazolam (1.0 mg/kg) and pentobarbitone (20 mg/kg) before intubated and placed on their backs for washing and sterile procedures. They were ventilated with a pressure controlled ventilator (Babylog 8000+; Drägerwerk, Lübeck, Germany) IMV mode. Anesthesia was maintained with fentanyl (50 mikrog/kg/h) and midazolam (0.25 mg/kg/h). As anesthetics have shown neuroprotective effects, we sought to standardize drug administration in all experiments [158;159]. Several different models for hypoxic-ischemic brain injury in the piglet have been developed [160-163]. We use a well established model of respiratory hypoxia where global hypoxia was induced by 8% of O$_2$ until BE was <-20mmol/L or mean arterial blood pressure (MABP) fell below 20mmHg. CO$_2$ was added during hypoxia, aiming for a PcO$_2$ of 8.0-9.5kPa. Piglets were randomized to sham or treatment with NACA (NACA-piglets) or saline. Based on previous animal experiments, 300mg/kg NACA was administered at end of hypoxia and again after 4.5 h. Blood samples were collected 6 times before harvest. At 9.5 h, animals were sacrificed by an overdose of pentobarbital (150mg/kg). Urine, CSF, and blood were frozen at -70°C. Brains were harvested and 1 hemisphere was stored on paraformaldehyde, while the other was dissected into subregions (cortex,
hippocampus, thalamus, striatum and cerebellum) and stored at -70°C for gene and protein analysis.

6.3 Laboratory Analyses

**TTC staining and blood sampling**

In the first report we examined infarction volumes 24 h after HI by TTC staining. TTC is a compound that stains living tissue red through a red-ox reaction. Animals were decapitated and by carefully holding the mice we were able to extract 50-100 microliters of blood from the wound surface in the neck that was mixed with 20 microliter of 0.5M Ethylenediaminetetraacetic acid (EDTA) and put on ice before centrifuged for 20 min. The plasma was removed and stored at -70°C.

The brain scull at P10 is still rather thin and can carefully be cut open with a small scissor without injury to the brain parenchyma. With a Young Mouse Brain Slicer (Zivic instruments), the neonatal brain was cut into 6-9 slices and immersed into 2% TTC solution for 30 min. Viable tissue then appears red and photographs under identical condition were taken with a Nicon Exlipse E 400. Infarction volumes were then measured as the relative loss of the contralateral hemisphere.

**Real time PCR**

In Paper I, measurement of gene expression 24 h after HI was carried out by dissecting out 5 different subregions of the brain; hippocampus, thalamus, subventricular lining, striatum and cortex. Total RNA was isolated and quantified and gene expression analyzed by real time polymerase chain reaction (PCR). In this method there is a reverse transcriptase (RT) that converts mRNA to cDNA. A
polymerase then amplifies the amount of cDNA in temperature regulated cycles. Sybergreen was used for fluorescent detection. Cycle threshold (CT) values were normalized to CT of the endogenous reference gene NAPDH.

In paper III, gene expression was analyzed in pig brains 9.5 h after hypoxia. 1 hemisphere was dissected into subregions (cortex, hippocampus, thalamus, striatum and cerebellum) and stored at -70°C. RT-PCR was performed in samples from prefrontal cortex. P0 was used as endogenous reference gene.

**Total mRNA sequencing**

In paper II, differences in gene expression in different regions of the brain were assessed 7 days after HI by total mRNA sequencing. Cortex, hippocampus and striatum were dissected out from ipsi and contralateral hemisphere and immediately placed in liquid nitrogen and stored at -80°C. AllPrep DNA/RNA/Protein Mini Kit (Qiagen) was used for total RNA isolation, and samples were treated with RNase free DNase 1 (Qiagen) to digest any remaining DNA. Ipsilateral regions of cortex, hippocampus and striatum of HI and sham animals of the three genotypes were joined making a total of 18 groups for comparison. Bioinformatics analysis of RNA sequencing data, to obtain differentially expressed genes (DEGs) was performed at Novogene. DEGs were filtered with log2 (fold change) > 1 and FDR-corrected p value q < 0.005. Ingenuity pathway analysis (IPA) was applied to identify enrichment of DEGs in canonical pathways, biological processes and upstream regulators.

