# Hypothermic Neuroprotection in Neonatal Inflammatory-Sensitized Hypoxic-Ischaemic Brain Injury

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# PREFACE

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## SUMMARY OF THE THESIS

### *INTRODUCTION*

A feared complication to birth at term includes injury to the brain of the newborn. The clinical neurological state is termed "encephalopathy". It is often presumed to result from insufficient oxygen (hypoxia) and blood (ischaemia) supply to the brain prior to or during birth, and is therefore termed "hypoxic-ischaemic (HI) encephalopathy", or "birth asphyxia". When birth asphyxia results in brain injury, current treatment options are limited. The only approved therapy is hypothermia (HT) treatment, where the asphyxiated term neonate is cooled to a core temperature of 33.5°C for 72 hours. Therapeutic HT was implemented into the international resuscitation guidelines in 2010. The treatment has reduced death and disability from 66% to 50%. However, the number of children with poor outcome is still too high. We do not know why some infants benefit from HT treatment, while others do not. This highly invasive treatment is frequently complicated by the need for sedative and vasoactive infusions for pain relief and blood pressure control. Additionally, breast milk production is delayed/suboptimal and contact between mother and child is highly limited during these important first days of life. There is a need to understand which of these patients are likely to have a neuroprotective effect from HT, and if there are patients for whom it is preferable to abstain from the treatment.

Perinatal infection and foetal inflammatory activation increase the risk of brain injury at birth by making the neonatal brain more vulnerable to HI. After an attempt to establish HT treatment in lower-resource settings in Uganda, the treatment failed to improve outcome. A hypothesis was established that higher infection rates would reduce the HT neuroprotective potential. Pre-clinical studies on rodent models using lipopolysaccharide (LPS) to trigger systemic inflammation prior to the administration of an HI insult, sensitised the neonatal brain, and resulted in increased injury. A lack of neuroprotective effect from HT in such a "double-hit" setting was confirmed in our laboratory, using the same model.

However, the rate and type of infection affecting pregnant women varies across world regions and countries. The animal model that uses LPS as inflammatory trigger represents only one sub-population of bacterial species (Gram-negative). The model does not reflect the clinical situation among newborn babies with infection in our part of the world, where Gram-positive pathogens are highly predominant.

Aims of the thesis were therefore:

1 To establish a neonatal rat model of Gram-positive infection, that is temporary Gram-positive systemic inflammatory activation, which would be useful for further investigations.
2 To investigate whether the model of Gram-positive type inflammatory activation sensitise the brain to HI.
3 To re-test the hypothesis that "systemic inflammation negates the neuroprotective effect of HT", in a model more relevant to our clinical scenario.
4 Additional aims that occurred during the work were to further characterize the two inflammatory models in terms of systemic inflammation as well as inflammatory responses in the brain, without the hypoxic-ischaemic insult.

## *METHODS*

The already well-established neonatal rat model of unilateral HI brain injury from 1981 had been modified to include inflammatory pre-sensitisation, using LPS. We built on this model to also include Gram-positive type inflammatory pre-sensitisation, by introducing a synthetic inflammatory trigger, $PAM_3CSK_4$ (PAM). Thereafter HT treatment was added, and compared with normothermic groups with and without the Gram-positive type inflammatory activation. After one week's survival, we examined cross-sectional brain slices using histology, and area loss on the injured relative to the healthy hemisphere was calculated. Area loss was also analysed on regional brain levels (hippocampus, cortex and thalamus). In addition we used immunohistochemistry to mark neurons in the hippocampus, and counted remaining viable pyramidal cells within an area of interest. By changing the duration of hypoxia, the same model was used for investigating both moderate and severe levels of injury severity, and the subsequent effect of HT treatment, with and without pre-sensitisation.

Systemic and central nervous system inflammatory characteristics of the two models of inflammation were examined. A single systemic injection of LPS or PAM, or NaCl (Vehicle, Veh) for control animals, were administered, without the HI insult. For quantification of brain inflammatory proteins (cytokines) after injections we used quantitative reverse transcription polymerase chain reaction (qRT-PCR). For measurement of cell death marker proteins (cleaved caspase 3) we used western blot (WB) technique. We used immunohistochemistry to

mark and quantify microglial cells (Iba1) in brain tissue. Core temperature development and body weight gain of the rat pups was monitored and analysed.

Statistical analyses were performed mostly using non-parametric statistics, where data are presented as median with 95% confidence interval. Temperature measurements are presented as mean ±SEM. A p-value of <0.05 (two-sided) was considered statistically significant.

*RESULTS*

We were able to establish a feasible model of Gram-positive type bacterial infection, using PAM as inflammatory trigger. Injection of PAM prior to the HI insult sensitized the neonatal rat brain to HI, and increased injury from 10.4% (95% confidence interval (CI) 2.1-37), to 35.8% (95% CI 20.4-48.6) (p=0.01).

Contrary to our hypothesis based on the previous LPS-studies, HT treatment provided neuroprotection and reduced brain injury by 80% in the pups pre-treated with PAM, from 35.8% (95% CI 20.4-48.6) to 6.6% (95% CI 4.4-18.8) (p=0.0002). Our findings on hemispheric area loss analysis correlated closely with regional area loss analyses.

In the more severe model we prolonged the hypoxia time from 50 to 90 min, and found that PAM (median 60% area loss) did not further increase injury when compared to the control group (median 58% area loss) at that level, nor did it increase mortality. HT was similarly neuroprotective in PAM and Veh animals (21.6% and 13.2% injury reduction respectively), although with a smaller effect size than in the model of moderate injury. Hippocampal area loss analysis showed the same pattern.

Upon analysing temporal changes in brain inflammatory cytokine expression after a systemic injection of LPS or PAM, we found significant differences. The two models displayed highly different temporal profiles of brain inflammatory cytokine development both in terms of when and to what magnitude the various cytokines were upregulated.

24 hours after systemic LPS injection, WB on crushed brain tissue revealed significant apoptosis (programmed cell death) compared to brains in the Veh group (p<0.0001), while no significant apoptosis was found in brains of pups that received systemic PAM.

Microglial activation was seen to a similar degree in LPS and PAM injected pups.

2 hours after LPS injection mean core temperatures had dropped by 4.3°C (95% CI 2.7-6.4). After a Veh or PAM injection, pups were able to maintain their body temperature at a steady level of 35-36ºC (p=0.01). It took 8 hours for LPS animals to re-establish normal core temperature.

Weight gain over one week was 138% (130.5-145.5) and 145.6% (134.1-137.1) in Veh- and PAM groups respectively, with no statistical difference between the two. The LPS-injected pups gained significantly less weight, at 115.2% (91.5-138.9) ($p<0.01$ for both comparisons).

## *DISCUSSION*

Our study shows that HT effectively reduces brain injury in a neonatal rat model of PAM-sensitised HI brain injury. The results are in stark contrast to the LPS-sensitised model, where HT neuroprotection was negated. Also in more severe injury, the neuroprotective effect of HT prevailed, however with a reduced effect size with increasing injury.

Several important factors impact injury severity in this model. The importance of core temperature *during* the HI insult has been described through decades, and multiple cell culture studies as well as animal studies have shown that administering an HI insult at a reduced temperature will lead to less cellular death. Likewise, elevating core temperature during an HI insult will lead to more severe injury. In this model, hypoxia is administered at 36°C. Our temperature study reveals that LPS pre-treated animals will have a mean core temperature of 31-32°C when they receive the HI insult, four hours after the injections. Administering hypoxia at 36°C means that we rapidly increase their core temperature by 4-5°C at the same time.

Mitochondria, the energy factory of the cell, has been devoted a pivotal role in the mechanisms of cellular death. The most acute and severe cellular death occurs when there is massive mitochondrial injury, when cellular energy stores are depleted and intracellular regulatory processes cannot be driven. Cellular death is thereby strongly related to metabolic rate. Metabolic rate increases with increasing temperature. It is therefore thought that when an injurious insult is administered at high temperatures with high metabolic rate, cellular death will occur faster and more widespread (necrosis). HT treatment will not be able to save a necrotic cell. There are however different types of cell death, but as the more controlled types of cell death depend on energy-demanding processes (apoptosis, necroptosis), the theory

holds. With lower intra-hypoxic temperatures, more of the controlled type cell death will be induced, minimizing the injury. HT has been shown to reduce apoptosis, and save neurons in such a stage of injury. This could explain why HT works in the PAM-sensitised model, but not in the LPS model.

Interestingly, both systemic PAM and LPS induced inflammation in the brain even in the absence of the HI insult, while only LPS induced apoptosis. This suggests that there is pro-inflammatory cross talk between blood and brain, which can act injurious in one setting, and seems to be harmless in the other, in the absence of a second hit (HI insult). Both inflammatory triggers induced a cytokine response in the brain, however, with different temporal profile as well as different composition. In PAM injected animals, the anti-inflammatory IL-10 was more prominent, while in LPS-injected animals we saw a rapid elevation in pro-inflammatory IL-6 and TNF-α. The balance of pro- and anti-inflammatory cytokines and combined signalling might be important. The demonstrated differences might partly explain why both LPS and PAM sensitises the immature brain to HI, but LPS-sensitisation runs a more detrimental route, with poorer general conditions, more cellular death, higher mortality and brain injury beyond the point of rescue.

## *CONCLUSIONS*

1. Systemic inflammatory activation through the Gram-positive route sensitises the neonatal brain to HI injury.
2. HT is neuroprotective in this double-hit setting, and reduces Gram-positive type inflammatory-sensitised HI brain injury.
3. There are significant differences in the neonatal inflammatory responses to Gram-negative pre-sensitisation (LPS) versus Gram-positive pre-sensitisation (PAM), in terms of systemic inflammatory parameters, brain inflammation and intracerebral cellular death.

Our findings suggest that HT neuroprotection in the setting of HI brain injury and concurrent infection and/or inflammatory activation, depends on the pathogen and route of inflammatory activation. This study points to challenges of interpretation form bench to bedside. The results

underline the importance of tailoring a pre-clinical model as accurately as possible to the clinical scenario we aim to mimic.

# ABBREVIATIONS

| | |
|---|---|
| BBB | Blood brain barrier |
| cCas3 | Cleaved caspase 3 |
| CI | Confidence interval |
| CIRBP | Cold-inducible RNA-binding protein |
| CSF | Cerebrospinal fluid |
| CNS | Central nervous system |
| CP | Cerebral palsy |
| DAMP | Damage-associated molecular pattern |
| EOS | Early onset sepsis |
| E. coli | Escherichia coli |
| GBS | Group B streptococci |
| h | Hours |
| HI(E) | Hypoxic-ischaemic (encephalopathy) |
| HT | Hypothermia |
| Iba1 | Ionized calcium-binding adapter molecule 1 |
| IL | Interleukin |
| IHC | Immunohistochemistry |
| i.p. | Intraperitoneal |
| JC | Juvenile controls |
| kDa | Kilodaltons |
| LPS | Lipopolysaccharide |
| Min | Minutes |
| NE | Neonatal encephalopathy |
| OR | Odds ratio |
| P | Postnatal day |
| PAM | $PAM_3CSK_4$ |
| PAMPs | Pathogen associated molecular patterns |
| PRRs | Pattern recognition receptors |
| qRT-PCR | Quantitative reverse transcription polymerase chain reaction |
| RIP | Receptor interacting protein |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Veh | Vehicle |
| WB | Western blot |

## INCLUDED ARTICLES

Article I: Hypothermic Neuronal Rescue from Infection-sensitised Hypoxic-Ischaemic Brain Injury is Pathogen Dependent.
**Falck M.**, Osredkar D., Maes E., Flatebø T., Wood T., Sabir H., Thoresen M. *Develomental Neuroscience*, 2017 Apr 10.1159/000455838.

Article II: Neonatal Systemic Inflammation Induce Inflammatory Reactions and Brain Apoptosis in a Pathogen Specific Manner.
**Falck M.**, Osredkar D., Maes E., Flatebø T., Wood T., Sabir H., Thoresen M. *Neonatology*, 2017 Dec 10.1159/000481980

Article III: Therapeutic Hypothermia is Neuroprotective after Severe Hypoxic-Ischaemic Brain injury in neonatal rats pre-exposed to $PAM_3CSK_4$
**Falck M.**, Osredkar D., Maes E., Flatebø T., Wood T., Sabir H., Thoresen M. [Submitted manuscript to *Developmental Neuroscience*, January 3rd 2018]

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# 1. INTRODUCTION

## 1.1 PERINATAL INFECTION

A perinatal infection refers to the passage of a pathogen from the mother or outside environment to the foetus, during or shortly after delivery, and is defined as disease caused by a pathogen (bacterial, viral, fungal or parasitic) in a newborn baby, from birth up to and including day 28 of life. The diagnosis is based on a combination of clinical presentation, pathogen isolation and biochemical markers.[1]

This work focuses primarily on the sensitization of the neonatal brain to hypoxic-ischaemic injury by invasive bacterial infections and inflammation occurring prior to and/or during birth (early onset sepsis, (EOS)).[1]

### *1.1.1 INCIDENCE AND EPIDEMIOLOGY*

In the developed part of the world, the incidence of EOS is 0.5-1/1000 term born babies.[2] In low resource settings, however, both the incidence and epidemiology is different, with as many as 6-18/1000 term born babies suffering from EOS. Also, local patterns of pathogens as well as antimicrobial resistance vary.[3–5]

According to large studies in developed countries, the organisms most frequently involved in EOS irrespective of gestational age and birth weight are group B streptococci (GBS) and Escherichia coli (E. coli). Importantly however, the epidemiology differs with gestational age. While GBS accounted for >70% of all EOS cases among term born babies in a study of North American infants,[6] and for 58% of EOS in a Norwegian population-based observational study,[7] pre-term born babies exhibit a different etiology pattern. When pre-term and very low birth weight neonates are considered as separate populations, E. coli and other Gram-negative species is a more common etiology of EOS.[8]

Moreover, the epidemiology of neonatal sepsis in term born neonates has changed over time.[9] The previously more predominant Staphylococcus Aureus is now less frequently isolated, and since the 1970s, GBS has been the leading cause of EOS in term born babies.[10] Strategies to reduce the incidence of GBS infections, like chemoprophylaxis in at-risk women to reduce vertical transmission,[11] and recommendations on antenatal screening in week 35-37 of pregnancy, have dramatically reduced the incidence.[12] However, in spite of these preventive

measures GBS and other Gram-positive species still remain the most frequently isolated pathogens in term infants with EOS.[13,14]

EOS continues to be a serious and feared birth complication, with a mortality in the western world of about 1.6% among term born neonates.[9,14] The organism contributing most to mortality is E. coli, despite fewer total infections than GBS. However, the overall burden of GBS disease is significant, with 25-30% of women being colonized.[13,15] The rate of vertical transmission is reported to be 50%, and infants born to GBS colonized mothers have an increased risk of being admitted to the Neonatal intensive care unit.[15]

### *1.1.2 Classification of bacteria based on Gram staining*

The Gram Stain technique was introduced by the Danish bacteriologist Hans Christian Gram in 1884. It is still the most frequently used differential staining technique in microbiology. This test differentiates bacteria into Gram-positive and Gram-negative bacteria. All though proper classification of bacteria is much more complex and includes bacterial shape, structure and oxygen requirements, Gram stain reveals some of the membrane traits of the bacteria. This is very important, as certain membrane structures have implications to how our immune system reacts to these as foreign, because different bacterial membrane molecules fit to specific receptors on the human immune cells (section 1.1.3 below).

During the Gram staining procedure, the primary stain Crystal Violet is fixed by a mordant (iodine). The cell wall of Gram-positive bacteria has a thick layer of protein-sugar complexes, peptidoglycans, and its lipid content is low (Fig. 1A). A decolorizing step with ethanol causes this thick cell wall to dehydrate and shrink, which closes its pores and prevents the stain from leaving the cell. Thus ethanol cannot remove the crystal violet-iodine complex that is bound to the thick layer of peptidoglycan of Gram-positive bacteria and they appear blue or purple in colour (Fig. 1B). Gram-negative bacteria also take up the crystal-violet-iodine complex, but due to the thin layer of peptidoglycan, and their double outer lipid layer (lipopolysaccharide, LPS) being destroyed by the ethanol, the coloured complex is washed off. The secondary stain, safranin (or fuchsine) is taken up by both Gram-positive and Gram-negative cells, but only the thus far unstained Gram-negative bacteria appear red or pink in color (Fig. 1B).

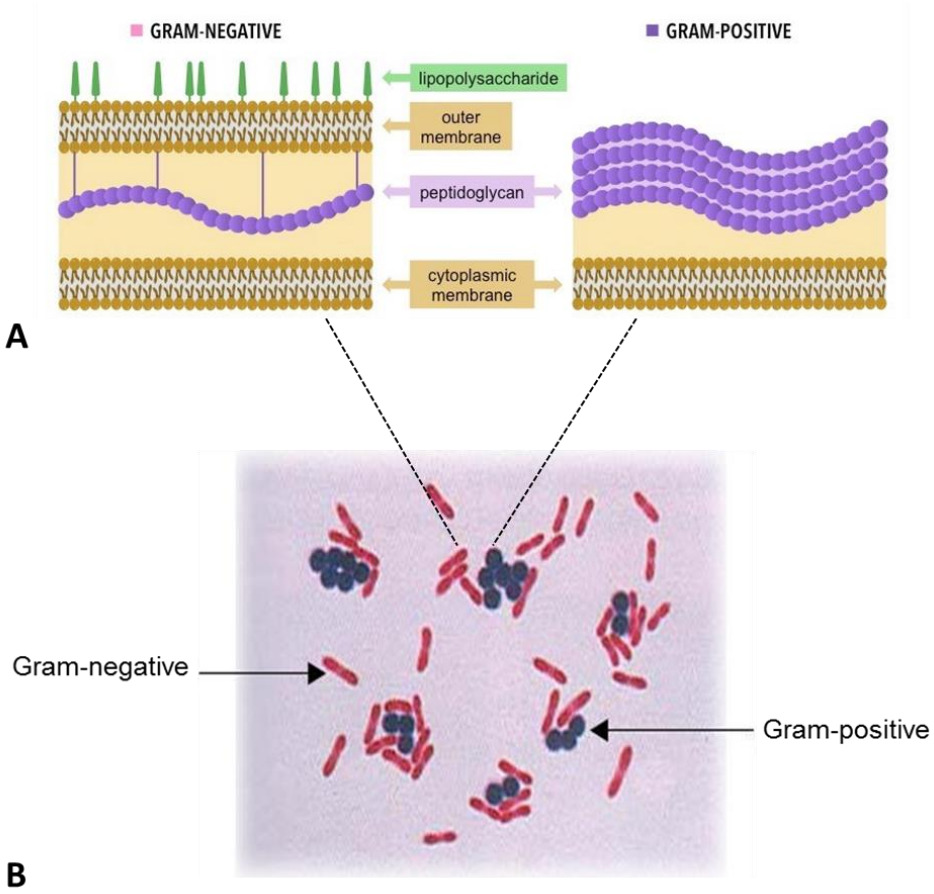


**Figure 1 Classification of bacteria based on Gram-staining**

**A:** Schematic demonstrating the differences in cell wall structure. **B:** Light microscope image of Gram-stained bacteria

### *1.1.3 IMMUNE RESPONSE ACTIVATION IN THE NEONATE*

Pattern recognition receptors (PRRs) are located on antigen presenting cells of the innate immune system (dendritic cells, macrophages, monocytes, neutrophils and epithelial cells). They provide the organism with intrinsic mechanisms to distinguish self from non-self-antigens, and defend against invading microbes.[16]

These receptors recognise different classes of pathogens via pathogen associated molecular patterns (PAMPs), as well as endogenous molecules released from damaged tissue; damage associated molecular patterns (DAMPs). PRRs are therefore involved in both infection-induced injury and inflammatory responses, e.g. those resulting from hypoxic-ischaemic injury. To date, the Toll-like receptor (TLR) family has been the most studied PRR type in the developing brain. With their different membrane structures, Gram-negative and Gram-

positive bacteria do not possess the same ligands for TLR's on mammal immune cells, and thus they activate immune reactions through different immunological pathways.[17] While LPS from Gram-negative membrane binds primarily to TLR-4, peptidoglycans and lipoteichoic acid in Gram-positive bacterial cell wall adhere to TLR-2, and have been shown to be TLR-4 independent (Fig. 2).[17,18]

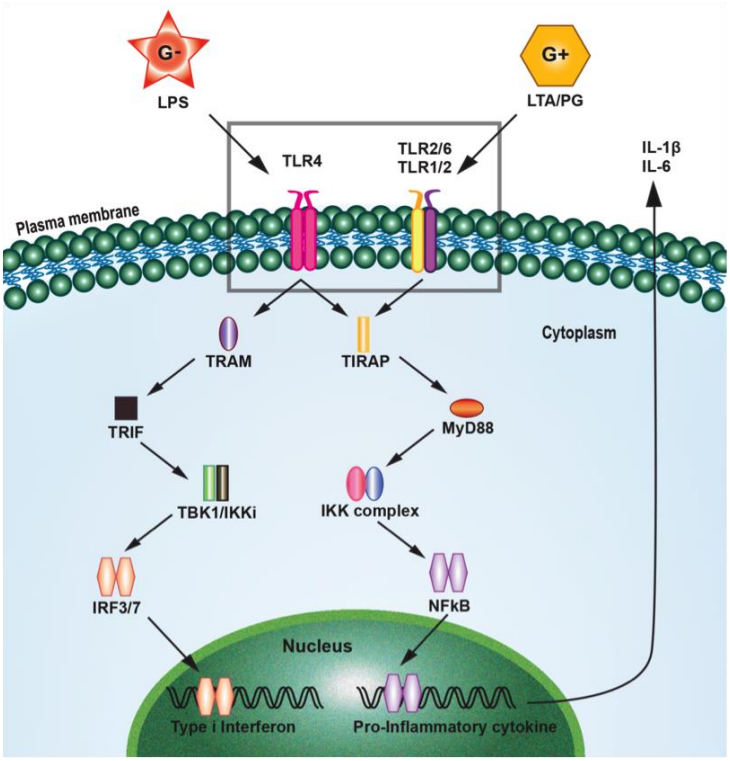


**Figure 2 Inflammatory activation by Gram-positive and Gram-negative bacteria**

Recognition of Gram-negative (LPS) and Gram-positive (LTA, PG, $PAM_3CSK_4$) bacterial pathogen associated molecular patterns (PAMPs) by membrane-localized TLR-4 and TLR-2 (TLR-2 forms a heterodimer with TLR-1 or TLR-6 to form a functional receptor complex). TLR-2 and TLR-4 both act through the MyD88-dependent signalling pathway, where the active IκB kinase (IKK) complex activates nuclear factor kappa B (NF-κB) subunits to initiate the transcription of inflammatory cytokines. TLR-4 also activates MyD88-independent signalling by recruiting TIR-domain-containing adaptor-inducing interferon-β (TRIF). Here activation of the TANK-binding kinase 1 (TBK1)/IKK inhibitor (IKKi) complex results in the production of inflammatory cytokines and type I interferons (Modified from Kumar *et al.*[16])

The foetus and newborn have a unique anatomical distribution and functional expression of innate immune molecules, including TLRs.[19] Although several studies have established that cord-blood monocytes express comparable amounts of TLRs to adult monocytes,[20,21] functional consequences of neonatal TLR activation are different from in adults, such as diminished production of tumour necrosis factor (TNF), and an elevated interleukin (IL)-6 to TNF ratio.[22]

Importantly, there is an age-dependent maturation of the immune response after birth. Given the limited exposure to antigens in utero, and thus a naïve state of the neonatal adaptive immunity,[23] newborns must to a significant extent rely on their innate immune system for protection.[19] Additionally, the innate component is immature, and differs from that of the adult.[23,24] Neonatal neutrophils show impairment of multiple functional aspects, including chemotaxis, rolling adhesion and transmigration. These differences begin to correct within the first weeks of life.[25] The non-classical pathways of complement activation are not fully developed in term infants, which is likely to exert important age-dependent effects on the inflammatory response.[26] The high risk of death from neonatal sepsis has been attributed to immaturity of innate immunity. For example, neonatal plasma concentrations of a series of acute phase reactants, as well as complement factors are diminished compared to those in adults (~10-70%).[27] This has been hypothesised to result in a reduced ability to limit the replication of many bacterial strains in the blood.[28]
Steady-state expression of TRIF-adaptor dependent genes was shown to be very low after birth, and increase gradually during the first year of life, shifting immune regulation toward the adult phenotype.[29] On the contrary, MyD88-dependent genes were elevated at baseline in neonatal monocytes compared with their expression in adult monocytes.[29] And stimulus-induced production of certain cytokines by neonatal monocytes have actually been shown to exceed those in adults.[30]

These differences might partly explain why newborn babies, and especially pre-terms, are more vulnerable to infections than adults. It might also partly explain why the foetal and neonatal brain, when exposed to inflammatory activation, result in neurodevelopmental disabilities, even without bacterial invasion of the central nervous system (CNS).[31] Already in 1976, Gilles showed how administration of systemic LPS caused leukoencephalopathy in neonatal kittens, but not in mature cats.[32] More recently, systemic inflammation occurring during the early neonatal period has been shown to be sufficient to dysregulate the ongoing process of neurogenesis in mice.[33]

As depicted above, Gram-positive and Gram-negative bacteria initially interact with our immune system through different receptors. The association between sepsis and cerebral injury, seems to be largely independent of the bacterial species involved,[34–36] suggesting that a detrimental final common pathway can be activated by diverse initial host-microbe interactions.

