

Hypothermia in preclinical models of neonatal hypoxic-ischemic encephalopathy

Doctoral Dissertation

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2. LIST OF ABBREVIATIONS

5CSRTT – Five-Choice Serial Reaction Time Test

ADH – Antidiuretic hormone

ADHD – Attention-deficit/hyperactivity disorder

ADP – Adenosine diphosphate

AED – Acute energy depletion

aEEG – Amplitude-integrated EEG

AIF – Apoptosis inducing factor

AMP – Adenosine monophosphate

Apaf-1 – Apoptotic protease-activating factor-1

ATP – Adenosine triphosphate

AVP – Arginine vasopressin

β -NTP – β -nucleotide triphosphate

BAK – Bcl2-antagonist/killer1

BAT – Brown adipose tissue

BAX – Bcl2-associated \times protein

Bcl2 – B-cell lymphoma 2 protein family

Bcl-XL – B-cell lymphoma-extra-large

BID – BH3 interacting-domain death agonist

bpm – Beats per minute

CA – Cornu ammonis

CBF – Cerebral blood flow

CNS – Central nervous system

CI – Confidence interval

CP – Cerebral palsy

Cx - Cortex

DAB - 3,3'-Diaminobenzidine

DD – Delayed Discounting

DG – Dentate gyrus

Diablo – Direct inhibitor of apoptosis binding protein with low Pi

EAA – Excitatory amino acid
EPM – Elevated Plus Maze
EPP – Exchangeable phosphate pool
FiO₂ – Fraction of inspired oxygen
HC - Hippocampus
HI – Hypoxia-ischemia
HIE – Hypoxic-ischemic encephalopathy
HR – Heart rate
IBA-1 – Ionized calcium-binding adapter molecule 1
ICAM – Intercellular adhesion molecule
IL – Infralimbic cortex
ITI – Intertrial interval
LH – Limited hold period
MABP – Mean arterial blood pressure
MRI – Magnetic resonance imaging
MRS – Magnetic resonance spectroscopy
MWM – Morris Water Maze test
NAA – N-acetyl-aspartate
NE – Neonatal encephalopathy
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NO – Nitric oxide
nNOS – Neuronal nitric oxide synthase
NTP – Nucleoside triphosphate
OF – Open Field
OFR – Oxygen free radical
³¹P-MRS – Phosphorus-31 Magnetic Resonance Spectroscopy
PaO₂ – Arterial partial pressure of oxygen
PaCO₂ – Arterial partial pressure of carbon dioxide
PBS – Phosphate-buffered saline
PCr - Phosphocreatine
PFA – Paraformaldehyde
PFK – Phosphofructokinase

Pi – Inorganic phosphate
PrL – Prelimbic cortex
RI – Resident-Intruder test
RNS – Reactive nitrogen species
RSG Cx – Granular part of the Retrosplenial Cortex
SD – Standard Deviation
SEF – Secondary energy failure
SEM – Standard Error of Mean
SI – Social Interaction test
Smac – Second mitochondria-derived activator of caspase
tBID – Truncated BH3 interacting-domain death agonist
TNF – Tumor necrosis factor
TO – Timeout period
TRAIL – TNF-related apoptosis-inducing ligand receptor
Trec – Rectal temperature
VGAT – Vesicular GABA transporter
VGLUT – Vesicular glutamate transporter

3. INTRODUCTION

3.1 Neonatal hypoxic-ischemic encephalopathy (HIE)

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of the most devastating diseases of the perinatal period. Approximately 1-2 newborns per 1000 live births are affected in the developed countries while its prevalence in the developing world is much higher, altogether amounting to around 700.000 neonatal deaths worldwide annually.¹ An additional 1.15 million newborns are estimated to develop neonatal encephalopathy and almost half a million will suffer lifelong neurodevelopmental impairments due to HIE.¹ These children present an enormous socio-economic burden to the families and to the whole of society as well. Looking at only cerebral palsy (CP) which is traditionally considered one of the most pervasive neurodevelopmental disorders related to perinatal hypoxic events, it can be estimated that HIE introduces a lifelong economic burden of \$1.9 billion every year in the US alone.² These statistics unambiguously show that neonatal HIE presents one of the most severe problems in perinatal care.

Our definitions of “birth asphyxia” and neonatal HIE have evolved in parallel with our understanding of these conditions over the past few decades. Originally “birth asphyxia” was the umbrella term used for all cases of depressed birth, whether a mild and transitory respiratory problem or genuine neonatal stillbirth.³ Additionally, from the middle of the 19th century such a broad diagnosis was thought to be causally linked to the development of CP in later childhood.⁴ This one-dimensional causal association have been called into question repeatedly⁵ and it has become clear that in order to efficiently design therapeutic interventions, the diagnostic criteria need to be more specific. In order to avoid confusion, it might be desirable to retain the term “birth asphyxia” for its literal meaning, i.e. a combination of (arterial) hypoxemia and hypercapnia present at birth. For the neonatal condition associated with certain cases of birth asphyxia, there is an ongoing debate whether hypoxic-ischemic encephalopathy (HIE), neonatal encephalopathy (NE), or some other term would be most adequate.⁶ Due to its widespread use, I will use the term HIE to indicate the clinical condition,

which is outlined in **Table 1**.

Table 1: Diagnostic criteria of hypoxic-ischemic encephalopathy.⁷

| | |
|--|---|
| <p>A) Infants \geq 36 completed weeks gestation admitted to the NICU with at least one of the following:</p> | <ul style="list-style-type: none"> • Apgar score of \leq 5 at 10 minutes after birth • Continued need for resuscitation, including endotracheal or mask ventilation, at 10 minutes after birth • Acidosis within 60 minutes of birth (defined as any occurrence of umbilical cord, arterial or capillary pH < 7.00) • Base Deficit \geq 16 mmol/L in umbilical cord or any blood sample (arterial, venous or capillary) within 60 minutes of birth |
| <p>B) Moderate to severe encephalopathy, consisting of altered state of consciousness (lethargy, stupor or coma) AND at least one of the following:</p> | <ul style="list-style-type: none"> • hypotonia • abnormal reflexes including oculomotor or pupillary abnormalities • absent or weak suck • clinical seizures |

HIE indicates infants, who fulfil two sets of criteria. The first category is related to the newborn's condition at birth (asphyxia, A criteria), including various markers of intrauterine or intrapartum hypoxia and a need for cardiorespiratory support. The second set of criteria is related to the infant's condition in the first hours after birth (encephalopathy, B criteria), i.e. whether neurological impairment can be observed following resuscitation and stabilization. Some clinical trials have employed a third set of criteria, which included abnormalities on amplitude-integrated EEG (aEEG),⁷ but in current clinical practice the diagnosis of HIE can be given without aEEG recording.⁸

Thus the definition of HIE is now rather narrow, compared to the historical use of birth asphyxia. On the one hand, infants who develop neonatal encephalopathy or CP in later life, but do not show signs of severe hypoxia at birth (thus they do not fulfil the

A criteria) are excluded, as the underlying cause of their impairment is likely to have been present prior to birth, e.g. genetic causes or placental insufficiency.⁵ On the other hand, approximately 10% of all newborns require some form of assistance at birth and 1% need vigorous cardiopulmonary resuscitation, but the majority of these infants do not develop encephalopathy or other neurological impairments later on.⁹ Thus the term HIE is used for infants, who demonstrably suffer from some level of neurological impairment in the first few hours of life and this is likely related to peripartum hypoxic events.

Historically, infants with HIE have had a highly variable prognosis. Approximately 35% of these infants died, 35% suffered some form of neurodevelopmental impairment and 30% survived with normal neurological outcome.¹⁰ Neurodevelopmental impairment is generally measured at 18 to 24 months of age, when mental and psychomotor development can already be assessed adequately.¹¹ However, the majority of children showing impaired neurological function at 2 years also display lower IQ-scores in school age.¹²

It is important to note that while infants with moderate to severe HIE have a highly variable prognosis, there is an even larger group of babies, who require some assistance at birth, but do not develop acute encephalopathy.⁹ These newborns have a highly favorable prognosis in terms of the severe neurodevelopmental impairments of asphyxic babies. However, a number of cohort studies have attempted to follow these newborns and some have suggested an increased risk for subtle neurodevelopmental impairments in later life.¹³⁻¹⁷

3.2 Animal models of neonatal HIE

In order to design rational therapeutic interventions for neonatal HIE, it was necessary to develop a working understanding of its pathomechanisms.¹⁸ Due to the imprecise timing of hypoxia, ischemia and hypercapnia in the peripartum period, as well as to the difficulties of indirectly measuring the severity of encephalopathy in newborns, most of the mechanistic understanding of HIE emerged from animal models.¹⁹ From 1955 to 1994 there were almost 300 preclinical papers published related to neonatal HIE.²⁰ The

earliest recorded experiments of neonatal asphyxia were conducted 1813 by LeGallois, who noted that respiratory efforts in newborn rabbits subjected to asphyxia persisted for 27 minutes compared to only 2 minutes in adult animals.²¹ In 1870 Bert et al. made similar observations when comparing newborn pups with 20-day-old juvenile rats.²² In addition to the inverse correlation between maturity and tolerance to asphyxia, some of the earliest observations on various animal species concluded that males are less tolerant to asphyxia than females and high temperature, thyroxin, insulin injection or adrenalectomy all reduce resistance to hypoxia.²³

In order to draw adequate conclusions from animal experiments, one needs to take into account a wide range of considerations regarding animal species, post-conceptual age, level of maturity and the method of inducing hypoxia or asphyxia. Some of the most frequently used animal species in investigations of HIE have traditionally been the immature rat²⁴ or mouse,²⁵ the newborn piglet,²⁶ the fetal sheep²⁷ and various species of non-human primates.²⁸ The optimal age of the animal at the time of hypoxia depends on the developmental dynamics of the specific species as well as the focus of the investigation. The developmental timing of particular markers of brain maturity (eg. brain growth spurt, neuronal migration, myelination, etc.) can be rather diverse in different species.²⁹ This makes it impossible – in principle – to develop animal models which reflect all or most aspects of human HIE and therefore requires researchers to take a holistic view of the strengths and limitations of various animal models.

Historically, the foundations of perinatal asphyxia research were laid down by the work of Myers and Brann on the rhesus monkey.³⁰ Throughout the 1970s these studies employed both fetal and neonatal models of HIE with various methods of achieving hypoxia and/or ischemia and investigated cardiovascular as well as neurological outcomes in both acute and chronic settings.³¹⁻³³ The major contributions of these studies to our understanding of perinatal HIE have been summarized by Raju as follows:³⁴ (1) the immature brain has a greater degree of tolerance to an asphyxic insult than the mature brain; (2) in addition to a reduction in PaO₂ (hypoxemia alone), ischemia is also required to cause measurable brain damage; (3) depending on the type of insult, two distinct patterns of neuropathological damage can be observed: acute nuclear damage in the brain stem in case of global ischemia (together with anoxia), and

oedema with neuronal necrosis in the cerebral hemispheres in case of prolonged partial asphyxia. Limitations of these primate models have also been pointed out, in particular that more severe maternal hypoxia is necessary to generate fetal CNS injury compared to humans, and also that the prevalent pattern of oedema with neuronal necrosis in the primate can rarely be seen in humans, while the human pathology of intra- and periventricular hemorrhage was scarcely observed in these animals. Additionally, by the 1990s primate experiments have become increasingly difficult to conduct due to strict regulations and prohibitive costs.

The fetal or neonatal sheep has been one of the most important experimental large animal models in the investigations of cerebral blood flow (CBF) and brain metabolism during and after HIE. A large number of these studies were published in the 1980s and they generally involved a Cesarean-section performed at varying periods of gestation and the instrumentation of the fetal lamb for later monitoring and manipulation.³⁵⁻³⁷ The uterus and the abdomen were closed and after 2-3 days of recovery either the mother or the fetus were subjected to hypoxia, hypercapnia, acidosis, anemia, polycythemia or other procedures. These studies played a major role in elucidating the specific effects of different components of asphyxia (hypoxia, acidosis, ischemia, etc) on CBF and brain energy metabolism, understanding the developmental aspects of CBF control and describing the extent and limitations of CBF autoregulation in the fetal lamb.³⁴ The major limitations of these models include the fact that most of the studies focused on acute changes in CBF and brain metabolism and less so on the middle to long term outcomes of HIE, and also that cortical neuronal maturation before birth is much more rapid in the sheep than in the human. While a fetal lamb at 120 days (86%) of gestation can be considered similar to a term human infant regarding brain maturity, the neonatal lamb already possesses an adult pattern of cortical maturity.³⁸ Additionally, maintaining this model requires resources in laboratory space, personnel and funding which only a few centers can provide.

Parallel to the development of ovine preparations, the newborn piglet has also emerged in the 1980s as an affordable and 'workable' model in numerous laboratories.³⁴ These studies also focused primarily on the acute effects of HIE on CBF and brain metabolism, and thus confirmed and elaborated on many of the findings of lamb studies.^{39,40} More recently, however, the piglet model has gained increasing popularity

among researchers investigating diseases of the neonatal period.⁴¹ This is partially due to the emergence of magnetic resonance imaging and spectroscopy (MRI and MRS) as invaluable tools for the *in vivo* investigation of the brain as well as potential bridging biomarker modalities, which could possibly provide direct links between animal studies and the human clinical condition.⁴² The neonatal piglet is ideally suited for such investigations, owing to its similarity to human neonates in terms of brain development, as well as its optimal size for imaging.⁴¹ In the clinical translation of therapeutic hypothermia, piglet studies have been invaluable in providing guidelines about the timing and the optimal depth of hypothermia,^{43,44} a work which is still ongoing, as will be highlighted later on.

With more than 1300 citations to the original 1981 paper, the single most widely used preclinical animal model of HIE is the Rice-Vannucci rodent model.²⁴ In this preparation 7-days-old rat pups are subjected to permanent unilateral carotid artery ligation followed by a transient period of hypoxia. The authors of the original article cite Levine, who conducted experiments regarding the tolerance of adult rats to anoxia and ischemia.⁴⁵ His 1960 paper reported that anoxia alone was unsuited for producing significant histological brain damage, as most of the animals either died or survived without lesions. Therefore, he introduced permanent unilateral carotid artery ligation to sensitize the forebrain to subsequent anoxia. Since these findings were in accordance with those of Myers et al. on rhesus monkeys,³⁰ Vannucci and colleagues adopted this preparation to the immature rat, successfully producing significant unilateral brain injury without acute signs of neuromotor dysfunction.²⁴ While numerous modifications to the original setup have been introduced since, including the occlusion of both carotid arteries⁴⁶ or using different degrees of hypoxia for various durations, the core of this model has been instrumental in uncovering many pathophysiological features of HIE, including the depletion of high-energy phosphate metabolites as well as the role of excessive excitatory amino acid release in the development of brain injury.⁴⁷

The advantages of this preparation – its relative cost-efficiency and ease of use – are well reflected in its widespread adaptation and usage even today. However, if one considers the fact that more than 500 pharmacological agents were found to be neuroprotective in preclinical models of neonatal HIE in the past decades, while none of these could be successfully translated to clinical care, the low predictive power of these

models becomes obvious.⁴⁸ The reasons for this are probably several fold, but likely include: (1) the difficulty of determining the optimal age for comparison with the term human newborn; (2) the major anatomical differences between the rodent and the human brain (eg. archicortex volume, axonal myelination, grey/white matter ratio, developmental velocity); (3) the absence of particular patterns of injury in the rodent model, which are regularly seen in the human (eg. injury to the parasagittal cortex, subcortical white matter and brain stem); (4) the absence of systemic level multi-organ involvement, which is also commonly observed in severe cases of human HIE;⁴⁹ (5) the invasive and permanent surgical ligation of one common carotid artery which has virtually no translational equivalent in human HIE.⁵⁰ Additionally, a number of investigators have noted lately that while this preparation reliably produces some level of injury on most surviving animals, the variability of injury is still relatively large, which consequently requires a great number of animals to be sacrificed for each study.⁵¹ While some researchers have tried to argue that this is in fact an advantage of the model, as such a variability is also seen in human infants with HIE,⁵² this difficulty of early patient stratification into meaningful prognostic groups is one of the reasons why human clinical trials in HIE require so prohibitively high number of subjects.⁴⁸

3.3 Neonatal HIE pathomechanisms

Despite their limitations, animals models have enabled us to gain a mechanistic understanding of HIE pathology.³⁰ An excellent paper by Michel J. Painter summarized the state-of-the-art of perinatal HIE research in 1995, which I will attempt to further expand using data from the last 20 years since that review was published.¹⁹

One of the earliest observations was that in addition to the direct detrimental effects of hypoxia and ischemia, neuronal death continued upon reperfusion.⁵³ It was recognized early on that, in principle, this late neuronal death could be ameliorated via post-insult neuroprotective interventions and hence it has been the focus of numerous investigations. More recent studies showed that while a number of neurons indeed die during the primary phase of the injury, the hypoxia-induced impairment of cerebral oxidative metabolism, cytotoxic edema and the accumulation of excitatory amino acids

(EAAs) typically recover, at least partially over approximately 30-60 minutes upon resuscitation.⁵⁴ This period is usually followed by a ‘latent phase’, when the EEG is still suppressed, but high-energy phosphates have recovered to almost baseline levels.⁵⁵ During this phase cerebral metabolism is believed to be actively suppressed, since tissue oxygenation is increased while cerebral perfusion is reduced.⁵⁶ In the case of moderate to severe HIE, this latent phase is generally followed by a ‘secondary energy failure’ (SEF), which is characterized by the accumulation of EAAs, cytotoxic edema, mitochondrial failure and spreading neuronal death.^{57,58} More severe HIE appears to produce higher levels of neuronal death during the primary phase and also an earlier and more severe SEF with more extensive neuronal loss.⁵⁷ Clinically, this is also the phase when stereotypic seizures usually occur.⁵⁹ Finally, brain injury and repair is believed to continue for weeks or months after the SEF. This tertiary phase has recently become the focus of neuroprotection and –regeneration studies.⁶⁰ **Figure 1** summarizes the phases of cerebral injury in HIE as well as the dominant pathomechanisms, which will be discussed below in more detail.