**Protein analyses**

In paper I, for measurement of IL 18 protein levels in blood 24 h after the insult, we used a mouse IL-18 enzyme-linked immunosorbent assay (ELISA) kit (R&D
systems). We also measured IL-1β levels but the concentration was too low in several samples to allow for statistical analysis. In paper III, protein concentration of TNF was measured in blood with ELISA and IL-1β, IL6, IL 8, IL10, IL18 and IFNγ with ELISA and phosphorylated p65 in prefrontal cortex by Western Blot.

**Liquid chromatography-Mass spectrometry analysis of 8-oxodeoxyguanine**

In paper III, we also investigated if NACA treatment affected levels of a DNA base modification, 8-oxodeoxyguanine by mass spectrometry.

**Immunohistochemistry**

For immunohistochemical analyses in mice, we harvested brains 3 h, 24 h, 72 h (paper I), and 7 days after HI (paper II). Mice pups were analgesized and perfused with 4% paraformaldehyde. Brains were removed and immersionfixed in paraformaldehyde for at least 24 h before embedded in paraffin. Using a microtome, the entire forebrain was cut into 4 micrometer thick sections. Sections were used for measurement of infarction volume and immunofluorescence staining. In paper III, brains of pigs were harvested 9.5 h after hypoxia and 1 hemisphere of each pig was immersionfixed in formalin. 5 mm thick blocks from the cortex were embedded in paraffin and cut with a microtome into 4 micrometer thick sections. Sections were stained with hematoxylin eosin for comparison of brain injury, and assessment of apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).
7. Summary of Results

Paper I:

**Early upregulation of NLRP3 in the brain of neonatal mice exposed to hypoxia ischemia: No early neuroprotective effect of NLRP3 deficiency.**

Neonatal hypoxic ischemic encephalopathy initiates a robust inflammatory response. Innate immune responses represent our first line of defense. We found upregulation of NLRP3 in the hippocampus, thalamus and striatum 24 h after HI and early expression of NLRP3 in hippocampus and thalamus in astrocytes. Later on, microglia also expressed NLRP3. There was however no effect of NLRP3 deficiency on infarction volume and levels of IL-18 in blood. We conclude that there is no effect of NLRP3 deficiency in the acute phase of neonatal hypoxic-ischemic brain injury in mice.
Neuromodulatory effect of NLRP3 and ASC in neonatal hypoxic ischemic encephalopathy.

Innate immune response following neonatal HI propagates beyond the early phase and shifts to an anti-inflammatory phase for resolution of brain damage. Since we did not find any early neuroprotective effect of NLRP3 deficiency we now assessed infarction volumes 7 days after HI injury by MAP 2 staining. These experiments showed that NLRP3 deficient animals suffered increased infarction volumes, while ASC deficient animals had smaller infarction volumes compared to Wt mice. Immunohistochemical analyses showed more activated microglia in NLRP3 deficient animals, while ASC deficient animals showed less activated microglia when compared to Wt mice. To further explore the mechanism of increased damage in NLRP3 deficient animals, we performed total RNA sequencing of hippocampus, striatum and thalamus. In the hippocampus we found that NLRP3 deficient animals showed an altered inflammatory transcriptional response and a unique pattern of increased expression of genes involved in protein translation as well as genes that are positive regulators of Epithelial Mesenchymal Transition (EMT). These possible non-inflammasome effects might explain the increased susceptibility to neonatal HI brain injury in NLRP3 deficient animals; while ASC deficient animals are neuroprotected.
N-Acetylcysteine Amide exerts possible neuroprotective effects in pigs after perinatal hypoxia

NACA, the amide form of NAC, has potentially better properties than NAC to cross the BBB and serve as neuroprotectant in neonatal hypoxic encephalopathy. Examination of NACA treated pigs 9.5 h after hypoxia with histopathological scoring with hematoxylin eosin and degree of apoptosis showed no neuroprotection compared to sham treated animals. However, in the cortex of piglets treated with NACA, IL-1β was reduced compared to sham treated animals. Furthermore, NF-κB expression was also reduced in these animals indicating a dampened inflammatory response. Indication of decreased inflammation was also found in the systemic circulation as NACA treated piglets showed steeper decline in levels of TNF during the first 30min after hypoxia. We speculate that NACA potentially serve neuroprotective effects by anti-inflammatory mechanisms.
8. Discussion

8.1 Methodological considerations

8.1.1 Vanucci model of neonatal hypoxic-ischemic brain injury

In our search for new neuroprotective strategies for neonatal hypoxic-ischemic brain injury, we applied the widely acknowledged Vanucci model, modified for use in neonatal mice (see chapter 6.1). By unilateral ligation of the common carotid artery followed by hypoxia, the model generates in all animals a brain infarction, with severity depending on length of hypoxia and temperature of the animal. It provides a method to study basic mechanisms of injury, with several advantages, but when performing these experiments, we also encountered several flaws that need to be taken into account.