### *1.1.4 Inflammatory sensitization of the immature brain*

The brain is generally referred to as an organ with poor regenerative capacity. However, this is far from absolute, and varies with age and brain region.[37] The blood brain barrier (BBB), preventing most circulating cells and molecules from entering the CNS, has been considered an advantage. However, Hagberg *et al.* describes how both peripheral and central immune signals can induce inflammatory responses within the immature CNS, and how once inflammatory responses are activated in the brain, its protective "immune-privilege" is undermined.[38] A growing body of evidence suggests an unfavorable effect of systemic inflammatory activation in the not yet mature neonatal organism, with less robust barrier functions and organ systems. The concept of *inflammatory sensitisation of the immature brain* is becoming increasingly accepted. It refers to the setting where an infection and/or systemic inflammatory activation, not severe enough by itself to induce significant brain damage, make the brain more susceptible to a second insult.[39]

In 1997, Grether and Nelson looked retrospectively at maternal pyrexia (>38ºC) and other signs of perinatal infection in term born children with unexplained severe cerebral palsy (CP). They found that maternal fever, as well as the clinical diagnosis of chorioamnionitis, was strongly associated with increased risk of CP (odds ratio (OR) 9.5, 95% CI 2.7-31). Among children with spastic quadriplegic type CP, 37% of mothers had one or more indicators of infection during labour, compared to only 3% in the control children (OR 19.0, 95% CI 6.5-56).[40] A few years later, a prospective cohort study of almost 5000 low-risk women in labour at or near term, found that maternal fever (>37.5ºC) was associated with an increased risk of neonatal brain injury (OR 10.8, 95% CI 4-29.3), and concluded that the relationship between maternal intrapartum fever and signs of an injured brain in the newborn, was independent of other known intrapartum risk factors.[41]

In its severest form, chorioamnionitis evolves to include the cord (funisitis), with vasculitis of the umbilical vessels. Vasculitis entails presence of foetal neutrophilic granulocytes indicating

activation of the foetal inflammatory response.[42] Multiple studies in term as well as preterm neonates suggest strong associations between fetal infection and inflammation, perinatal brain damage and neurological disability.[43–46]

It is believed that inflammatory activation can modulate the vulnerability of the immature brain to hypoxic insults, and lower the threshold against hypoxia-induced brain injury. During life in the womb a maternal systemic infection can lead to a trans-placental systemic inflammatory activation in the fetus, with CNS inflammation as a downstream consequence, even without direct contact with the pathogen.[47] As when a peripartum hypoxic-ischaemic injury leads to CNS inflammation, it propagates into a longer-lasting activation of inflammatory repair mechanisms, which in the immature brain might exert injurious effects (see section 1.2.3) (Fig. 3).

The molecular mechanism of inflammatory sensitisation has however not been fully mapped. The choroid plexus has been considered a possible access route for peripheral immune signals and cells into the CNS. In neonatal mice, systemic immune stimulation with TLR ligands altered the expression of mRNAs encoding the tight junction protein occludin, and systemic administration of the TLR-2 ligand, $PAM_3CSK_4$ (PAM), dramatically increased leukocyte diapedesis into the cerebrospinal fluid (CSF).[48]

Altogether, vast amounts of pre-clinical and clinical data support the hypothesis of inflammatory sensitisation of the developing brain to injurious events around the time of birth. This complex process contribute significantly to the neurological prognosis of CNS injury, and the timing of infection might be important in this equation.[49]

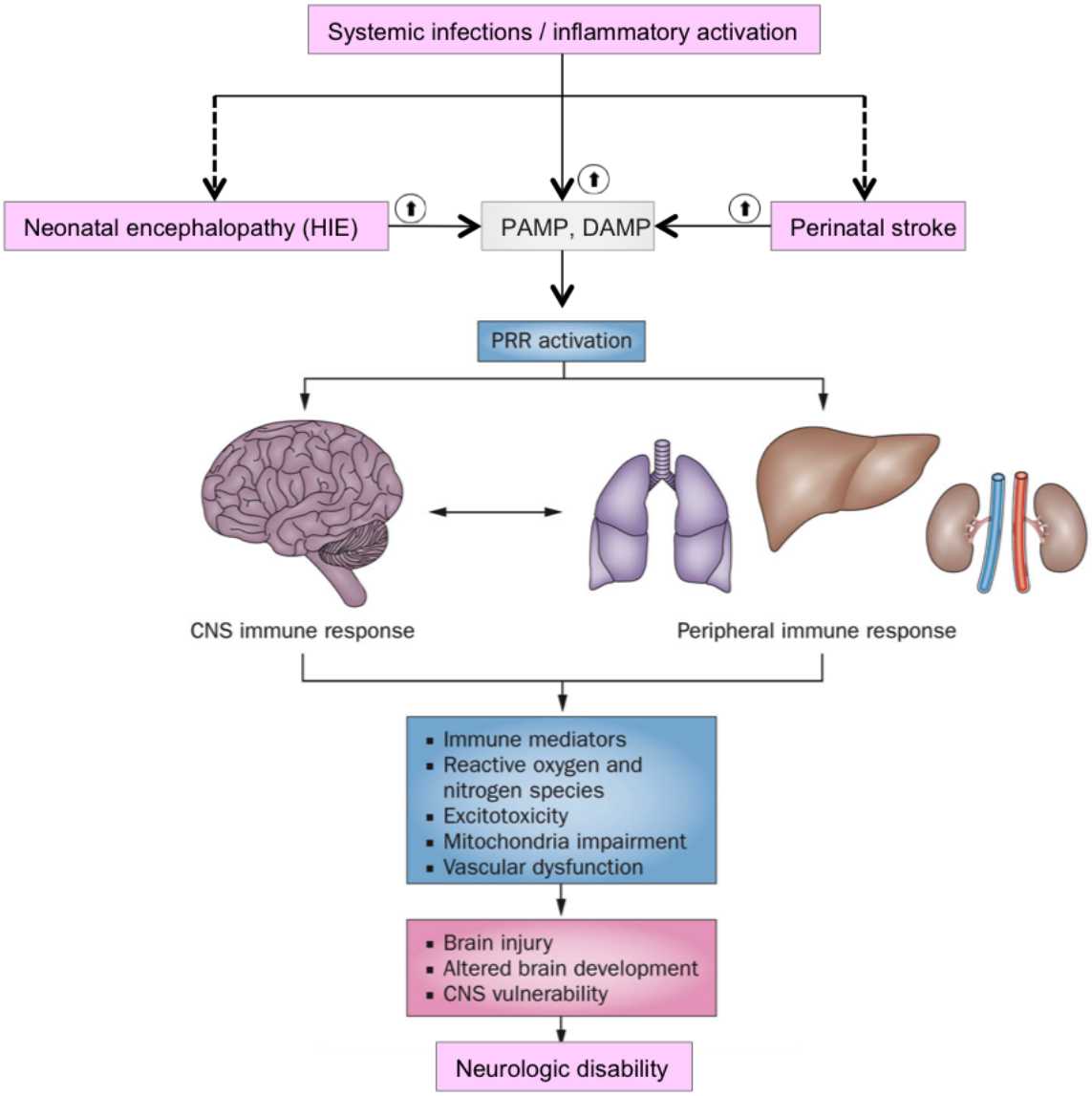


**Figure 3 Suggested pattern of inflammation in the developing brain**

Neonatal encephalopathy, perinatal stroke and systemic infections trigger release of PAMP and DAMP molecules, which activate PRRs. Systemic infection can also be an antecedent of the other insults (dashed arrows). PRRs trigger inflammation in the periphery and in the brain. Inflammatory activation can act in concert with HI to induce activation of immune mediators, reactive oxygen and nitrogen species, excitotoxicity, mitochondrial impairment and vascular disruption. These effectors can cause brain injury directly, interfere with brain development, and modulate CNS vulnerability, all of which may contribute to neurological disease. Figure from Hagberg *et al*.[38]

## 1.2 NEONATAL ENCEPHALOPATHY

Perinatal hypoxic-ischemic brain injury accounts for 25% of all cases of developmental disabilities in children worldwide.[50] Neonatal encephalopathy (NE) is a term designating an infant with clinical signs of cerebral dysfunction at, and around the time of, birth. Most often, the exact aetiology is unknown, and no sentinel event can be documented.[51,52] However, the most commonly *defined* antecedents include cord prolapse, uterine rupture, abruptio placentae, placenta praevia, maternal hypotension, obstructed labor, breech presentation or shoulder dystocia. All of these cover events where blood flow and/or oxygen delivery to the baby's brain and body is compromised ("hypoxia-ischaemia", HI). The HI insult to the brain is underlying to the clinical picture of cerebral dysfunction in the neonate, referred to as "encephalopathy". The expression "hypoxic-ischaemic encephalopathy" (HIE) is frequently used as HI often is the presumed cause. Which of the two terms (NE or HIE) is best fit in the clinical setting has been an ongoing discussion for more than two decades.[49,51,53,54] This thesis concerns brain injury of HI origin in a pre-clinical model, and the term HIE will be referred to.

Intrapartum related HI lead to permanent brain injury or death in approximately 8.5/1000 term born babies worldwide per year.[55] Importantly, the global burden of HIE is highly influenced by factors associated with socio-economic status, such as availability of pre-natal care and hospital proximity, as well as functioning maternity clinics and neonatal intensive care units. In 2010 an estimated 96% of babies with HIE were born in low- and middle income countries. In the western world, 1-3/1000 term born babies suffer from perinatal asphyxia,[56] and this incidence have been stable over the last decade.[57] With intensive care support at normal temperature, 66% of asphyxiated babies will suffer death or moderate-to-severe long-term disability.[57]

### *1.2.1. ANIMAL MODELS OF HIE*

Considerable progress has been made in understanding the pathogenesis of HIE thanks to pre-clinical research, by the use of cell cultures and animal experiments. The patients in our target population suffer from a global insult where the whole body has been subjected to HI, meaning these are sick newborns with complex comorbidities such as cardiac, hepatic and renal injury in addition to brain injury. Therefore, whole animal models where complex organ interactions are at play are often a more clinically relevant choice than cell cultures in this context.

#### 1.2.1.1 Models of HIE in larger animals

A number of different animal models were adapted to model brain injury in the newborn human, ranging from neonatal rodents and rabbits to monkeys, sheep and pigs.

Myers categorized patterns of brain injury and correlated them with systemic abnormalities studying primates in the 1970'ies.[58,59] Term monkey fetuses were exposed to asphyxia by covering their heads with a rubber sac and clamping their umbilical cord at delivery. This insult lead to immediate rise in fetal blood pressure, followed by profound fetal bradycardia within 20 seconds, and an accompanying drop in arterial pressure, oxygen content and pH to approximately 6.9. At least 12 minutes (min) of total asphyxia were required to produce any sign of neuropathology after survival. This is similar to what was found in clinical studies on fetal heart rate deceleration, where at least 18 min or more would result in morbidity.[60] Myers developed the model further to include partial ischemia in utero, combined with a terminal total asphyxia event, where both clinical and pathologic changes closely resemble what we observe in human perinatal HIE.

Inder *et al.* developed a baboon model of white matter injury, primarily based on premature birth, where baboons were delivered by hysterotomy at 125, 140, or 160 days of gestation (term, 184 days), equivalent to human gestational ages 26-28 weeks, 30-32 weeks, and 34-36 weeks respectively.[61] Premature baboons were treated in an intensive care setting, designed to reflect the human neonatal intensive care unit. Before delivery, pregnant mothers received dexamethasone, and the premature baboons were intubated, received surfactant therapy, and were ventilated for 2-4 weeks, after which they were sacrificed. The neuropathological patterns of brain injury displayed close resemblance to those seen in prematurely born humans.[62,63]

Thorough studies by Gunn's group on cord occlusion, modeling intrauterine asphyxia at term equivalent gestation in the sheep, has helped us understand fetal cerebral and systemic metabolic effects to acute and chronic insults.[64,65] Using this model, they have examined and described in-depth, the resulting neuropathology.[66–68] The sheep fetus model has been adjusted to examine repeated brief episodes of in utero HI as well as prolonged HI episodes.

The Thoresen group developed a global model of hypoxia-induced encephalopathy using newborn pigs in the early 1990's.[69] This model allows the animals to recover for up to four days after the insult and is shown to be clinically, electrophysiologically, and neuropathologically similar to that in the asphyxiated term infant. This model is also suitable

for further examination of mechanisms of injury, and exploration of potential protective therapies after birth asphyxia.[70,71]

#### 1.2.1.2 The Rice-Vannucci Rat model of unilateral HIE

However, large animal models are technically challenging, time consuming, and costly. As demands for research animals expanded, Russell and Burch defined the three R's to improve humane treatment of animals in research in 1959: Replacement (methods which avoid or replace the use of animals should be used when possible), Reduction (use of methods reduce the number of animals used to obtain a comparable level of information) and Refinement (use of methods that minimize potential pain, suffering or distress to the animal).[72] For sake of practicality in gross-anatomical questions, while keeping these principles closely in mind, smaller animal models serve a better purpose when confirming or discarding a hypothesis requires a certain sample size.

An important discovery by Hicks already in the 1950s was that with severe hypoxic insults (anoxic), high injury severity gives a narrow margin between a degree of asphyxia sufficient to kill the animal, and one that will just fail to produce brain lesions.[73] Also Levine's group experienced that after 20 min of anoxia, mortality was high, and survivors rarely have brain injury.[74] To be able to explore a wider set of mechanical (and later therapeutic) questions, Levine and co-workers titrated the injury to be of moderate severity by increasing vulnerability of the forebrain. This was achieved by ligating the common carotid artery on one side. Unilateral carotid ligation by itself did not produce lesions. The "Levine model of unilateral HI brain injury" in the adult rat was described and published in 1960.[74] Hicks discussed and investigated the vulnerability of the *neonatal* brain to anoxia.[75] Twenty years later, Rice and Vannucci modified the Levine model to explore questions of neonatal morbidity, where they used a combination of unilateral common carotid artery ligation with 8% oxygen in postnatal day 7 (P7) rat pups.[76] This modification stimulated interest in rodent brain maturation in relation to humans. Subsequently, learning about brain maturation processes in our target model have been pivotal for translational reliability (Fig. 5).[77]

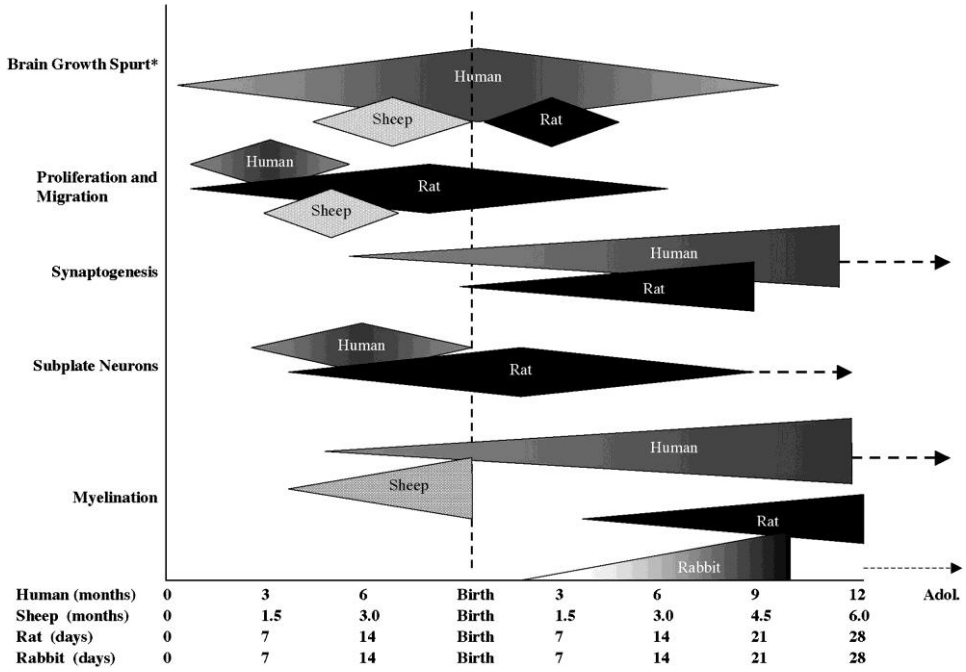


**Figure 5 Brain maturation**

Comparison of parameters of brain development for several species. Note difference in duration, with timing in months for humans and sheep, and in days for rats and rabbits. From Yager *et al* 2009.[78]

With the Vannucci model large variability of injury within experimental groups has been accepted. Although more demanding to work with, it does reflect the clinical situation better than a narrow range model. Furthermore, after demonstrating experimental usefulness and translational potential for half a century, the rodent model's variability should be interpreted a strength. The last decades, the use of genetically modified animals has gained an increasingly important role in research, and the model have been modified to fit neonatal mice,[79] as well as several levels of brain maturation reflecting different human gestational ages (GA).[80] The P7 Rice-Vannucci model, which corresponds to a GA of 34-36 weeks – that is late preterm neonates – is still the most frequently used and referred to of the rodent models. The last decade, the Vannucci group have modified the model to use P10 pups, arguing that it is closer to the term-born neonate.[81] A main advantage of this rodent model is the wide use and characterization over the years.[78,82,83] Lately, the element of inflammatory pre-sensitisation of the immature brain to HI injury have been added to the model (see below).[84–87]

Importantly, in the rodent models the ischaemic insult is administered solely to the brain, and not to the rest of the body. Although hypoxia is global, hypoxia alone does not produce organ injury in the rats. When we bypass the concept of global HI, ultimately some information

resulting from organ-to-organ intercommunication is lost, and mechanisms of cerebral injury as well as inflammatory responses cannot be readily translated without keeping this major difference in mind. In order to improve the relevance of preclinical studies to clinical practice, the STAIR (Stroke Therapy Academic Industry Roundtable) criteria were set up in 1999,[88] describing how considerable findings in rodent models should be tested also in larger animal models before moving to humans. Interpretation across species is perhaps increasingly challenging as we move towards finer details, such as molecular mechanisms and studies on gene expression.[89] Yager and Ashwal stated in their review on animal models of perinatal HI brain injury from 2009: "A combination of appropriately suitable animal models to conduct these studies will bring us closer to understanding human perinatal brain damage and the means to treat it."[78]

### *1.2.2 Clinical Diagnosis*

The manifestations of HIE in early postnatal life include poor umbilical cord gases (pH ≤ 7.0 or base deficit ≥ 12-16 mmol/L), the need for respiratory support for more than the first 10 min of life, initial bradycardia, and thereby low Apgar scores. Additionally, a clinical neurological examination gives basis for the diagnosis of "encephalopathy". Different scoring systems have been developed, and one of the first and most frequently referred to is the Sarnat&Sarnat scoring system, which gives the state of neurological dysfunction three grades of severity, from 24 h of life onwards (Table 1).

| | *Stage I* | *Stage II* | *Stage III* |
|---|---|---|---|
| **Level of consciousness** | Hyper alert | Lethargic or obtunded | Stuporous |
| **Neuromuscular control** | | | |
| Muscle tone | Normal | Mild hypotonia | Flaccid |
| Posture | Mild distal flexion | Strong distal flexion | Intermittent decerebration |
| Stretch reflexes | Overactive | Overactive | Decreased or absent |
| Segmental myoclonus | Present | Present | Absent |
| **Complex reflexes** | | | |
| Suck | Weak | Weak or absent | Absent |
| Moro | Strong; low threshold | Weak; incomplete; high threshold | Absent |
| Oculovestibular | Normal | Overactive | Weak or absent |
| Tonic neck | Slight | Strong | Absent |
| **Autonomic function** | ***Generalized sympathetic*** | ***Generalized parasympathetic*** | ***Both systems depressed*** |
| Pupils | Mydriasis | Miosis | Variable; often unequal, poor light reflex |
| Heart rate | Tachycardia | Bradycardia | Variable |
| Bronchial and salivary secretions | Sparse | Profuse | Variable |
| Gastrointestinal motility | Normal or decreased | Increased; diarrhea | |
| **Seizures** | None | Common; focal or multifocal | Uncommon (excluding decerebration) |
| **Electroencephalogram (EEG) findings** | Normal; awake | Early: low-voltage continuous delta and theta.<br>Later: periodic pattern (awake).<br>Seizures: focal 1-1½Hz spike-and-wave | Early: periodic pattern with isopotential phases<br>Later: totally isopotential |
| **Duration** | Less than 24 hours | 2-14 days | Hours to weeks |

**Table 1 The Sarnat&Sarnat scoring system for Neonatal Encephalopathy**

The table from Sarnat&Sarnat[90]distinguishes features of the three clinical stages of encephalopathy at 24 h of age.