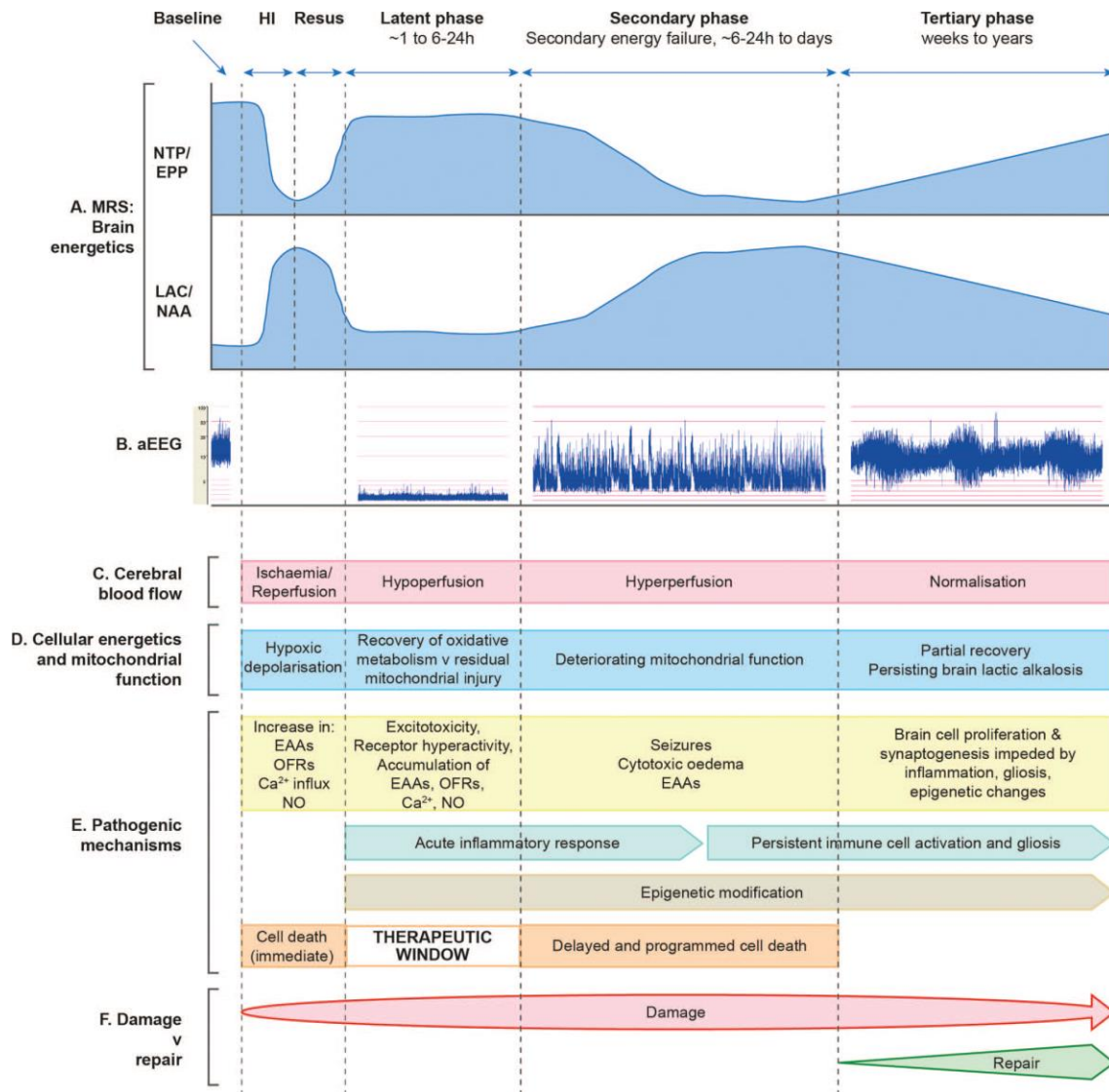


Figure 1: Schematic diagram illustrating the different pathological phases of cerebral injury after cerebral HI, modified from Hassell et al, 2015.⁶⁰ The primary phase (acute HI), latent phase, secondary energy failure phase and tertiary brain injury phase are shown. (A) Magnetic resonance spectra showing the biphasic pattern of NTP/EPP (high energy phosphates) decline and lactate/NAA (anaerobic metabolism) increase during primary and secondary phases following HI insult. Persisting brain alkalosis with high levels of lactate is shown in tertiary phase. (B) Amplitude-integrated EEG showing normal trace at baseline, flat tract following HI, burst-suppression pattern in latent phase, emergence of seizures in secondary phase and normalization with sleep–wake cycling in tertiary phase. (C) After HI, there is a period of hypoperfusion associated with hypometabolism during latent phase, followed by relative hyperperfusion in secondary phase. (D) Cellular energetics and mitochondrial function are reflected in the biphasic response shown on magnetic resonance spectroscopy (A), with a period of recovery in latent phase followed by deterioration in secondary phase. There is partial

recovery in tertiary phase. (E) The most important pathogenic changes are shown for each, including generation of toxic free radical species, accumulation of EAAs, cytotoxic edema, seizures and inflammation. Cell lysis occurs immediately following HI, while programmed cell death occurs in secondary phase; latent phase provides a therapeutic window. Persisting inflammation and epigenetic changes impede long-term repair. (F) Damage is maximal in the secondary phase, but persists into the tertiary phase as inflammation and gliosis evolve. HI, hypoxia-ischemia; EAAs, excitatory amino acids; EPP, exchangeable phosphate pool; NAA, N-acetyl-aspartate; NO, nitric oxide; NTP, nucleoside triphosphate (this is mainly ATP); OFRs, oxygen free radicals;

In most cases of HIE the primary pathological event is a disruption of maternal-fetal gas-exchange, either due to acute or chronic causes (eg. placental abruption or chronic placental insufficiency, respectively).^{*61} This leads to decreased oxygen levels (hypoxemia) and accumulation of carbon-dioxide (hypercapnia) with associated respiratory acidosis in the blood. On the cellular level reduced oxygen availability impedes the operation of the mitochondrial respiratory chain, which in turn results in the accumulation of reduced electron carriers NADH and FADH and the interruption of oxidative phosphorylation. Without the mitochondrial electron transport chain the oxidation of NADH can only be accomplished via anaerobic respiration, during which pyruvate is converted to lactate by the enzyme lactate dehydrogenase, which also oxidizes equimolar amounts of NADH to NAD⁺. The process of anaerobic respiration, however, only produces 2 ATP molecules per glucose molecule, which is only about 5% of the amount produced via aerobic respiration (theoretically 38 ATP molecules per glucose molecule). This leads to the depletion of other intracellular energy stores, such as phosphocreatine, and eventually to the decline of intracellular ATP levels and the parallel accumulation of ADP and AMP.⁵⁵ This decrease in the ATP / (ADP + AMP) ratio induces phosphofructokinase (PFK), which is the rate-limiting enzyme of glycolysis.¹⁹ The resulting overdrive of glycolysis will sharply increase the demand for glucose in order to at least partially compensate for the lack of high-energy phosphates.

* I will refer to J.J. Volpe's Neurology of the Newborn textbook without repeated citations in the next paragraphs describing the biochemical features of HIE.

The initial increase of cerebral blood flow in response to hypoxia can, to some extent, satisfy this demand.¹⁹ However, a sustained lack of O₂ will lead to a decline of heart muscle contractility and heart rate, and thus to a subsequent decrease in cardiac output, which will produce hypotension and cerebral ischemia.³⁵ In turn, ischemia will again reduce the supply of oxygen as well as glucose to neurons, even further tipping the balance of energy metabolism. The accumulation of lactate and protons will create an intra- and extracellular acidosis, which will consume a large amount of plasma buffers, primarily HCO₃⁻. Henceforth, babies with severe HIE characteristically present with a combined respiratory and metabolic acidosis at birth, typically with low plasma pH, high pCO₂, low standard HCO₃⁻ and high lactate levels.

Intracellular acidosis, however, can also have protective effects during hypoxia/ischemia. PFK, the rate-limiting enzyme of glycolysis is highly sensitive to intracellular pH and acidosis strongly inhibits its function.⁶² Additionally, neuronal excitability is also highly dependent on intra- and extracellular pH via a variety of mechanisms, the sum effect of which can be strikingly different in various neuronal populations.⁶³ Acidification can increase excitability in certain neuronal types, like chemosensitive neurons in the brain stem, while other neuronal types, such as hippocampal pyramidal neurons are strongly inhibited by acidosis. As hippocampal neurons are among the most sensitive to hypoxic injury, this protective effect of acidosis can have an important role in HIE pathophysiology.⁶⁴

While the failure of cellular energy metabolism is one of the most important and well-studied phenomena during hypoxia-ischemia, other pathomechanisms also play a significant role. One such process is the generation of free radicals. As discussed above, the shortage of oxygen supply halts oxidative phosphorylation and forces the cell towards anaerobic respiration. However, the slowing down of the mitochondrial ATP synthesis also implies that the reduction of O₂ will be only partial and non-enzymatic free radical generation will be stimulated.⁶⁵ Additionally, the hypoxia-induced accumulation of arachidonic acid and the calcium-induced activation of phospholipase A₂ will result in the activation of prostaglandin synthesis, which also generates free radicals.⁶⁵ Finally, the failure of cellular energy metabolism will lead to the accumulation of breakdown products from high-energy phosphate compounds. This process will give rise to the build-up of hypoxanthine, a product of AMP degradation.⁶⁶

Under physiologic conditions, hypoxanthine is converted to xanthine and further on to uric acid by xanthine-dehydrogenase which utilizes NAD⁺ as a cofactor. During hypoxia, however, xanthine-dehydrogenase is converted to xanthine-oxidase via calcium-induced proteolysis and this enzyme utilizes molecular O₂ instead of NAD⁺ as a cofactor, thus generating superoxide anions.⁶⁵ In addition to oxygen free radicals, there is also compelling evidence to suggest the detrimental role of nitrogen free radicals (RNS) in HIE pathology, primarily via the generation of nitric oxide (NO•) by neuronal nitric oxide synthase (nNOS).⁵⁴ The reaction of NO• with superoxide in the cytosol and mitochondria can lead to the generation of highly reactive peroxyxynitrite and other RNS compounds.⁶⁷ Even though free radical generation is induced during and following hypoxia-ischemia, its scavenging enzymes such as superoxide dismutase and glutathione peroxidase are insufficiently expressed during the perinatal period.⁶⁸ The net effect is an increased free radical load, which can exert a variety of detrimental effects, from lipid peroxidation to DNA/RNA-fragmentation and thus contribute to HIE neuropathology.⁵⁴ Accordingly, preclinical experiments demonstrated the benefits of preventive maternal administration of allopurinol, an inhibitor of xanthine oxidase as a potentially neuroprotective intervention.⁶⁹

As described previously, cellular energy metabolism initially attempts to compensate for the lack of O₂ during hypoxia-ischemia via increasing the rate of anaerobic respiration, but this mechanism rapidly becomes insufficient and intracellular high energy phosphate levels begin to fall.¹⁹ Under physiological conditions the largest consumers of ATP are protein synthesis and the Na⁺/K⁺ pump. While non-essential protein synthesis is quickly blocked upon hypoxia,⁷⁰ the function of the Na⁺/K⁺ pump is essential even under such conditions, since it constantly maintains the high potassium and low sodium concentrations intracellularly. The transmembrane Na⁺ gradient is the primary driver of a number of other ionic processes, contributing to the maintenances of pH- and calcium-homeostasis. The K⁺ gradient is essential for the repolarization of membrane potential in excitable cells. During uncompensated hypoxia, the lack of ATP will compromise the operation of the Na⁺/K⁺ pump and lead to membrane depolarization via Na⁺ and Ca²⁺ entry into the cells.⁵⁴ One immediate effect of the influx of ions is the concomitant entry of water and subsequent cell swelling.⁶¹ While this so called cytotoxic edema can lead to direct neuronal death in extreme conditions, usually

upon reperfusion these swollen neurons recover, at least temporarily.⁵⁴ After the latent phase, however, more severe cytotoxic edema will develop, which can be measured *in vivo* via diffusion-weighted magnetic resonance imaging, which is a sensitive marker of the extent of hypoxic-ischemic injury.⁷¹

Failure of the Na⁺/K⁺ pump and subsequent membrane depolarization will lead to the release of excitatory neurotransmitters to the synaptic cleft, primarily glutamate.⁵⁴ Excessive release of EAAs will trigger the activation of NMDA, AMPA and other receptor-channels on post-synaptic neurons, which will cause disproportionate calcium and further sodium entry into these cells.⁷² This intracellular calcium surge will activate a number of downstream pathways, including the over-activation of enzymes such as calpains and other proteases, protein kinases, calcineurin, endonucleases and nitric oxide synthase, which in turn can further increase free radical generation.⁷² In this context, EAAs and intracellular calcium appear to be important mediators of cellular injury in HIE.⁵⁴

As shown in **Figure 1**, a number of pathological features of hypoxia-ischemia recover to almost baseline levels within 30-60 minutes after resuscitation.⁶⁰ Cytotoxic edema subsides, EAAs are removed from the synaptic cleft and intracellular high energy metabolites recover. This so-called latent phase usually lasts between 6 and 24 hours after recovery and is considered the primary therapeutic time window, when intervention could prevent or ameliorate subsequent injury.⁶⁰ Some studies suggest that during this phase, cerebral metabolism might be actively suppressed by endogenous protective mechanisms, as indicated by the simultaneously present decreased cerebral perfusion and increased tissue oxygenation, consistent with decreased oxygen consumption.⁵⁶ This suggested controlled hypometabolism has important implication for both clinical practice and preclinical research, as will be discussed later.

Approximately 6-24 h after the initial insult, a secondary energy failure has been described both in preclinical⁵⁷ and clinical studies⁷³ using phosphorus-31 MRS (³¹P-MRS). The ³¹P-MRS spectra consists of phosphorus-containing metabolites, including phosphocreatine (PCr), inorganic phosphate (Pi) and nucleoside triphosphates (NTP). There are three NTP peaks in the ³¹P spectra, α-, β-, and γ-NTP, corresponding to the three phosphate groups on NTP molecules (primarily ATP, but also including guanosine triphosphate and uridine triphosphate).⁷⁴ During the SEF, levels of cellular high energy

metabolites (PCr and NTP) fall and Pi increases. Additionally, persistently high levels of intracerebral lactate⁷⁵ and an alkalotic shift in intracellular pH can also be observed,⁷⁶ which are associated with later neurodevelopmental impairments. This secondary phase is also marked by secondary cytotoxic edema, accumulation cytokines and excitotoxins, mitochondrial failure, and clinically the onset of seizures (see **Figure 1**).⁵⁴ A tertiary cerebral hyperperfusion takes the place of the previous, actively suppressed cerebral metabolic state.⁷⁷ These various processes likely contribute to the spreading neuronal death continuing for days or even weeks after the initial insult.⁶⁰

The precise pathomechanisms involved in this secondary energy failure are not fully understood yet, but they likely involve excessive calcium influx, inflammatory mediators, and activation of pro- and anti-apoptotic proteins as well.⁵⁴ Mitochondrial failure appears to be a central feature of the SEF, upon which a number of these processes converge. **Figure 2** summarizes some of these processes.⁵⁴

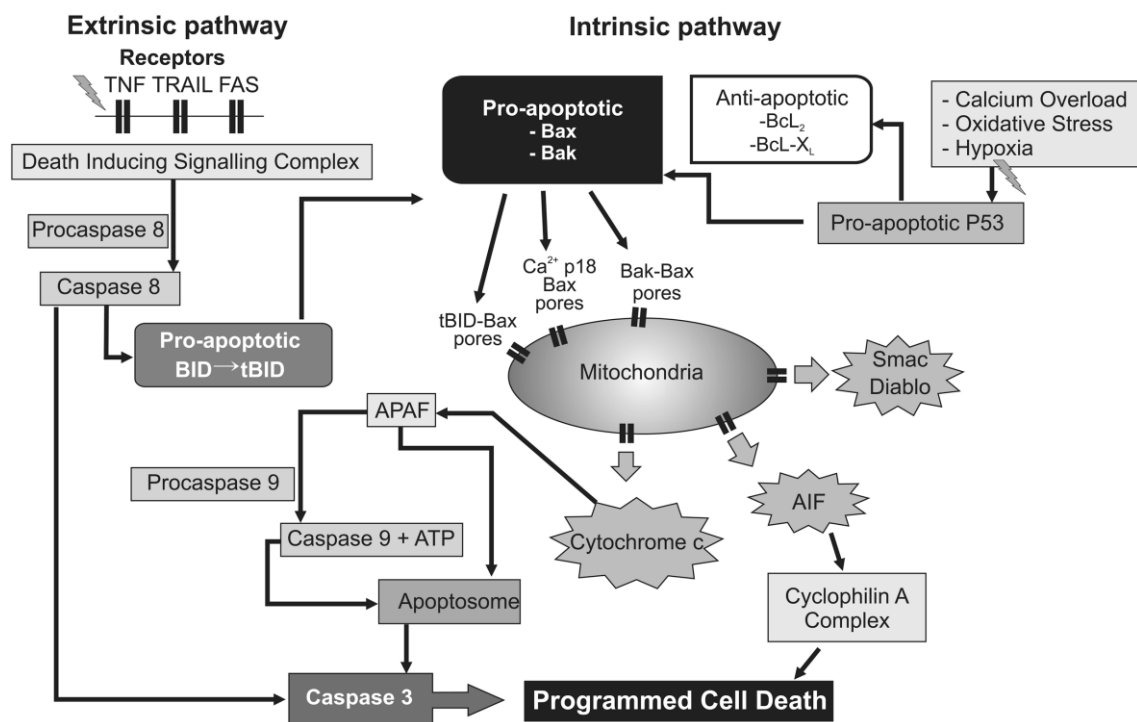


Figure 2: Intracellular mechanisms associated with the permeabilization of the mitochondrial membrane leading to apoptosis, modified from Wassink et al.⁵⁴ Upstream triggers such as inflammatory mediators and withdrawal of trophic support can activate the extrinsic pathway of apoptosis, while calcium accumulation and ROS can induce the intrinsic pathway. Some downstream members, such as Bid creates cross-activation between these two main pathways.