Advantages of mouse models in pre-clinical research are many. Mice are relatively easy to house and breed with large litters and short gestation. The mouse genome is approximately 14% smaller than the human (2.5 GB versus 2.9 GB) [164]. However approximately 99% of mouse genes have a human homologue. Furthermore around 96% of the homologues are located similarly as in the human genome [164;165]. With the capability of editing genes in mice by depletion or insertion, knock out and transgenic models can be generated, allowing assessment of the effect of specific genes on basic injury mechanisms.

The Vanucci model modified for mice does however have some weaknesses and flaws. First, in the model of unilateral ligation and hypoxia, hypocapnia ensues, counteracting metabolic acidosis [166]. This is not the case in the clinical setting of
most neonates with HIE. Second, the model produces a unilateral infarct as opposed to the clinical setting of a more global hypoxia ischemia. Third, even though the contralateral hemisphere in the Vanucci model has been proposed to be an internal control, it has suffered from hypoxia and both short and long term alterations also occur in the contralateral side [167-169]. Finally, outcome and reproducibility will be affected by operational skills, the ability to control temperature and litter differences. In our quest to minimize these effects we bred mice in a heterozygous pattern (paper I). We generated heterozygous parents by mating a NLRP3\(^{-/-}\) mouse with a wildtype (WT) mouse. The heterozygous littermates were then bred to generate litters with pups consisting of WT, heterozygous and NLRP3\(^{-/-}\) animals. This reduces interlitter difference at the cost of doubling the amount of animals required and costs and workload of further analysis. When we introduced a second knockout mouse, the ASC\(^{-/-}\) mouse, in paper II, heterozygous breeding with all three genotypes were unfortunately no longer achievable and represents a weakness of this study.

Another major possible confounding factor in these experiments is temperature control. In our first experiment, the only available hypoxia chamber was custom made with heating from the bottom of the chamber and the possibility to address the pups if needed during hypoxia was difficult without affecting oxygen level and temperature inside the chamber. Daily differences in the ambient room temperature, as well as need to handle the pups during experiment could impact on the temperature of the pups. To further optimize our experiments we therefore invested in a hypoxia chamber (Invivo 500, Thermo Scientific) to better control oxygen concentration and temperature of animals used in paper II.
Figure 5. Custom made hypoxia chamber (left) and hypoxia chamber Invivo 500 from Thermo Scientific (right)

In this chamber temperature is regulated by the chosen air temperature administered. The chamber is completely air sealed and there are two gates in front through which the investigator can reach the animals and an air sealed slot were animals can be introduced and removed with minimal changes of the interior milieu. As temperature control is used for treatment, controlling it is of outmost importance, and mice rendered encephalopathic during hypoxia and ischemia will lose their temperature rapid unless the environment is tightly controlled.

8.1.2 Piglet model of neonatal hypoxic-ischemic brain injury
The anatomy and physiology of the piglet brain resembles the human brain making it a good model for pre-clinical examination of the effects of hypoxic-ischemic damage and possible interventions [170]. Brain maturation, growth, myelinisation, distribution of grey and white matter of a newborn piglet resembles that of 36-38 week human newborn [162;171;172]. Furthermore, the piglet shows similar cerebral blood flow response to hypoxia ischemia, similar vulnerability of different cerebral regions, and a similar pattern of energy failure as the human. However the cerebral blood flow and metabolism in piglets are higher [173], with a lower limit of cerebral auto regulation.
around 40 mmHg [174]. The approximate same size as a newborn fetus makes it possible to use standard intensive care equipment for experiments. A disadvantage to the model is that newborn piglets have already accommodated to extra uterine life at beginning of experiments. Even though only healthy pigs are used in the experiment, the animal model shows huge inter individual difference increasing variation in data. This has to be taken into account during power estimation. In addition, the model is relatively resource demanding, making longer end points from hypoxia reperfusion difficult to accomplish. In our model, 9.5 h was the latest end point. Although we identified differences in inflammatory markers at this time point, the inflammatory process has only just begun in the neonatal HI brain.