### *1.2.3 PATHOPHYSIOLOGY OF NEONATAL ASPHYXIA*

Asphyxia, meaning impaired oxygen delivery and gas exchange, may occur before, during or after delivery. Its pathophysiology is extremely complex, and can be a result of factors related to the mother, the placenta, and/or the fetus/neonate, leading to interruption of adequate fetal or neonatal blood flow. When the intricate process of circulatory adaptation from fetal to neonatal form is unsuccessful it can be an additional challenge. Moreover, the phathophysiological effects of a hypoxic-ischemic insult progress over time.[91]

#### 1.2.3.1 Primary Energy failure in the immature brain

The impairment of cerebral blood flow entails inadequate supply of oxygen and metabolic substrates (i.e. glucose, fatty acids, and ketone bodies) to the tissue. Reduced glycolysis due to eliminated supply leads to accumulation of lactate. At the same time, with insufficient oxygen for mitochondria to produce energy (ATP), anaerobic glycolysis takes over ATP production, with lactate as its end product. This process cannot meet the demand of cellular energy need. The failure to produce sufficient ATP, together with an increased lactate production in neurons and supportive glial cells, leads to acidosis which initiates a cascade of cellular injury. Maintenance of membrane potential is crucial for the cell to uphold cellular homeostasis. Without sufficient ATP levels mechanisms that preserve and resets ion concentrations across the membrane, like the Na+/K+ pumps and Na+/Ca2+ exchangers, fail. With pump failure, sodium and water move into cells, leading to cell swelling. Increasing intracellular calcium concentration initiates an excessive release of excitatory neurotransmitters, like glutamate. Reuptake mechanisms are also energy-dependent, and they become compromised, resulting in accumulation of glutamate in the extracellular space. Over-excitation further increases calcium influx via the glutamate N-methyl-D-aspartate (NMDA) receptors, fostering an excitotoxic cycle.[92] Thereafter, further consequences include formation of free radicals, production of nitric oxide, and cell membrane lipid peroxidation. Cytotoxic cellular edema results in acute cell lysis and ultimately cellular death (Fig. 6).[93]

These changes affect all cell types in a hypo-perfused region, including components of the neurovascular unit. Tight-junction proteins, basement membrane, endothelial cells, astrocytes, pericytes and neurons suffer cellular death, leading to a disruption of the BBB. A failure of the BBB opens up to further intracerebral injury, like oedema and haemorrhage.

Importantly, the neonatal and still immature brain relates differently than the adult brain to an HI insult. The neonatal brain harbors disadvantageous characteristics making it more vulnerable to ischaemia. Transporter proteins for glucose (GLUT 1 and 3) as well as for lactate and ketone bodies (MCT 1 and MCT2) are necessary for transport of these substrates from the circulation and into the brain. The transporter expression, especially neuronal GLUT3, increase with neuronal maturation and synaptogenesis. Immature brains therefore have a reduced uptake of glucose compared to the adult brain. Low transport rate across the BBB in combination with reduced glucose supply in case of ischaemia, leads to a drop in brain glucose level very quickly.[94]

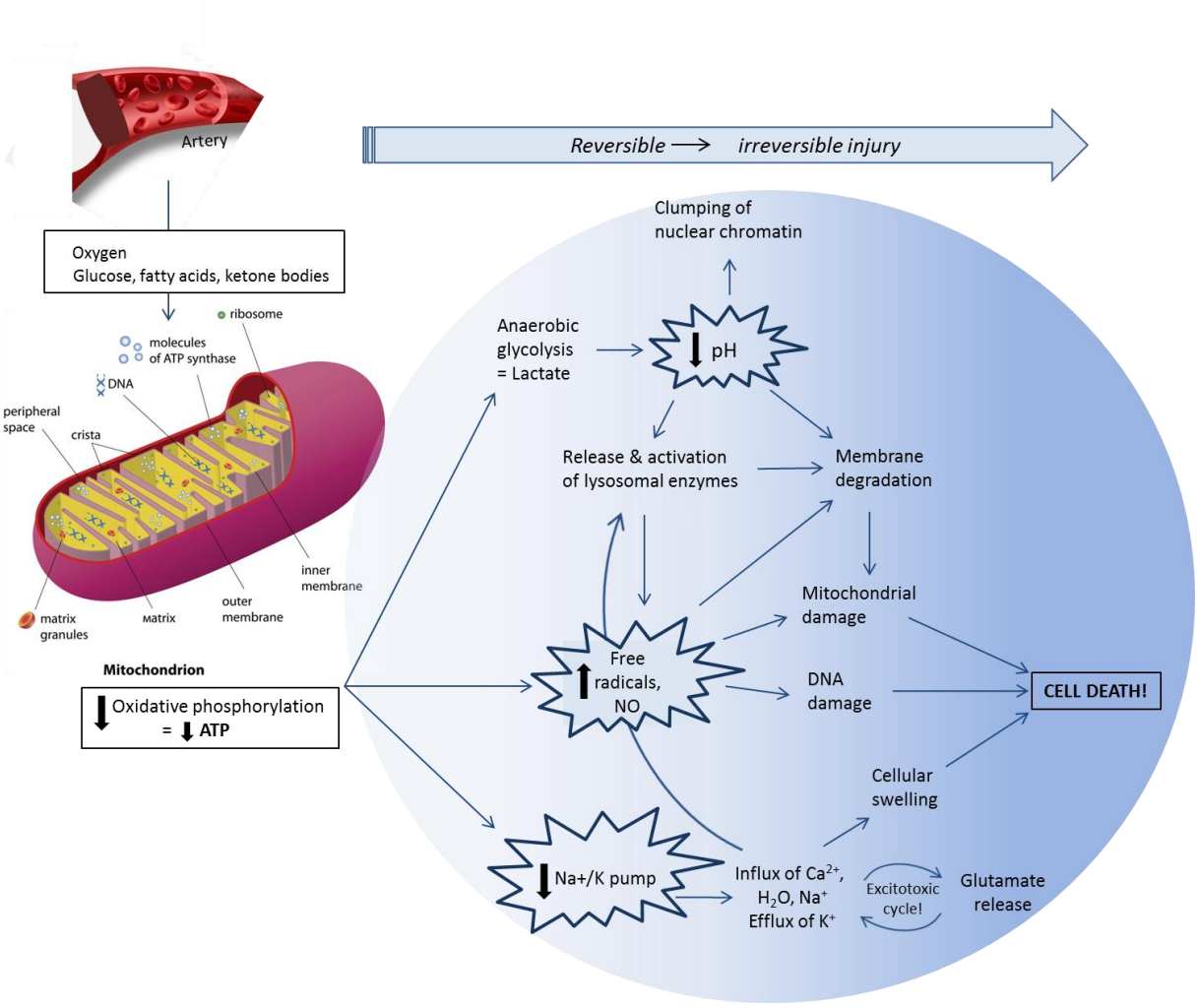


**Figure 6 Hypoxia-ischaemia induced energy failure**

The severity of the insult and consequential injury is illustrated by the colour gradient; the less severe damage processes are still reversible, while the most severe injury ultimately leads to acute cellular death.

On the other hand, other characteristics of the neonatal brain increase its resistance towards energy failure during HI. The immaturity entails immature neurons and low synaptic activity, requiring a lower rate of cerebral energy metabolism.[95,96] Additionally, the newborn brain can to a higher degree than the adult brain utilize substrates other than glucose to satisfy their cerebral energy requirements, with MCT1 being highly expressed in all cell types.[95] This phenotype results in an immature brain with a low capacity for glucose transport, but a high capacity for both ketone body and lactate transport. Thereby, during HI, when glucose supply is insufficient, increasing cerebral lactate is rapidly either cleared or utilized during the immediate recovery period, providing more time before tissue acidosis becomes critical.[97]
In addition to the above-mentioned differences between the adult and the immature brain, injury in the neonate may trigger certain molecular processes normally intended to shape developing brain circuitry.[98] One example is involvement of the glutamate NMDA receptor, which is enhanced in early brain developmental stages. With enhanced capacity for $Ca^{2+}$ flux, the complex plays a prominent role in activity-dependent shaping of neuronal circuitry, and in sculpting of the developing brain by apoptotic removal of redundant or damaged neurons.[99]

#### 1.2.3.2. Secondary Energy Failure – prolonged cell death and inflammation

After resuscitation of the neonate, cerebral perfusion and oxygenation are rapidly restored, along with partial restoration of energy sources.[100] During this "latent phase", also referred to as the "early recovery phase", with normalization of glucose supply and ATP production, cellular pH is re-balanced. There is still a phase of mitochondrial dysfunction, and due to the deprivation of high-energy phosphate stores during the insult a conversion of phosphocreatine is necessary to maintain ATP levels. When also creatine and phosphocreatine levels fall 24 to 48 hours (h) later, there is a subsequent progressive decrease in high-energy phosphates (ATP). This is known as the "secondary energy failure".[103] During this period, reperfusion injury occurs owing to extended reactions from the primary insult. This phase is multifactorial, and is characterized by inflammation, cytotoxic oedema, mitochondrial failure and generation of reactive oxygen species (ROS) and free radicals, and importantly programmed cell death.[97] With severe cerebral injury cerebral autoregulation is abolished, there is vasoparalysis (cerebral blood flow is directly proportional to arterial blood pressure) and cerebral hyperperfusion.[101,102] Clinically this phase is marked by the onset of seizures (Fig 7).
Although the inflammatory response induced by the acute HI injury is thought to contribute to tissue recovery and repair, it can also exacerbate brain injury,[104] much like what is described

above (section 1.1.4, Fig 3). The injured brain stimulates innate immune responses leading to activation of microglia (the monocyte cell line of the CNS) and circulating leukocytes. The immune cells release various molecules, including ROS, proteases and pro-inflammatory cytokines. These molecules activate more inflammatory cells, leading to a vicious cycle of further immune activation and cell death.

Mitochondrial injury has been described as the center stage in the response to HI in the neonatal brain, and the pivotal stage for when injury develops from reversible to irreversible.[105] When mitochondrial impairment leads to bio-energetic failure, generation of ROS and dysregulation of calcium homeostasis culminates in an auto-reinforced cycle of further injury, via mitochondrial permeabilisation and apoptotic cell death. In addition, death receptors are activated that could lead to mitochondrial fission, excessive ROS production and cell death with a predominately necrotic phenotype.[106]

The severity of the primary and secondary phases are directly related,[107] and the severity of secondary energy failure has been shown to be closely related to the risk of death and severe neurologic disability.[108]

A *tertiary phase* is also described, where injurious processes persist for weeks to months or even years after the initial insult. Findings from human pathology studies as well as experimental animal studies suggest that there are persistent active mechanisms that prevent regeneration or exacerbate brain damage, or both.[109] Persistent inflammation by over-activation of microglia and astrocytes, and epigenetic changes have been proposed as mechanisms.[110] Whether this tertiary phase is another window for therapeutic intervention is under discussion in the academic community, and investigations are ongoing (Fig. 7).[38]

It is important to be aware that the active processes of both the secondary and the tertiary phases of injury include immune system mechanisms which are also crucial in repair and regeneration. For example, microglia have been shown to participate in normal developmental proliferative processes, by phagocytosis of new cells in the subgranular zone of the dentate gyrus in the hippocampus.[111] On the other hand, inflammation and over-activation increases this phagocytic capacity, and concentrations of activated microglia correlate with a reduction in hippocampal long-term potentiation and thereby reduced memory formation in adult rats.[112] Glial responses are generally thought to be repair mechanisms, while at the same time, products of persistently activated astrocytes inhibit axonal regrowth and remyelination.[113]

This fine balance of beneficial and injurious immune system events can be challenging to intervene with, especially when results are not visible on short term. Robust and accurate experimental investigations must be convincing before moving to clinical trials on this matter.

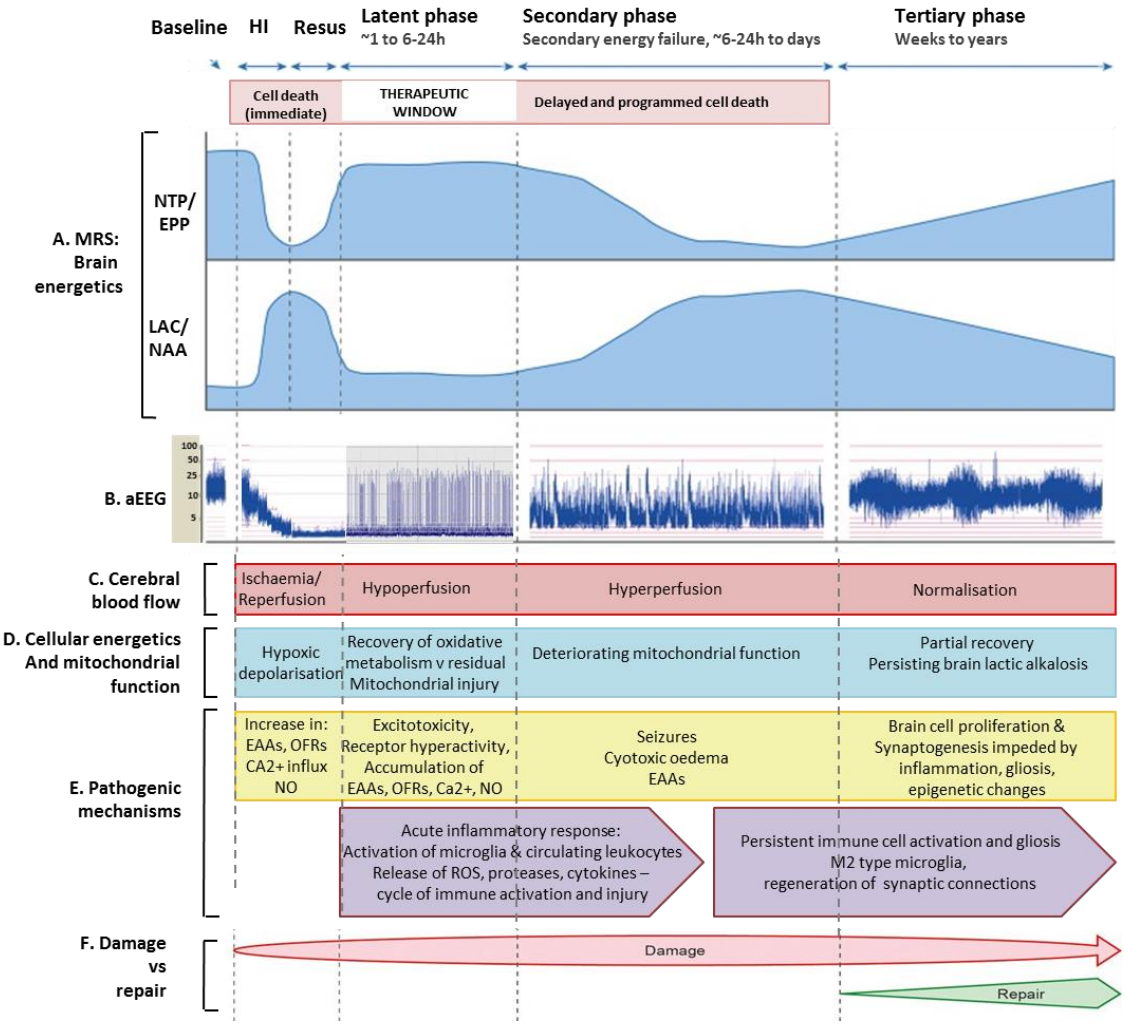


**Figure 7 Phases of brain injury after hypoxia-ischaemia**

The primary phase, latent phase, secondary energy failure phase and tertiary brain injury phase are shown. (A) MRS showing the biphasic pattern of high-energy reserves (NTP/EPP) and brain tissue acidosis (LAC/NAA) during primary and secondary phases following HI insult. (B) aEEG showing normal trace at baseline, HI induced lowering of amplitude and severely low voltage trace, followed by burst-suppression pattern in the latent phase, seizure activity in secondary phase and normalization in tertiary phase. (C) Following HI there is a period of hypoperfusion associated with hypometabolism during latent phase, and relative hyperperfusion in the secondary phase. (D) Cellular energetics and mitochondrial function show a period of recovery in the latent phase followed by deterioration in secondary phase. There is partial recovery in tertiary phase. (E) Pathogenic mechanisms are shown for each phase. Inflammatory activation is initially injurious, and can lead to a cycle of immune activation and cell death. With time, reparative mechanisms take over. (F) Damage is maximal in the secondary phase, but persists into the tertiary phase as inflammation and gliosis evolve. EAAs, excitatory amino acids; EPP, exchangeable phosphate pool; NAA, N-acetylaspartate; NO, nitric oxide; NTP, nucleoside triphosphate (this is mainly ATP); OFRs, oxygen free radicals. Modified from Hassell *et al*.[114]

## 1.3 THERAPEUTIC HYPOTHERMIA

Therapeutic hypothermia (HT) is our only current validated treatment option for infants with moderate to severe HIE. It has since 2010 been standard of care according to international guidelines published by the International Liaison Committee on Resuscitation (ILCOR).[115] When a neonate suffers HIE of moderate or severe severity and treatment criteria are fulfilled (table 2) cooling must be initiated within 6 h of birth. The baby's core temperature is lowered first passively, by avoiding overhead heating during resuscitation. As soon as possible active cooling should be started. The most widely used method is by placing the patient in a cooling blanket with circulating water connected to a water tank. This system lets a rectal thermometer give continuous feedback to the cooling machine, which maintains the set temperature at 33.5 ± 0.2 ºC. Other methods include cold water bags or pads. After 72 h of cooling, slow rewarming is commenced, by elevating core temperature with a maximal rate of 0.5 ºC per h.

| |
|---|
| **Inclusion Criteria for Hypothermia Treatment in Term Neonates (>36 w), weighing at least 2000 g, with HIE:**<br><br>A) 1 or more are fulfilled:<br><br>• Apgar score ≤ 5 at 10 minutes<br><br>• The need for ventilatory support at 10 minutes<br><br>• Acidosis in arterial cord blood or in any blood gas sample within the first 60 minutes of life, with pH ≤ 7.0, or a base deficit of ≥ 16 mmol/L |
| B) When ≥ 1 conditions listed in A are present, the child should be examined for:<br><br>• Physical examination consistent with moderate to severe encephalopathy (table 1)<br><br>• Seizures are an automatic inclusion criterion |
| C)<br><br>• Evidence of encephalopathy suggested by amplitude integrated EEG (aEEG) is considered a diagnostic aid in cases of doubt |

**Table 2 Inclusion criteria for hypothermia treatment in neonates with HIE**

### *1.3.1 HISTORY AND PRE-CLINICAL RESEARCH*

HT has a long history in the treatment of birth asphyxia. Centuries ago, unresponsive infants were given cold baths to induce spontaneous respiration through reduced core temperature. Studies in the 1940s compared time to last gasp in newborn animals after total anoxia. Reducing environmental temperature to 15ºC increased the duration of survival by up to 12 times.[116] In the 1960s Westin and colleagues claimed immersion of the asphyxiated neonate into cold water induced reduction in brain damage, but the studies were not controlled.[117] After Silverman *et al* had reported increased mortality in premature infants who were hypothermic over 5 days,[118] HT as a therapeutic for newborn babies, was not further studied.

Meanwhile, clinical data were published on surviving adults who suffered from accidental HT with cardiac arrest, and who exhibited low degree of long-term neurological sequelae.[119,120] This again supported the idea of HT as a neuroprotectant, and pre-clinical research groups revisited the hypothesis using adult animal models. In the 1980's several publications demonstrated the protective effect of decreased brain temperature *during* the administration of an HI insult. Busto *et al* showed that mild intra-ischaemic HT conferred a reduction in brain injury, as well as inhibition of glutamate release.[121,122] Deep HT was shown beneficial during complete occlusion of the middle cerebral artery.[123] The phenomenon was reviewed by Ginsberg *et al* in 1992 after comprehensive pre-clinical work.[124] The findings led to HT being introduced as a method of tissue preservation during major surgical procedures necessitating circulatory interruption.[125,126]

Over the next decades, large amounts of pre-clinical research in vitro as well as in vivo showed benefit from therapeutic HT post-HI injury. HT treatment was applied to already existing well-described animal models of HI brain injury (section 1.2.1). First in adult animal models (gerbil[127] and rat[128]), and later in neonatal animal models in the rat,[129] sheep,[130,131] and pig.[100] The Vannucci group found a reduction in injury, first with decreasing intrahypoxic temperatures in the neonatal rat.[132] Subsequently they applied post-hypoxic HT after a 3 h delay, but found no effect. On the contrary, Thoresen *et al* demonstrated a clear and long-lasting neuroprotective effect in the Vannucci model when HT was applied immediately after the HI insult.[129,133] The importance of timing – the window of treatment opportunity – has been demonstrated for HT in several animal studies (Fig. 8). Gunn's group showed how HT treatment was no longer neuroprotective when delayed with 8.5 h in fetal sheep, and the Thoresen group found very similar results in the rat model.[134,135] The same phenomenon was later indicated by clinical data showing neuroprotective effect when cooling commenced

within 4-6 h of life, but with still improved motor outcome if cooling was commenced before 3 h of life.[136]

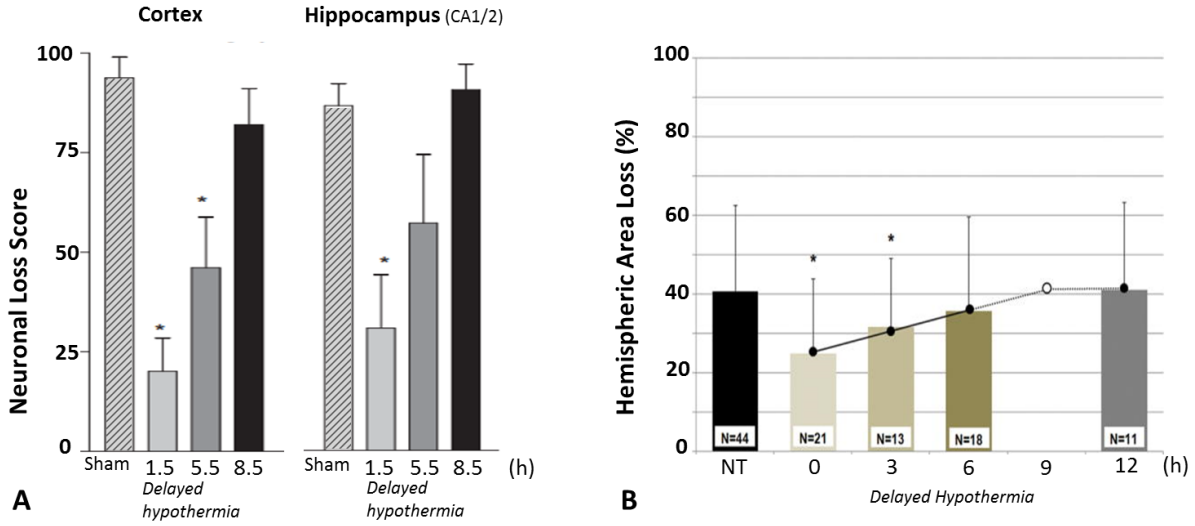


**Figure 8 Diminishing neuroprotective effect with delayed cooling**

**A)** After 30 min of hypoxia in fetal sheep, cooling was maintained for 72 h. Brains were harvested after 5 days' survival. Cooling started 1.5 h after reperfusion (n=7) was protective in cortex and hippocampus compared to sham cooling (n=13). When cooling was delayed until early secondary phase (8.5 h) it was not neuroprotective. Cooling started at the end of the latent phase (5.5 h) was partially protective. CA1/2: cornu ammonis fields 1 and 2 of the hippocampus. *p<0.005; data are Mean ± SEM. **B)** 5 h of cooling administered to neonatal rats after carotid artery ligation and 90 min of hypoxia. Cooling was induced immediately (n=21), with a 3-h delay (n=13), with 6-h delay (n=18), and with 12-h delay (n=11). Significant protection was seen in the immediate HT and HT with 3-h delay groups compared with the NT-Mod group (p=0.05). Neuroprotection was lost when HT was delayed with 12 h. Modified from Gunn and Thoresen (A)[134] and Sabir *et al.* (B).[135]

The model has since then been utilized for extensive investigations on mechanisms of action of HT in neonatal HI injury. As described above, perinatal infection and inflammatory activation elicits many of the same injurious processes as an HI insult, and potentiates injury (Fig. 3). Over the last decade, both the sheep model and the rat model have been modified to include systemic inflammatory activation by LPS prior to the HI insult.[84,85] Inflammatory pre-sensitisation with LPS has also been shown in the newborn pig.[137] The Mallard group in Gothenburg have thoroughly investigated mechanistic questions on the overlap between the

immune response from HI and the inflammation induced by an infectious stimulus.[84,85,87,138] In the LPS-sensitised rat model, the P7 rat pup receives a systemic injection of LPS four h before HI is administered. The LPS sensitized immature rat brain is more vulnerable to the HI insult than control animals, leading to more severe injury, as well as greater mortality.[85] Our group have added the question of HT neuroprotection to the setting of inflammation-sensitised HI injury, and applied HT to the modified Vannucci model.[139,140]

### *1.3.2 Mechanism of hypothermic neuroprotection*

Pre-clinical studies have investigated a number of mechanistic explanations of the neuroprotective effect of HT on the molecular level (for reviews: ref.[141,142]). A complete overview of its mechanism of action, however, has not yet been fully elucidated. Therapeutic HT is perhaps the most robust neuroprotective mechanism studied, with its ability to act on multiple pathways simultaneously, and over time (Fig 9).