AIF, apoptosis inducing factor; Apaf-1, apoptotic protease-activating factor-1; ATP, adenosine triphosphate; BAK, Bcl2-antagonist/killer1; BAX, Bcl2-associated × protein; Bcl2, B-cell lymphoma 2 protein family; Bcl-XL, B-cell lymphoma-extra-large; BID, BH3 interacting-domain death agonist; Diablo, direct inhibitor of apoptosis binding protein with low Pi; P53, p53 tumor suppressor protein; Smac, Second mitochondria-derived activator of caspase; tBID, truncated BH3 interacting-domain death agonist; TNF, tumor necrosis factor receptor; TRAIL, TNF-related apoptosis-inducing ligand receptor

A central feature of secondary energy failure appears to be a cellular accumulation of calcium due to excessive glutamatergic activation. This calcium build-up can be buffered by the mitochondria up to a certain level.⁷⁸ Above this, however, it can inhibit the function of the electron transport chain, uncouple oxidative phosphorylation and lead to a permeabilization of the mitochondrial membrane, which results in the activation of the intrinsic apoptotic pathway.⁷² This process occurs via the translocation of the cytosolic pro-apoptotic Bax to the mitochondrial membrane, where it forms transition pores together with Bak and Bid, all members of the apoptotic Bcl-2 family.⁷⁹ This allows the leakage of pro-apoptotic proteins, such as the second mitochondria-derived activator of caspase (Smac), the direct inhibitor of apoptosis binding protein with low Pi (Diablo) and apoptosis inducing factor (AIF), as well as cytochrome c to the cytosol.⁷⁹ While the loss of cytochrome c oxidase itself could impair energy metabolism, these processes also trigger further downstream effector mechanisms. These include the activation of the initiator caspase 9 and the subsequent formation of the apoptosome, which in turn will activate effector caspase 3 and ultimately lead to DNA fragmentation, membrane blebbing and apoptotic cell death.⁸⁰ There is a good amount of evidence showing that apoptotic cell death is indeed an important contributor to HIE neuropathology.⁸¹ Additionally, a number of other pathological processes during this secondary energy failure stage can result in, or contribute to apoptotic neuronal death, including the loss of trophic support by astrocytic growth factors,⁸² inflammatory mediators,⁸³ and the induction of the extrinsic apoptotic pathway via the activation of death receptors Fas and TRAIL.⁸⁴ Recent consensus suggests, however, that the classification of injury as apoptotic vs. necrotic might be inadequate and instead there is probably a continuum of phenotypes between these two categories present after neonatal HIE.⁸⁵

There is now accumulating evidence to show that following the SEF, a tertiary phase of active pathological processes takes place, which continues for weeks, months, even years.⁸⁶ This is consistent with the observation that high levels of cerebral lactate and brain alkalosis can persist for over a year in babies with severe neurodevelopmental impairment.⁷⁵ Pathomechanisms during this phase are yet poorly understood, but likely involve the proliferation of astrocytes and the formation of glial scar, persistent inflammatory activation and epigenetic changes.

3.4 Experimental neuroprotective interventions

Owing to the growing understanding of the pathomechanisms involved in neonatal HIE, there has been a number of experimental therapies designed to prevent or ameliorate its neurodevelopmental sequelae. Hypothermia, as the only therapeutic intervention to date with clinically proven safety and efficacy, will be described comprehensively in the following section. Before that, a brief overview is provided regarding the experimental therapies investigated in recent years.

One of the most intensively researched interventions has been the application of inhaled xenon.⁶⁹ Xenon is a non-competitive NMDA-antagonist with a wide range of effects on downstream intracellular pathways, ultimately providing a strong anti-apoptotic effect.⁶⁹ Several groups have demonstrated its neuroprotective effects in preclinical models.⁸⁷⁻⁸⁹ A recent clinical trial, however, failed to show the additional benefit of 30% xenon administration in addition to hypothermia.⁹⁰ There are two other ongoing trials investigating the benefits of different xenon administration protocols (clinicaltrials.gov, NCT01545271 and NCT02071394).

As described before, the inhibition of xanthine-oxidase during hypoxia has been suggested as a potential way to decrease oxidative damage during HIE. Preclinical experiments demonstrated the benefits of preventive maternal allopurinol administration, an inhibitor of xanthine oxidase.⁶⁹ Recent clinical trials, however, could not provide sufficient support for these claims.⁹¹

Another suggested experimental intervention has been the administration of melatonin.⁶⁰ In addition to its well-known role as a regulator of circadian rhythms,

melatonin influences a wide range of physiological functions, including growth and development, reproduction and the immune response.⁶⁰ Its neuroprotective effects have been demonstrated in a number of preclinical models^{92,93} and the mechanisms likely involve anti-oxidant, anti-apoptotic and anti-inflammatory effects.⁶⁰ Clinical trials are now underway to provide information about optimal dosing regimens and potential neuroprotective effects (clinicaltrials.gov, NCT02621944).

Finally, erythropoietin (EPO) has also been suggested as a potential neuroprotective agent after neonatal HIE.⁶⁰ In addition to its role as a hematopoietic growth factor, EPO is known to be highly important for normal brain development *in utero* and appears to be an important mediator of the brain's endogenous protective mechanisms via anti-apoptotic, anti-inflammatory and anti-oxidative effects.⁶⁰ A number of clinical trials involving various protocols of EPO administration are currently underway.⁹⁴

Until today, however, the only intervention proven to be safe and effective was therapeutic hypothermia.

3.5 Therapeutic hypothermia

It is probably no overstatement that therapeutic hypothermia has been the most important recent development in the care of infants with HIE. The concept itself, however, is probably almost as old as medicine itself. The first description of intentional cooling to reduce neurological injury comes from Hippocrates, from approx. 400 BC, who suggested packing wounded soldiers in snow and ice.⁹⁵ The first record of therapeutic hypothermia in newborns dates back to the 17th century, when Sir John Floyer described the immersion of a still-born infant into ice water in order to stimulate the onset of respiration.⁹⁶

“Sarah Parks . . . gave still-birth to a baby boy . . . A young doctor assisting the Parks’ regular physician begged for an opportunity to experiment with an idea he had to rouse the lifeless infant. A tub of ice was ordered and the young doctor plunged the baby into it. Out came the screaming little Parks and he was named Gordon after the doctor who

prodded him to life.”

Sir John Floyer, 1697

The first scientific attempt to use hypothermia in the resuscitation of asphyxiated newborns was made by James Miller from New Orleans and Björn Westin from Stockholm in the 1950s. After conducting a series of animal experiments to demonstrate the beneficial effect of cooling on survival from asphyxia,⁹⁷ they organized the first pilot study of therapeutic hypothermia. Ten severely depressed newborns who did not respond to resuscitation were immersed in a cold water bath until spontaneous respiration commenced or their rectal temperature decreased to 27 °C.^{98,99} Four of these infants also received oxygenated blood transfusions. After resuscitation, the infants were dried and allowed to rewarm spontaneously. One of the 10 infants died due to respiratory distress syndrome, but the other nine survived and did not show neurological impairment in a 10-year follow-up study, despite the fact that their periods of apnea ranged from 8 to 79 minutes.¹⁰⁰ After their initial success Miller and Westin proceeded to conduct a larger study of cooling on 65 infants unresponsive to standard resuscitation, 52 of whom survived, which was an outstanding result at the time.⁹⁷ Small scale clinical trials were also conducted elsewhere in the 1960s, from California to Switzerland and Finland.¹⁰¹⁻¹⁰⁴

Despite these hugely promising results and the championing of cooling for severely depressed newborns by Westin and Miller, which they continued in the 1970s, the general international medical community did not adopt the concept of therapeutic hypothermia. One of the primary reasons for this was likely the publication of another scientific paper almost simultaneously with Miller and Westin’s first study. In 1958 Silverman and colleagues reported a study of 182 premature infants, who were kept in normothermic (31.9 °C) or hypothermic (28.9 °C) incubators for the first 5 days of life.¹⁰⁵ The overall survival was 68% in the hypothermic group versus 83% in the normothermic group. Despite the fact that later re-analysis showed that most of the mortality in the hypothermic group occurred in infants <1000g and that there was a sampling bias between the two groups, which together could explain at least some of the results, this study propelled a large amount of research into neonatal thermoregulation and motivated clinicians to adopt strict monitoring and maintenance of rectal

temperatures of newborns.⁹⁶ Even though the Silverman study was conducted on preterm babies, the extrapolation about the dangers of hypothermia to term newborns was generally accepted by clinicians.

It was only in the 1990s that neonatologists started to question the dogma concerning the dangers of hypothermia in the neonatal period. The causal relationship between postnatal hypothermia and mortality in preterm infants, suggested by the Silverman paper was called into question by a more detailed study by Hazan and colleagues in 1991.¹⁰⁶ By the late 1990s a sufficient amount of data from animal studies have emerged which all supported the potentially beneficial effects of mild to moderate hypothermia after resuscitation from hypoxia-ischemia (HI).¹⁸

The first pilot safety study of selective head cooling in asphyxiated newborns was published in 1998 by Gunn and colleagues.¹⁰⁷ Whole-body cooling was also successfully tested in a clinical pilot safety study two years later.¹⁰⁸ The first randomized multicenter study evaluating the efficiency of selective head cooling for neonatal HIE was the CoolCap trial, the results of which were published in 2005.¹⁰⁹ Even though that study only found significant improvement in neurological outcome in infants with moderate HIE, two other studies using whole-body hypothermia were published the same year, both of which found significant improvement in the risk of death or disability in infants with both moderate and severe HIE.^{110,111} The largest trial of whole-body hypothermia published interim results in 2008⁷ and the final outcomes in 2009.¹¹ After enrolling 325 infants, the TOBY study concluded that cooling to 33.5 C for 72 hours, if started within the first 6 hours of life, leads to improved neurological outcome in survivors, even though it could not identify significant reduction in the risk for death or severe disability which was the study's primary outcome. A subsequent meta-analysis of hypothermia trials, encompassing 1320 infants was necessary to demonstrate with adequate statistical power that moderate hypothermia consistently improves both survival and neurological outcome at 18 months of age in infants with moderate HIE, but this effect was still not significant for infants with severe HIE.¹⁰ Only in 2012 and 2013 could two revised meta-analyses conclude that infants with severe HIE also benefit from therapeutic hypothermia and unequivocally put hypothermia in the standard protocol of care for infants with HIE.^{112,113} Still, concerns about the long-term benefits of hypothermia persist. The school-age follow ups of all

three major clinical trials has already been published and while all suggest benefits of hypothermia in this age group as well, the studies were not powered to draw such conclusions with statistical significance.^{12,114,115} Undoubtedly, an ongoing data collection from many centers employing similar standard protocol for cooling is necessary to further understand the benefits and limitations of therapeutic hypothermia.

3.6 Mechanisms of action in therapeutic hypothermia

The mechanisms of hypothermic neuroprotection are several-fold. Hypothermia reduces cerebral metabolism by approximately 5% for every 1 °C decrease in body temperature.¹¹⁶ Hypothermia during HI delays the onset of anoxic depolarization, reduces the amount of EEAs and potentially suppresses ROS and RNS formation.⁵⁴ If hypothermia is initiated upon reperfusion/resuscitation, it appears to accelerate the normalization of extracellular EEAs, ROS sequestration and recovery of high-energy phosphates.¹¹⁷

Clinically, however, hypothermia treatment can be started realistically only a few hours after birth.¹¹⁸ The effect of hypothermia on secondary (and tertiary) injury is thus highly important. Microarray studies suggest that hypothermia can suppress a large number of gene responses to ischemia, many of which are involved in calcium homeostasis, cellular and synaptic integrity, inflammation and apoptosis.¹¹⁹ As described before, pro-apoptotic processes are incredibly complex and involve a large number of redundancies, therefore it is difficult to conduct and interpret studies including the manipulation of individual mediators. However, the caspase-dependent activation of apoptosis does generally converge on caspase-3 as a final executioner, which makes it a reasonably good indicator of upstream processes. Accordingly, delayed hypothermia was found to decrease caspase-3 activation after HI in the near-term fetal sheep.¹²⁰ This finding was confirmed in both rodent¹²¹ and in piglet models.¹²² There is also evidence from adult animal studies that post-reperfusion hypothermia can suppress the mitochondrial permeability transition and prevent the release of cytochrome-c and other pro-apoptotic mediators.¹²³

The inflammatory cascade induced by HI can cause injury via the activation of apoptotic cell death, or via direct damage from inflammatory cells, such as microglia or leukocytes.¹²⁴ This inflammatory signaling appears to be interrupted by hypothermia at a number of sites, as cooling was found to decrease the levels of various pro-inflammatory cytokines as well as to directly suppress microglial activation.¹²⁵

The fact that therapeutic hypothermia needs to be maintained for at least 48 hours in order to be effective suggests that a continued suppression of programmed cell death and inflammation is necessary until normal homeostasis can return.¹²⁶ Additionally, hypothermia results in suppressed cerebral hyperperfusion, edema formation and seizure activity that could all indicate or contribute to neuroprotection.⁷⁷ As these effects on a wide range of deleterious processes are combined, it is not hard to appreciate the level of protection offered by hypothermia. Hence it is reasonable to ask what may be the optimal setting for administering hypothermia in order to achieve maximal protection.

3.7 Application of therapeutic hypothermia

One important aspect of therapeutic hypothermia is the method of administration. One of the large clinical trials, the CoolCap study utilized selective head cooling,¹⁰⁹ which was previously successfully used in large animal models as well.⁵⁹ This method can produce selective head cooling with only mild systemic hypothermia. However, as discussed before, the CoolCap trial could only identify potential benefit of hypothermia in a selected subgroup of infants with HIE. Insight into the potential reasons for this was provided by piglet studies showing that different CNS structures have different optimum temperatures for protection.¹²⁷ In these experiments optimal protection for cortical neurons was achieved at 35 °C, while deep gray matter structures required 33.5 °C for maximal neuronal sparing. These results, together with previous studies showing that it is challenging to achieve adequate cooling of deep brain structures by surface cooling alone,¹²⁸ suggest that systemic hypothermia may offer a more reliable and effective protection.¹²⁹

Another important aspect of the administration of therapeutic hypothermia is the initiation and the length of cooling. Inclusion criteria of the large multi-center trials involved the initiation of cooling within 6 hours after birth.⁷ This time was chosen as a practical compromise between the clinical reality of birth asphyxia (many such children are born out-of-hospital and require transport to a tertiary center) and the results of animal studies, which showed that delayed hypothermia can be neuroprotective, but only if started before the occurrence of seizures, i.e. during the latent phase.¹³⁰ The length of cooling was set to 72 hours in order to achieve maximum protection, as preclinical studies indicated an inverse relationship between the depth and length of cooling necessary to achieve neuroprotection.¹³¹ 72 hours of cooling was found to be safe and effective in translational large animal models of HIE.⁵⁹ The potential benefits of stretching both ends of the cooling period are now being evaluated. Earlier start of cooling, for example during neonatal transport has been studied at a number of centers. It appears to be feasible and safe using servo-controlled cooling devices¹³² and while definitive results regarding neurological outcomes are still lacking, preliminary data suggest improved neuroprotection with earlier initiation of cooling.¹³³ On the other hand, the potential prolongation of cooling was evaluated in two recent clinical trials. One of these trials was halted interim due to the lack of observed benefit in survival from either 120 hours vs 72 hours of cooling, 32 °C vs 33.5 °C target temperature, or both.¹³⁴ The other trial examining the effect of late, prolonged cooling initiated between 6-24 hours after birth and maintained for 96 hours has not published its results yet.¹³⁵

Although these recent results show a lack of benefit with cooling to 32 °C instead of 33.5 °C, animal studies have indicated that deeper cooling might offer better protection.¹³⁶ However, it appears that there is a clear trade-off between the depth of cooling and the systemic-side effects of hypothermia. For example, deep hypothermia (15 °C) after cardiac arrest in the adult dog was found to be detrimental,¹³⁷ while moderate hypothermia (28-32 °C) offered no protection¹³⁸ and only mild hypothermia (34-36 °C) resulted in consistently improved neurological outcomes.¹³⁹ The question of whether deeper hypothermia might offer better protection is especially important now that a large number of physicians start to adopt therapeutic hypothermia as standard of care for infants with HIE, while currently no other neuroprotective interventions are

available. Therefore, it is essential to determine the optimal cooling temperature for infants with HIE.

3.8 Hypothermia as an endogenous protective mechanism

Even though recent successes with ‘artificial’ therapeutic hypothermia has raised a large amount of interest in the past decade, the first observations about ‘endogenous’ hypothermia as a response to hypoxia were made more than 50 years ago. In 1955 Cross, Tizard and Trythall observed that infants breathing 15% O₂ had reduced metabolic rate and heat production.¹⁴⁰ While their experiments could not show decreases in rectal temperature during the brief period of 15% hypoxia, another report showed that maintaining this O₂ level for a week resulted in a significant decrease in temperature as well.¹⁴¹ Instead of this approach of induced hypoxia, Burnard and Cross decided to study infants after birth asphyxia, as natural model of hypoxia and found that asphyxic babies had significantly reduced body temperatures (approx. 35 °C) for several hours after birth (**Figure 3A**).¹⁴² This finding has also been confirmed more recently in a trial of therapeutic hypothermia under low-resource settings in a developing country, where this ‘natural cooling’ was present in the normothermic asphyxia group for an average of 15 hours, despite attempts of active rewarming (**Figure 3B**).¹⁴³

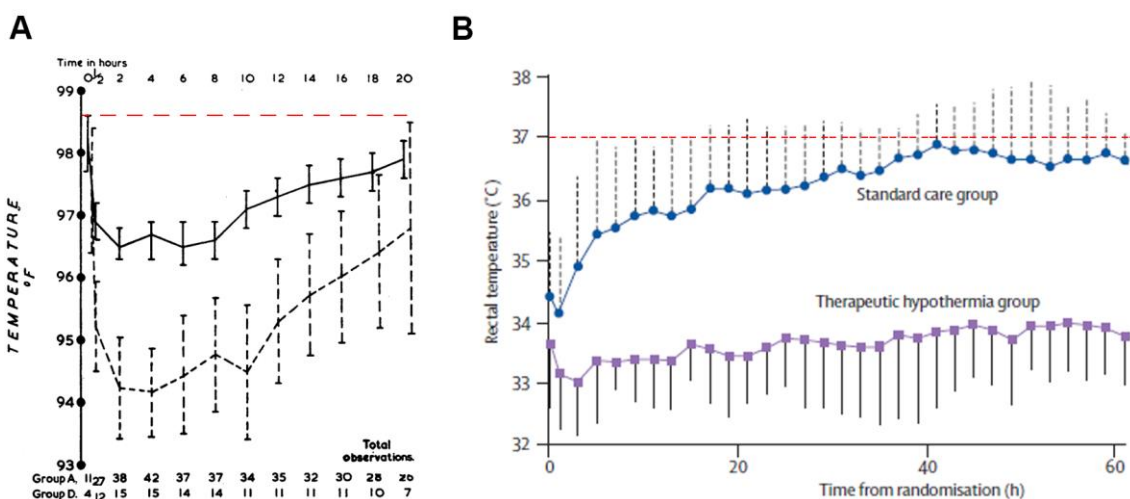


Figure 3: Endogenous hypothermia in neonates after birth asphyxia. (A) shows the seminal work of Burnard and Cross from 1958, where they demonstrated that asphyxic infants (dashed

line) had significantly lower rectal temperatures than healthy newborns (solid line) for several hours after birth.¹⁴² Note the lack of quick rewarming even in healthy newborns. Data is displayed as mean and 95% confidence intervals. (B) is adopted from a recent clinical trial of hypothermia in low-resource settings, which shows that the normothermic control asphyxia group (blue line) remained at sub-normal temperatures for several hours after resuscitation, despite efforts of rewarming.¹⁴³ Data is displayed as mean \pm SEM. The dashed red line shows 37 °C rectal temperature on both graphs. Adopted from Burnard and Cross, 1958¹⁴² and Robertson et al, 2008¹⁴³.