8.2 Discussion of Major Findings

8.2.1 Is NLRP3 expressed in the brain following neonatal HI in mice?
Neonatal HI initiates a robust inflammatory response [175]. As part of this response, innate immune actions are the earliest[175]. Several important PRRs have previously been investigated using the neonatal mouse model of HI brain injury [57], but NLRP3 has still not been examined. In our first report we therefore aimed to detect early NLRP3 expression, namely 3 h following HI. Our hypothesis was that microglia would show the earliest activation of NLRP3, but after evaluating several different antibodies, to our surprise, early NLRP3 expression was only found in astrocytes in the hippocampus and lining the ependymal wall. Microglia are well known initiators of inflammation, but astrocytes have gained increasing interest in the pathogenesis of HI brain injury [176]. Microglia and astrocytes are the two primary effector cells in neuroinflammation and moreover, their intercommunication is highly important in
brain injury [130]. NLRP3 expression has been shown in neurons in cortical slides obtained from humans suffering from stroke [177], but it remains debated whether NLRP3 is expressed by neurons [130]. Yang et al demonstrated that NLRP3 expression occurred in microglia and endothelial cells, rather than neurons and astrocytes 24 h after MCAO in adult mice [178]. This coincides with our finding that microglia started showing NLRP3 expression 24 h and 72 h after neonatal HI. As described in chapter 4.3.5 most cells, including macrophages, do not constitutively express NLRP3, and a priming signal is necessary to increase its expression. This priming signal is typically mediated by other PRRs such as TLR4. Thus, it is tempting to hypothesize that the lack of microglial NLRP3 expression in the early stages reflects that these cells have not yet responded to the DAMPs required for NLRP3 upregulation, but that this is the case in the later stages after HI.

To find antibodies that specifically bind to NLRP3 protein can be difficult, and different antibodies have been used in these investigations. So far, several different cell types have been implicated in early NLRP3 activity and for further description of NLRP3 activity in neonatal HI brain injury, it would be interesting to examine cell culture analysis. Applying total mRNA sequencing to isolated microglia, astrocytes and neurons could give valuable cell specific information about innate immune responses following neonatal hypoxia ischemia. Our finding of upregulated NLRP3 mRNA in hippocampus, striatum and thalamus of Wt mice 24 h after HI, further support the finding that NLRP3 is involved in the pathogenesis of neonatal hypoxic-ischemic brain injury. In line with our finding, NLRP3 expression was increased in ipsilateral hemisphere of Wt mice 24 h after MCAO [178]. Unfortunately we did not have material to examine protein levels of
NLRP3 at this time point, but increased NLRP3 was shown by western blot 24 h after MCAO [178]. Upon NLRP3 activation, IL-1β and IL-18 are cleaved from their proforms. We did not have brain material for protein analysis, but managed to gather enough blood from the mice during decapitation to examine protein levels of IL-1β and IL-18 in blood and it is well known that brain injury initiate a peripheral immune response[175]. From the analysis of IL-1β, unfortunately, too many of our samples showed too low levels to give reliable results. IL-18 levels were evaluable, but did however show no difference between wt and NLRP3⁻/⁻ mice 24 h following neonatal HI. To assess IL-1β and IL-18 response in brain of NLRP3 deficient animals would clearly be interesting to deepen our understanding of the inflammatory response following neonatal HI.

**8.2.2 Does NLRP3 play a role in neonatal HI brain injury in mice?**

Since we could not find any difference in brain infarction volumes of NLRP3 deficient and Wt mice 24 h after neonatal HI, and no difference in cytokine response, we now concluded that although NLRP3 is expressed early in astrocytes, it did not influence early outcome in neonatal HI. This is supported by the report of Denes et al. where ASC contribute to brain injury in conjunction with other inflammasomes such as NLRC4 and AIM2, but independently of NLRP3 [179]. However, support of a damaging role of NLRP3 activation in cerebral stroke is reported by others at this time point [178;180]. Differences in neonatal and adult innate responses are however extensive and the adaptations that occur after birth are massive[175]. Immune cells in peripheral blood represent one example as human newborns have a higher total blood count than adults. Neutrophils increase dramatically during the first 3 days of life, but show impaired functions i.e. chemotaxis and antigen presentation [181]. Importantly,
neutrophils secrete proteolytic enzymes that are able to activate IL-1β independently of inflammasomes [182]. Also lymphocyte counts increase after birth, but with a different population, where newborns have a higher percentage of naïve T cells and lower effector and memory T cells, compared to adults. Furthermore, studies have demonstrated that T cell capability of IL-1β and TNF production reaches adult capacity first at 2 years of age [175]. Thus, as both the brain and immune system is still developing during the perinatal period, multiple examples of opposite findings between neonatal and adult experiments exist, when challenging the brain to injury, as previously described [175].