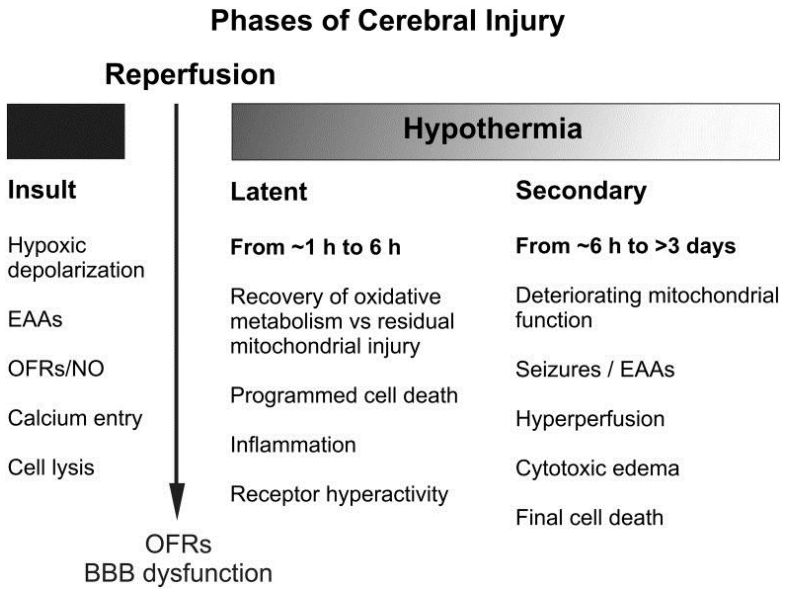


**Figure 9 Temporal and mechanistic aspects of hypothermic neuroprotection**

Therapeutic HT initiated during the latent phase reduces metabolic demand from dysfunctional mitochondria, reduces excitotoxic effects of EAAs, stabilizes CBF, and inhibits cell death. OFRs: oxygen free radicals; BBB: blood-brain-barrier; EAAs: excitatory amino acids; NO: nitric oxide. Figure from Wassink *et al*..[143]

HT has been shown to alter the neurotransmitter release in both grey and white matter brain regions, measured as a reduction in glutamate and in the citrulline/arginine ratio compared

with normothermic controls in the piglet model, indicating a reduced production of nitric oxide.[70] Drury *et al.* argued that extracellular accumulation of excitatory amino acids (EAAs) and excess free radical production largely resolve during reperfusion after the insult, and seem to have returned to normal values during the latent phase (~1-24 h). Thus, the ability of HT to reduce release of excitotoxins cannot fully account for the protective effects of cooling. Rather they suggest that the critical effect of HT is to block the *intracellular sequelae* of depolarization and EAA exposure, such as programmed cell death, secondary inflammation, abnormal receptor activity and mitochondrial preservation.[142,144]

Importantly, HT reduces metabolic rate during HI by slowing the breakdown of high-energy phosphate compounds (PCr, ATP, ADP and AMP) (Fig. 10).[100,145–148]

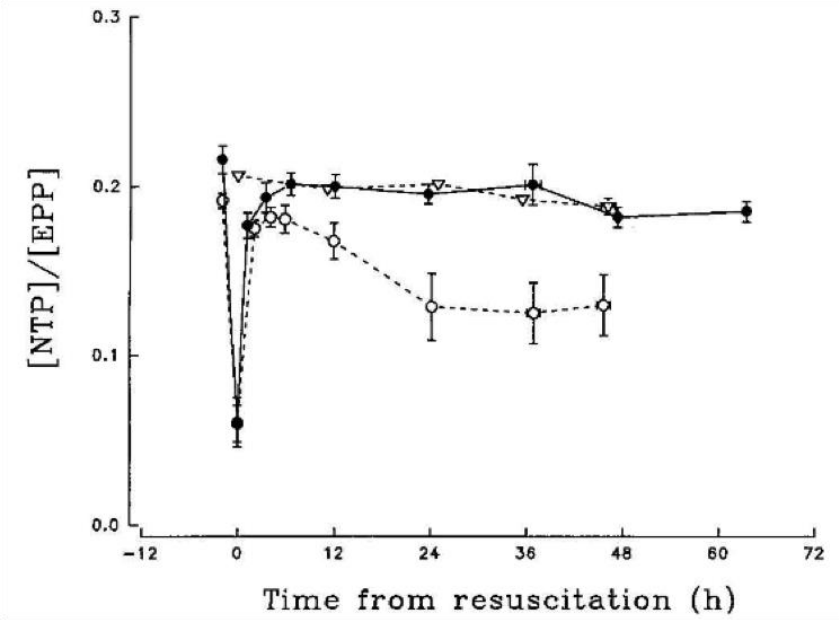


**Figure 10 Secondary energy failure after HI is abolished by HT**

Changes in [NTP]/[EPP] in sham operated control piglets (triangle) and piglets subjected to a cerebral hypoxic-ischemic insult, which were subsequently maintained hypothermic (filled circle) or normothermic (clear circle). Normothermic piglets had significantly lower values of [NTP]/[EPP] between 24 and 48 h than the hypothermic and control piglets, whereas no differences were detected between hypothermic piglets and the control group ($p < 0.05$, analysis of variance). [NTP]: nucleoside triphosphate (mainly ATP); [EPP]: exchangeable phosphate pool. The ratio represents cellular energy reserves. Figure from Thoresen *et al.*[100]

Studies from Gunn's group revealed that the post-hypoxic hypoperfusion during the latent phase after HI injury is an effect of reduced metabolism in the sheep fetus, which can be prolonged by HT to reduce neuronal injury.[149] Erecinska and Thoresen reviewed the effects of HT on CNS metabolism. The authors describe that global cerebral metabolic rate for glucose, lactate and oxygen metabolism decrease in parallel, 2- to 4-fold per 10°C reduction in temperature. They conclude that HT, both during and after HI, helps to preserve brain ATP supply and level.[150]

HT reduces levels of various molecules involved in injurious processes induced by HI, and a number of studies have shown how the molecule in question was expressed at a lower level when compared to a normothermia treated control group. However, most of these effects are downstream consequences of a suppressed metabolic rate and decrease in protein expression. HT-induced reduction in metabolic rate is comparable to what happens in hibernating animals, which utilize the same biological phenomena for tissue preservation during winter. Overlapping mechanisms include suppressed protein synthesis, excitotoxicity, inflammatory responses, oxidative stress and activation of cell death pathways.[151] In the hibernating animal, this state of energy conservation also entails profound reduction in cerebral blood flow, but causes no lasting brain injury. Interestingly, these animals have shown relatively increased tolerance to hypoxia during and after the hibernation period.[152]

Lowering of core temperature also prevent injury through active processes, by regulation of certain proteins involved in cell survival and growth. Similar to in bacteria that can withstand decreased temperatures, mammals respond to cold by upregulating "cold-shock proteins" that induce arrest of the cell cycle and inhibition of protein translation and gene transcription, as an active self-protective mechanism. Two of these genes, cold-inducible RNA-binding protein (CIRBP) and RNA-binding motif protein 3 (RBM3) are specifically induced by mild HT.[153] CIRBP mRNA in the cortex and hippocampus was found to increase after brain ischemia in rats, with even higher increments in ischaemic brains of rats that had been pre-exposed to HT.[154,155] An *endogenous neuroprotective mechanism* has been hypothesized, referring to a spontaneous drop in core temperature as a consequence of HI brain injury. This was hypothesized already in the late 1950's, first by Moore who looked at temperature changes in hypoxic kittens and puppies,[156,157] Then by Burnard and Cross, who monitored the core temperature of newborn healthy babies and self-ventilating babies with moderate birth asphyxia for 24 h after birth (Fig. 11).[158]

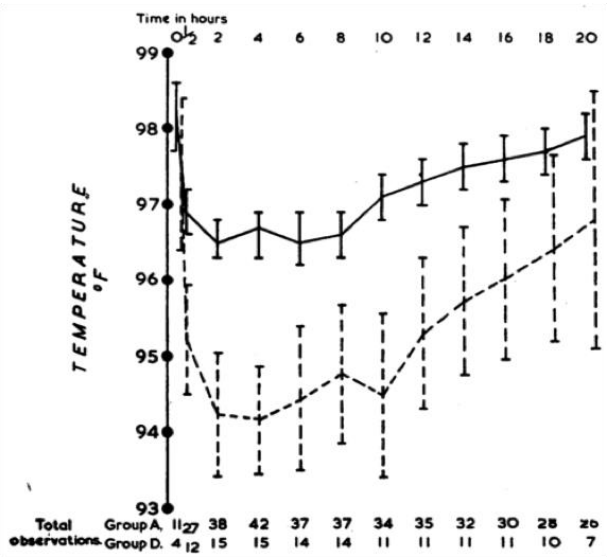


**Figure 11 Post-hypoxia–ischemia temperature**

24 h following HI, the core temperature of newborn babies was monitored. Complete line: healthy babies; dotted line: asphyxiated babies. Panel A from Burnard and Cross.[158]

The hypothesis of an endogenous HT response to HI brain injury has later been demonstrated in rodents, and shown to confer neuroprotection.[159]

Also important is the finding that neuroprotective effect of induced HT is modulated by cold induced sympathic stimulation in unsedated animals. The impact of sedation and paralysis on HT effect was shown in newborn pigs, where mild HT after HI was not neuroprotective without sedation,[160] whereas a marked neuroprotective effect was seen after 3-12 h cooling in anaesthetized animals.[71]

Among the effects of HT is modulation of the immune response triggered by HI injury. Many molecular pathways have been investigated. Although largely suppressive effects on inflammation have been shown (reduced neutrophil numbers and activated microglia, reduced levels of ROS,[161] cytokines,[104] NF-κB[162]), evidence of a suppressive effect on anti-inflammatory molecules (IL-10[163], TGFβ[164]) have been demonstrated as well. Again, the mechanism of HT seems to be unspecific and global, mitigating the inflammatory cascadic processes.[165] The anti-inflammatory properties of HT may serve as a major protective mechanism in a setting of ischemia and reperfusion. However, HT was not beneficial in perinatal asphyxia in a low-resource setting, where infection rates are higher.[4] In a Danish

study, therapeutic HT administered to adults with severe bacterial meningitis increased morbidity and mortality, and the study was stopped after interim analyses.[166]

These findings point to the fact that there is no simple explanation to the mechanism of HT, and there is an urgent need to find the balance of when actively reducing the core temperature, slowing metabolic rate and diminishing immune responses of a severely ill patient is beneficial, and when it can be detrimental.

### *1.3.3 Clinical trials of HT Treatment after neonatal HIE*

Based on findings from pre-clinical animal studies, several small clinical studies on therapeutic HT were conducted showing beneficial effects of HT as a neuroprotective therapy in the encephalopathic newborn.[167–170] Feasibility studies from Gunn *et al* in New Zealand,[171] and Thoresen *et al* in the UK, [172] showed the safety of cooling for three days. Thereafter, three large randomized clinical trials were run; the NICHD (National Institute of Child Health and Human Development) and the CoolCap studies published in 2005, conducted in the United States and England respectively.[173–175] The third was the TOBY (Total Body Hypothermia of Neonatal Encephalopathy) study, published in 2009.[176–178]

On meta-analysis in 2010, HT showed improved outcome,[179] and also the ILCOR guidelines suggested therapeutic HT as standard of care.[115]A large review was published from the Cochrane study group in 2013, concluding that HT does have a neuroprotective effect, and the overall number needed to treat to reduce mortality and severe neurological disability was 7-8.[57]

Even though these data by now are robust, and therapeutic HT has become standard of care in the western world as well as becoming available in less developed countries, half a million babies still suffer from severe disability or death each year. Infection rates in the respective clinical trials have been high at 6-13%, as compared to 0,5-1% in the general population.[57] These figures are supportive of the hypothesis of infectious sensitization of the immature brain to HI insults. How infection as co-morbidity to HI injury influence the neuroprotective effect of HT is unrevealed, and is addressed in this thesis.

# 2. AIMS OF THE STUDY

The overall aim originated from the hypothesis that perinatal infection as co-morbidity to HIE results in more severe brain injuries,[85] and that this "double hit" could ameliorate the neuroprotective potential of HT treatment.[4] Perinatal infections in term born neonates of the western world are largely caused by Gram-positive pathogens (> 90% in a Norwegian study).[7,13] We previously reproduced the Vannucci model with LPS-based inflammatory pre-sensitisation, where HT neuroprotection was negated.[139] As LPS represents Gram-negative bacteria exclusively, we asked if HT would be inefficient as a neuroprotectant also if the inflammatory activation prior to HI was triggered through the Gram-positive route.

Our first objective was to expand the model, by exchanging LPS for a Gram-positive synthetic analog (PAM), and thereby investigate whether Gram-positive perinatal infection sensitise the immature brain to HI. Secondly, we aimed to examine whether a Gram-positive sensitisation of the brain prior to HI injury influence the neuroprotective effect of HT (Article I).

Within this frame we examined if systemic inflammation and neuro-inflammatory responses differed depending on which type of inflammatory pathway was elicited (LPS vs. PAM). We asked if systemic triggering of TLR-2 and -4 induce a neuro-inflammatory response over the same timescale, by analyzing cerebral pro- and anti-inflammatory cytokines. We also looked at microglial activation and induction of cerebral apoptosis. To examine systemic inflammation in these neonatal rats we monitored core temperature changes and weight gain (Article II).

Our group previously showed that HT is not neuroprotective after severe HI brain injury in the Vannucci model.[135] Having shown neuroprotective effect after PAM-sensitised moderate brain injury, we proceeded to investigate HT neuroprotection after a prolonged hypoxic insult producing a more severe inflammation-sensitised HI brain injury. We hypothesized that HT would no longer be neuroprotective in PAM-sensitised animals subjected to a more severe HI insult, and that mortality would be high (Article III).

# 3. METHODS

The model of unilateral HI brain injury was originally described in adult rats by Levine in 1960.[74] The work described in this thesis was based on primary assessments using the model as adjusted to the immature brain, referred to as the Vannucci neonatal rat model.[76] At P7, brain maturity correspond to the a human neonate of approximately 34-36 weeks gestational age.[180] The modified Vannucci model with inflammatory pre-sensitisation was established by the Mallard-group in 2001.[85] The established model of pre-sensitisation with LPS 4 h prior to unilateral HI brain injury was the starting point of this work. The double hit model was re-established in our laboratory in 2014, with HT added to the question.[139] HT was not neuroprotective in the setting of LPS-induced inflammatory activation prior to the HI injury. As LPS represent Gram-negative infections only, we wanted to modify the model to include inflammatory activation through the Gram-positive route, and retest the hypothesis.

To further examine and adjust the modified model investigations on the cellular and molecular levels were performed. Details of the methods are described below, with in-depth descriptions provided in each of the individual articles.

We used Wistar rats from Charles River laboratories in Sulzfeld, Germany. The rat pups used for experiments were always of age P7. They were always randomised across litter, sex and weight according to the pre-determined experimental protocol. Prior to commencement of the experiments, all pups were given non-permanent colour-coded markings based on their randomisation to allow for rapid allocation to treatment groups during the experiments.
All experiments were approved by the University of Oslo's Animal Ethics Research Committee.

## 3.1 INFLAMMATORY PRE-SENSITISATION

As the main question concerned hypothermic neuroprotection, the insult was titrated to produce an area loss in the range of moderate severity (~35-50%), where HT traditionally has provided effective neuroprotection in the Vannucci model.

In this project we pursued the question of HT effect after inflammatory pre-sensitisation, exchanging LPS for a trigger of inflammation that mimics a Gram-positive infection, through TLR-2. To allow for a comparison to the LPS-model, we aimed to change the model as little

as possible, keeping the age of the pups, injection method, surgical and anaesthetic procedure, hypoxia time and administration method, as well as treatment protocol.

### *3.1.1 INJECTIONS*

As TLR-4 agonist we used the same LPS solution made from E. coli 055:B5 (Sigma) as in previous experiments with LPS, at the same dose (0.1 mg/kg body weight).[139] Dilutions were made with sterile physiologic saline (0.9% NaCl). To trigger inflammation through the TLR-2 pathway we used the synthetically manufactured TLR-2/1 agonist $PAM_3CSK_4$ (N-palmitoyl-S (2.3-bis(palmitoyloxy)-(2R,S-propyl)-®-cysteinyl-seryl-(lysyl)3-lysine, *$PAM_3CSK_4$ Vaccigrade, Sigma-Aldrich*) (PAM) in a dose of 1 mg/kg, dissolved in sterile LPS-free water, further diluted in 0.9% NaCl.

Based on previous publications on this agonist used in neonatal rodents,[48,181,182] we compared pre-sensitisation with 3 different doses of PAM; 0.1, 0.5 and 1.0 mg/kg in a pilot study (n=22-25). One control group received a single dose of vehicle (sterile physiological 0.9% NaCl) (Veh) (n=20), and another control group received LPS, 0.1 mg/kg (n=9). All pups received the injection 4 h prior to an HI insult with 50 min hypoxia, and 5 h normothermia treatment at 37°C (as in the LPS model) (Fig. 12B). The PAM dose (1.0 mg/kg) producing results most similar to the LPS-injected group in this experiment was chosen for further experiments.

For all injections, the pups were restrained in a firm grip, abdomen facing upwards, and the needle injected into the lower left abdominal quadrant (Fig. 12A). All injections were given intraperitoneally (i.p.) in a volume of 10 µl/g body weight.

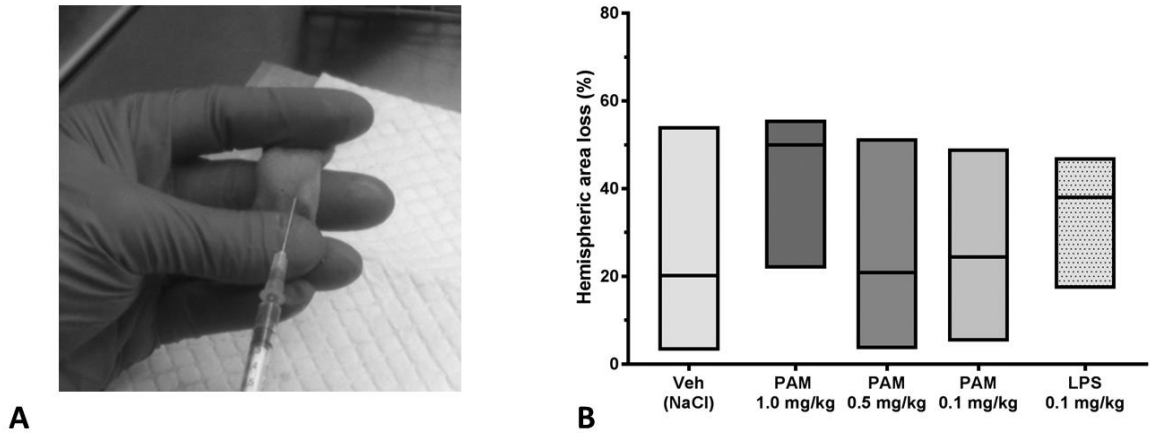


**Figure 12 Intraperitoneal injection and PAM dose response curve**

**A**: Intraperitoneal injection to the P7 rat pup. The needle is aimed at the left lower quadrant, carefully avoiding the urinary bladder and the inguinal ligament. **B**: One control group had veh injection (n=20), one group had LPS injection in the same dose as previously used in our LPS-model,[139] and three groups had decreasing doses of PAM injected; 1.0 mg/kg (n=25), 0.5mg/kg (n=22), and 0.1mg/kg (n=24). All pups were subjected to the same HI insult (50 min) and 5 h normothermia treatment (37°C).

### *3.1.2 Brain Cytokine expression level by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)*

The time course of systemic inflammatory activation after TLR-2 versus TLR-4 ligation is not elucidated. In the LPS model injections were administered 4 h prior to commencement of the surgical procedures. This incubation time was shown by Eklind *et al* to be pivotal for the sensitising effect of the neonatal brain.[87] For PAM sensitisation, however, this had not been elucidated.

We used qRT-PCR to study the time course of brain tissue expression of pro- (IL-6, IL-1ß, TNF-α) and anti-inflammatory (IL-10) cytokines after systemic LPS-injection (n=50), over a 48 h period. Subsequently, the same cytokines were examined in brain tissue over time after a systemic injection of PAM (n=50), as well as after systemic injection of Veh (n=50). Nine post-injection time points were selected for analysis in LPS- and PAM-injected animals (0, 2, 4, 6, 12, 18, 24, 36 and 48 h). An elevation of inflammatory cytokines in brain tissue was not expected in the control group, and only four time points (0, 4, 8 and 24 h post injection) were selected in the Veh groups. Animals received injections as described, and were sacrificed by

decapitation at the selected time points. Brains were harvested and snap frozen in liquid nitrogen, before storage at -80°C (Fig. 13).