Following the initial human observations in the 1950s, hypoxic hypometabolism was also studied in newborn lambs, puppies, kittens and rats.^{144,145} It appeared that since many of these species are born at a lower degree of maturity, endogenous protection against accidental and transient hypoxia might be evolutionarily beneficial. In the pursuing decades experimental research about the mechanism and role of hypoxic hypometabolism have expanded greatly and this body of knowledge have been the subject of a number of excellent reviews.¹⁴⁶⁻¹⁵⁰

Neonatal responses to hypoxia, including hypometabolism and a biphasic ventilatory response have often been considered as peculiarities by physicians, comparing them to adult physiology. It is important to note, however, that these processes have very similar analogues in lower species, which possess a greater tolerance to hypoxia than adult mammals.¹⁴⁷ Studies confirmed that the decrease in metabolism in response to hypoxia is not due to the reduced oxygen availability, since lactate production was not increased, therefore anaerobic metabolism did not have to compensate for the reduced O₂-supply.¹⁴⁶ Another potential explanation of hypoxic hypometabolism might be the effect of decreasing temperature itself, which is known to slow down metabolism and result in a decreased metabolic rate. However, the drop in metabolic rate actually precedes the decrease in temperature, so hypothermia itself cannot cause the hypometabolism.¹⁴⁸ Additionally, it was found that the magnitude of hypometabolic response to hypoxia is primarily determined by the level of baseline metabolic rate, which in turn depends on several factors, including body size, age and ambient temperature.¹⁵¹ Since newborns have limited thermal insulation, they require a higher basal metabolic rate and heat production to maintain a stable body temperature.

Consequently, this also allows for a potentially higher magnitude of hypometabolic response compared to adults.¹⁵¹

In addition to the decreased metabolic rate and O₂ consumption, a decrease in thermogenesis was also found to be an important contributor to the hypoxic response.¹⁵² In newborns thermogenesis is primarily non-shivering and is mediated via brown adipose tissue (BAT), therefore this appears to be the center of this effect.¹⁴⁶ Hypometabolism and decreased thermogenesis by BAT can both contribute to endogenous hypothermia. A normal reaction to decreased body temperature in newborn animals could involve huddling by the pups, but this behavioral response is also suppressed during hypoxia.¹⁵³

In summary, the controlled decrease in O₂-consumption (hypometabolism) and the reduction of BAT- and behavioral thermogenesis together suggest a central neurohormonal regulatory mechanism closely coordinating these responses.¹⁴⁸ This theory is confirmed by the fact that denervation of peripheral chemoreceptors does not decrease the hypometabolism response.¹⁵² The regulatory mechanisms responsible for this coordination are still unknown, but the decreased concentration of O₂ not as a substrate, but as a messenger has been discussed repeatedly.¹⁴⁸ Another suggested mediator might be arginine-vasopressin (AVP, previously called anti-diuretic hormone or ADH),¹⁵⁴ an important stress-hormone in the perinatal period, the level of which is greatly increased in asphyxic babies.¹⁵⁵

While the exact mechanism of the controlled decrease in metabolism and body temperature is not clear, their synergistic role in protection from hypoxia is well-established, partially due to the mechanisms discussed in the previous parts. Accordingly, it is important to note that in a hypoxic newborn, artificial increase of body temperature (as standard postnatal care) results in tachypnea and a drop in systemic vascular resistance which would indicate the newborn's attempts to dissipate heat.¹⁵⁶ This suggests that the 'normothermic' temperature of an asphyxic newborn might be lower than that of a healthy neonate.

All this highlights the importance of taking a more physiological and evolutionary point of view by clinicians and taking into consideration the lessons learned from comparative physiology instead of solely relying on, and striving to achieve 'normal ranges' of physiological parameters, many of which are directly and

incorrectly extrapolated from adult standards. A similar lesson could be drawn from the slow acceptance of resuscitation with room air instead of 100% O₂, when the former practice is supported by a large body of physiological and experimental data, while the latter was a direct adaption of adult resuscitation guidelines.¹⁵⁷ Instead of considering hypothermia as an exogenous neuroprotective therapy, it might be more relevant to think of it as a measure supporting and securing an endogenous protective mechanism in order to maximize its benefits. This has over-arching implications for neonatologists working directly with infants just after suffering a period of asphyxia.

3.9 Endogenous hypothermia in preclinical research

As we have seen, most of our understanding regarding hypoxic hypometabolism and the resulting hypothermia comes from well-designed animal physiology studies from a number of species. Preclinical drug research, however, has mostly ignored this phenomenon. One example of this are the previous and highly successful animal studies using the NMDA-antagonist MK-801, which have failed to consider endogenous hypothermia resulting from the administration of the drug and thus included a significant confounder.^{46,158} There has been more than 500 neuroprotective agents which showed efficacy in preclinical trials but failed in the clinical translation process.⁴⁸ Therefore we should consider all possible sources of variation or noise potentially contributing to this unfortunate phenomenon. One such confounder is endogenous hypothermia.

As we have seen previously, hypoxic hypometabolism is a highly conserved mechanism, even though its magnitude depends on a number of factors, including postnatal age, weight and ambient temperature.¹⁴⁸ Thus, in order to exclude or control for the effect of hypoxic hypometabolism, one has to closely monitor both ambient temperature and rectal temperature in the experimental animals. A strict control of rectal temperature is generally part of protocols with large animal models, as these systems aim to closely mirror the clinical situation.¹⁵⁹ On the other hand, monitoring of rectal temperature in neonatal rodent models is more cumbersome, as it inevitably leads to significant stress and these animals generally have to be excluded from later analysis.

Some groups have adopted a sentinel monitoring method, where only one animal from the experimental group is monitored.¹⁶⁰ Others are attempting to assess thermogenesis directly by measuring skin temperature from above the inter-scapular BAT.¹⁶¹ Without direct measurement of core temperature or BAT thermogenesis, it becomes very difficult to closely monitor and control the thermal homeostasis of neonatal rodents, especially during hypoxia. Ambient temperature and baseline metabolic rate are major determinants of the magnitude of hypoxic hypometabolism.¹⁴⁸ Using higher ambient temperatures can minimize the baseline metabolic rate and thus could also minimize the magnitude of hypometabolic response.¹⁶¹ Ambient temperature monitoring, however, can be also challenging, as the type, response-rate and placement of the sensors likely varies greatly between laboratories.

Reviewing a set of publications citing the original Rice-Vannucci paper²⁴ one finds that some groups appear to use a strict control of rectal temperature,¹⁶² while others only monitor ambient temperature to some degree.¹⁶³⁻¹⁶⁵ This raises the possibility that imprecise control of experimental temperatures, without specific attention to the effects of hypoxic hypometabolism, could conceivably influence outcome and decrease the reliability and reproducibility of preclinical experimental findings.

4. OBJECTIVES

The objectives of this thesis can be summarized as follows:

- 1. To investigate the effects of cooling to different target temperatures in a preclinical piglet model of HIE. In particular, to examine the safety and efficacy of deep hypothermia (30 °C) utilizing clinical outcome markers and neuro-pathological indicators.**
- 2. To examine the confounding effect of inadvertent hypothermia during and after hypoxia in a rodent model of HIE.**
- 3. To characterize the novel rodent model of HIE adopted from Prof. Kai Kaila.¹⁶⁶**

5. METHODS

5.1 *Piglet experiments*

Detailed descriptions of methods and procedures can be found in the corresponding publications.^{122,159,167}

5.1.1 **Animal preparations**

All experimentation was conducted under UK Home Office Guidelines (Animals (Scientific Procedures) Act 1986) and approved by the institutional animal care and use committee of the University College London Biological Services and Institute of Neurology. Twenty-eight large white male piglets, less than <24 h old were anesthetized and surgically prepared as described previously.⁵⁷ Briefly, the animals were sedated by an intramuscular injection of midazolam (0.2 mg/kg) and arterial O₂ saturation monitoring was started (Nonin Medical, Plymouth, MA). Isoflurane anesthesia (4% vol/vol) was established via a facemask to facilitate tracheostomy and intubation. Isoflurane was maintained at 3% during surgery and at 2% afterwards. Piglets were mechanically ventilated so as to maintain temperature corrected arterial pressures of PaO₂ (8-13 kPa or 60-100 Hgmm) and PaCO₂ (4.5-6.5 kPa or 33-48 Hgmm). An umbilical venous catheter was introduced to administer maintenance fluids (10% dextrose, 60 ml/kg/d), fentanyl (3–6 µg/kg/h), and antibiotics (benzylpenicillin 50 mg/kg and gentamicin 2.5 mg/kg, every 12 h). An umbilical arterial catheter was also inserted for continuous monitoring of heart rate (HR) and mean arterial blood pressure (MABP) and for 6-h blood sampling to measure PaO₂, PaCO₂, pH, electrolytes, glucose, and lactate (Abbot Laboratories, UK). Bolus infusions of colloid (Gelofusin; B Braun Medical, Emmenbrucke, Switzerland) and inotropes (dopamine and dobutamine 5–20 µg/kg/min, noradrenaline 0.1–5 µg/kg/min) were used to maintain MABP above 40 mm Hg. Hyperglycemia of more than 10 mmol/l was treated by changing from 10% to 5% glucose infusion; hyperglycemia of more than 20 mmol/l was treated by changing to

saline infusion. Severe metabolic acidosis (base excess > -10 mmol/l) was corrected with sodium bicarbonate (8.4% wt/vol). All animals received continuous physiological monitoring (SA instruments, New York), and intensive life support throughout experimentation. Arterial lines were maintained by infusing 0.9% saline solution (Baxter, 1 ml/h) with heparin sodium (1 IU/ml) to prevent line blockage. Both common carotid arteries were surgically isolated at the level of the fourth cervical vertebra and encircled by remotely inflatable vascular occluders (OC2A; In Vivo Metric, Healdsburg, CA). After surgery, piglets were positioned prone in a plastic pod with their heads immobilized.

5.1.2 Induction of cerebral hypoxia-ischemia

A magnetic resonance spectroscopy surface coil was secured to the cranium, and the animal was positioned in a 9.4 Tesla Varian magnetic resonance spectrometer. While in the spectrometer, transient HI was induced by inflating the vascular occluders and reducing fractional inspired oxygen (FiO₂) to 12% (vol/vol). During HI, cerebral energetics were monitored every 2 min by phosphorus (³¹P) MRS, and the β-nucleotide triphosphate (β-NTP, consisting mainly of adenosine triphosphate) peak height was automatically measured. When β-NTP had fallen to 40% of baseline levels, FiO₂ was adjusted in order to stabilize β-NTP at that level for 12.5 min, after which the occluders were deflated and FiO₂ normalized. ³¹P spectra were acquired for a further 1 h to monitor recovery from HI. The time integral of the decrement of β-NTP/EPP (EPP = exchangeable phosphate pool = inorganic phosphate + phosphocreatine + high energy phosphates) during HI and the first 1 h of resuscitation quantified the acute energy depletion (AED) as described previously.⁵⁷

5.1.3 Experimental groups

Following HI and resuscitation piglets were randomized into four groups: (I) normothermia (rectal temperature (T_{rec}) 38.5 °C throughout), or whole-body cooling between 2–26 h after the insult to (II) T_{rec} 35 °C, (III) T_{rec} 33.5 °C, or (IV) T_{rec} 30 °C (n = 7 in all groups). Normothermic piglets were maintained at their target T_{rec} using a

heated water mattress above and below the animal; hypothermic piglets were cooled by reducing the water mattress temperature to their target Trec over 90 min, starting 2 h after HI. At 26 h after HI, cooled piglets were rewarmed to normothermia with a maximum speed of 0.5 °C/h using the water mattress. Forty-eight hours following HI, piglets were euthanized with pentobarbitol, the brain was fixed with cold 4% paraformaldehyde (PFA) via cardiac perfusion and removed along with major organs and processed for histology and immunohistochemistry.

5.1.4 Serum cortisol & troponin measurements, pathological examinations

The measurement of serum cortisol and troponin levels from arterial blood samples, as well as the pathological examination of the major organs were conducted by my colleagues. I only reference the corresponding publication here, which contain detailed methods description and discuss their results later on.¹⁵⁹

5.1.5 *In Situ* Hybridization, mRNA Quantification and immunocytochemistry

The investigation of brain injury using *in situ* hybridization and immunohistochemistry were conducted by my colleagues and our collaborators. Therefore, I only reference the relevant publications here, which contain the detailed description of methods and discuss the results briefly later on.^{122,167}

5.1.6 Data analysis

Three periods were defined for HR and MABP analysis to obtain information about ideal cooling kinetics: cooling induction (2 to 3.5 h post-insult), cooling maintenance (3.5 to 26 h post-insult) and rewarming/normothermia (26 to 48 h post-insult). Blood chemistry analysis was performed at baseline, at the end of HI, and at 12, 24, 36, and 48 h post-HI. Intergroup statistical comparison of physiological measures and blood chemistry used linear regression and one-way ANOVA with adjustment for group baseline differences. Secondary “pairwise” analyses comparing the 30 °C group to each

of the other groups included Bonferroni correction. Differences between the 30 °C group and the other groups are reported with a 98.3% confidence intervals (CI). For non-normally distributed data, the Kruskal–Wallis rank test was used. Results are mean (\pm SD) unless stated otherwise; statistical significance was assumed for $p < 0.05$.

5.2 *Rodent experiments*

5.2.1 Animal preparations

All experiments were carried out under the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary. Wistar rat pairs (Charles River Laboratories, Hungary) were time-mated and housed under 12/12 light-dark cycles in a temperature ($22 \pm 2^\circ\text{C}$) and humidity ($60 \pm 10\%$) controlled environment with ad libitum access to laboratory rat food (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water. Fathers were removed from the cages on E19 of the pregnancy. On the day of birth litters were culled to 8-12 pups. A total of 194 pups were used for the experiments. On postnatal day 3 (P3) pups were tattooed on their feet for identification. On P7 pups were separated from their dams (except for unseparated controls) and transferred to temperature-controlled chambers with individual cells maintained at $37 \pm 0.5^\circ\text{C}$ ambient temperature (except for pilot experiments, see below). The rectal temperature of one sentinel animal from each asphyxia group was continuously monitored (Physitemp Instruments, Clifton, NJ), which was later excluded from the analysis. Preliminary experiments showed that temperature variability within experimental groups was minimal, hence the sentinel temperature could be used as a reliable surrogate marker for the whole group. Following a 20-minute accommodation period the pups were subjected to control or asphyxic gas mixtures for 15 mins (room air or 4% O₂, 20% CO₂, 76% N₂, respectively) and were allowed to recover for 60 mins before returning to their dams. This model procedure was originally developed by Prof Kai Kaila's group.¹⁶⁸ Early outcome groups were sacrificed 3, 8 or 24 hours after the insult, while others were allowed to reach adulthood.

Surviving rats were weaned from their dams at P21 and housed in groups of three or four male animals in plastic cages measuring 59.5 × 38 × 22 cm (1354G, Eurostandard Type IV) prior to experiments. Females were excluded from behavioral experiments due to the large variability in behavioral phenotypes resulting from the estrous cycle. The animals were kept under reversed day–night schedule (lights off at 10:00 h, lights on at 22:00 h) since rats are naturally active during night hours. Habituation to the reverted light/dark cycle lasted at least 2 weeks.

5.2.2 Blood biochemistry and tissue cytokine measurements

Rats were sacrificed either on P7 (3 or 8 h post-HI), or on P8 (24 h post-HI) for cytokine and blood biochemistry analysis. Following intraperitoneal injection of ketamine-xylazine (100 mg/kg and 10 mg/kg, respectively) and subsequent thoracotomy, approximately 500 uL of blood was collected from the right ventricle into heparinized syringes and centrifuged at 4 °C, 200 RCF for 5 minutes. Serum was separated, frozen and stored until analysis.

After transcardial perfusion with phosphate-buffered saline (PBS), liver, kidney, spleen, lung and small intestine samples were collected. Urine was also collected when possible via direct bladder puncture. All samples were frozen and stored until analysis.

Serum samples were analyzed for Na⁺, K⁺, Cl⁻, glucose, carbamide, creatinine, total protein, albumin, GOT, GPT levels and urine samples for Na⁺, K⁺, Cl⁻, glucose, carbamide, creatinine, and total protein content.

Tissue samples were homogenized and analyzed for IL-6 and CXCL-1 levels using ELISA (DuoSet ELISA kits, R&D Systems, Minneapolis, MN), and IL-1 β and TNF- α levels using cytometric bead assays (CBA Flex Sets, BD Biosciences, New Jersey, NJ). Total protein content was quantified using the Pierce BCE Protein Assay (Thermo Scientific, Waltham, MA). All tests were carried out according to manufacturer's instructions.

5.2.3 Histology and immunohistochemistry

Rats were sacrificed either on P7 (3 h post-insult) or on P8 (24 h post-insult). Following

i.p. injection of a ketamine-xylazine mixture, the animals were perfused transcardially with PBS and cold 4% PFA. Brains were removed and placed in 4% PFA with 20% sucrose for 24 h post-fixation. Afterwards brains were transferred to 20% sucrose in PBS and cut on a sliding microtome within 48 hours. Sequential brain slices of 40 μ m thickness were kept in antifreeze solution at -20°C until staining. The following markers were used for immunohistochemistry: rabbit anti-IBA1 (1:500, Wako Chemicals, Germany), mouse anti-NeuN (1:1000, Merck Millipore, Darmstad, Germany), biotinylated lectin (1:100, Sigma-Aldrich - Merck, Darmstad, Germany), rabbit anti-cleaved caspase 3 (1:500, Cell Signaling Technology, Danvers, MA), rabbit anti-annexin-V (1:500, Novus Biologicals, Littleton, CO), rabbit anti-MBP (1:500, Abcam, Cambridge, UK), mouse anti-SMI-32 (1:1000, BioLegend, San Diego, CA), goat anti-ICAM-1 (1:250, R&D Systems, Minneapolis, MN), rabbit anti-NF κ B (1:500, Abcam, Cambridge, UK), rabbit anti-c-FOS (1:500, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-HIF-1a (1:500, R&D Systems, Minneapolis, MN), mouse anti-CD-45 (1:200, BD Biosciences, New Jersey, NJ). After washing and blocking with 2% normal goat or donkey serum (plus 0,3% H₂O₂ for peroxidase quenching in case of 3,3'-Diaminobenzidine staining), incubation with primary antibodies was done overnight. Slices were washed and incubated with secondary antibodies (either fluorescent or biotin-conjugated) for 2 hours. After washing, fluorescently labelled slices were mounted on glass slides and covered with Moviol solution under cover slips. 3,3'-Diaminobenzidine (DAB) staining was completed by incubating the slices with avidin-biotin complex for 1 hour and exposure to DAB for 20 minutes. Slices were mounted on glass slides and covered with gelatine solution under cover slips.

Histological analysis was performed blindly by me and two other experimenters, using standard light microscopy for DAB stained slices and epifluorescent or confocal microscopy for fluorescently labelled slices. Positive cells were manually counted on all the slices which contained the pre-designated areas of interest.