Our results indicate that the pathogenic role of NLRP3 is limited in the early phase of neonatal HI. However, brain injury following neonatal HI is developing beyond 24 h. From our next report in mice (Paper II), we were once more surprised to find increased brain damage in NLRP3 deficient mice compared to Wt, while ASC deficient mice were neuroprotected 7 days after HI. ASC deficiency is thus protective in both neonatal HI and adult MCAO [179]. As ASC is also a component of inflammasomes such as NLRC4 and AIM2, this suggests that either ASC or other inflammasomes could serve as anti-inflammatory and neuroprotective targets in neonatal HI. At the same time point, the amount of activated microglia was increased in NLRP3 deficient animals, and these cells are proposed to have damaging effects through excessive production of pro inflammatory cytokines [175]. However, microglia depletion worsens brain injury in both neonatal and adult experiments indicating that these cells also have a protective role. We used the morphological appearance to differentiate activated from surveilling microglia. The shift from pro inflammatory to anti-inflammatory cytokine production is vital to limiting and resolution of brain injury [57]. Besides microscopic investigation of microglial
morphology, it would be interesting to further analyze microglial antigen presentation such as CD11b+, CD45+ to gain interest in their pro or anti-inflammatory properties.

With the evolution of totalRNA sequencing techniques, we were able to examine mRNA expression in different regions of the brain. Differentially regulated genes in hippocampus of NLRP3 deficient mice indicated an impaired inflammatory response in these mice following HI, possibly resulting in the increased damage. Again we were surprised to see a marked down regulation of genes related to protein translation, with upregulation of ribosomal genes indicating an impaired translational response. In addition, several of the down regulated genes have been shown to be positive modulators of EMT. EMT is the process in which epithelial cells transform into mesenchymal cells to migrate, before transforming back to epithelial cell through mesenchymal to epithelial transition (MET) to reconstitute injured areas. EMT is furthermore essential in resolution of BBB damage, as arachnoideal cells undergo transformation and migrate to injured areas through mechanisms involving TGF-β signaling [110]. Other reports of such NLRP3 inflammasome independent effect have been found in renal ischemia reperfusion injury [183] and colon cancer, again through TGF-β signaling [184]. Moreover, we found that positive regulators of EMT, such as argonaute-2, GSK-3β, PDK1 and ISR ½ [185-188], were down regulated in NLRP3 deficient mice. This could potentially implicate an inflammasome-independent effect of NLRP3, resulting in increased brain damage in these animals. In our search to find proof of altered BBB response in NLRP3 deficient animals we stained for albumin extravasation, but unfortunately, results turned out difficult to analyze (data not shown).
8.2.3 Affecting inflammatory response in newborn pigs with NACA

To gain further insight into inflammatory responses in neonatal brain injury, we used another animal model, the piglet model of neonatal asphyxia. In neonates increased levels of inflammatory cytokines such as IL-1β, IL-8, IL-9, TNF and RANTES are found in children who develop cerebral palsy [189;190]. NACA carries two important properties with potential to ameliorate brain damage and dampen the inflammatory response. First its antioxidant capacity can decrease amount of damaged tissue, thereby reducing the inflammatory stimuli. Second, by inhibition of NF-κB activity, NACA may serve to reduce the essential priming of NLRP3 that is needed for assembly of the NLRP3 inflammasome and further maturation of IL-1β and IL-18. We therefore aimed to prove that treatment with NACA would exert neuroprotective and anti-inflammatory effects by examining hypoxia induced brain damage and inflammatory cytokines in newborn piglets.