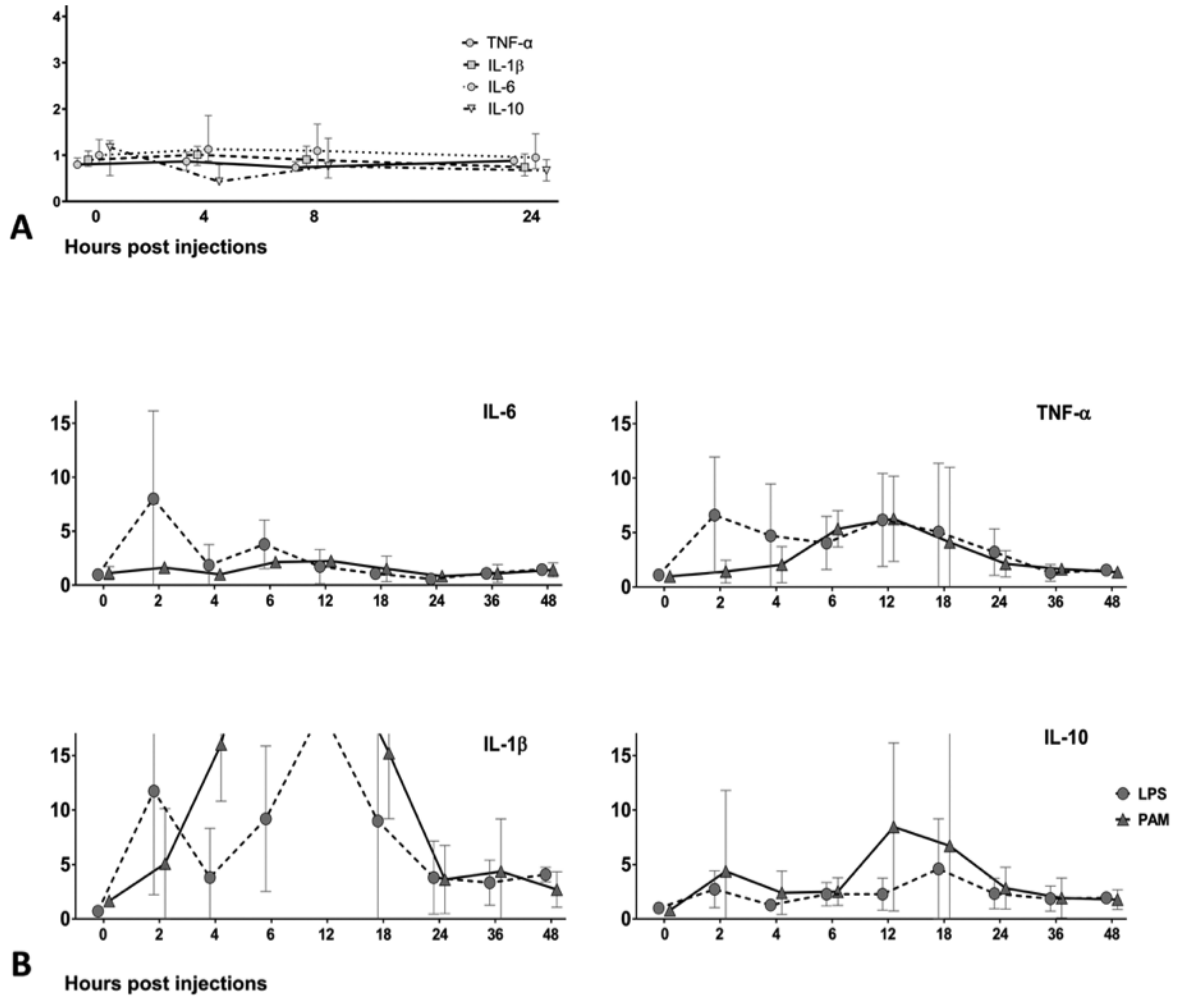


**Figure 13 Cytokine expressions in brain tissue**

Y-axis values are cytokine expression relative to expression of a house keeping protein (GAPDH) in the same tissue sample (arbitrary units). The lines are drawn through the median for each time point, with error bars showing 95% CI. **A**: Temporal expression of IL-6, TNF-α, IL-10 and IL-1β after i.p. injection of Veh (n=7-14 per time point). **B**: Graphs show temporal profiles of specific cytokines (IL-6, TNF-α, IL-10 or IL-1β) after a single i.p. PAM- (triangles, complete line) or LPS- (circles, dotted line) injection (n=5-6) (Fig 4 in article II).

LPS and PAM injected animals had different temporal profiles for intracerebral inflammatory activation. As the peak of the pro-inflammatory response seemed to appear 4-6 h later after

PAM than after LPS, a choice was made to extend the "incubation time" – that is the time between injection and commencement of the surgical procedures. We tested three different incubations times, 4, 8 and 24 h (Fig. 14). The results from the 8 h incubation-group were found most likely to be reproducible with the desired sensitisation effect (area loss of ~35-50% after 50 min hypoxia).

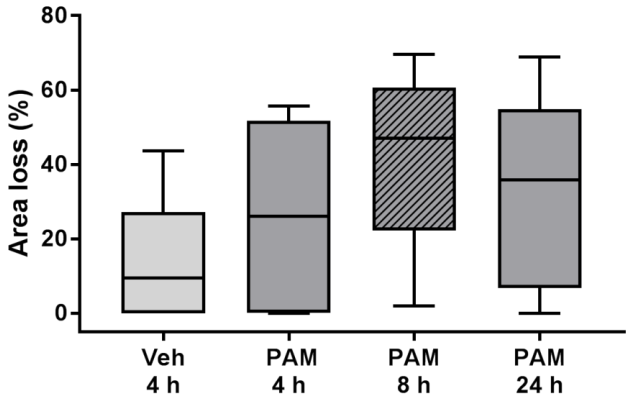


**Figure 14 Different pre-insult sensitisation time after PAM injection**

Inflammatory pre-sensitisation with PAM was induced 4, 8 or 24 h prior to the hypoxic-iscaemic insult (n=10-11/group). Error bars show 95% CI of hemispheric area loss (%) after carotid artery ligation and 50 min hypoxia (8%$O_2$ at 36°C). Veh 4 h: Veh injected 4 h prior to the insult. PAM 4 h, PAM 8 h and PAM 24 h: PAM injected 4, 8 or 24 h respectively, prior to the insult.

## 3.2 UNILATERAL HI BRAIN INJURY

Upon establishing the model, both Hicks in 1950[73] and Levine in 1960[74] found that administering a hypoxic injury to the rat brain proved challenging, as mortality was high, and survivors had no lesions. When hypoxia alone was not sufficient, Levine *et al* increased the vulnerability of the forebrain by unilateral common carotid artery ligation. Importantly, unilateral carotid ligation alone did not result in lesions. With the combination of hypoxia and ischaemia, a wider range of injury was achieved, and allowed for a shorter duration of anoxia, which again entailed lower mortality.

### *3.2.1 SURGICAL PROCEDURES*

To ensure similar total time away from the dams, all pups were removed from their dams at the same time before surgical procedures were started, and maintained under a heating lamp.

Deep sedation was induced and maintained via a nose cone using a 3% isoflurane in a 2:1 mixture of $NO_2/O_2$. Under a dissecting microscope the left common carotid artery was identified and dissected out from the surrounding tissue. One proximal and one distal suture of 6-0 surgical silk secured against hemorrhage before permanent ligation of the artery between the two knots (Fig. 15). The wound was closed by 5-0 surgical silk. After fully recovering under the heating lamp, pups were collectively returned to their respective dams for a minimum of 30 min.

In order to control experimental variables that may influence the variability of the model, median anaesthesia time was maintained under 6 min, and the total time from ligation to induction of hypoxia was maintained under 180 min (including the 30 min recovery period with the dam).[183]

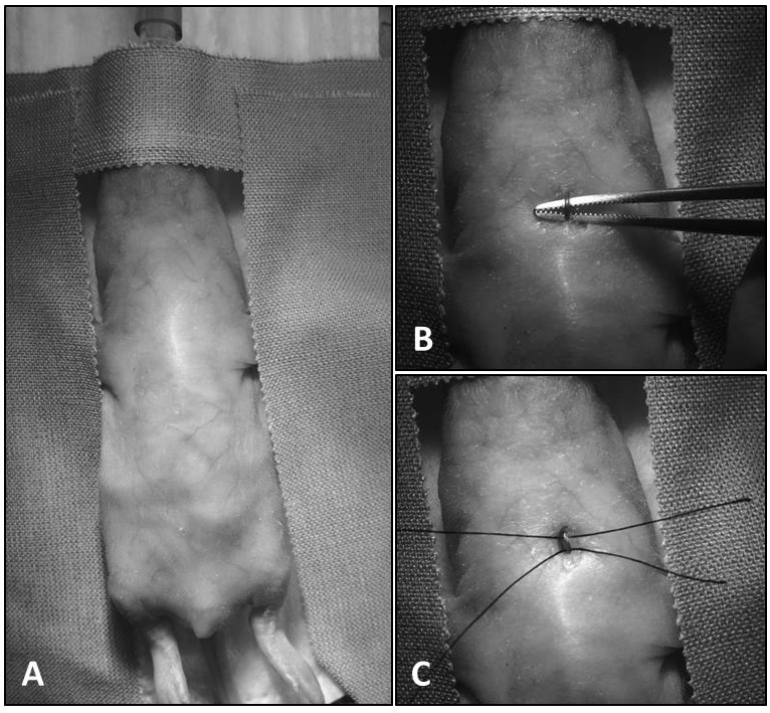


**Figure 15 Ligation of left common carotid artery in the P7 rat**

**A**: The rat pup is anaesthetized and restrained under the light microscope. **B**: After an incision to the skin just to the left of the trachea, the left common carotid artery is localized and dissected out. **C**: Proximal and distal sutures are placed before permanent ligation of the artery.

### *3.2.2 HYPOXIA*

The rat pups were placed in individual cells within a specially-designed metal cage, each cell with its own gas supply. The cage was then placed on top of a water-filled mat inside a closed polycarbonate chamber. Body temperature was continuously measured in two sentinel animals in each chamber – one carrying a custom rectal probe (IT-21, Physitemp Instruments, Clifton, NJ), and the other with a skin probe attached to the abdomen. Feed-back from these recordings servo-controlled the temperature of the water circulating inside the mat, and thereby the temperature of the chamber. Both probes were calibrated to ±0.2°C using a certified mercury-in-glass thermometer (BS593; Zeal, London, UK). The temperature management system (CritiCool, MTRE, Yavne, Israel) used to control the insult temperature had a custom-built temperature management algorithm provided by the manufacturer to prevent large variations in temperature during correction of over- or under-heating. Once the chamber was sealed, medical air was supplied while temperatures stabilised at a set point of 36°C. Thereafter the gas was switched to 8% $O_2$ (92% $N_2$). Oxygen concentration within the chamber was continuously monitored, and when it reached 8% this was maintained for 50 (article I and II, "moderate injury") or 90 min (article 3, "severe injury"), depending on the aim of the study.

Comparing results from different experimental protocols are challenging, and in our laboratory we have administered the insult with three different severity levels, depending on the aim of the experiment. Therefore, we re-characterised the model as run in our laboratory by performing a series of two-group experiments without inflammatory pre-sensitisation, administering 50 or 90 min of hypoxia (Fig. 16).

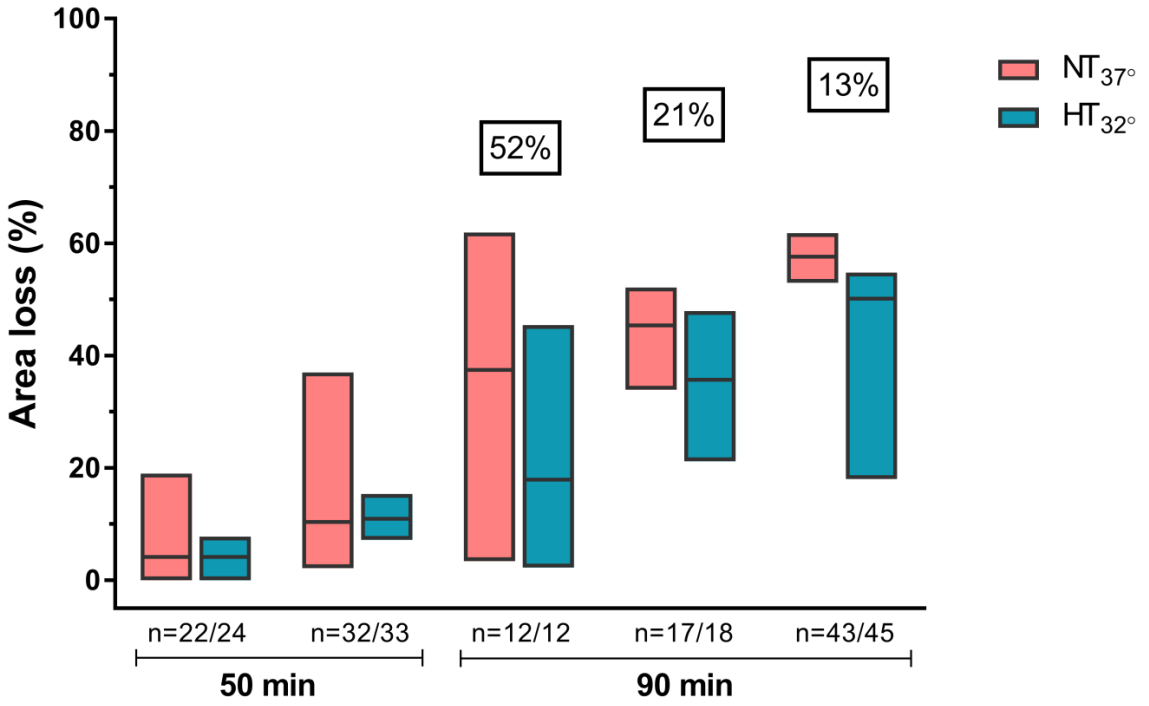


**Figure 16 Model variability; Hemispheric area loss (%) after 50 or 90 min of hypoxia**

The floating bars show median area loss with 95% CI from a series of experiments with different hypoxia times (the first two: 50 min, the next three: 90 min), producing a mild and moderate injury, respectively, with a degree of injury along a spectrum of severity. The red bars display normothermia (NT) treated groups, and the blue bars display HT treated groups in the corresponding experiments. The HT-mediated reduction in median injury degree (%) per experiment is indicated by the numbers in squares (Fig. 3 in article III).

When inflammatory pre-sensitisation is added to the model, pups are more vulnerable, the injury is more severe, and the "mild insult" in the original model will result in a moderate injury with pre-sensitisation (table 3).

| **Hypoxic insult:** | **50 min 8% $O^2$ @ 36ºC** | **90 min 8% $O^2$ @ 36ºC** | **150 min 8% $O^2$ @ 37ºC** |
|---|---|---|---|
| Injury degree without pre-sensitisation | Mild injury: < 20% area loss | Moderate injury: ~ 40% area loss | Severe injury: ~ 60% area loss |
| Injury degree with inflammatory pre-sensitisation | Moderate injury: ~ 40% area loss | Severe injury: ~ 60% area loss | |

**Table 3 Relationship between insult severity and degree of injury**

The table shows the median degree of injury we have seen traditionally in our lab after a mild, moderate or severe hypoxic-ischaemic insult. First row shows the standard model, the second row shows how the degree of injury changes with inflammatory pre-sensitisation (Table 1 in article III).

### *3.2.3 POST-HYPOXIC TEMPERATURE TREATMENTS*

Immediately after the hypoxic insult, the chamber was opened to allow for rapid re-oxygenation in room air (21% $O_2$), and pups were separated according to the 2 allocated treatments: 5 h of NT ($T_{rectal}$ 37.0°C) or HT ($T_{rectal}$ 32.0°C). Pups pre-randomised to NT treatment remained in the same chamber, and the target temperature elevated by 1°C. The HT groups were moved to an identical chamber, pre-heated to a target temperature of 32°C. Two probe animals were pre-selected for temperature regulation as described above in the second chamber. Medical air was supplied throughout the treatment period.

## 3.3 HISTOPATHOLOGY AND AREA LOSS ANALYSES

As done originally, the outcome measure of this model was based on crude unilateral tissue loss assessment after 1 week's survival. The method is relatively quick, it allows for large group sizes to be analysed, and therefore speeds up the investigation process. Percent hemispheric area loss at this level has previously been shown to be highly correlated with a formal neuropathology score and global degree of injury in this model.[135] Furthermore, the model and method of analysis has already translated well into clinical practice.[184]

Additionally, tissue loss has been assessed on regional level, and sub regionally on the cellular level (article I and III).

### *3.3.1 TISSUE PROCESSING*

At P14, animals were sacrificed by trans-cardiac perfusion-fixation with saline, followed by 4% neutral-buffered formalin under isoflurane/$N_2O$-anaesthesia. Brains were harvested and further fixated in 10% neutral-buffered formalin. Three mm coronal blocks were cut using a standard rat matrix (ASI instruments Inc., Warren, MI, USA), and embedded in paraffin. Five µm slices were cut from the two neighbouring blocks best representing cortex, hippocampus, basal ganglia and thalamus, mounted on glass slides, and stained with haematoxylin and eosin (H&E).

### *3.3.2 HEMISPHERIC AREA LOSS*

Brain size is variable with body weight. Performing the coronal cross sectional blocks at the exact same place for all animals is not feasible, and due to the small size of the neonatal rat brain, a small variability in location would impact the cross-sectional area. A measure of absolute cross-sectional area in the injured (or non-injured) hemisphere is therefore not useful. The model of unilateral HI brain injury always leaves the investigator with an internal control per animal – the non-injured hemisphere on the un-ligated side, Area loss relative to the unligated side can then be calculated.

For area loss analyses, four H&E stained sections (two from each of two coronal blocks cut 3 mm apart), were scanned (Epson Perfection V750 Pro) and exported as high-resolution (600 dpi) image files. Using ImageJ computer software (ImageJ, version 1.46r,

National Institutes of Health, Bethesda, MD, USA), optical density and hemispheric area was used to calculate a percent area loss of the ligated (left) side compared to the uninjured (right) side (Fig. 17). The average of the four sections from each brain was used as representative for injury severity in that brain.

### *3.3.3 Regional area loss*

The hippocampus, cortex and thalamus are the most vulnerable brain regions to HI injury, as well as being particularly sensitive to the neuroprotective effect of TH.[133,185,186] Analyses of area loss in these regions were therefore used as an additional proxy to degree of injury, and were correlated to hemispheric area loss. Slides were rescanned at higher resolution (1200 dpi) and the hippocampi, cortices and thalami manually identified. Evaluation of regional area loss was performed using the same calculation as for hemispheric area loss (Fig. 17)

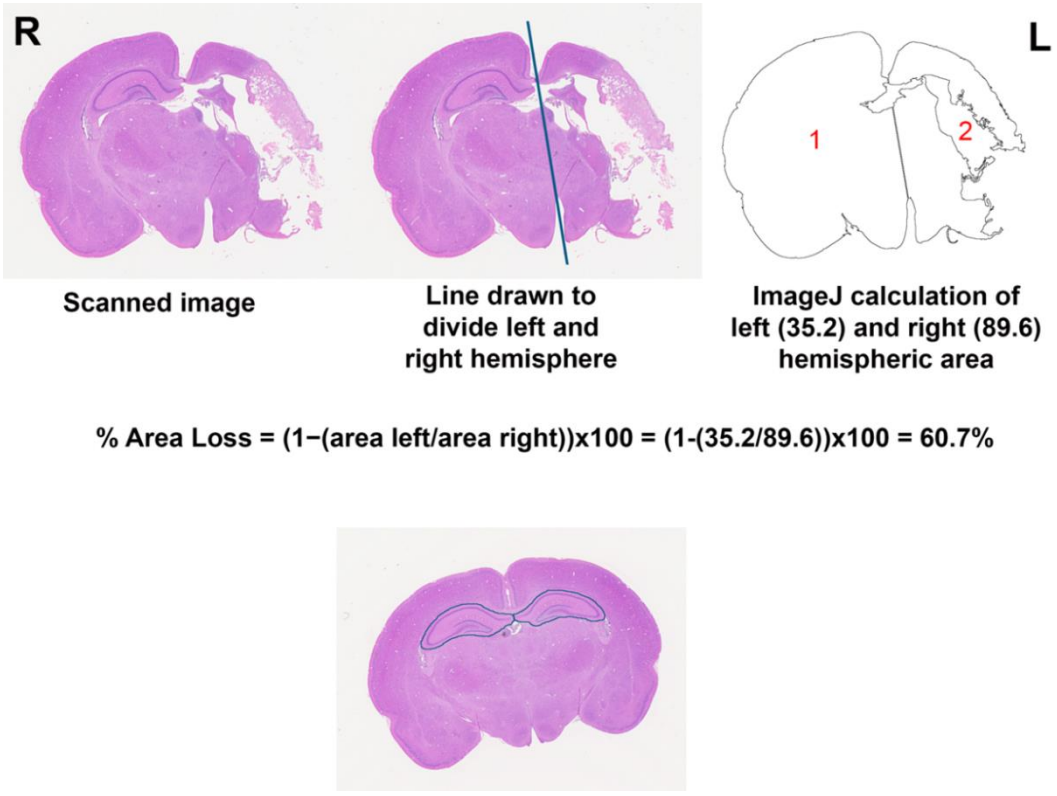


**Figure 17 Calculation of hemispheric and hippocampal area loss**

Method and calculation of hemispheric area loss in ImageJ. In this example, 60.7% area loss was seen. The image below demonstrates calculation of hippocampal area loss. Lines were manually drawn around the left and right hippocampi and area loss calculated using the same formula. Figure from Smit et al.[187]

### *3.3.4 Neuron count in the hippocampal CA1 region*

To confirm the findings of area loss on a cellular level, immunohistochemical staining and counting of viable neurons was performed in the CA1 region of the hippocampus, in a representative subset of sections. The staining method is described below in section 3.4.4, and in-depth in the articles. All cell counting was performed by two independent observer blinded to the treatment groups, and the average of the two used in the analysis, after checking for inter-rater correlation. Three consecutive non-overlapping regions of interest (ROIs) were assessed. Cells located within the pyramidal layer, which were visible within the plane of focus, and positive for both NeuN (neuronal marker) and DAPI (marker of cell nuclei), were counted as neurons. Healthy neurons were identified as those with large, round nuclei, and paler areas indicating unstained nucleoli. The total number of viable pyramidal neurons across the three ROIs from the CA1 hippocampal region was then summed to allow comparison across experimental groups (Fig. 3 and 6, article I and III respectively).

## 3.4 Inflammatory activation

We examined differences in systemic and neuroinflammatory activation upon triggering the neonatal immune system through TLR-2 (PAM) versus TLR-4 (LPS), compared to a control group (Veh). After randomization and marking, P7 rat pups received a single i.p. injection of one of the three substances, and were thereafter returned to their dams. No HI insult was applied.

### *3.4.1 Core temperature monitoring after injections*

Core temperature change, either hyperthermic (fever) or hypothermic, is a physiological response to infection with systemic inflammatory activation. Adult rats respond to LPS with temperature change.[188] To examine thermoregulatory changes in the neonatal rat in response to LPS and to PAM as compared to Veh, we performed monitoring of core temperature at 0, 1, 2, 4, 6, 8, 10, 12, and 24 h after single injections.

All groups were handled in a similar fashion throughout the experiment. To get the individual nesting temperature at a given time point, one pup at a time was removed from the dam to have the core temperature measured, before returning to the dam. Using the same temperature probes employed during hypoxia and temperature treatments, the measurements were

performed in a temperature-controlled room at 21°C. Between measurements the dams were kept in the same room.

### *3.4.2 WEIGHT GAIN ANALYSES*

Weight gain is a marker of wellbeing in the neonatal mammal. In a separate study, P7 pups (n=36) received a single injection as described, and returned to their dams. At P14 all pups were weighed separately. Weight gain was calculated as percentage gain from P7 to P14, and compared across groups.

### *3.4.3 WESTERN BLOT ANALYSES*

To examine protein expression of markers of neuro-inflammation and programmed cellular death (apoptosis) after a single injection, western blots (WB) were performed on frozen crushed brain tissue (-80°C in liquid nitrogen) at 24, 36 and 48 h's survival post injection, without an HI insult. Cleaved caspase 3 (cCas3) was our marker of apoptosis (Fig 18). Although neuro-inflammation was examined by immunohistochemistry (IHC) as well, (section 3.4.4), quantification on protein expression level is still desirable, as it is a more reliable method, and the combination of the two methods strengthens the findings. For WB as well as IHC we examined for ionized calcium binding adaptor molecule 1 (Iba1), which is a marker of microglia, and is upregulated upon microglial activation (article II).

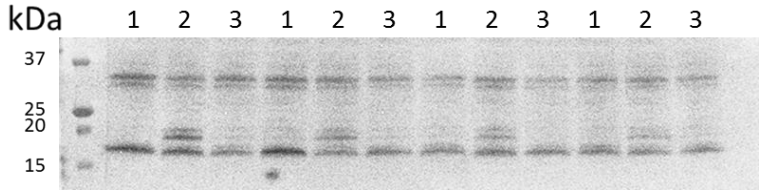


**Figure 18 Western blot of cleaved caspase 3**

Protein extracts from brains in 3 different groups were loaded repeatedly; 1: Veh, 2: LPS, 3: PAM. cCas3 forms two bands with molecular weights of 17 and 19 kDa.