5.2.4 Behavioral testing

Each animal performed in 2 behavioral tests, while weighing 250–400 g (at the age of 40–100 days). All testing was conducted during the early hours of the dark phase under

dim red illumination, except for the Delayed Discounting test.

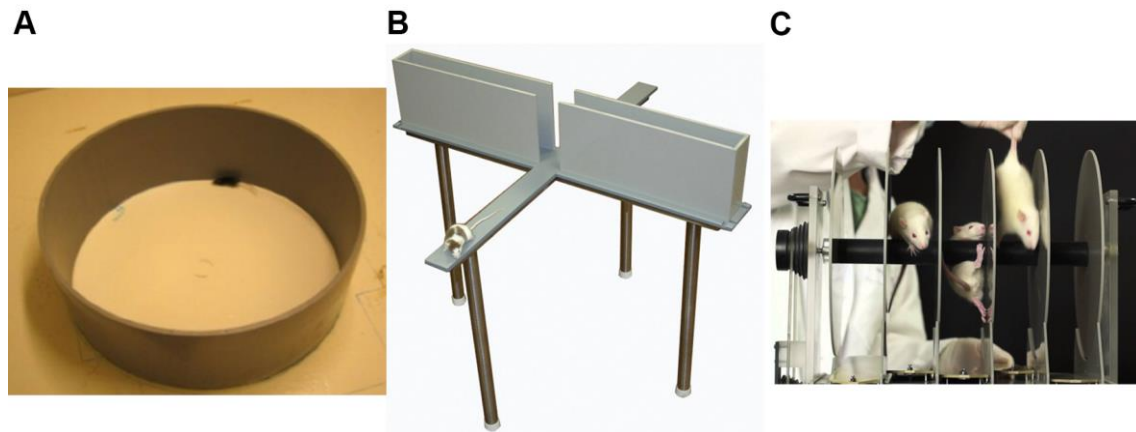


Figure 4: Illustrations of neuromotor tests. (A) Open Field; (B) Elevated Plus Maze; (C) Rotarod.[†]

The neuromotor test paradigms are illustrated on **Figure 4**. The Open Field (OF) test was used to evaluate locomotion and anxiety. In this test, the animals are placed in the middle of a round box and their behavior is observed for 10 minutes. The OF box was a round wooden area (diameter 90 cm) surrounded by a metal wall (40 cm), painted dark grey.

The elevated plus-maze (EPM) test was also used to investigate locomotion and anxiety. In this test, the animals are placed in the middle of a plus-shaped maze and their behavior is observed for 5 minutes. The EPM field was made of wood (painted dark grey) and consisted of two opposite open arms (50 × 20 cm) and two enclosed arms (50 × 20 × 30 cm), elevated to a height of 80 cm above the floor. The junction area of the four arms (central area) measured 20 × 20 cm. Rats were placed on the central area facing a closed arm at the beginning of the test.

For both tests, the testing apparatus was cleaned thoroughly between subjects with wet and dry towels. Behavior was video-recorded by an overhead light sensitive camera placed 2 m above the testing apparatus and analyzed by a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

Motor coordination was assessed using a Rota-rod apparatus (InsightR, Brazil)

[†] Image sources: (A) Spruijt BM et al, J Neurosci Methods. 2014 ¹⁶⁹; (B) <http://www.stoeltingco.com>; (C) <https://www.youtube.com/watch?v=v56MtrmWAs0>

according to Rojas et al.¹⁷⁰ In this test, the animals are taught to maintain their balance on a rotating horizontal rod (3 cm diameter). Animals were exposed to one habituation session in five trials of maximum 3 min each with the apparatus on slow velocity (4 rpm). In the test session, 24 h later, animals' motor ability was evaluated. The rota-rod test was performed by placing rats on the rotating drums and measuring the time each animal was able to maintain its balance on the rod. The speed of the rota-rod accelerated from 4 to 25 rpm over a 3 min period. Variables recorded were: latency of the first downfall, number of falls (maximum 5).

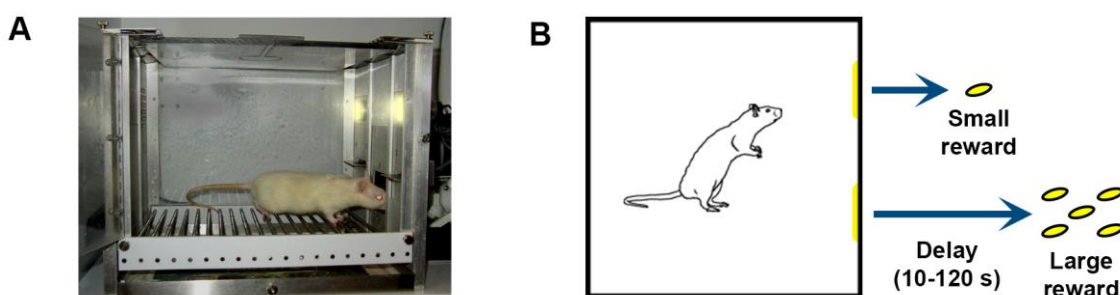


Figure 5: The Operant Learning – Delayed Discounting test. (A) Photograph showing the Operant learning apparatus (“Skinner Box”). (B) The procedure of the Delayed Discounting test.

The delay discounting test (DD) was used to assess learning and impulsive behavior (**Figure 5**). This test was conducted using automated operant chambers equipped with two nose-poke holes with infrared sensors and LED lights, a chamber light and a feeder device with a magazine into which food pellets were dropped (Med Associates, St. Albans, VT, USA). Chambers were placed inside sound-attenuated wooden cubicles and were controlled via computers running Med-PC IV software (Med Associates, St. Albans, VT, USA). Rats were always trained and tested in the same operant box because even small changes in the environment can have deleterious effects on the training. After each session ended, the chambers were cleaned with tap water and were dried with paper towels. All experiments were conducted in the early hours of the dark phase.

5.2.4.1 Delayed Discounting procedure

A schematic drawing of the test paradigm is shown in **Figure 5B**. The feeding of

subjects was restricted throughout the study to increase their motivation for food rewards. During the training phase, animals were placed inside a chamber for 30 min daily for 5 days. A response on one of the nose-poke holes was rewarded with one 45 mg food pellet (small reward), while a response on the other hole resulted in five 45 mg food pellets (large reward). Both types of reward were presented immediately after the response and were followed by a 25 s timeout (TO) with the chamber light switched on. Chamber light was used as a cue which could be associated with the reward after responding on one of the nose-poke holes.¹⁷¹ During the timeout period, responses were not rewarded but were registered. To avoid side preference, the nose-poke hole on which responding was rewarded with five food pellets was randomly assigned to either the left or the right side between animals. Animals were placed in the same chamber with the same nose-poke hole side assignment throughout the experiment. At the end of the training phase, the animals were expected to respond on the nose-poke hole that was paired with the large reward in approximately 90% of all trials.¹⁷¹

After two days of rest, the animals underwent the test phase. During this phase, each animal was placed in a chamber for 30 min daily for 8 days. The procedure was similar to that described for the training phase, but a delay was inserted before the large reward (**Figure 5B**). The delay was fixed for each daily session and was increased progressively over subsequent days (10, 20, 30, 45, 60, 80, 100 and 120 s). Responses during these delays were not rewarded, but they were recorded by the software. Sessions of the test phase were conducted at the same time as sessions of the training phase. During the test phase, subjects were expected to shift their preference from the nose-poke hole rewarded by the delayed large reward to the nose-poke hole rewarded by the immediate small reward.¹⁷¹

During the training sessions, we recorded the preference of the nose-poke hole paired with the large reward (large reward preference) to assess learning capabilities. Increases of greater magnitude in large reward preference indicated quicker learning. During the test phase, large reward preferences were indicative of non-impulsive choices. This variable is negatively associated with choice impulsivity, which refers to an inability to prefer a larger, delayed reward over an immediate smaller one.¹⁷² The number of inadequate responses (the sum of responses during timeouts and delays), which reflects the number of premature, impulsive responses, was also evaluated. With

this measure, we were able to assess motor impulsivity, which is defined as the inability to inhibit inappropriate actions.¹⁷²

All behavioral tests were analyzed by automated tracking software (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands) or the H77 event recorder software.

5.2.5 Statistical analysis

Data were processed and analyzed using the GraphPad Prism 5 software. Where normal distribution could be assumed, Student's t-test was used and the data is displayed as mean + standard error. In cases where normality could not be assumed, the Mann-Whitney U-test and the Kruskal-Wallis rank test were used and data is displayed as median + inter-quartile range. Where in addition to treatment, another repeated factor was also present, repeated-measures two-way ANOVA was used with post-hoc testing by Dunett's method. Significance was assumed with $p < 0.05$.

6. RESULTS

6.1 Cooling to different target temperatures in a piglet model of HIE

These experiments were conducted at the University College London, UK, using a preclinical piglet model of neonatal HIE.

6.1.1 General characteristics

We found no differences between the groups regarding body weight, postnatal age, insult severity or baseline physiological and biochemical parameters (**Table 2**). Six piglets cooled to 30 °C suffered an episode of cardiac arrest and five of these died before 48-h post-HI due to a combination of pulmonary edema, fluid in the thorax or abdominal cavities, severe metabolic derangement, or profound hypotension. One animal cooled to 33.5 °C suffered two cardiac arrests but survived to 48 h. There was one recorded incidence of persistent arrhythmia in the 30 °C group.

Table 2: Physiological parameters for piglets in each temperature group. Mean (SD) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and post-hoc analysis were carried out on comparisons between groups with Tukey's and Dunnett's method. Time zero was set at the start of resuscitation after the hypoxic–ischemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 min of resuscitation. AED, acute energy depletion; HI, hypoxia–ischemia; HR, heart rate; MABP, mean arterial blood pressure; Trec, rectal temperature; a: $P < 0.05$ vs. normothermia at the same time point or during the same time period; b: $P < 0.05$ vs. 35 °C at the same time point or during the same time period; c: $P < 0.05$ vs. 33.5 °C at the same time point or during the same time period; d: $P < 0.01$ in cross-group comparisons.

| Variables | Normothermic | 35 °C | 33.5 °C | 30 °C |
|--------------------------|--------------|-------------|-------------|-------------|
| Postnatal age (h) | 22.3 (1.2) | 22.6 (1.1) | 22.7 (0.9) | 22.5 (1.1) |
| Body weight (g) | 1771 (132) | 1786 (90) | 1714 (146) | 1864 (180) |
| AED (h, insult severity) | 0.07 (0.04) | 0.05 (0.03) | 0.06 (0.04) | 0.08 (0.07) |
| HR (bpm) | | | | |

| | | | | |
|--------------------------|------------|--------------------------|----------------------------|------------------------------|
| Baseline | 158 (26) | 162 (32) | 152 (28) | 144 (21) |
| End of insult | 182 (28) | 184 (30) | 187 (32) | 172 (29) |
| 2-3.5 h after time zero | 155 (28) | 130 (26) | 132 (30) | 130 (11) |
| 3.5-26 h after time zero | 156 (15) | 121 (28) | 114 (21) | 123 (16) a |
| 26-48 h after time zero | 151 (22) | 146 (33) | 140 (17) | 149 (4) |
| MABP (mmHg) | | | | |
| Baseline | 51 (6) | 53 (7) | 53 (9) | 51 (6) |
| End of insult | 51 (10) | 59 (16) | 58 (11) | 55 (6) |
| 2-3.5 h after time zero | 45 (6) | 43 (5) | 44 (8) | 47 (7) |
| 3.5-26 h after time zero | 51 (6) | 48 (6) | 47 (6) | 43 (7) |
| 26-48 h after time zero | 52 (6) | 51 (7) | 47 (6) | 48 (11) |
| Trec (°C) | | | | |
| Baseline | 38.4 (0.4) | 38.2 (0.6) | 38.1 (0.8) | 38.4 (0.4) |
| End of insult | 38.2 (0.4) | 38.1 (0.3) | 38.1 (0.4) | 38.2 (0.4) |
| 6-26 h after time zero | 38.5 (0.4) | 34.9 (0.4) a d | 33.2 (0.5) a b d | 30.2 (0.4) a b c d |
| 26-48 h after time zero | 38.4 (1.3) | 37.7 (1.2) | 37.0 (1.8) a b d | 34.4 (2.6) a b c d |

6.1.2 Cardiovascular parameters

During the induction of cooling and the rewarming/normothermia phases, HR and MABP were similar in all of the groups (**Table 2**). The baseline heart rate averaged over all groups was 154 (17) beats per minute (bpm). In the 35 °C and the 33.5 °C groups, HR was unchanged during hypothermia as well, but in the 30 °C group, HR was significantly lower during cooling than in normothermic animals ($p < 0.001$, **Figure 6A**).

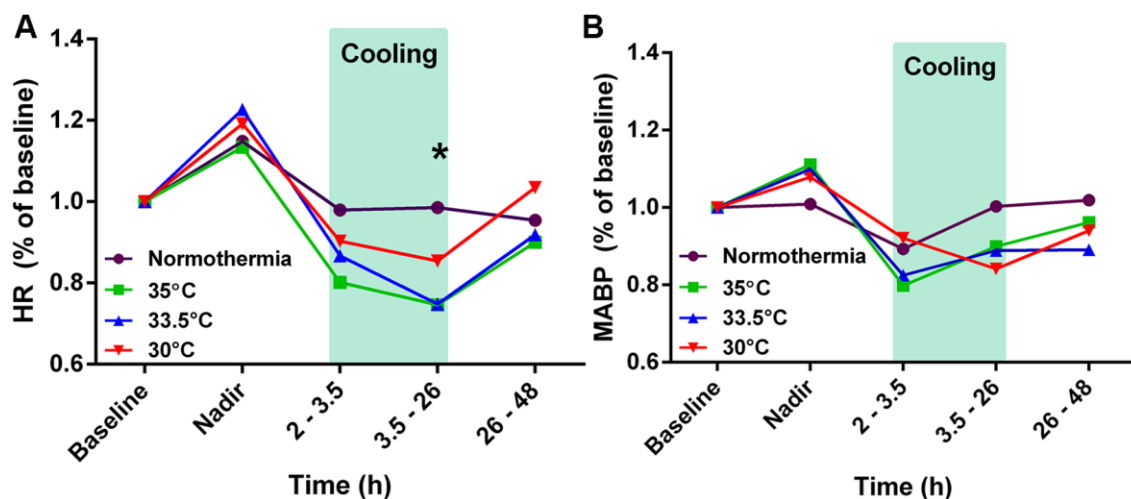


Figure 6: Summary of heart rate and mean arterial blood pressure changes. Mean % change in (A) heart rate (HR) and (B) mean arterial blood pressure (MABP) during and after 24-h whole-body hypothermia for each temperature group: 38.5 °C (normothermia, purple circles), 35 °C (green squares), 33.5 °C (blue triangles) and 30 °C (red upside-down triangles). The blue box represents the duration of the cooling period. Error bars are omitted for clarity. *Significant difference between the 30 °C and the normothermia groups ($p < 0.0001$). Nadir: hypoxia mid-point; 0 h: end of hypoxia-ischemia.

We found periods of profound hypotension (~30 mm Hg) in the 30 °C group that lasted for several hours. These periods usually preceded a cardiac arrest and were generally seen in the five 30 °C animals which died before 48 hours. However, when averaged over the analysis periods, these hypotensive episodes did not alter the overall picture significantly and the MABP of the 30 °C animals was similar to the other cooling groups (**Figure 6B**).

Two of the normothermic animals required volume replacement after HI; these animals required no inotropes. The median overall volume replacement (saline and Gelofusin combined) was significantly higher in the 30 °C group compared to the normothermic group ($p = 0.05$, **Table 3**). The median dopamine infusion dose over 48 h was higher in the 30 °C group than in the normothermia ($p = 0.01$) or in the 33.5 °C hypothermia ($p=0.05$) groups. The median dopamine infusion dose was also higher in the 33.5 °C group compared to normothermic animals ($p = 0.05$). Additionally, multiple inotrope infusions (dopamine, dobutamine and noradrenaline) were required during hypothermia and rewarming in the 30 °C group, while only dopamine use was

necessary in all other groups.

Table 3: Summary of volume replacement and inotrope use. Median (Interquartile range) total volume replacement (ml/kg) and inotrope dose (ug/kg/min) are shown over the 48-hour period following hypoxia-ischemia according to each temperature group. Kruskal-Wallis equality-of-populations rank test was used and a '0' value was assumed for periods when no inotropes were given; a: $p < 0.05$ vs normothermia at the same time point or during the same time period; b: $p < 0.05$ vs 35°C at the same time point or during the same time period.

| | Normo-thermic | 35 °C | 33.5 °C | 30 °C |
|-----------------------------------|----------------------|--------------|-----------------------------|--------------------------------|
| Volume replacement (ml/kg) | 15 (0, 31) | 19 (0, 38) | 18 (0, 82) | 70 (46-108) a |
| Inotropes (ug/kg/min) | | | | |
| Dopamine | 0 (0, 0) | 0 (0, 4.8) | 5.5 (0.7, 11.1) a | 13.8 (8.2, 18.6) a b |
| Dobutamine | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) | 16.0 (0, 18.7) |
| Noradrenaline | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) | 0.1 (0, 0.3) |

6.1.3 Changes in blood biochemistry parameters

Blood pH was lower in the 30 °C group at 36 h compared to the normothermic group ($p = 0.05$) and also at 12 h with borderline *post hoc* significance ($p = 0.05$, **Table 4** and **Figure 7A**). Base deficit was increased in the 30 °C group at 12, 24 and 36 h compared to all other groups (all $p < 0.05$, **Table 4** and **Figure 7B**), while it was similar among all other groups at these time points.