From hematoxylin eosin staining and apoptosis examination, we could not prove any effect of NACA treatment. As the piglet model is resource demanding, further follow up beyond 9.5 h was not possible in our experiments. One could however speculate that a longer follow-up could allow for histological differences between intervention groups to be manifested. Despite no early effect on immunohistochemical staining, levels of IL-1β in cortex of piglets were significantly reduced in the NACA treated group. Furthermore, when looking at NF-κB activation in the brain, levels of phosphorylated p65 were significantly lower in NACA treated piglets compared to saline treated piglets. These two findings support that NACA exerts anti-inflammatory effects. To further examine this mechanism it would have been elegant if we could assess brain expression of NLRP3 at time points earlier than 9.5 h. Also our own result from mice exhibiting NLRP3 expression 3 h after hypoxia ischemia would strengthen such a finding.
Major surgical interventions alone induce cytokines release in blood such as IL-1β and TNF [191] and the cross talk between peripheral immune response and brain injury has been recently described in a review by Lai *et al.* [175]. From adult mice, we know that peripheral immune response peaks 4 h after stroke, while brain inflammation peaks after 24 h [192]. When examining systemic blood levels, we were not able to find absolute differences in levels of IL-1β, IL-6 or IL-8. When examining systemic pro-inflammatory effect of hypothermia versus normothermia in a hypoxia-ischemia piglet model, Rocha-Ferreira *et al.* could neither find any effect of hypothermia on IL-1β, IL-6 or IL-8, but noted a huge variability in cytokine status, even at baseline [193]. We as well noted huge variance in cytokine levels. This is a clear limitation to the study, but at the same time, possibly reflects the clinical situation in human neonates. In our piglet model, levels of TNF decreased during the first 30 min, and when looking at the reduction of TNF, NACA treated piglets showed a steeper decrease in TNF than saline treated piglets. Early decline in TNF has also been seen in cortex of piglets treated with hypothermia [194] and the hypothermia group in the study of Rocha-Ferreiro also showed lower TNF levels the first 12 h of observation [193]. In previous work from our institute, Østerholt *et al.* also examined the effect of NAC in the same piglet model of hypoxia, but resuscitated pigs with 100% oxygen [51]. In accordance with our findings, they report reduction in inflammatory markers in pigs treated with NAC, namely TNF and IL-1β. We speculate that decreased levels of IL-1β in cortex, and a steeper decrease in TNF in peripheral blood, indicate that NACA treatment serve anti-inflammatory effect and potential neuroprotective capacity.
9. Conclusions

1. Activation of NLRP3 occurs following HI in neonatal mice as evidenced by:

- Astrocytes are the earliest cell type to show NLRP3 expression following neonatal HI, while microglia show expression later in the course.

2. NLRP3 deficiency aggravates neonatal hypoxic-ischemic brain injury:

-deficiency of NLRP3 does not influence early (24 h) outcome, but aggravates injury later in the course (7 days) accompanied by more activated microglia and an impaired inflammatory transcriptional response.

3. ASC deficiency is neuroprotective in neonatal HI:

-deficiency of ASC reduces brain injury accompanied by less activated microglial response.

4. Possible neuroprotective effect of NACA through anti-inflammatory mechanism in hypoxia induced brain damage in newborn piglets:

-There are decreased levels of IL-1β in cortex in NACA treated piglets with a steeper decrease in TNF in plasma than saline treated piglets.
10. Future Perspectives

From our results following experiments in neonatal mice and piglets there are several interesting directions to follow in further experiments. First of all, it should be obligatory that our findings are reproduced, preferentially by another research group. Additionally, when using the Vanucci model modified for newborn mice, longer follow-up with behavioral testing is warranted. This would in particular be important for gaining further understanding of the potential damaging effects of NLRP3 deficiency. Effect of NACA in conjunction with hypothermia also needs to be addressed as it serves as the only available treatment for HIE today. Furthermore, using NACA treatment in mice, could give us a possibility to investigate potential neuroprotective effects in a longer follow-up. Since our experiments indicate that ASC deficiency is neuroprotective in neonatal HI, it would be interesting to further characterize the activation profile of ASC, both by co-staining to identify cellular location as well as time profile by using different time points. Pharmacological inhibition of ASC is currently not available, however further studies using inhibitors of caspase-1 is a natural step while waiting for other approaches. To identify mechanism of neuroprotection, levels of inflammatory markers, ideally at different time points, could indicate a possible anti-inflammatory effect. Obviously, IL-1β and IL-18 warrant investigation, but also measuring IL-6, IL-8 and TNF would be of uttermost clinical relevance. As our experiments have demonstrated a possible NLRP3 independent effect, future experiments should also address this in more detail by protein examination to compare them with the mRNA findings.


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