### *3.4.4 IMMUNOHISTOCHEMISTRY*

For IHC analysis, P7 rat pups (n=30) received single injections, and were sacrificed at P14 by transcardiac perfusion. Paraffin-embedded 5 µm sections were used. Primary antibodies against Iba1 (microglial marker), or NeuN (neuronal marker), were applied overnight at 4°C. In control brain sections, the primary antibodies were omitted. After incubating with secondary antibodies the slides were cover slipped with ProLong Gold with DAPI (cell nuclei). Sections were scanned with a virtual microscopy scanner (Axio Scan.Z1; Carl Zeiss, Jena, Germany) using the fluorescence mode with plan apochromatic 20X lens. Virtual slides were exported as high-resolution tiff images for further analysis. The method of identifying and counting of hippocampal neurons is described above in section 3.3.4. Iba1 positive cells were separated from background and analysed by ImageJ. The summed colour intensity detected was calculated as a L/R hemispheric ratio and normalised to cross-sectional area before comparison across groups. The correlation of results from two assessors blinded to treatment groups was calculated to validate the method, and found satisfactory, before comparison across the three groups (Fig. 19).

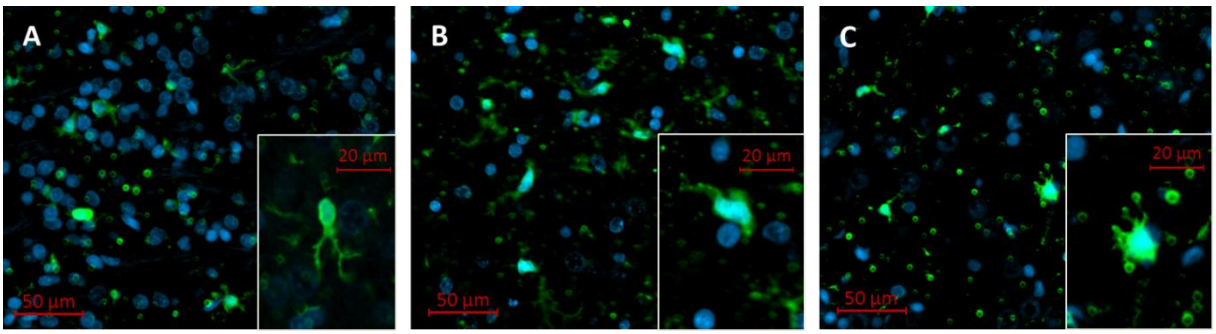


**Figure 19 Iba1 expressions after systemic injections**

Representative IHC images from the Veh-group (**A**), the LPS-group (**B**) and the PAM-group (**C**). Iba1 expression is seen as green. DAPI (blue) stains nuclei. Magnified in picture A is a typical ramified resting microglia. Image B and C show microglia in the activated state, with larger rounded somata and withdrawn dendritic processes.

## 3.5 STATISTICAL DATA ANALYSES

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, Ca, USA) and SPSS software version 22 (SPSS Inc., Chicago, IL, USA). Temperature measurements are presented as mean ±SEM. As the remaining data were not

normally distributed, non-parametric statistics were applied, and median with 95% confidence intervals (CI) are presented, calculated by the exact method (Clopper & Pearson).[189] Due to the variable spread in cytokine expression data, Kolmogorov-Smirnov test was used to compare groups. Wilcoxon-Mann-Whitney tests were performed for comparing two groups to get exact two-tailed p-values, and Kruskal-Wallis test was used for multi-group comparisons. Linear regression analysis was used to confirm correlation between hemispheric area loss (dependent variable) and hippocampal area loss (independent variable). A p-value of $<0.05$ (two-sided) was considered statistically significant.

# 4. SUMMARY OF RESULTS

## ARTICLE I

We examined whether the synthetically manufactured TLR-2 agonist would sensitise the neonatal rat brain and make it vulnerable to HI, and whether HT is neuroprotective in the setting of Gram-positive type pre-sensitised HI brain injury.

In this study we found that administering the TLR-2 agonist, PAM, sensitised the neonatal rat brain and made it more vulnerable to HI injury, increasing median area loss from 10.4% (95% CI 2.1-37) in the normothermic Veh group (n=32), to 35.8% (95% CI 20.4-48.6) in the PAM sensitised normothermic group (n=35) (p=0.01). Furthermore, we found that in spite of increased damage, the pups that were pre-sensitised clearly profited from 5 h of HT treatment, with an 80% reduction in median area loss (median 35.8% to 6.6% (95% CI 4.4-18.8) (n=32)) (p=0.0002). This was in stark contrast to our findings in the LPS-model, where hypothermic neuroprotection was abolished.

Our findings on hemispheric area loss analysis were confirmed on regional area loss analyses; hippocampal (HIP) as well as cortical (Cx) area loss was significantly higher in PAM-sensitised pups (HIP: 55.5% (95% CI 26.3-69.2); Cx: 50.4% (95% CI 25.1-67.6)) compared to the Veh group (HIP: 13.4% (95% CI 3.1-40.2); Cx: 18.5% (95% CI 2.4-54.2)) (HIP: p=0.03; Cx: p=0.03). PAM was less sensitising in the thalamic region (Th), and the increment in median area loss was not statistically significant when comparing the normothermic Veh group (14.2%, 95% CI 7-32.1) to the normothermic PAM group (28.8%, 95% CI 13.2-46.3). Significant hypothermic neuroprotection in PAM-sensitised animals was seen in all three regions as a reduction in area loss; HIP: 85% (p=0.003); Cx: 77% (p= 0.0003) and Th: 67% (p=0.01). Hippocampal neuroprotection was further confirmed on analysis of remaining viable neurons in the pyramidal layer of the hippocampal CA1 region, by similar distribution across groups.

Iba1 upregulation relative to amount of remaining tissue was more pronounced in PAM-treated animals compared to Veh animals (p=0.035), indicating a stronger neuroinflammatory activation in the double hit model. This was reversed in the PAM-HT group, with significantly reduced Iba1 immunolabelling (p=0.006) (n=10/group).

## ARTICLE II

This article we published with regards to the development of the model of inflammatory sensitisation through TLR-2 (PAM). This was done by analysing changes of brain cytokine expression over time after a systemic injection of PAM to P7 rat pups. We had previously found after a dose response study, which dose of PAM resulted in a similar injury severity to 0.1 mg/kg LPS. Using this PAM dose (1mg/kg), we analysed the same cytokines at the same time points after an LPS injection, as well as after a systemic injection of Veh for control. We found significant differences both in which cytokines were upregulated at what time point, as well as in the magnitude of upregulation. Describing a different temporal pattern of cytokine upregulation, we then investigated CNS inflammatory parameters. On WB analysis of crushed brain tissue, we found significantly increased apoptosis (cCas3) in brains of pups who received LPS, but not in those receiving Veh or PAM ($p<0.0001$ for both comparisons). Iba1 expression, a marker of microglial activation, was elevated to a similar level in PAM and LPS injected pups as compared to pups injected with Veh.

We also monitored core temperature after respective injections, and found that after both Veh- and PAM-injection, pups are able to maintain their body temperature at a steady level of 35-36ºC. After LPS injection, however, core temperatures dropped significantly after only 1-2 h. We analysed weight gain one week after injections, and found that Veh- and PAM-injected pups were similar, 138% (130.5-145.5) and 145.6% (134.1-137.1) respectively, and gained weight satisfactorily, while LPS-treated animals had a poorer weight gain, 115.2% (91.5-138.9) compared to the Veh-group ($p=0.02$) and the PAM-group ($p<0.01$).

## ARTICLE III

In this study we repeated the four-group design from the first study, but with a slightly different protocol. To produce a more severe insult we increased the hypoxia time from 50 to 90 min, at the same intrahypoxic temperature. We found that with the severe hypoxic insult, Gram-positive inflammatory pre-sensitisation does no longer have an additional injurious effect, and injury was similar to that of the Veh group. Area loss analyses were done on hemispheric (HEM) and hippocampal (HIP) areas. The normothermic PAM animals had a comparable median area loss (HEM: 60%; HIP: 61%) to Veh animals (HEM: 58%; HIP: 60%), a level of area loss, which is defined as severe brain injury. Mortality was low and similar in the two groups (Veh 4.5% vs PAM 6.6%). HT significantly reduced injury in the

Veh groups from 57.6% (95% CI 53-61.8%) (n=43) to 50.1% (95% CI 18-54.7%) (n=45) (p=0.048), as well as in the PAM groups, from 60% (CI 43.9-63.5%) (n=46) to 47% (CI 33.8-54.8%) (n=45) (p=0.03). Hippocampal area loss showed the same pattern. These results are again in stark contrast to the LPS-model, where also the injury level is saturated with increasing hypoxia time,[85] but with an increasing mortality. Surprisingly, the neuroprotective effect of HT prevailed, however with a reduced effect size with increasing injury.

# 5. DISCUSSION

The discussion includes methodological considerations, as well as a general discussion of the results and their implications on the clinical setting.

## 5.1 CHARACTERISATION OF THE MODEL

One of the strengths of the Vannucci model is its reproducibility.[83] An often less valued feature, on the other hand, is that the degree of injury induced by a given hypoxia length has great variability.[190] Comparing results across laboratories are challenging, because the smallest differences in protocol or environment can have impact on injury severity. Some have attributed it to variability in communicational blood flow. Edwards *et al.* published a reproducible modified model where arterial ligation is done to the external carotid artery as well as the common carotid artery.[190] However, the modification did not reduce the injury variability. Others have documented a stress response in rodents handled by male, but not by female researchers,[191] and stress response has been shown to modify injury severity.[192]

In our laboratory we saw an increased vulnerability in the pups when the 2005 changes in EU-regulation for animal transport were enforced from 2015. The amendments led to rat pups being cross-fostered to a dam which is not biologically theirs.[193] We have several times had to re-establish the model, before experimental testing of a hypothesis. As described in article III we performed a series of experiments with identical protocol with 90 min of 8% hypoxia, and compared to previously performed milder insults of only 50 min of 8% hypoxia, conducted by the same researchers (Fig. 16). All experiments were followed by 5h of NT or HT and 1 week survival before analysis of relative hemispheric area loss. As shown by the graph, both 50 and 90 min of hypoxia induced a variable injury degree after NT survival. Importantly, the corresponding HT treated groups showed less neuroprotection with increasing injury severity.

Also HT neuroprotective effect is variable. We see a 25-50% degree of neuroprotection in moderate brain injury.[133,194] In the mildest injuries, the measurement method of hemispheric area loss is unable to identify small changes. HT appears here to be neuroprotective over a continuum of injury severity in this model, and the effect tapers off with increasing area loss (Fig. 16). On the other hand, in a study from our laboratory performed in 2014, median area loss after 90 min of hypoxia was 54%, and HT reduced it to 34%, that is a 37% reduction after quite severe injury.[194] Most likely there is more than one explanation to the variability in

injury severity, and HT neuroprotection variability is affected by parameters that we are not yet aware of. Some individual differences might exist in the immune response elicited, and the balance of injurious versus reparative downstream effects.

High variability does reflect the clinical situation, where we still cannot explain why some babies seem to endure severe insults without significant cellular injury, and some display a graver clinical picture than expected from the events of birth. The variability of pre-clinical models should therefore be valued, and considered a strength rather than a disadvantage, especially if treatment effect is shown, and the model is reproducible.

In the rodent model, the HI insult is applied solely to the brain, which is distinctly different from the clinical setting. In most cases of human babies suffering HI around the time of birth, the flow in the umbilical cord will be compromised, leading to global whole-body HI. Usually, several organ systems are affected, and it is therefore reasonable to assume that the proportion of systemic inflammatory activation is more substantial than in a model of isolated cerebral HI. Inflammatory activation from cellular injury in multiple organ systems might further modulate the intracerebral inflammatory state, and thereby affect vulnerability and potential for neuronal rescue by HT. As mentioned in the introduction, direct translation from rodent studies to humans should be avoided, and the same hypothesis should be tested also in larger animal models allowing for better monitoring of vital parameters, as recommended by the STAIR criteria developed for stroke research.[88]

## 5.2 The model of inflammatory pre-sensitisation

The association between sepsis and cerebral injury in very premature infants have been suggested to be largely independent of the bacterial species involved,[34–36] suggesting that a detrimental final common pathway can be activated by diverse initial host-microbe interactions. Our findings in a model equivalent to late preterm to term, on the other hand, suggest some differences. Both LPS and PAM induced intracerebral inflammation, and sensitised the brain to HI injury. However, the intracerebral inflammatory response was not similar for the two models, and as HT was neuroprotective in one but not the other, it is likely that the different inflammatory responses exert different downstream effects.

The role of LPS in the pathomechanism of Gram-negative sepsis is well described,[195,196] and the use of LPS in pre-clinical research in various sepsis models well-established.[197–199] In the laboratory, LPS has the advantage of being a potent trigger of inflammatory activation. It is relatively cheap, and readily available. On the other hand, LPS is an unstable biological product with high variance in potency between batches. Behind the LPS-sensitised modification of the Vannucci model lie a number of investigative experiments to find the suitable dose and timing of injection.[85–87] The fact that LPS exclusively represents a subpopulation of bacteria (Gram-negative species), however, is rarely addressed. The heterogeneity of the Gram-positive bacterial wall makes it more challenging to find a corresponding representative Gram-positive immune trigger. Lipoteichoic acid, peptidoglycans, and lipoproteins have different cytokine- and chemokine inducing properties.[200,201] TLR-2 has been demonstrated to be required for host defense against GBS, and previous literature use PAM to model GBS infection.[202] The fact that GBS is the most commonly isolated pathogen in neonatal sepsis in the developed parts of the world,[203] made PAM a natural choice as our trigger of the TLR-2 mediated immune response.[204] PAM is a highly stable synthetically manufactured product, where the TLR-2-triggering potency of each batch is tested before it is sold.

One of our first aims was to establish a model of Gram-positive type inflammatory sensitised HI brain injury. We managed to do so, based on cytokine investigations, as well as pilot experiments on dose and incubation time (section 3.1). It is debatable how valid it is to compare brain cytokine response after injection of such different doses of the two triggers. On the other hand, to be able to test HT effect after pre-sensitised HI, we needed to reach a level of injury within the moderate range (30-50% area loss) (table 3). The investigation of cytokine expression responses was not quantitative, but rather a comparison of their temporal pattern of expression for 48 h following injections, as this was important to how the model would be run.

As described in the introduction, the high mortality in neonatal sepsis has been attributed to immaturity of the innate immune system. On the contrary, the clinical observation of hyper-inflammatory courses of neonatal sepsis contradicts the concept of an impaired neonatal innate immune system.[205] In our study, TLR-4 induced upregulation of certain brain cytokines was rapid, while that of TLR-2 activation was somewhat delayed in comparison (article II).[206] The explanation for this is not known. While TLR-2 activation only involves the MyD88-pathway, TLR-4 activation involves the TRIF-pathway in addition to the MyD88-

pathway (Fig. 2). However, the cytokines we have measured are described as MyD88 dependent, and the differences we observed can therefore not be explain by that.[18,207] Cytosolic endogenous TLR-4 ligands, like S100A8/9 (calprotectin) might play a role, as they can be rapidly released and induce a self-reinforced pro-inflammatory activation.[29] The S100 alarmins are abundant cytoplasmic proteins of phagocytic cells,[208] They have been shown to act as endogenous activators of TLR-4, by amplifying endotoxin (LPS)-triggered inflammatory responses of phagocytes through promotion of the MyD88-dependent pathway. Thereby they promote lethality during sepsis and septic shock.[209] Whether S100A8/9 also act through TLR-2 (or other PRRs), which also activate the MyD88-pathway,[16] has to our knowledge not been investigated, but cannot be excluded.[210] Ulas *et al* measured high levels of S100 alarmins (S100A8 and S100A9) in plasma of neonates. They also assessed the response of adult and cord blood monocytes to LPS, and found altered activation of MyD88-dependent signalling in neonatal monocytes. TRIF-adaptor-dependent regulatory genes remained unaffected by perinatal S100 programming and responded strongly to LPS, but were barely expressed.[29] They suggest that neonates are characterised by a selective, transient microbial unresponsiveness that prevents harmful hyperinflammation, while allowing for sufficient immunological protection.

Upon adjusting the Vannucci model to include LPS-sensitisation, Eklind *et al.* found increasing damage in the Veh groups with increasing hypoxia duration. LPS pre-treatment increased the area of infarction in combination with 20, 30 and 40 min of hypoxia compared to Veh. With 50 min of hypoxia, however, the area of infarction was similar in the Veh and the LPS treated groups, and degree of injury seems to be saturated, to a point where more injury will result in death (Fig. 20A).[85] Similarly, in the PAM sensitised model we found increasing hypoxia duration to increase injury in the Veh groups, while with 90 min of hypoxia injury severity was similar in the Veh and the PAM treated groups (Fig. 20B).

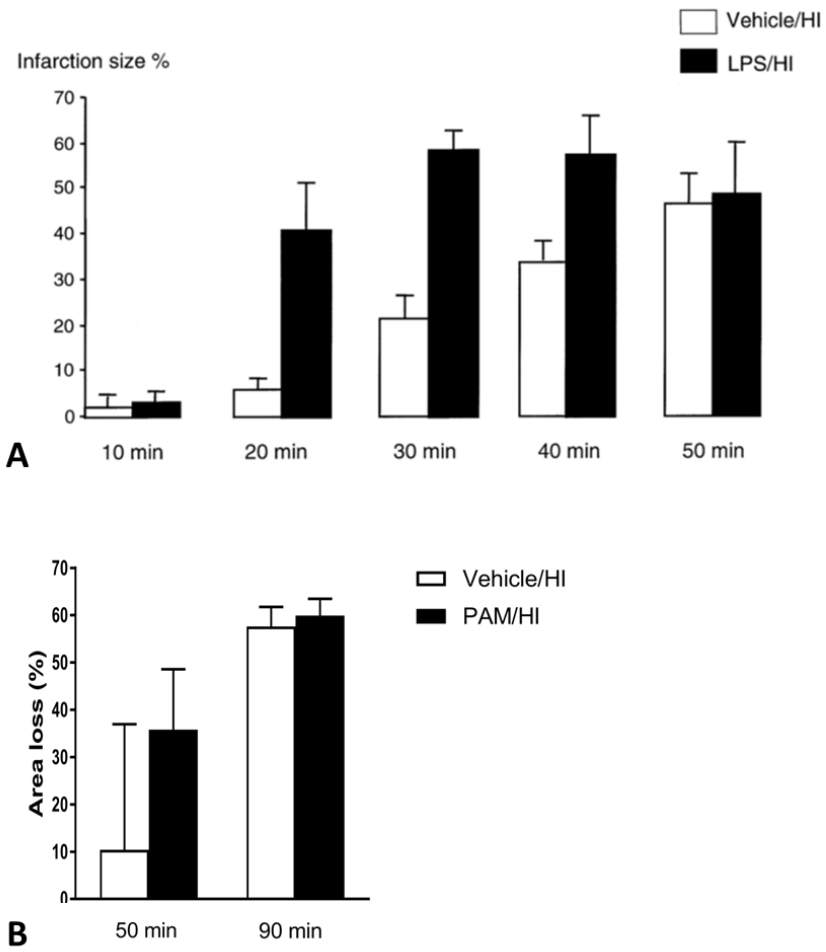


**Figure 20 Inflammatory pre-sensitised HI brain injury after different hypoxia durations**
Panel **A** is from Eklind *et al*. and shows infarction size (%) after LPS-sensitised HI injury, with increasing duration of hypoxia from 10-50 min. Panel **B** shows our data on PAM-sensitised HI injury from a mild insult of 50 min (article I) and the moderate insult of 90 min (article III).

The P7 rat pups appear to be more robust against HI injury in our laboratory setting when comparing hypoxia duration and degree of injury. This again points to the variability of the Vannucci model (section 5.1).

A major difference between the LPS- and the PAM-sensitised models is the mortality. In LPS studies, mortality increases with hypoxia duration, from 0% at 10 min HI to 44% at 50 min HI.[85] LPS studies in our lab resulted in a mortality of 42% after 50 min HI.[139] In the PAM sensitised model mortality is negligible after 50 min of hypoxia, and low at <7% after 90 min of hypoxia. The explanation to the difference in mortality from LPS and PAM is not known. Interestingly, LPS without HI induced brain apoptosis (article II), while PAM was similar to Veh, and did not (Fig. 21).

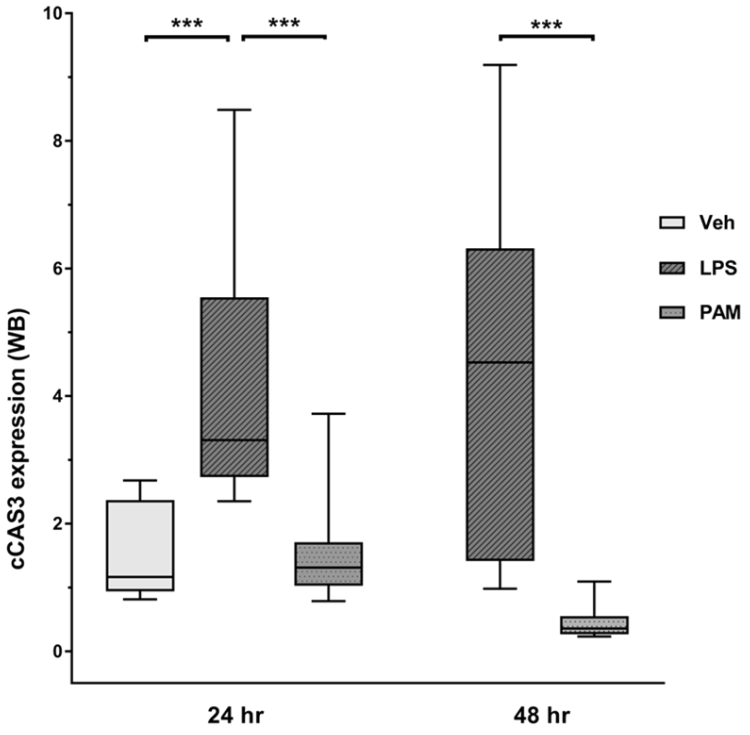


**Figure 21 Apoptotic activation in brain after systemic injections**

Box-&-Whiskers plot of cCas3 expression (WB) in brain tissue at 24 and 48 h after injections. *** $P < 0.001$.

Clinically, sepsis from Gram-negative species run an undistinguishable course from Gram-positive sepsis in adults, with similar mortality and outcome.[211,212] In preterm neonates, however, Gram-negative sepsis has significantly higher mortality than does sepsis caused by the most commonly isolated group of Gram-positive organisms, coagulase-negative staphylococci.[203]

### *5.2.1 CROSS-TALK BETWEEN BLOOD AND BRAIN*

Only a small proportion of cerebral morbidity from clinical neonatal infection is caused by bacterial infections of the neonatal brain, and substantial neonatal brain injury can occur without entry of bacteria into the CSF.[47,213] Culture negative asymptomatic, but histologically confirmed, chorioamnionitis, and even maternal GBS colonization without chorioamnionitis,

lead to a persistent activation of the neonatal inflammatory response, and is associated with adverse short- and long-term neonatal outcomes.[15,35,214]

The vulnerability of the neonatal brain to systemic inflammation was shown pre-clinically already in 1976 by Gilles, who induced leukoencephalopathy in neonatal kittens after systemic administration of LPS.[32] Later, intrauterine exposure to LPS induced microglial activation and caused leukomalacia-like lesions in brains of both pre-term and near-term fetal sheep.[215] As a result of these pre-clinical and clinical findings, the term "infectious pre-sensitisation" is gradually being exchanged for "inflammatory pre-sensitisation". This supports the use of models of inflammatory activation through a one-time-inflammatory-activation rather than a model with real bacterial infections.