Table 4: Blood gas variables for piglets in each temperature group. Mean (SD) or median (Interquartile range) values are presented. Linear regression with adjustments to baseline and one-way ANOVA and post-hoc analysis was carried out on comparisons between groups with Tukey's and Dunett's method. Time zero was set at the start of resuscitation. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 min of resuscitation. HI, hypoxia-ischemia; PaCO₂, partial arterial pressure of carbon dioxide; PaO₂, partial arterial pressure of oxygen; a: $P < 0.05$ vs. normothermia at the same time point or during the same time period; b: $P < 0.05$ vs. 35 °C at the same time point or during the same time period; c: $P < 0.05$ vs. 33.5 °C at the same time point or during the same time period; d: $P < 0.01$ in cross-group comparisons; e: $p < 0.05$ within group comparisons vs baseline; f: $p < 0.05$

within group comparisons vs 24h after time zero.

| Variables | Normo-thermic | 35 °C | 33.5 °C | 30 °C |
|----------------------|-------------------------|-----------------------|----------------|-------------------------|
| PaO2 (kPa) | | | | |
| Baseline | 9.3 (1.1) f | 10.5 (1.7) | 9.4 (1.9) | 8.3 (1.7) |
| nadir of the insult | 3.3 (1.0) e f | 3.1 (1.1) e | 3.1 (1.2) | 3.0 (1.0) e |
| 12 h after time zero | 10.3 (1.2) | 8.8 (1.2) | 9.2 (5.3) | 8.0 (2.9) |
| 24 h after time zero | 11.2 (2.6) e | 12.3 (6.3) | 9.8 (3.3) | 7.5 (2.2) |
| 36 h after time zero | 10.9 (2.2) | 10.0 (3.6) | 11.5 (4.5) | 8.9 (1.7) |
| 48 h after time zero | 11.7 (2.2) | 11.6 (3.0) | 14.2 (6.3) | 11.7 (1.9) |
| PaCO2 (kPa) | | | | |
| Baseline | 5.46 (0.74) | 5.26 (1.05) | 5.26 (0.65) | 4.98 (0.76) |
| nadir of the insult | 4.36 (0.49) | 4.88 (1.22) | 5.27 (0.94) | 5.02 (0.82) |
| 12 h after time zero | 5.29 (0.93) | 4.81 (0.71) | 6.17 (0.86) | 5.83 (2.13) |
| 24 h after time zero | 4.23 (1.03) | 6.08 (1.71) | 4.96 (1.34) | 4.82 (2.43) |
| 36 h after time zero | 5.29 (1.11) | 5.68 (1.36) | 6.80 (1.26) | 6.82 (1.35) |
| 48 h after time zero | 5.21 (1.28) | 5.15 (0.71) | 6.15 (1.43) | 4.47 (0.51) |
| pH | | | | |
| Baseline | 7.49 (0.04) | 7.50 (0.09) | 7.52 (0.06) | 7.51 (0.07) |
| nadir of the insult | 7.47 (0.09) | 7.44 (0.05) | 7.43 (0.16) | 7.44 (0.08) |
| 12 h after time zero | 7.57 (0.08) | 7.54 (0.09) | 7.45 (0.05) | 7.37 (0.20) |
| 24 h after time zero | 7.59 (0.09) | 7.45 (0.14) | 7.48 (0.14) | 7.37 (0.28) |
| 36 h after time zero | 7.47 (0.10) | 7.45 (0.10) | 7.37 (0.10) | 7.29 (0.11) a |
| 48 h after time zero | 7.45 (0.11) f | 7.47 (0.07) | 7.38 (0.09) | 7.52 (0.09) |

| | | | | |
|---------------------------------|-------------------------------|------------------------------|------------------------------|----------------------------------|
| Base Excess (mmol/L) | | | | |
| Baseline | 8.2 (3.6) | 7.8 (2.1) | 9.6 (5.4) | 7.5 (4.5) |
| Nadir of the insult | 0.7 (5.4) e f | 0.0 (4.0) e f | 2.2 (8.0) | 1.2 (3.0) |
| 12 h after time zero | 11.7 (3.3) | 9.7 (1.0) | 9.3 (2.7) | 0.6 (8.8) a b c d |
| 24 h after time zero | 8.5 (3.7) | 7.2 (2.6) | 3.6 (5.5) | -5.5 (8.8) a b c d |
| 36 h after time zero | 6.1 (3.0) | 5.6 (1.5) | 3.6 (3.8) | -3.1 (6.0) a b c |
| 48 h after time zero | 3.6 (4.0) | 5.0 (2.6) | 2.1 (4.0) | 4.5 (5.4) |
| Lactate (mmol/L) | | | | |
| Baseline | 3.4 (2.6, 4.3) | 3.7 (2.9, 4.0) f | 2.7 (2.4, 5.5) | 3.3 (2.8, 3.8) |
| Nadir of the insult | 7.6 (6.47, 8.5) e f | 7.4 (6.7, 8.4) e f | 6.9 (6.0, 9.7) e f | 8.2 (6.7, 9.0) |
| 12 h after time zero | 1.8 (1.3, 1.9) | 1.6 (1.1, 1.9) e | 1.3 (0.7, 1.7) | 2.2 (1.7, 10.0) |
| 24 h after time zero | 2.4 (1.6, 3.0) | 1.6 (0.9, 1.7) e | 1.8 (1.5, 3.3) | 3.6 (2.1, 12.0) |
| 36 h after time zero | 1.0 (0.8, 1.7) | 1.5 (1.5, 1.6) | 1.1 (1.0, 1.4) | 2.7 (1.5, 3.8) |
| 48 h after time zero | 1.3 (1.2, 1.4) | 1.3 (1.1, 2.1) e | 1.2 (1.1, 1.8) | 2.6 (1.8, 3.2) |
| Glucose (mmol/L) | | | | |
| Baseline | 7.5 (6.1, 8.1) f | 6.8 (6.1, 7.5) | 6.2 (5.7, 6.5) | 7.6 (6.9, 8.3) f |
| Nadir of the insult | 9.8 (8.3, 10.4) e f | 7.8 (7.7, 8.9) | 8.4 (7.5, 9.8) | 8.5 (8.5, 9.5) |
| 12 h after time zero | 5.2 (5.0, 6.2) e | 5.5 (4.4, 7.0) | 9.3 (4.7, 10.7) | 17.2 (9.7, 28.7) a b e |
| 24 h after time zero | 5.4 (4.8, 6.2) e | 5.4 (4.5, 9.6) | 5.5 (5.1, 8.5) | 23.9 (12.2, 25.9) e |
| 36 h after time zero | 4.9 (4.1, 6.2) | 6.4 (6.1, 7.4) | 5.5 (4.3, 11.6) | 15.9 (4.8, 15.9) |
| 48 h after time zero | 4.8 (3.9, 5.2) | 4.9 (4.7, 5.2) | 4.3 (3.0, 5.5) | 5.8 (4.3, 6.4) f |

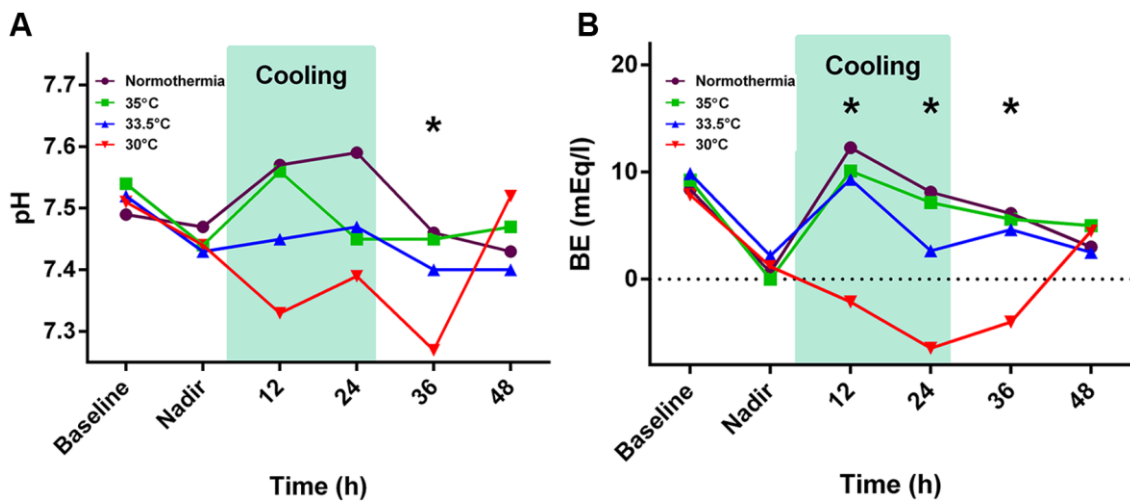


Figure 7: Summary of blood pH and base excess values. Mean blood pH (A) and base excess (B) are displayed at baseline, nadir, 12, 24 36 and 48 h post-HI for each temperature group: 38.5 °C (normothermia, purple circles), 35 °C (green squares), 33.5 °C (blue triangles) and 30 °C (red upside-down triangles). The blue box represents the duration of the cooling period. Error bars are omitted for clarity. *Significant difference between the 30 °C and all other groups ($p < 0.05$) at 12, 24 and 36 h. Nadir: hypoxia mid-point; 0 h: end of hypoxia-ischemia.

Blood glucose was significantly higher in the 30 °C group at 12 h compared to either the normothermic or the 35 °C groups ($p = 0.05$ in both cases). Since findings of hyperglycemia were always treated according to clinical protocols outlined in the Methods section, the glucose levels of the 30 °C group normalized by the end of the cooling period at 24 h (**Table 4** and **Figure 8A** and **B**). Blood lactate reached its highest levels at 24 h in the 30 °C group, but it was not significantly different from the other groups (**Table 4** and **Figure 8C** and **D**). We found lower blood potassium levels in the 30 °C group at 12 h as compared to the 35 °C and the 33.5 °C groups ($p = 0.01$ and $p = 0.05$, respectively). Hematocrit was found to be higher in the 30 °C group at 24 h compared to the normothermic and 33.5 °C groups ($p = 0.05$ in both cases) and hemoglobin was also greater in the 30 °C group at 12 h as compared to the 35 °C group ($p = 0.05$) and at 24 h as compared to the 33.5 °C group ($p = 0.05$, **Table 5**).

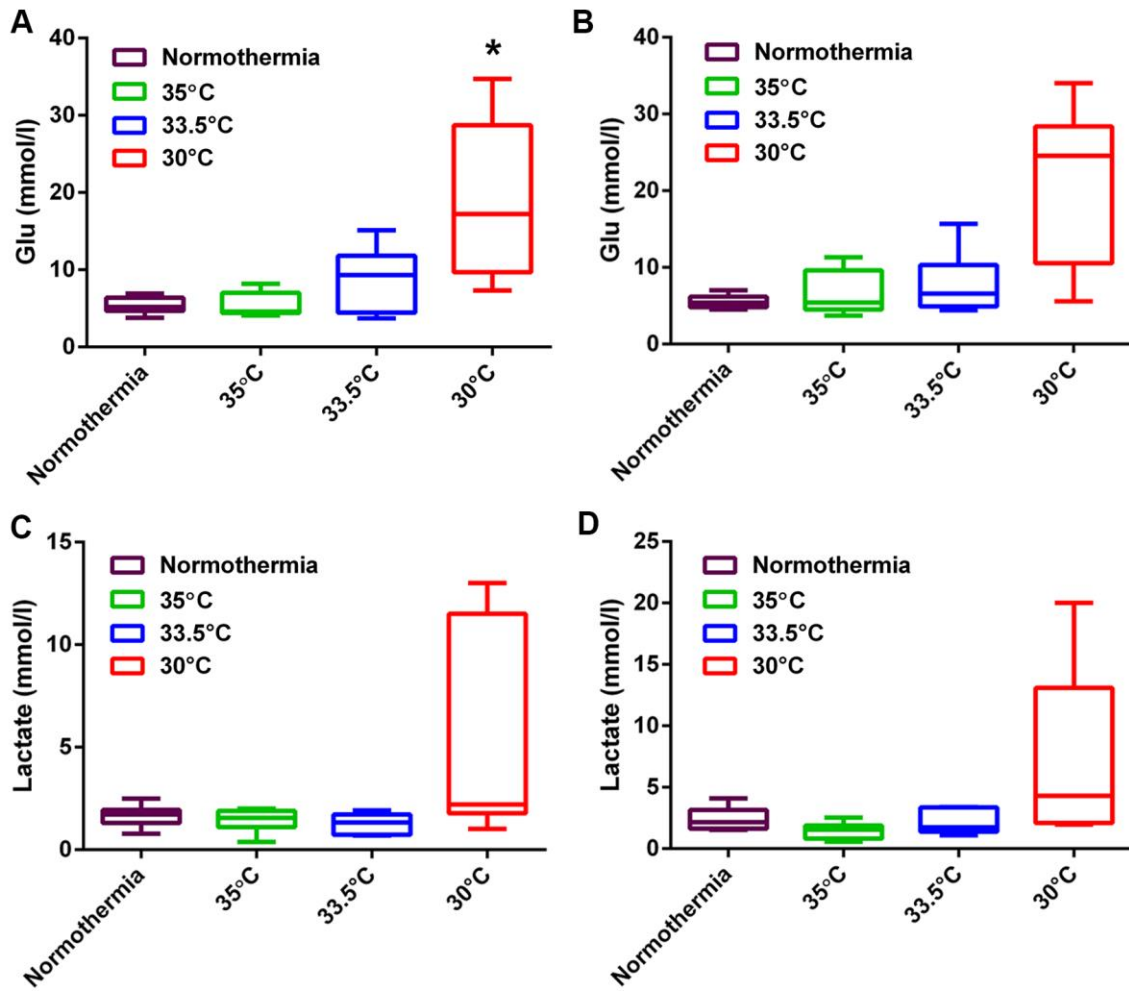


Figure 8: Glucose and lactate values. Levels of median (A & B) blood glucose and (C & D) lactate are shown 12 and 24 h after HI. *Significant difference between the 30 °C group and the normothermia and 35 °C groups for glucose only ($p < 0.05$). HI: hypoxia-ischemia. Values are displayed as median & quartiles.

Table 5: Blood chemistry for piglets in each temperature group. Mean (SD) or median (Interquartile range) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and post-hoc analysis was carried out on comparisons between groups with Tukey's and Dunnett's method. Time zero was set at the start of resuscitation after the hypoxic–ischemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 min of resuscitation. HI, hypoxia–ischemia; a: $P < 0.05$ vs. normothermia at the same time point or during the same time period; b: $P < 0.05$ vs. 35 °C at the same time point or during the same time period; c: $P < 0.05$ vs. 33.5 °C at the same time point or during the same time period.

| Variables | Normothermic | 35 °C | 33.5 °C | 30 °C |
|---------------------------|---------------------|--------------|----------------|---------------------------|
| Sodium (mmol/L) | | | | |
| Baseline | 133.4 (3.4) | 131.3 (3.6) | 130.2 (2.6) | 130.7 (2.9) |
| Nadir of the insult | 132.0 (3.6) | 131.3 (4.7) | 130.2 (3.7) | 130.8 (3.5) |
| 12 h after time zero | 128.3 (3.2) | 125.7 (3.6) | 126.3 (2.7) | 128.7 (8.5) |
| 24 h after time zero | 127.9 (3.0) | 124.3 (3.6) | 125.0 (3.7) | 124.6 (6.9) |
| 36 h after time zero | 128.4 (6.9) | 122.8 (5.7) | 125.4 (2.2) | 127.1 (3.3) |
| 48 h after time zero | 128.1 (8.0) | 125.8 (1.9) | 126.1 (4.5) | 130.3 (3.6) |
| Potassium (mmol/L) | | | | |
| Baseline | 3.76 (0.69) | 4.49 (1.11) | 4.96 (0.54) | 3.87 (1.42) |
| Nadir of the insult | 3.92 (0.82) | 4.55 (1.15) | 5.22 (1.07) | 3.68 (1.10) |
| 12 h after time zero | 4.95 (0.77) | 5.76 (0.71) | 5.25 (0.77) | 3.89 (0.92) |
| 24 h after time zero | 4.77 (0.89) | 5.31 (1.24) | 5.47 (1.32) | b c 4.38 (1.13) |
| 36 h after time zero | 4.93 (0.09) | 6.02 (0.14) | 5.84 (0.14) | 5.14 (0.28) |
| 48 h after time zero | 5.36 (1.03) | 5.08 (1.44) | 5.60 (1.53) | 4.75 (0.50) |
| Chloride (mmol/L) | | | | |
| Baseline | 99.6 (3.3) | 99.7 (2.8) | 99.4 (3.2) | 97.5 (2.5) |
| Nadir of the insult | 101.0 (4.4) | 100.7 (4.0) | 99.8 (2.9) | 97.4 (2.9) |
| 12 h after time zero | 98.3 (3.9) | 96.9 (2.8) | 96.5 (3.2) | 97.3 (8.1) |
| 24 h after time zero | 97.7 (3.9) | 94.7 (3.1) | 97.4 (1.9) | 95.0 (2.3) |
| 36 h after time zero | 100.1 (7.0) | 95.8 (4.0) | 98.0 (2.3) | 100.0 (5.8) |
| 48 h after time zero | 100.0 (7.4) | 96.3 (2.4) | 99.0 (2.3) | 101.0 (3.7) |
| Hematocrit | | | | |
| Baseline | 28.6 (10.6) | 24.6 (3.3) | 24.7 (4.7) | 23.6 (6.0) |
| Nadir of the insult | 22.8 (4.0) | 29.6 (9.3) | 29.6 (9.3) | 28.4 (5.7) |
| 12 h after time zero | 23.8 (7.4) | 22.3 (4.4) | 27.7 (3.3) | 29.3 (10.4) |
| 24 h after time zero | 22.6 (3.4) | 27.6 (5.8) | 22.6 (5.1) | 31.7 (6.7) |

| | | | | |
|----------------------------|-------------|-------------|-------------|-------------|
| | | | | a c |
| 36 h after time zero | 21.0 (3.8) | 22.4 (3.2) | 19.4 (5.0) | 23.4 (5.5) |
| 48 h after time zero | 22.0 (4.5) | 23.5 (7.3) | 18.4 (5.3) | 19.3 (5.6) |
| Hemoglobin (g/dL) | | | | |
| Baseline | 6.7 (3.6) | 8.3 (1.1) | 8.4 (1.6) | 8.1 (2.0) |
| Nadir of the insult | 7.8 (1.4) | 7.8 (1.4) | 10.0 (3.2) | 9.6 (1.9) |
| 12 h after time zero | 8.1 (2.5) | 7.6 (1.5) | 9.4 (1.1) | 11.1 (2.2) |
| | | | | b |
| 24 h after time zero | 7.7 (1.2) | 9.4 (2.0) | 7.7 (1.7) | 10.8 (2.3) |
| | | | | a |
| 36 h after time zero | 7.1 (1.3) | 7.6 (1.1) | 6.6 (1.7) | 8.0 (1.9) |
| 48 h after time zero | 7.5 (1.6) | 8.0 (2.5) | 6.7 (1.4) | 6.6 (1.9) |
| Creatinine (mmol/l) | | | | |
| Baseline | 0.60 (0.13) | 0.59 (0.07) | 0.65 (0.24) | 0.56 (0.07) |
| Nadir of the insult | 0.58 (0.17) | 0.53 (0.08) | 0.64 (0.18) | 0.52 (0.16) |
| 12 h after time zero | 0.73 (0.27) | 0.90 (0.16) | 0.95 (0.31) | 0.80 (0.26) |
| 24 h after time zero | 0.94 (0.61) | 1.20 (0.23) | 0.99 (0.55) | 1.2 (0.42) |
| 36 h after time zero | 1.17 (0.61) | 1.38 (0.26) | 1.50 (0.61) | 1.24 (0.18) |
| 48 h after time zero | 1.53 (0.73) | 1.54 (0.79) | 1.57 (0.71) | 1.00 (0.37) |

Shivering episodes unrelated to seizures occurred with similar frequency in all groups. The cortisol measurements conducted by my colleagues did not reveal any differences between the groups at any timepoints, although there was a trend-like increase of cortisol levels 2 h after HI and a similar decrease at 12 h (during the cooling phase) after HI. The cardiac troponin-I measurements conducted by my colleagues showed decreased levels in the 30 °C group compared to other groups over the 12-48 h period following HI ($p \leq 0.01$). The figures for this data can be found in the original publication.¹⁵⁹

6.1.4 Ex vivo investigations

These studies were conducted by my colleagues and collaborators using the tissue samples generated from the experiments detailed before. Macroscopic organ pathology was evaluated by an expert pathologist. Any kind of macroscopic organ pathology was noted in 25%, 33%, 50% and 33% of the animals in the normothermic, 35 °C, 33.5 °C

and 30 °C groups, respectively (**Table 6**). Patchy sinusoidal congestion of the liver was the most common pathological finding across all groups. Evidence of pneumonia was observed in all groups except the 30 °C group and vacuolated kidneys in all but the normothermic group. The occurrences of acute tubular necrosis and liver steatosis were limited to the 33.5 °C and the 30 °C group only. No evidence of macroscopic pathology was noted in the hearts of the 33.5 °C and the 30 °C group. No organ pathology was noted in two naïve piglets, who did not undergo any experimental procedures.