Our findings on systemic inflammatory activation leading to an intracerebral inflammatory response (cytokine response and microglial activation) and cellular death (cCas3) were without HI, thereby with an intact BBB. LPS, a 60 kilodaltons (kDa) glycoprotein, only crosses the intact BBB to a minimal degree.[216] Whether PAM crosses the BBB has not been investigated, but is unlikely to cross by diffusion due to its size of 1.5 kDa.[217] It is believed that there is immune system cross-talk between blood and brain in the neonate, also across the intact BBB. Although the BBB is highly restrictive, its non-permeability is not absolute. The absence of tight junctions between endothelial cells in the choroid plexus makes it one of the few places within the CNS devoid of a BBB. It allows the movement from blood to brain of proteins such as albumin, binding proteins, immunoglobulins and complement factors.[218,219] Inhibiting complement (C3) was shown in a study to block microglial activation and cytokine production induced by systemic LPS,[220] indicating an important role of complement system in inflammatory sensitisation. Additionally, there is active transport of blood-borne cytokines across the BBB. However, not all cytokines are transported and, for those which are, transport rates differ among cytokines, among brain regions, with physiological circumstances, and with disease.[219,221] There are transport systems for IL-1β, IL-6 and TNF-alpha which are distinguishable from each other.[222] These are saturable systems, demonstrated by radioactive iodine-labelled cytokines acting as competitive auto-inhibitors of transport from blood to brain.[222] They do not affect each other's transportation. The amount of blood-borne cytokines entering the brain is modest, but thought to be sufficient to affect brain function.

Furthermore, peripheral circulating immune cells cross the endothelial cells of the BBB, by a process called diapedesis, entailing a combination of invagination of the endothelial cell and

podocytosis of the immune cell.[223] Studies suggest that leukocyte entry through the choroid plexus is an important mechanism.[48]

In our study on systemic LPS or PAM injections without HI, increased Iba1positive cells were seen after PAM and LPS injections to a similar degree.[224] This supports the idea that inflammatory activation in blood leads to activation of the monocyte line in the CNS.[225,226] TNF-α was shown to play a major role in recruitment of monocytes from blood to brain,[227] and IL-6 is a key factor stimulating microglial activation and proliferation.[228] Both LPS and PAM induced significant elevations of TNF-α and IL-6 well within the time point where we analysed microglial activation, and could therefore explain the similar Iba1 densities. Interestingly, systemic PAM has been shown to strongly induce leukocyte trafficking into the neonatal brain, of which mainly neutrophils and monocytes.[229] For systemic LPS induced inflammation, data are somewhat conflicting. Montero-Menei *et al.* found 80% of immune-labelled cells in the brains of adult rats to be recruited monocytes 24 h after an intracerebral injection of LPS.[225] On the contrary, and with a more clinically-relevant model to our field, Smit *et al.* argue that 48 h after a systemic injection of LPS in the neonatal mouse, there is a significant increase in number and density of Iba1 positive cells in the hippocampus, to which peripheral monocytes did not contribute.[33]

The pathophysiological role of microglia continues to be debated, and when they contribute to injury versus when they exert a repair mechanisms is not clear.[38,230] The divergent results could relate to differing microglial phenotypes, depending on their phagocytic activity. This difference in ratio of residential microglia to recruited monocytes from peripheral blood in the setting of systemic inflammation might be significant to downstream intracerebral inflammatory effects. Ritzel *et al.* showed in a model of ischaemic stroke, that resident microglia adopted a largely pro-inflammatory phenotype and produced higher levels of ROS and TNF, compared to monocytes which were the predominant IL-1β producer, and primarily were involved with early debris clearance of dying cells.[231]

Activation of microglia and their release of pro-inflammatory molecules induce cellular death.[232] Kim *et al*. attributed LPS-induced neurotoxicity and apoptosis to microglial density.[232] In light of these different origins and qualities of Iba1 positive cells, the lack of induction of apoptosis after PAM injection as compared to a clear apoptosis induction after LPS (Fig. 21) supports their idea; While PAM induces higher rates of monocyte infiltration

into the CNS, which contribute to anti-inflammatory processes and repair, LPS induces more of a local microglial proliferation and activation, with pro-inflammatory properties.

Ådén *et al*. found that TNF-α knock-out mice were not sensitized by IL-1ß to an excitotoxic insult of intracerebral ibotenate.[233] The authors attributed a key role to TNF-α in the mediation of brain damage after combined inflammatory and excitotoxic insults. The hypothesis is supported by the fact that TNF-α is central in temperature regulation,[234] in combination with the impact of intra-ischaemic temperature on HI brain injury (discussed below). Unfortunately, core temperatures were not reported in their article.[233] However, as addressed in article II, PAM also induced elevated TNF-α intracerebrally, but without the corresponding loss of thermoregulation. Ådén *et al.* consider the imbalance of pro- and anti-inflammatory cytokines important, and underline the absence of IL-10 elevation in their experiment. IL-10 is traditionally known to have anti-inflammatory or immune modulatory effects. In a neonatal pig study on LPS-sensitised HIE, recombinant IL-10 counteracted LPS-induced metabolic deterioration and tissue oxygenation.[137] In another study, IL-10 abrogated the stimulatory effect of IL-1 beta on superoxide dismutase activity and ROS production.[235]

We found that systemic PAM injection induced significantly elevated intracerebral IL-10 after 2 h, and it remained elevated until 18 h post injection. IL-10 was only briefly elevated in LPS-injected animals at the 6 h time point before returning to baseline. In combination with our temperature data, which demonstrated a loss of temperature regulation from LPS but not from PAM, our findings also indicate an importance of the balance of combined cytokine signaling, and support their hypothesis.

## 5.3 INTRA-ISCHAEMIC TEMPERATURE

As referred to in the introduction (section 1.3.1), HT was first shown to be neuroprotective when administered *during* the HI event,[122,132] but Yager *et al.* did not find HT neuroprotection from immediate post hypoxic cooling to 31 or 34°C in P7 rats. Colbourne described in 1997 intraischaemic HT as remarkably neuroprotective, but at the same time the value of post-ischaemic cooling was viewed with skepticism.[236] Notably, Yager *et al*. administered the HI insult at a slightly raised temperature of 37°C in the control groups. Sabir *et al.* demonstrated HT neuroprotection after moderate HI injury, while after severe injury HT failed to be neuroprotective. The severe insult was administered at 37°C.[135]

Normal core temperature in nesting healthy P7 rat pups is 35.5°C, gradually increasing to 36°C around P10.[194] Traditionally, the moderate HI insult in the Vannucci model is administered at 36°C, that is at or just above their normothermic core temperature. In the model, the relationship between hypoxia duration and injury severity is not linear. When the rats have not been pre-exposed to systemic inflammation, producing a severe brain injury in P7 pups demands both an increased duration of the hypoxic insult, as well as an elevation of intrahypoxic temperature (to 37°C).[135]

A linear relation between brain temperature and the rate of energy utilization was described in newborn pigs.[147] Depletion of cellular energy supply and mitochondrial dysfunction are pivotal to the choice of cell death pathway.[237,238] The mechanistic relationship between cellular energy reserves and neonatal HI brain injury was addressed by Northington *et al.* The immature brain is primed to respond to injurious stimuli by activation of apoptotic pathways,[239] and neonatal brain HI is known to induce apoptosis.[240,241] Northingtons group, however, described a continuum of cell death phenotypes,[242] where the cellular energy available determines the cell's ability to drive the apoptotic pathways to completion.[243] The time from start of HI until high-energy reserves are exhausted will be directly related to the metabolic rate.[244] When a higher intra-ischaemic temperature alters the metabolic rate and increases energy demands during the injurious processes, the compromise in cellular energy reserves is greater, shifting more cells towards the necrotic side of the cell death spectrum.

With changing the temperature by 1°C in our model, although medians remain the same, we see increased mortality, as well as a clear reduction in variability of injury among those who received the insult at a higher temperature (Fig. 22). There are no surviving pups with injury in the mild range, and fewer pups with very severe area loss, probably due to death.

Both hypoxia- and temperature-regulated induction of cell-preserving proteins, such as CIRBP, are dose dependent. The dose dependence of hypoxia-induced endogenous protective mechanisms is supported by Xue *et al.* showing how very high levels of ROS and $H_2O_2$ actually reduces CIRBP in cell cultures.[245] Upregulations of cell-preserving proteins could be another self-protective mechanism (in addition to controlled cell death) that requires a certain remaining level of cellular energy. Hagberg described a central role of the mitochondria, the main source of high-energy compounds, in HI injury. He suggest that the degree of mitochondrial injury and ROS production is the pivotal step where injury becomes irreversible.[105]

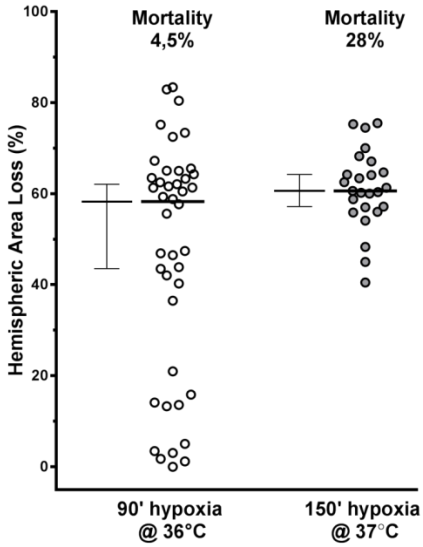


**Figure 22 Different mortality and distribution of injury with elevated intrahypoxic temperature**

Horizontal lines represent the median hemispheric area loss, bars show median with 95% CI. P7 rat pups were subjected to left carotid artery ligation before 90 or 150 min of 8% hypoxia, administered at 36°C or 37°C. The left bars (clear dots) display current data from the Veh-Normothermia group (hypoxia administered at 36ºC). The right bars (filled dots) show unpublished data M. Thoresen Bristol laboratory, where hypoxia was administered at 37ºC. Both insults induced a median area loss of around 60%, but with different distribution and mortality (4.5 vs 28%).

## 5.4 PATHWAYS OF CELLULAR DEATH

The classification of distinctly different forms of cell death originated from their different morphological appearance under a light microscope. Necrosis is defined by cytoplasmic swelling, nuclear dissolution (karyolysis), and cell lysis.[246] It is thought to be caused by rapid and severe failure to sustain cellular homeostasis, including rapid mitochondrial damage and energetic collapse. Apoptosis is a form of programmed cell death. The process is ATP-driven and sometimes gene transcription-requiring and caspase-dependent. It is recognized by orderly and compartmentalized dismantling of cells into consumable components for nearby intact cells, and has a distinctive appearance.[247] Apoptosis occur during nervous system development, and frequently in pathophysiological settings of neonatal nervous system injury.[248,249]

Necrosis was initially described as the most prominent form of cellular degeneration following neonatal HI.[58,186] Later, biochemical assays and laboratory methods for detection and manipulation of the apoptotic pathway became available, and research emphasis switched to the study of apoptosis. Pathological evidence for necrosis has been well documented following HI.[248,250] However, except from with the use of microscope studies, the significance of necrosis in neonatal HI has been difficult to assess because of the presumed lack of a measurable regulatory pathway. We now know that necrosis can be regulated, and the previously described dual classification of cell death is rather more of a continuum, where many components of the regulatory pathways are shared between different types of cell death.[243] It has been proposed that programmed necrosis (also called necroptosis) contributes significantly to neurodegeneration following HI.[251] Drugs have been designed that specifically modulate necroptosis, and attenuates HI injury.[251,252]

The mitochondria are described to be central in the HI induced injurious process (Fig. 23).[253,254] While selective mitochondrial outer membrane permeabilisation (MOMP) predominantly induce apoptosis, opening of the mitochondrial permeability transition pore (permeabilises outer and inner membranes), results in mitochondrial swelling and lead to necrotic cell death.[106,255] Necroptosis, a highly regulated cell death, morphologically resembles necrosis.[256] It is activated in situations where the caspase-dependent apoptotic pathway is inhibited by ATP deficiency. Necroptosis is commonly induced by death receptor ligands such as TNF-α, Fas, TRAIL or TLR-4 signalling,[257] and can occur following HI in the immature brain.[251] Once ligand-death receptor binding occurs, an adaptor protein (i.e. TRADD) promotes the interaction between two kinases, receptor interacting protein (RIP) 1 and RIP 3, forming the key signalling complex, the necrosome. RIP3 was shown to induce a shift in metabolism, leading to excessive ROS production and subsequent cell death.[257] Mixed Lineage Kinase Domain-Like protein (MLKL), a key effector protein of the necroptotic cascade, is activated by necrosome mediated recruitment and phosphorylation.[258]

Certainly, neonatal HI injury evolves through several types of cell death, influenced by factors like susceptibility of the developing brain,[239,259] insult severity, and most likely temperature (indirectly metabolic rate) and available high energy compounds during and after the insult.[260]

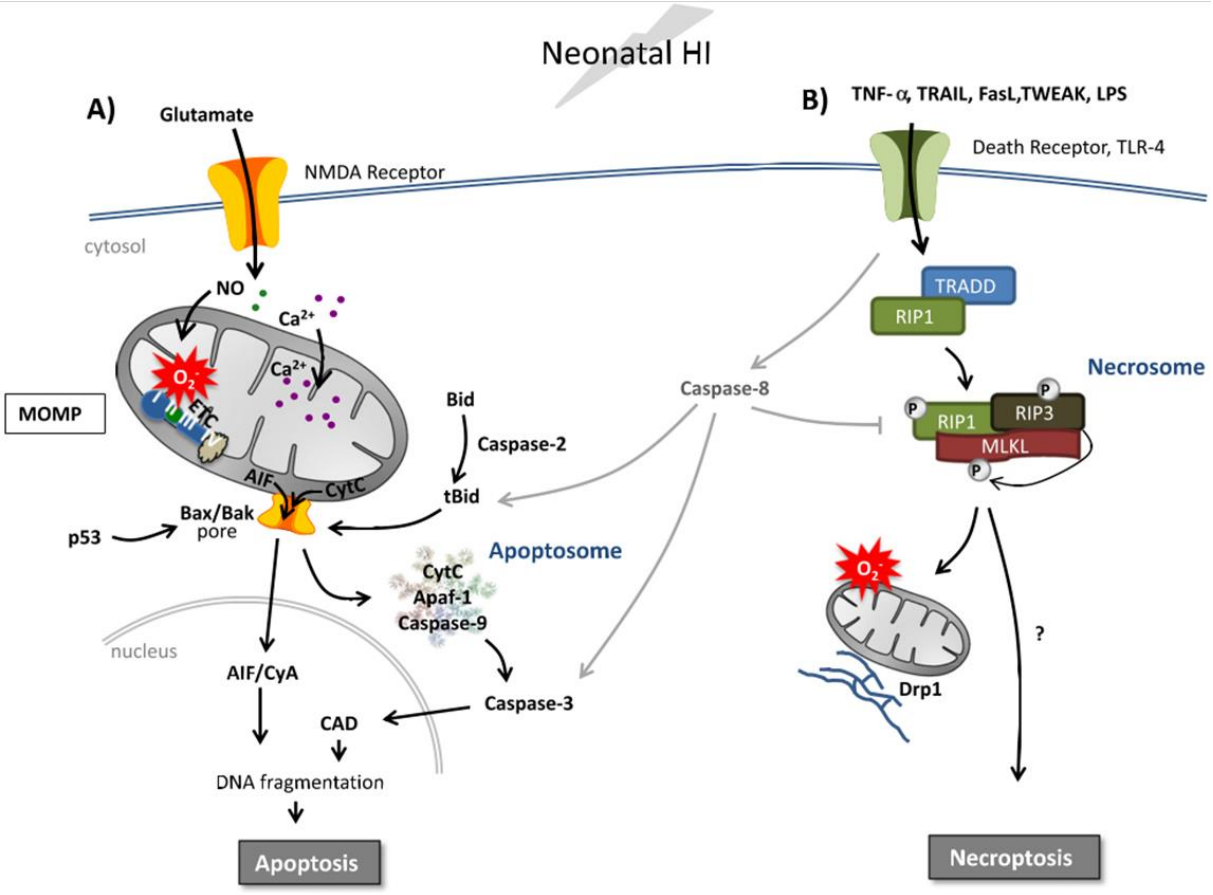


**Figure 23 Role of mitochondria in apoptotic and necroptotic cell death after HI in the developing brain**

**A)** Neonatal HI induces mitochondrial accumulation of calcium, increased production of ROS, and suppression of mitochondrial respiration that culminates in mitochondrial outer membrane permeabilisation (MOMP). Changes in Bcl-2 family proteins induce Bax-dependent MOMP leading to the release of cytochrome c (cyt c) and apoptosis-inducing factor (AIF). Cyt c induces apoptosome formation leading to caspase-3 activation, caspase-activated DNase (CAD) and DNA degradation. AIF forms a complex with cyclophilin A (CyA) which translocates to the nucleus and induces chromatinolysis and apoptotic cell death. **B)** Concomitantly, inflammatory microglia and astroglia will release TNF-α or other ligands, leading to the activation of death receptors, which in turn can induce both apoptosis and necroptosis depending on the availability of caspases. Recruitment of TRADD and RIP1 will lead to caspase-8 activation and cleavage of Bid leading to apoptotic cell death. Alternatively, under conditions when caspase-8 is inhibited, TRADD facilitates the interaction and activation of RIP1 and RIP3. RIP3 phosphorylates and recruits MLKL to the necrosome which can then be targeted to both plasma and mitochondria-associated endoplasmic reticulum membranes triggering increased reactive oxygen species, fission and necroptosis. Alternative non-mitochondrial mechanisms may also play a role in the induction of necroptosis. Figure from Thornton *et al.*[106]

The pathways of cell death have been thoroughly investigated in the setting of neonatal HI brain injury, the classical Vannucci model.[260–262] How these molecular pathways are modified by inflammatory activation is not known. HI induces rapid activation of microglia in the rodent brain.[230] Both intrinsic and infiltrating cells to the CNS produce pro-inflammatory cytokines and chemokines, ROS and death receptor agonists.[263,264] The evidence of TNF-α or TLR-4 mediated cascades in the pathways of cell death along the continuum, such as necroptosis, might play an important role. The TNF receptor 1 is critical for LPS-mediated sensitization to oxygen glucose deprivation in vitro.[265] Furthermore, deletion of the TNF gene cluster abolishes LPS-mediated sensitization of the neonatal brain to HI.[266]

The various contributions of various inflammatory mediators to brain inflammation and cell death mechanisms in the immature brain are not fully revealed, and investigations are ongoing.

### *5.4.1 Examining markers of cellular death*

After an HI insult administered at 37°C to the neonatal rat, HT is not neuroprotective. The variability is reduced in the severe model and mortality is high.[135,194] Temperature clearly affects markers of apoptosis.[267,268] The LPS-sensitised pups act similarly on several levels; the mortality is high; apoptosis markers are markedly affected,[140,224] and they are not susceptible to HT rescue.[139,140]

After a moderate insult in the Vannucci model, HT effectively reduces brain tissue loss, but only if administered immediately or within 3 h after hypoxia.[135] On this background we hypothesised that the differences between the types of injury that allows HT neuroprotection (moderate insult in the traditional model (90 min at 36°C), and PAM sensitized HI injury), or negates HT neuroprotection (the traditional severe insult (150 min at 37°C), or LPS-sensitised HI injury), occurs at an early time point following the injurious insult. Furthermore, that the cell death phenotype initiated within that time window (0-3 h) matters to the degree of neuroprotection we will see from HT. We aimed to investigate pathways of cellular death in the models of moderate and severe HI injury at early time points post-HI; immediately after the insult, as well as after 5 h of temperature treatments (NT/HT). We planned to examine the same after LPS and PAM injection without HI.

The investigations of cell death were done using two related methods. The cleaving, and thereby activation, of caspase 3 is a well-described step in the apoptotic pathway.[269] Quantifying cCas3 has long been used for this purpose and its two parts with molecular

weight of 17 and 19 kDa respectively are well-known. Quantification of cCas3 was therefore done by standard WB technique, as described in article II.

Demonstrating necrosis is not as straight forward. We used the high mobility group box 1 protein (HMGB1), which has been used to demonstrate necrosis,[269] however, it is not clear how accurate this method is for that purpose. HMGB1 is an intracellular protein that can translocate to the nucleus where it binds DNA and regulates gene expression of, among other proteins, pro-inflammatory cytokines. Necrotic cell lysis membranes are permeabilised and intracellular HMGB1 is released and evoke inflammatory responses.

The pathway of necroptosis is a more recently described phenomenon.[270] For this pathway, a methodological challenge is the lack of known regulatory proteins which are significantly upregulated or activated by cleavage. A step that allow for quantitative measurements has not been identified. The activating steps in the pathways of necroptosis rather rely on formation of protein-protein complexes (Fig. 24), with unknown total molecular weights. It is thought that the complex formation between RIP1 and RIP3 is an irreversible activating step, resulting in the phosphorylation and activation of Mixed Lineage Kinase Domain-Like protein (MLKL).[271] Oligomerised MLKL translocates to the plasma membrane, where it mediates TNF- induced necroptosis in a calcium influx-dependent way.[272]

By the use of immunoprecipitation, we isolated the RIP1-RIP3 protein complex for quantification. Anti-RIP1 antibodies were attached to protein A/G sepharose beads for precipitation from tissue lysate. Thereafter, protein-to-protein bands were broken by SDS-buffer and boiling steps, before WB were run on the protein precipitates with anti-RIP3 antibodies (Fig. 24).