Table 6: Macroscopic organ pathology following hypoxia-ischemia and survival to 48 hours according to temperature group. A subset of 27 piglets and 2 naïve piglets organs (lungs, liver, kidney, spleen, pancreas and heart) were assessed for macroscopic pathology (x4 and x40 magnification) and incidence of remarkable pathology per individual were noted. * Only 4 hearts were available for analysis (33.5 °C, 30 °C, 2x naïve).

| Group | Heart * | Lung | Liver | Kidney | Spleen and pancreas |
|-----------------------|-------------------|-------------------|---|---|------------------------------------|
| 38.5 °C n=6 | n/a | 1x pneumonia | No pathology seen | no pathology seen | no pathology seen |
| 35°C n=7 | n/a | 1x pneumonia | no pathology seen | vacuolated | no pathology seen |
| 33.5°C n=7 | No pathology seen | 3x pneumonia | 2x steatosis | vacuolated 2x acute tubular necrosis | no pathology seen |
| 30°C n=7 | no pathology seen | no pathology seen | 1x steatosis 1x acute tubular necrosis | vacuolated 1x acute tubular necrosis | Severe patchy necrosis in pancreas |
| Naïve n=2 | no pathology seen | no pathology seen | no pathology seen | no pathology seen | no pathology seen |

The in situ hybridization studies were conducted by our collaborators. The results were published elsewhere and the reader is referred to that article for the detailed description.¹⁶⁷ Briefly, these studies focused on eight mRNA transcripts relevant for perinatal brain injury (BDNF, MANF, GFAP, MAP2, HSP70, NgR, LDH-A, LDH-B) and found that most of them were significantly affected by HI. Hypothermia counteracted this affect to a certain degree in most brain areas. In some instances it was

indicated that cooling to 33.5 °C conferred the most benefit when compared to 35 °C and 30 °C. Deep hypothermia to 30 °C made the effect of HI even more severe in certain brain regions.¹⁶⁷

The histological and immunohistochemical analysis of the collected brain samples were performed by my colleagues and published separately; the details of this study can be found in that article.¹²² Briefly, markers of necrotic and apoptotic neuronal death as well as microglial activation markers showed that cooling to 35 °C and 33.5 °C both effectively ameliorated hypoxic-ischemic brain damage, while this effect was almost completely lost with 30 °C hypothermia.¹²²

6.2 Investigating endogenous hypothermia in a rodent model of HIE

These experiments were conducted at the Institute of Experimental Medicine, Budapest, Hungary, using a novel rodent model of neonatal HIE adopted from Prof Kai Kaila's group.¹⁶⁶

6.2.1 Effect of ambient temperature on neonatal asphyxia tolerance

During our pilot experiments, we first examined the effect of ambient temperature on the asphyxia tolerance of neonatal rat pups. We found that chamber water temperature was the best surrogate marker for ambient temperature, as various air temperature monitors displayed significantly different values and were slow to respond. We used three chamber temperature ranges: 30-31 °C, 33-34 °C, and 36-37 °C. We also performed one experiment at room temperature (21.2 °C) without heating, but excluded it due to the lack of observable signs of distress (gasping, laborious breathing, respiratory arrest) after 45 minutes of asphyxia. All experiments were conducted under otherwise similar conditions. The neonatal ventilatory response to hypoxia consists of the following stages: (1) immediate hyperventilation; (2) hypoventilation and gasping; (3) fast and shallow gasping; (4) terminal apnea.¹⁷³ The asphyxic insult was terminated when at least two animals entered the third stage of respiratory failure and this duration was considered the experimental group's asphyxia tolerance.

We found that on average, insult duration could be extended to 45 minutes at 30-31 °C, 27.5 minutes at 33-34 °C and 15 minutes at 36-37 °C ambient temperatures (**Figure 9A**). Since these were pilot experiments and the 30-31 °C and 33-34 °C groups involved only 3 and 2 sentinel animals respectively, this study was not powered to determine statistical differences between the temperature groups. Considering the fixed temperature environment of intrauterine asphyxia, where the fetal temperature cannot decrease below the maternal,¹⁷⁴ we chose to conduct all subsequent experiments at 37 ± 0.5 °C chamber temperatures.

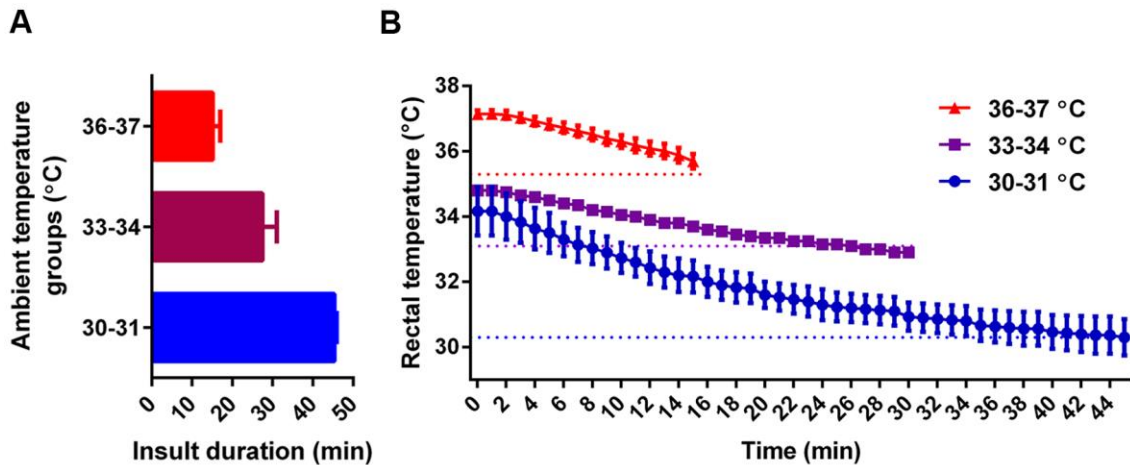


Figure 9: Summary of the preliminary temperature experiments. (A) shows the average length of the asphyxic insult in ambient (chamber) temperatures between 36-37 °C ($n=20$), 33-34 °C ($n=2$) and 30-31 °C ($n=3$). (B) shows the observed reduction of rectal temperature in the different ambient temperature groups during asphyxia. The dotted line shows the average chamber temperature. Values are displayed as mean + SEM.

6.2.2 Role of endogenous hypothermia

In the 36-37 °C group the average chamber temperature in the preliminary experiments was 35.3 (1.44) °C. Upon transfer to the chambers, pups had a Trec of 33.8 (0.97) °C. At the end of the 20 min baseline accommodation period, the mean Trec was 37.1 (0.5) °C. During 15 minutes of asphyxia, we observed a gradual decrease of mean Trec to 35.8 (1.03) °C (**Figure 9B**), consistent with the hypoxic hypometabolism response. A similar decrease in rectal temperature during asphyxia was observed in both other temperature groups (**Figure 9B**).

In order to determine the influence of chamber temperature on this phenomenon, we calculated the difference between the chamber temperature and the Trec for each litter. At the beginning of asphyxia, the animals were on average 1.78 (1.13) °C warmer than the set chamber temperature. However, this difference was significantly reduced by the end of asphyxia (0.48 (1.14) °C, $p < 0.0001$, **Figure 9B**, dotted red line). We have also made similar observations in the other ambient temperature groups, consistent with hypoxic hypometabolism and endogenous hypothermia in this paradigm.

6.3 Characterization of a novel rodent model of HIE

Following the experiments concerning endogenous hypothermia, we proceeded to characterize this novel rodent model of neonatal HIE.

6.3.1 Preliminary experiments

We have conducted a set of preliminary experiments in order to identify the most sensitive biomarkers of HI injury. Serum and urine samples did not reveal any significant differences between control and asphyxia samples. Cytokine analysis of tissue homogenates displayed high variability and no discernable trends between the two groups. We also conducted preliminary immunohistochemistry using a number of antibodies, which are widely employed in perinatal asphyxia research. Some of these antibodies did not show significant alterations in response to asphyxia (eg. CD-45, Annexin-V, HIF-1), and therefore were not used for randomized experiments. Other antibodies, which appeared to be more sensitive to asphyxia (eg. IBA-1) were included in the randomized experiments.

6.3.2 Randomized experiments

Pups were randomized into control or asphyxia groups on P7. Chamber temperature was set to 36.5 – 37.5 °C. The baseline characteristics of the animals were similar, as summarized in **Table 7**. The higher portion of males used are due to the fact that behavioral analysis can be most reliably performed on males and thus we attempted to exclude females when litters were culled. The asphyxia group had an overall mortality of 20.7 % (17/82) during asphyxia, while no animals died after reoxygenation. The control group had no mortality during the observation period.

Table 7: Baseline characteristics of the experimental animals used in the studies. Values are displayed as total number or mean (SD).

| | Control | Asphyxia | p-value |
|-------------------|----------------|-----------------|---------|
| Number of animals | 68 | 82 | |
| Male:female ratio | 43:25 | 51:31 | 1.000 |

| | | | |
|---------------------------------|-------------|-------------|-------|
| Weight (g) | 15.1 (2.04) | 15.7 (2.18) | 0.157 |
| Mortality during the experiment | 0 | 17 | |

6.3.3 Early brain histology

Brains collected 24 h after HI were used to investigate early brain injury after HIE. Nissl staining was conducted first in order to assess overt neuronal death throughout various brain regions (**Figure 10**). No areas of severe neuronal death or necrosis could be identified.

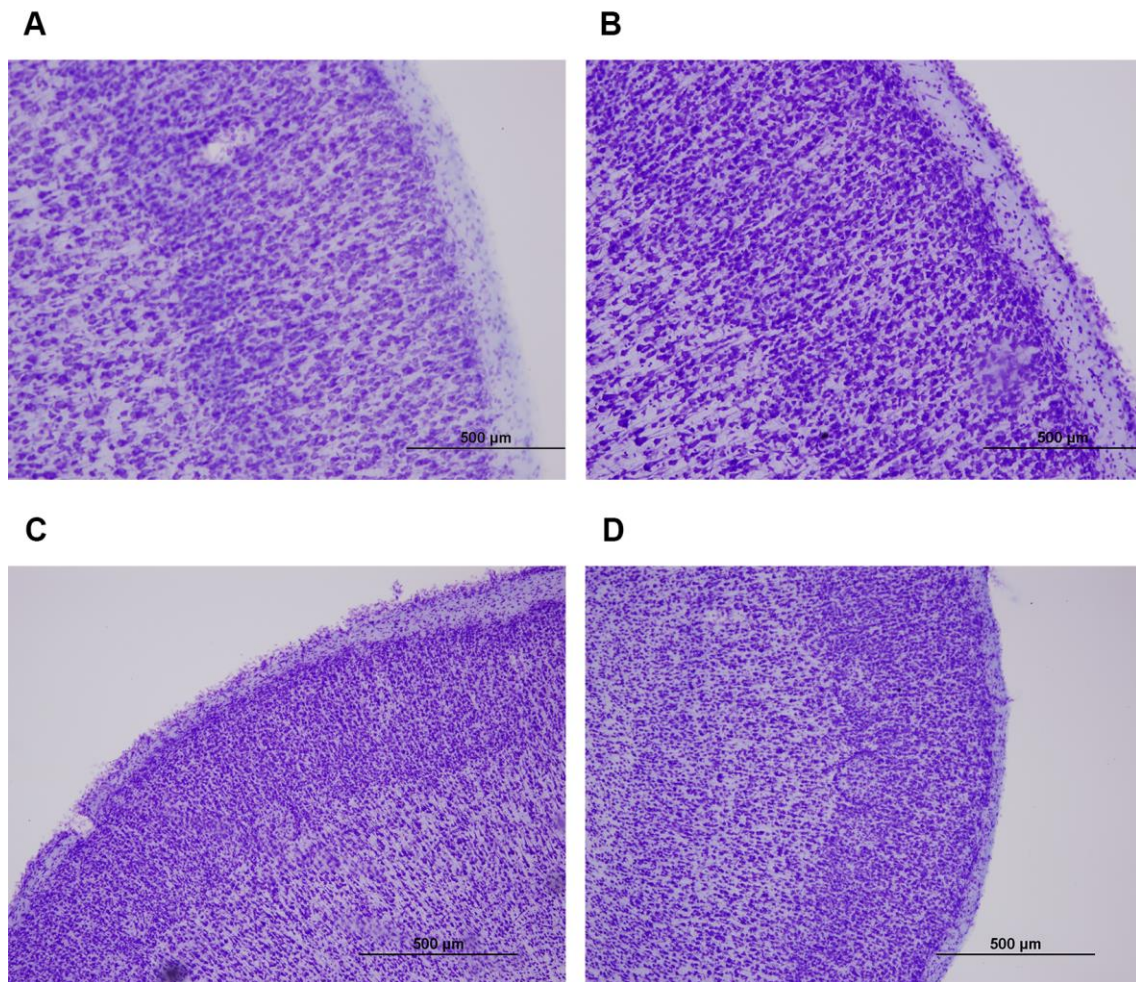


Figure 10: Nissl staining. Representative photomicrographs of Nissl staining from the (A-C) control and the (B-D) asphyxia groups. All images show intact cortical structure in the somatosensory cortex.

In addition to neuronal death, white matter damage and axonal injury was assessed using MBP and SMI-32 immunohistochemistry, respectively (**Figure 11**). We found no difference between the control and the asphyxia group with either staining.

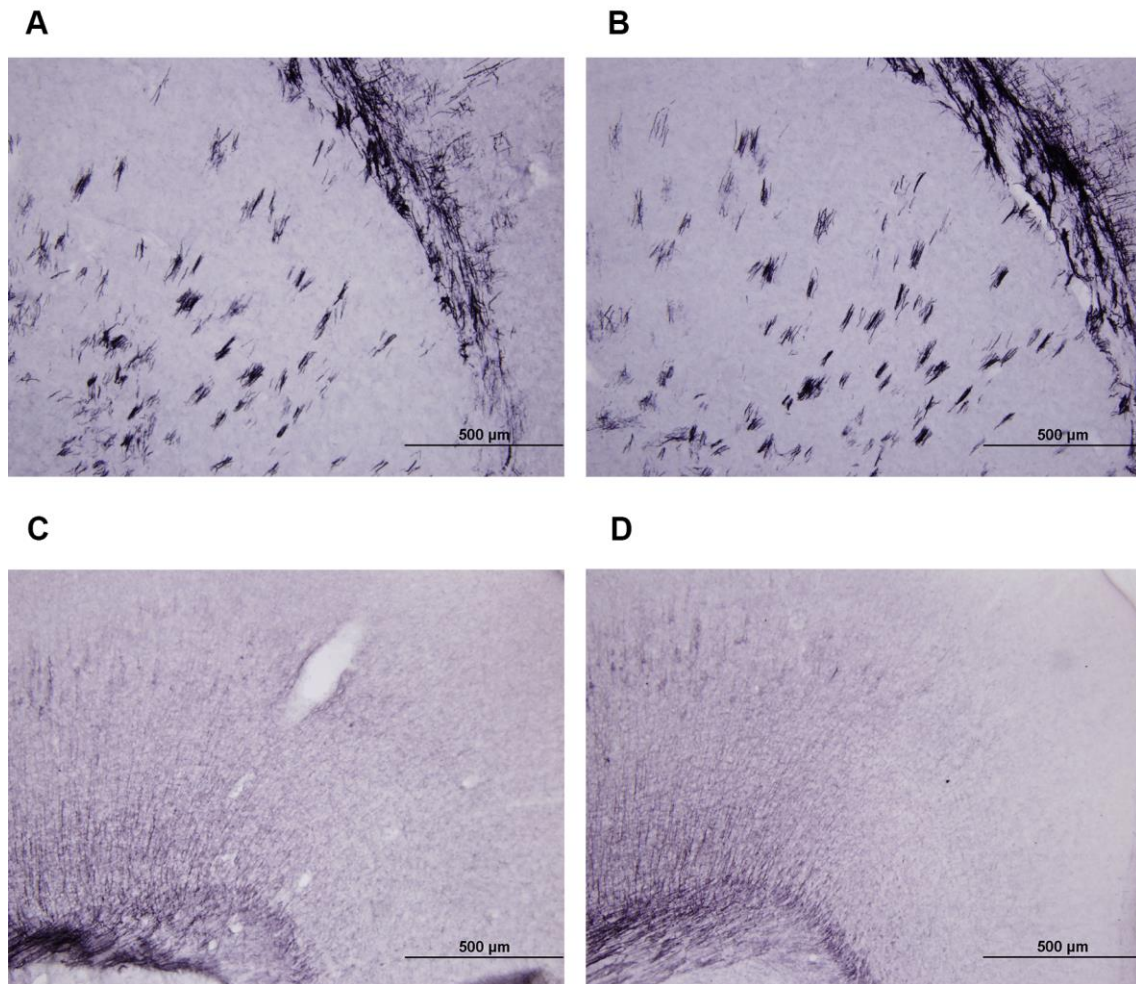


Figure 11: MBP and SMI-32 immunohistochemistry. (A-B) MBP immunohistochemistry of the striatum and external capsule showing no difference between (A) control and (B) asphyxic animals. (C-D) SMI-32 staining showing no signs of axonal injury in either the (C) control or the (D) asphyxia group. MBP: myelin basic protein; SMI-32: Neurofilament H Non-Phosphorylated.

In order to investigate inflammatory effects of HIE, IBA-1 immunohistochemistry was used to study microglial activation (**Figure 12**). We found an overall increase in the number of activated IBA-1 positive microglia in both the prelimbic- and infralimbic areas of the prefrontal cortex (PrL and IL, respectively), as well as in the cornu

ammonis and dentate gyrus of the hippocampus (CA and DG, respectively).