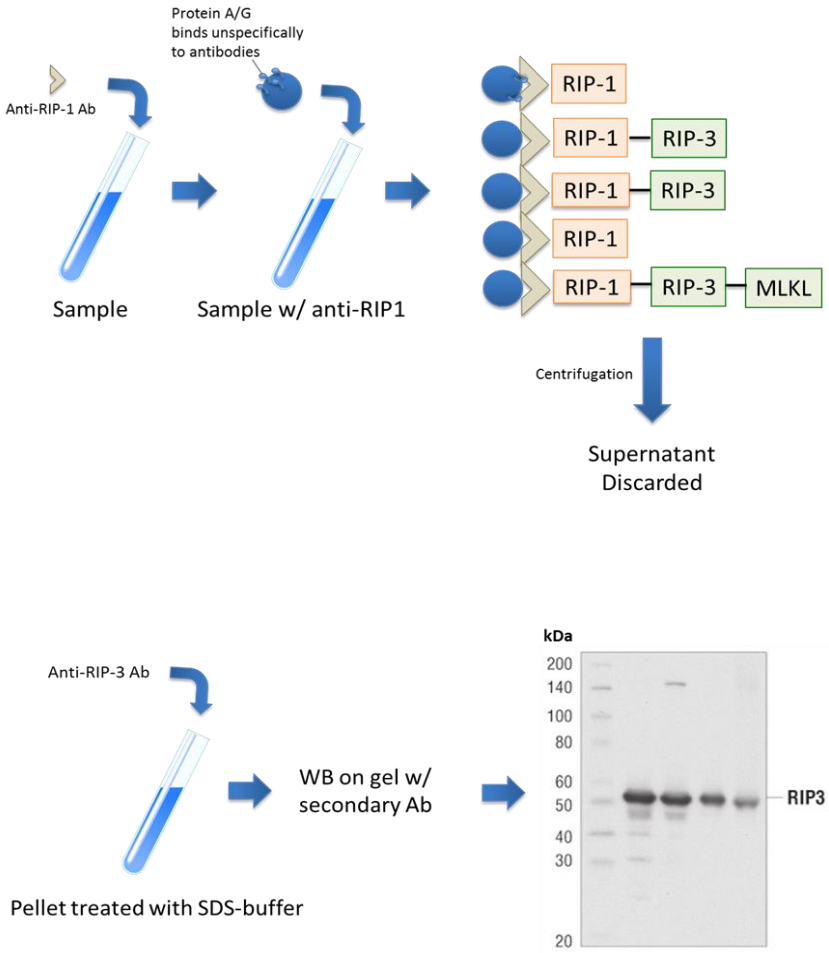


**Figure 24 Immunoprecipitation**

Anti-RIP1 antibody is mixed into a tissue sample. After time has allowed specific antibody-antigen binding, protein A/G-coated sepharose beads are added to the solution. When the beads bind the antibodies with attached proteins and protein complexes, centrifugation will bring them down in a pellet. The supernatant will contain the remaining proteins from the sample, and is discarded. When protein-protein interactions are broken by SDS and boiling, anti-RIP3 antibody is added, and secondary antibodies will bring forth RIP3 on the WB, exclusively representing the complex bound RIP3.

cCas3 was elevated in ligated hemispheres after both moderate and severe HI injury, compared to juvenile control animals (JC) and to the right hemisphere. We found a similar increment of cCas3 after the moderate and the severe insult. This could be explained by the time point of the investigation, as apoptosis has previously been shown to peak at 24 h after injury,[273] and would be expected to develop further.

We were not able to show elevated levels of RIP3 in injured compared to non-injured control brains. The same was true for HMGB1. This might also be a question of timing. However, due to these methodological difficulties these investigations did not lead to results eligible for publication (Fig. 25), and further investigations of animals with inflammatory activation (LPS vs PAM) were not completed.

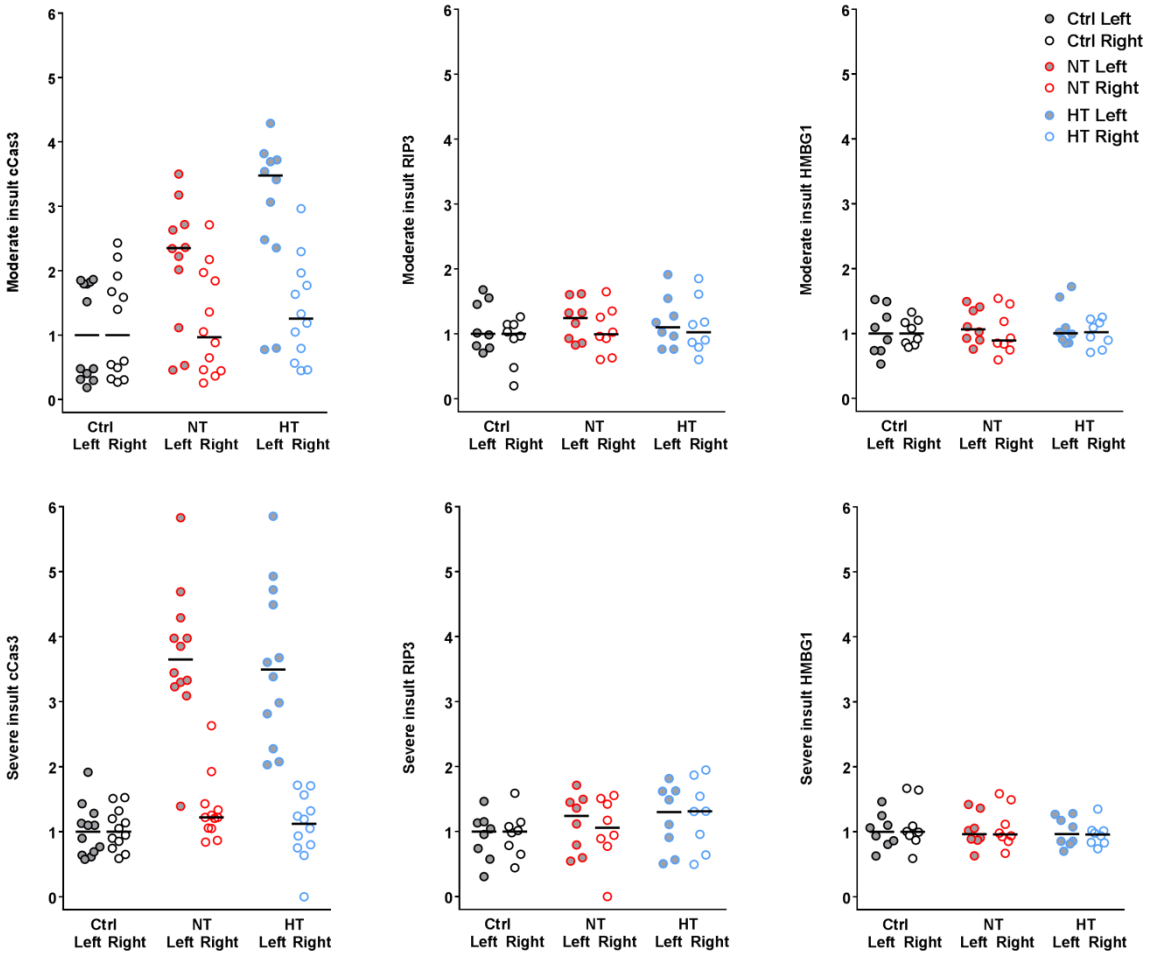


**Figure 25 Markers of apoptosis (cCas3), necroptosis (RIP3) and necrosis (HMGB1) after a moderate and severe HI insult**

P7 rat pups were exposed to either a moderate (90 min@36°C) or a severe (150 min@37°C) HI insult, and treated with 5 h of NT or HT. After precipitation of RIP1/RIP3 complex bound RIP3 protein by immunoprecipitation, we used WB to quantify RIP3, cCas3 and HMGB1. All values were quantified relative to ponceau stain in the same band, and compared to JC animals. Right (un-ligated) and left (ligated) hemispheres were analysed separately, as an additional internal control.

Chevin *et al.* investigated RIP3 immediately after 4 h of treatment in a P12 rat model of severe LPS-sensitised HI injury. They were also not able to demonstrate differences between NT and HT groups.[274] They do show findings that indicate increased levels of enzymes responsible for ROS clearance (superoxide dismutas-1 and glutathione peroxidas-1) in the HT treated group. However, RIP3 should be proven as part of the RIP1-RIP3 complex to be part of the necrosome, and demonstrating the free RIP3 could indicate the opposite, namely less of the protein bound to the complex and taking part in necroptosis. The results are therefore difficult to interpret.

## 5.5 Hypothermic Neuroprotection – modulation by CNS inflammation

Approaches to the mechanistic explanation of hypothermic neuroprotection appear to carry expectations of finding a pivotal molecular switch, with the goal of revealing events that can explain how HT imposes something like a phase transition in the curve of injury evolution. What is often actually elucidated are the mechanism of HI injury itself, which as discussed above, at a certain severity becomes a cycle of self-reinforcing deleterious changes. Some findings might rather than an HT-induced reduction, be a not-so-marked increase in the cooled groups, like e.g. a reduction of apoptotic pathways by HT,[240,275,276] or a decreased activation of immune transcription factors.[162] Few HT mechanisms have been studied without the HI injury beforehand. Hägerdal *et al* found that oxygen consumption was reduced, while high energy phosphates remained steady upon induced HT, without a preceding HI insult.[277,278] An interpretation with a greater overview is more useful, like suggesting that HT reduces injury in a dual fashion;

1) By actively inducing up-regulation of cold-shock proteins, intrinsic survival mecanisms are initiated.

2) By reducing metabolic rate, HT has impact on most of the consequences of the initial HI injury, prolonging the latent phase and extending the protective hypoperfusion period. Thereby, mechanisms leading to molecular cascades that will in turn become vicious circles are delayed, including inflammatory activation.

Both of these two main effects are induced by the endogenous neuroprotective mechanism (spontaneous hypothermia after HI brain injury). Our lab recently published the association between post-hypoxic core temperature drop in the classic Vannucci neonatal rat model, and

severity of injury. More severe brain injury induces a greater endogenous drop in core temperature (Fig 26).[279]

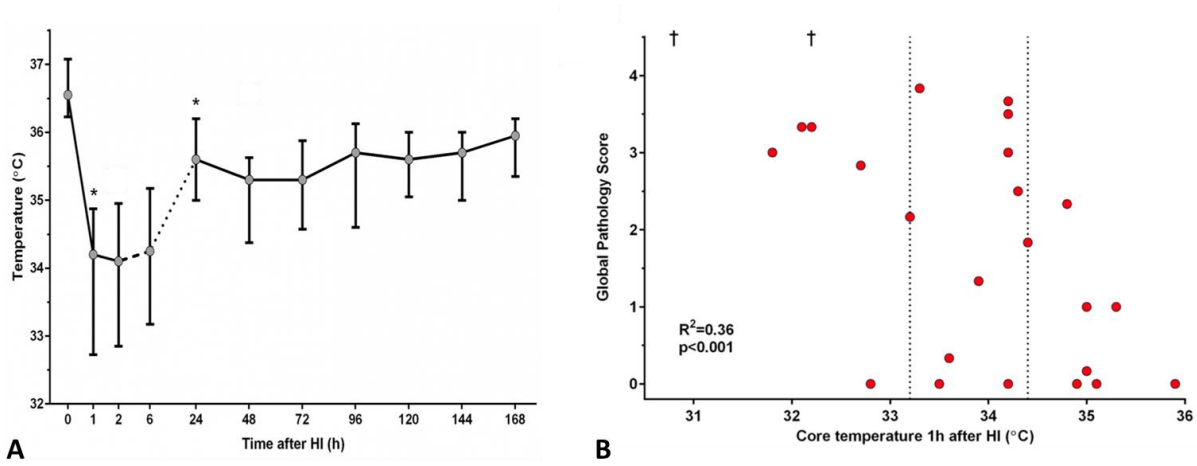


**Figure 26 Post-HI rectal temperature and global pathology score in the neonatal rat**

**A**: Median (IQR) rectal temperature after HI. One hour after HI core temperature dropped significantly, and recovered over the subsequent 24 h. * Denotes significant difference compared to the preceding time point ($p<0.0001$). **B**: One h after HI core temperature of P7 rats is significantly correlated with global pathology score at P14 ($R^2=0.36$, $p<0.001$). † Indicates an animal that died during the survival period. Modified from Wood *et al*.[279]

LPS triggered systemic inflammation induces a drop in core temperature (Fig. 27A), an effect not observed after PAM.[206] Interestingly, LPS has also been shown to induce cold-shock proteins.[280] To our knowledge, it has not been investigated in the setting of PAM induced inflammation. An induced elevation of core temperature has the opposite effect, and reduces expression of these protective proteins. Also re-heating has been shown to reverse upregulation of CIRPB and RBM3 induced by cold.[281,282] Postnatal hyperthermia or induced normothermia in a newborn with HI brain injury would oppose the endogenous neuro-protective mechanisms. The correlation between post-HI hyperthermia and bad outcome was also supported by clinical data from the big RCTs of therapeutic HT for term newborns with HIE (section 1.3.3), where an increased risk of adverse outcomes was found among babies in the control groups with elevated core temperature.[283,284] Both hypoxia- and temperature-regulated induction of cell-preserving mechanisms are subjected to cell vulnerability,

demonstrated by regional differences in cold-shock protein expression in the brain.[285] A regional vulnerability, both to HI injury and HT rescue, has been well documented, and regional variability in expression of cold-shock proteins might partly explain that.[129,134,286]

When HI injury is induced in rats at an elevated intra-hypoxic temperature, HT treatment does not exert its beneficial neuroprotective effect.[135,194] The same negation of HT neuroprotection was seen after pre-sensitisation with LPS,[139] where temperature drops prior to the HI insult, and HI is therefore administered at a relatively elevated temperature. This indicates that intra-hypoxic hyperthermia inhibits CIRBP, and thereby reduces the neuroprotective potency of HT.

To investigate the effect intrahypoxic temperature has on the LPS-sensitised HI injury degree, a separate study was performed (unpublished data) using the following 3-group design experimental protocol; All pups received a single i.p. $LPS_{0.1mg/kg}$ injection 4 h prior to HI (left carotid artery ligation before hypoxia in a temperature controlled chamber. Group 1 (n=29) received 50 min $hypoxia_{8\%O2}$ at 36°C, as in the original LPS study in our laboratory.[139] Group 2 (n=29) received 50 min $hypoxia_{8\%O2}$ at 32.5°C, with one degree elevation from pre-insult core temperature (Fig 27A). Group 3 (n=30) received 90 min $hypoxia_{8\%O2}$ (as in our traditional experimental protocol without inflammatory pre-sensitisation), at 32.5°C. All groups received 5 h of normothermia treatment immediately after HI, before returning to their dams. After one week's survival, pups were sacrificed and hemispheric area loss analysis was performed according to previously described protocols (3.1-3.3).

The group 1 protocol is identical to the normothermia group in the regular LPS-sensitised model, and results fit nicely with previous findings with median area loss of 47%. In LPS-sensitised neonatal rat pups with HI brain injury, avoiding the relative overheating by lowering intrahypoxic temperature from 36°C to 32,5°C reduced median hemispheric area loss from 47% (95% CI 36-56) to 23% (95% CI 15-59), although not statistically significant (p=0.08). Increasing hypoxia duration from 50 to 90 min at this low intrahypoxic temperature brought the median back up to 44% (95% CI 15-51). The variability within group 1 is limited, similar to in the traditional severe model where hypoxia is run at 37°C. For both groups which received HI at 32.5°C (group 2 and 3), the variability is large and similar to the traditional moderate model, where hypoxia is run at a temperature close to their normothermia (36°C) (Fig. 27 B). Interestingly, reducing the intrahypoxic temperature did not reduce mortality, which was high and similar in all three groups at 58.6, 58.6 and 46.7% respectively, indicating other factors than temperature are also important in LPS-induced cell death.

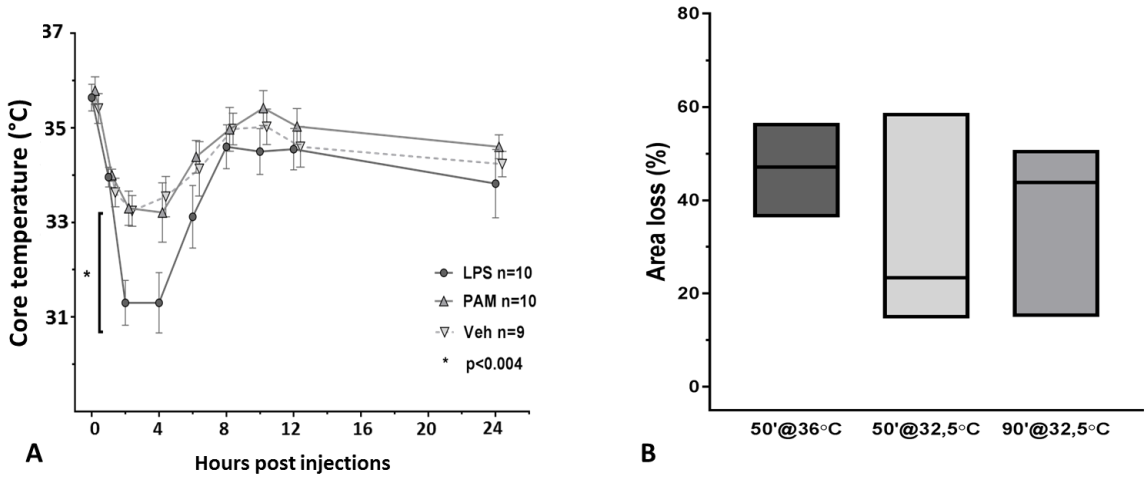


**Figure 27 Core temperature responses after Veh, PAM or LPS and impact on HI injury**

**A**: Sequential core temperature measurements (°C) of P7 rat pups over 24 h following i.p. injections of Veh (n=9), LPS (n=10), or PAM (n=10) expressed as mean ± SEM. ** $p < 0.01$. **B**: LPS sensitised HI injury after three different protocols; 50 min hypoxia at 36°C, 50 min hypoxia at 32.5°C, and 90 min hypoxia at 32.5°C. Bars show median with 95% CI.

The clinical counterpart to elevated intra-ischaemic temperature would be maternal pyrexia during labour. The most common cause of fever in otherwise healthy adults is viral infections.[287] In pre-clinical research, in addition to bacterial type pre-sensitisation through TLR-2 (PAM) or TLR-4 (LPS), pre-sensitisation with Poly I:C (acts through TLR-3 and mimics a viral infection) prior to an HI insult, also sensitises the immature brain to HI.[139,288] It is therefore reasonable to hypothesise that any systemic inflammatory activation with an elevated core temperature during an HI insult, could lead to a more severe and definite type of neuronal injury, with less potency for hypothermic rescue. We have not investigated whether administering the LPS-sensitised HI insult at a lower temperature would allow neuroprotection from HT treatment. It is an interesting question, as this could imply that strictly controlling maternal temperature at normothermia (36.5-37°C) during labour could be beneficial in terms of reducing the risk for HI injury to the neonatal brain, and/or maintain the neuroprotective potential of therapeutic HT if HIE was to incur, even in case of concurrent Gram-negative infection.

In the neonatal rat, we find that HT is neuroprotective in neonatal inflammation sensitised HI injury, if the inflammatory activation is through the Gram-positive route. If we are to translate this from bench to bedside, a highly relevant question that follows is whether therapeutic HT is hazardous in the setting of sepsis. In adult patients with severe bacterial meningitis, a clinical trial on therapeutic HT against the associated intracerebral inflammation and edema was stopped due to increased mortality.[166] Jenkins *et al.* compared circulating leukocytes and serum chemokines between infants under therapeutic HT and a normothermic group.[289] In the HT group, total white blood cells and certain subclasses of leukocytes were markedly depleted, and levels of chemokines, CCL2 and IL-8 were correlated negatively with leukocyte count. These data could indicate that HT is immunosuppressive, which would be hazardous with concurrent neonatal sepsis. Importantly, it is standard protocol to administer antibiotic treatment parallel to therapeutic HT, which reduces the likelihood of new infections incurring within the 72 h of HT treatment.

Very recent data (unpublished) reveal a dichotomous outcome among severely asphyxiated babies at time of birth (Apgar 0-2 at 10 min), where children either died or had great benefit from HT treatment, with normal neurodevelopmental scores at 18-24 months of age. 3 babies of the 9 who died had GBS or Klebsiella sepsis. (*unpublished data from the Bristol cohort of cooled infants*). The timing of the infection and the stage of inflammatory response in the neonate at the time of the HI challenge(s) are likely to be important parameters for vulnerability. This is supported by Tann *et al.* who found that neonatal bacteraemia and funisitis increased the risk of NE, while chorioamnionitis per se did not.[290]

The consistently increased proportion of GBS disease among infants with neonatal encephalopathy and significantly increased risk of mortality provides evidence that GBS infection contributes to NE.[291] Increased information regarding this and other organisms is important to inform interventions, especially in lower-resource settings.[291] In a meta-analysis including seven trials, comprising a total of 567 infants in low- and middle-resource contexts, found no statistically significant reduction in neonatal mortality from cooling (risk ratio: 0.74, 95% CI: 0.44 to 1.25). Neonatal morbidities and long-term neurological outcomes were insufficient to conclude on.[292] The Robertson group published a pilot study in 2011 on the feasibility of HT treatment and follow-up of asphyxiated infants in Uganda.[293] They suggested that in such low-resource settings, future studies need to focus on identifying risk factors for NE, and they particularly outline the potential role of perinatal infection in the aetiology and outcome of encephalopathy. With the use of blood culture and species-specific real-time PCR

assays to identify bloodstream pathogens, significantly more pathogenic bacterial species (both Gram-negative and Gram-positive species) were detected among encephalopathic infants than in controls, again supporting the role of neonatal bacteraemia as an important risk factor for NE in this setting.[294] However, our pre-clinical results suggest high degree of neuroprotection from HT in neonatal HI brain injury with infection as co-morbidity. Their conclusion is still highly relevant, as bacterial flora differs between countries.[5,203] Importantly, some of these countries have a higher proportion of infections from Gram-negative species at term, than in western countries. Several other factors might explain the lack of HT neuroprotection in these trials, like poorer prenatal care, proximity to hospitals, equipment and cooling method in the neonatal intensive care unit.
We do not have enough pre-clinical or clinical data to support a change in current clinical practice, where term infants with moderate or severe encephalopathy are treated with HT, regardless of concurrent infection status.

A number of immunomodulatory interventions that target inflammation have proved effective in experimental models and might have translational potential.[38] The road of translation for these strategies is long, and sets focus to the limitations of our pre-clinical models. Inhibiting a part of the immune response which results in reduced neuronal damage in cell culture, or in a rodent cerebral HI model, does not provide us with information on what effect the same intervention would have after a global HI insult. Our understanding both of the advantageous roles of inflammation in normal brain function and development, as well as when inflammation is deleterious during the perinatal period, is incomplete. Thus, treatment must achieve a delicate balance between possible beneficial and harmful effects.

# 6. MAIN CONCLUSIONS

6.1 ***Does Gram-positive inflammatory activation sensitise the neonatal brain to HI injury?***

We managed to expand the model, and exchange LPS for a Gram-positive synthetic analogue, PAM. Systemic PAM injection sensitised the neonatal brain to HI, and resulted in a moderately severe brain injury from a mild hypoxic insult (article I).

6.2 ***Is HT neuroprotective after Gram-positive type inflammatory-sensitised HI brain injury?***

HT significantly reduced PAM-sensitised HI brain injury. HT neuroprotection prevailed with increased injury severity. As this is in stark contrast to the LPS-sensitised model, we conclude that HT neuroprotection after inflammatory sensitized HI brain injury depends on the pathogen and route of inflammatory activation, in this neonatal rat model (article I and III).

*6.3* ***Are inflammatory responses different after systemic Gram-negative (LPS) compared to Gram-positive (PAM) inflammatory activation?***

In this neonatal rat model, there are significant differences in the inflammatory responses to systemic LPS versus that of PAM, in terms of systemic inflammatory parameters like temperature regulation and weight gain.

We also found significant differences between the brain inflammation, in terms of upregulation of pro- and anti-inflammatory cytokines and intracerebral cellular death, induced by LPS and PAM respectively (article II).

Altogether, our findings suggest that HT neuroprotection in the setting of HI brain injury and concurrent infection and/or inflammatory activation, depends on the pathogen and route of inflammatory activation.

LPS does not adequately model general inflammation or sepsis. Based on our findings, conclusions from LPS studies should include the specific detail that only Gram-negative infection, sepsis or inflammatory activation has been modelled. We do not currently have any data suggesting that we should not cool HI injured neonates with infection as co-morbidity, in fact, our findings through this work supports cooling of these patients, in western countries. This study points to challenges of interpretation form bench to bedside. Our immune responses in various clinical settings are not fully mapped, and therefore a humble approach should be taken when concluding on treatment effects based on pre-clinical studies which involve effects of the immune system.

The results underline the importance of tailoring a pre-clinical model as accurately as possible to the clinical scenario we aim to mimic, and epidemiological factors should be taken into account.

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