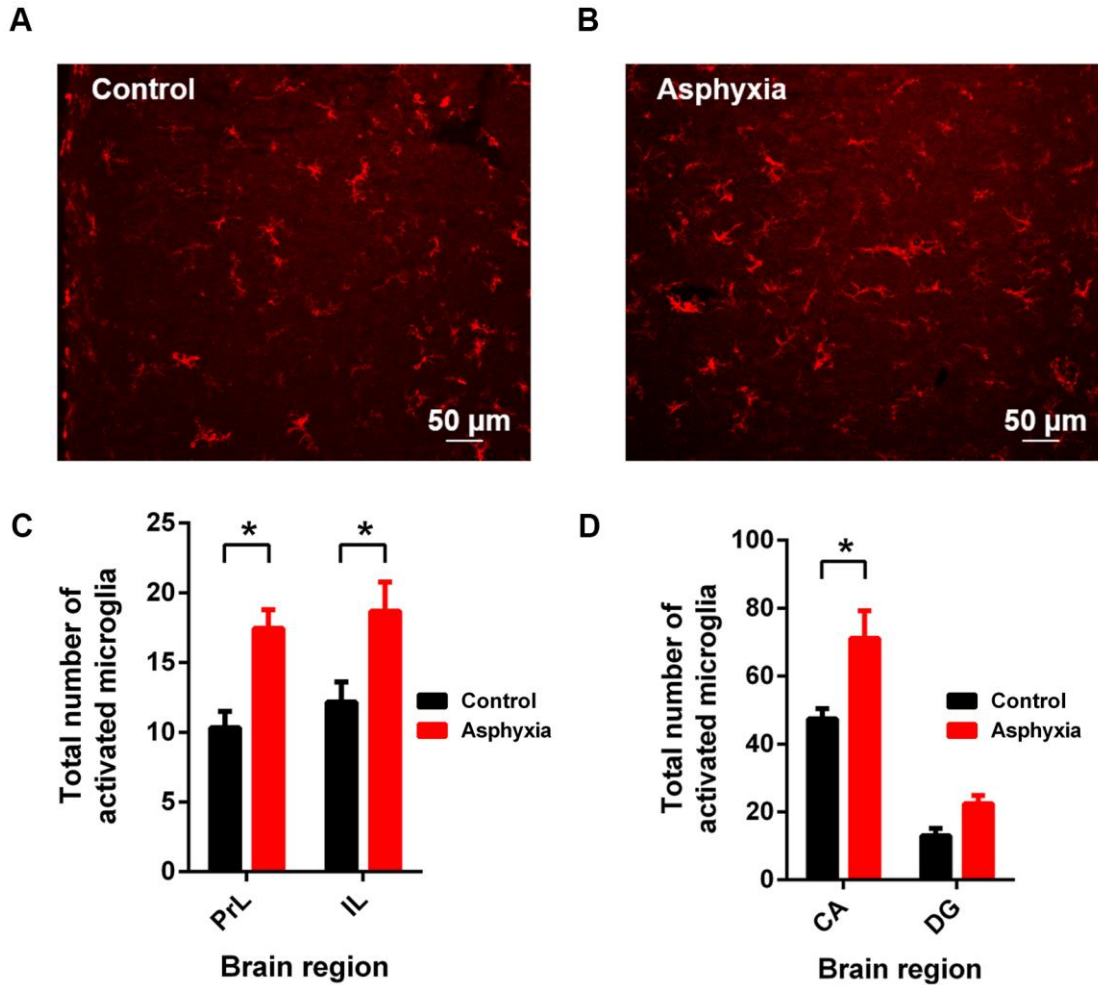


Figure 12: IBA-1 immunohistochemistry. (A-B) Representative photomicrographs showing an increased number of IBA-1 positive microglial cells in the hippocampus. (C-D) Quantification of the increased microglial activation in regions of the prefrontal cortex (PrL and IL) and the hippocampus (CA & DG), respectively ($n=5-10$ per group). *Significantly increased number of IBA-1 positive microglial cells in the asphyxia group compared to the control group ($p < 0.05$). The quantification of prefrontal microglial staining was conducted by my colleague, Barbara Orsolits. Values are displayed as mean + SEM. PrL: prelimbic cortex; IL: infralimbic cortex; CA: cornu ammonis; DG: dentate gyrus.

Brains collected 3 h post-HI were also stained for cFOS and NF κ B in order to potentially identify regions activated directly by asphyxia-induced stress. Early investigations using cFOS and NF κ B did not reveal significant differences between the groups in the studied brain regions. While we were able to observe trend-like

differences in certain areas, due to the high level of variance in both groups, no significant conclusions could be drawn.

6.3.4 Behavioral tests

First, in order to identify the most relevant control group for behavioral studies, we conducted the sensitive elevated plus maze test (EPM) using two control groups (unseparated and separated from the dam), in the interest of investigating the potential effect of maternal separation in addition to asphyxia. **Figure 13A-B** shows the results of the EPM test. In terms of the number of open arm entries, which is an indicator anxiety, (**Figure 13A**), we found that maternal separation had an effect of comparable size as asphyxia, even though this was not statistically significant. Since this could possibly become a confounding factor in these behavioral experiments and since there was no way to remove this confounder (i.e. conduct asphyxia without maternal separation), we opted to use maternally separated controls for all later experiments.

The results of the Open Field test are shown in **Figure 13C-D**. We found no significant differences in locomotion between the control and the asphyxia group in either the EPM (number of closed arm entries, **Figure 13A**) or the OF tests (total distance travelled, **Figure 13D**). In the EPM test the asphyxia group displayed a lower ratio of open arm entries compared to the separated control group (**Figure 13B**), indicating increased anxiety after asphyxia. Both groups spent similar time in the center in the OF test (**Figure 13C**). The Rotarod test also did not display any differences in motor function between the two groups (**Figure 13E**).

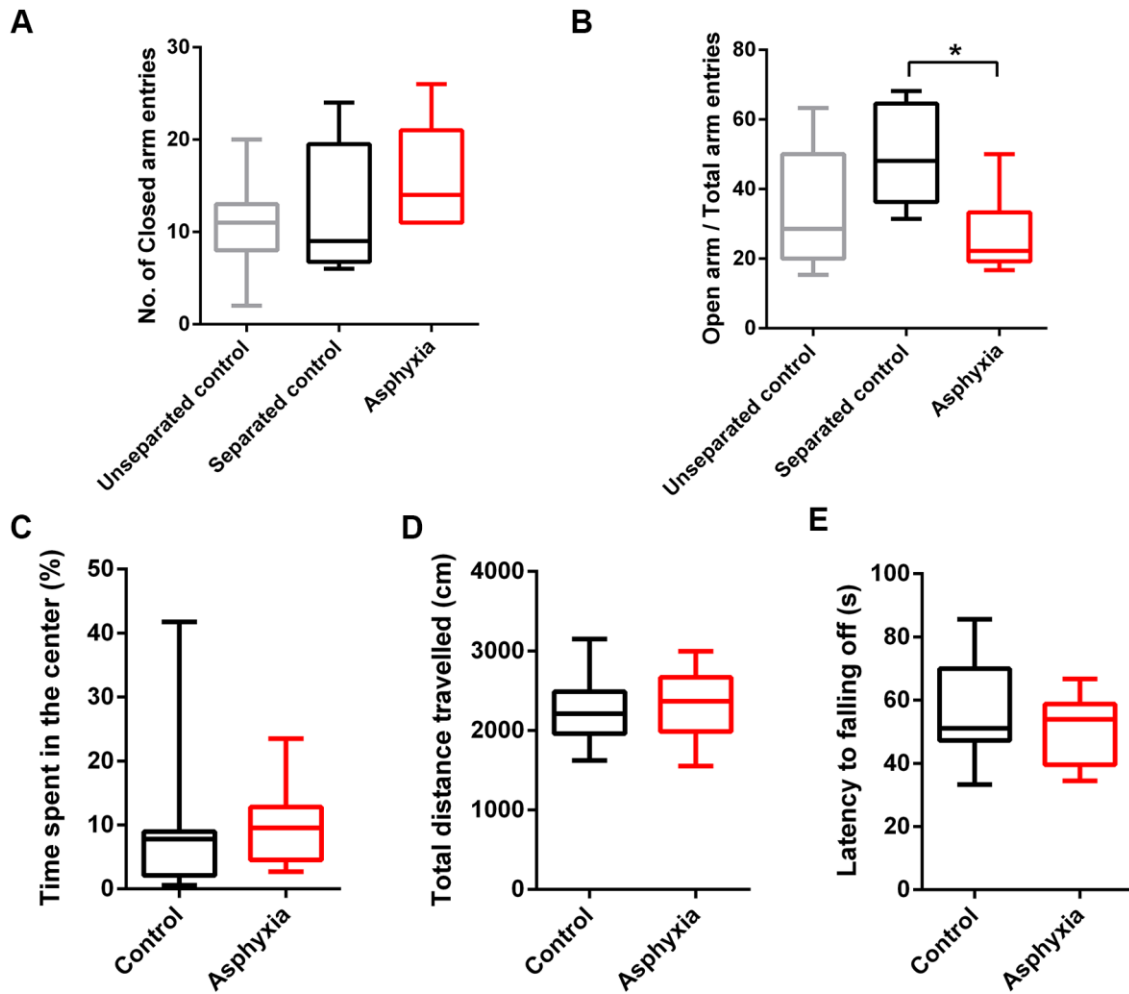


Figure 13: Summary of the Elevated Plus Maze, Open Field and Rotarod tests. (A) The number of closed arm entries was similar between all groups in the EPM test. (B) The asphyxia group showed a decreased ratio of open arm entries compared to the maternally separated controls in the EPM test ($p < 0.05$). (C) In the Open Field test, the control and asphyxia groups spent similar time in the center, (D) and travelled similar distances in total. (E) The Rotarod test did not reveal any significant difference in the latency to falling off between the two groups. Values are displayed as median & quartiles.

We used the Delayed Discounting paradigm to investigate learning and impulsivity in the model. During the training phase of the operant learning paradigm, we did not find significant differences between the learning curves of the two groups. (Figure 14A, left side). Similarly, when the increasing delay between the response and the large reward was introduced, large reward preference decreased similarly in the two groups (Figure 14A, right side). During this phase, however, the number of inadequate

responses (made during the delay between the nose-poke and the reward, or the subsequent time-out period) increased faster in the asphyxia group compared to the control group ($p=0.08$ for the interaction between the treatment and the delay, **Figure 14C**), consistent with increased motor impulsivity.

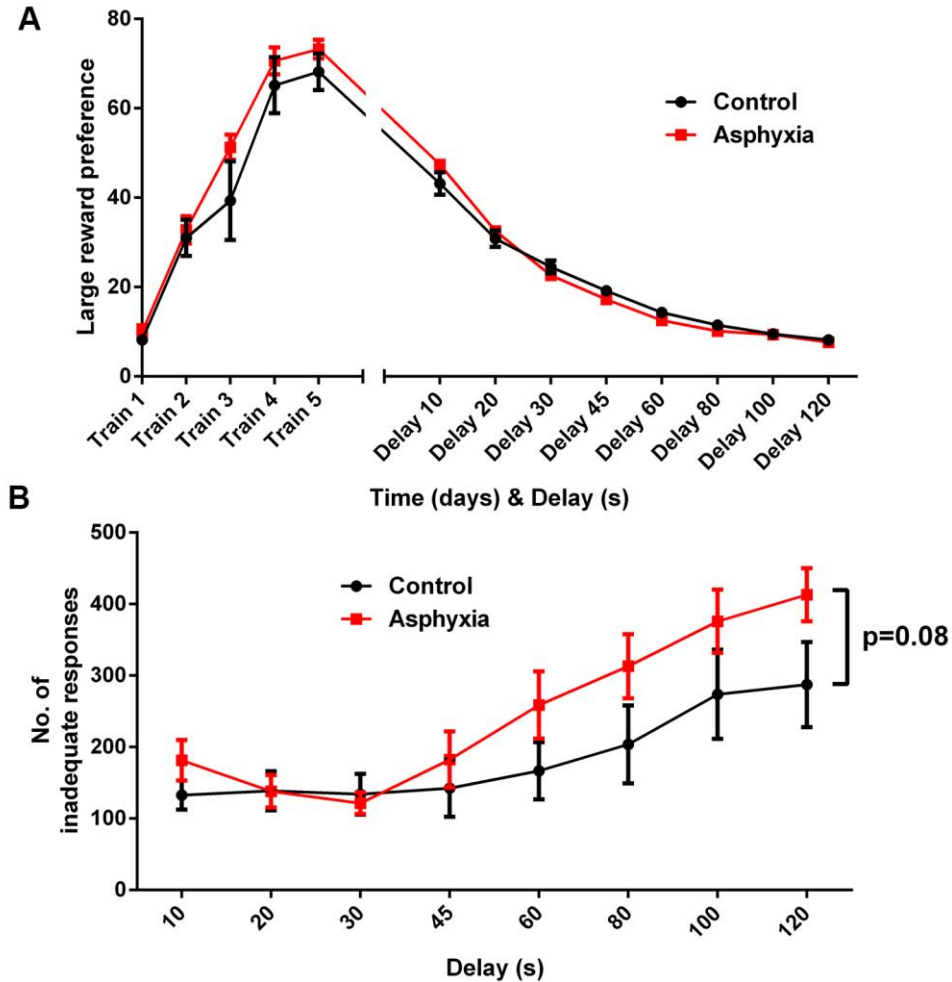


Figure 14: Results of the Delay Discounting test. (A) In the Operant Learning – Delayed Discounting paradigm, the learning curves of the control and the asphyxia group were similar, as the large reward preference increased during the training phase (left side) and decreased during the delay discounting phase (right side) similarly in both groups. (B) During the Delay Discounting phase of the test, however, the asphyxia group displayed a steeper increase in the number of inadequate responses with increasing delay between the response and the reward (borderline significance, $p=0.08$). Values are displayed as mean \pm SEM.

7. DISCUSSION

7.1 Deep hypothermia in the piglet model of HIE

Our objective was to investigate the effects of deep whole-body hypothermia in a preclinical newborn piglet model of HIE. We have demonstrated that deep hypothermia of 30 °C leads to an abnormal metabolic homeostasis, including lactic acidosis, hyperglycemia and hypokalemia; an increased need for volume replacement and inotrope support in order to maintain normal MABP; and more fatalities compared to either mild hypothermia (33.5 °C or 35 °C) or normothermia. Our results reinforce the need for strict control of body temperature during hypothermia even under conditions of intensive care with special attention to avoiding inadvertent overcooling. Such hypothermic overshoot may occur during passive cooling as well as servo controlled active cooling.^{175,176} Our results are similar to some of the earliest clinical hypothermia trials, which used deep hypothermia (30 °C) for the treatment of head injuries and were discontinued because of side effects and uncertain benefits.¹⁷⁷

The beneficial and neuroprotective effects of hypothermia have been discussed in detail in the Introduction and in a number of excellent reviews.^{54,60} Our experiments, however, have highlighted some of the potentially detrimental effects of deep hypothermia. Cooling has well-documented effects on the cardiovascular system, including peripheral vasoconstriction, sinus bradycardia with prolonged QT time, reduced cardiac output and decreased ejection fraction.¹⁷⁸ Deep hypothermia is known to decrease heart muscle contractility by interfering with Ca²⁺ signaling in cardiomyocytes.¹⁷⁹ However, the only cardiovascular side effect of mild cooling was benign sinus bradycardia in the large clinical studies of hypothermia for HIE.¹¹³ In our study, we have identified sinus bradycardia in the 30 °C group, but we have also found an increase in arrhythmias and in mortality, suggesting a more severe, uncompensated effect of deep hypothermia. Accordingly, our experiments also showed that the 30 °C deep hypothermia group required a significantly higher amount of volume replacement and inotrope support to maintain MABP. Current clinical protocols with moderate hypothermia did not result in an increased need for these measures,¹¹³ suggesting that

the current practice of using moderate hypothermia (33-34 °C) is unlikely to elicit more severe cardiovascular side effects.

Blood biochemistry and laboratory parameters have also been reported in clinical hypothermia trials, but the effect of cooling on these outcomes have been inconsistent and mild at most.¹¹³ Some trials, such as the early pilot studies of whole-body cooling reported mild hypokalemia in the hypothermia group¹⁰⁸ and this finding has also been confirmed in a number of preclinical and clinical reports.¹⁸⁰ Some publications have asserted that this effect might be due to the increased adrenergic tone during the induction of cooling and the resulting intracellular shift of potassium.¹⁸¹⁻¹⁸³ However, the exact mechanism of this adrenergic effect is largely unknown.¹⁸³ In our piglet experiments we have also observed hypokalemia during the cooling period in the 30 °C group. This finding was rather unexpected as this group has also received the highest amount of volume replacement, but it is likely that adrenergic activation was highest in this group due to the deep hypothermia and the administered β -adrenergic agonists. Additionally, we have observed hyperglycemia in the 30 °C group, which might be also secondary to the increased adreno-sympathetic tone and the decreased metabolic rate during cooling. In adults, hyperglycemia produces increased cerebral injury in both human and animal studies.¹⁸⁴ Neonatal studies are more equivocal, as a number of publications showed protective effects of hyperglycemia during ischemia, possibly due to increased substrate availability.¹⁸⁵ Early hypoglycemia in infants with HIE appears to be a strong predictor of poor outcome.¹⁸⁶ Recently, one of the large hypothermia trials were re-analyzed and both hypoglycemia and hyperglycemia were found to be independent predictors of bad outcome.¹⁸⁷ The most recent meta-analysis of hypothermia trials found a significant reduction in the prevalence of hypoglycemia in the hypothermia group, while hyperglycemia was not observed.¹¹³

The increased sympathetic tone during cooling is likely unavoidable and probably results from the decrease in cardiac output. At the same time, maintaining adequate anesthesia is necessary for neuroprotection. In a preclinical trial of hypothermia without anesthesia, the protective effects of hypothermia were lost in unsedated piglets.¹⁸⁸ In our study serum cortisol levels were not increased in either treatment group, indicating adequate sedation. It should also be noted that we found decreased levels of cardiac troponin-I at 12 and 48 hours in the 30 °C group compared

to all other groups. Since cardiac troponin-I is a robust and sensitive marker of cardiomyocyte injury,¹⁸⁹ our results concur with other recent findings indicating that hypothermia reduces injury to cardiomyocytes.¹⁹⁰ 30 °C hypothermia resulted in an even larger decrease in troponin-I levels than 35 °C and 33.5 °C in our study, suggesting that deep hypothermia might be more protective for the heart muscle. However, this appears to come at the price of significantly depressed cardiac function and potential arrhythmias.

Certain limitations to our study should be noted. Some animal species, such as the dog and the piglet might be more susceptible to systemic side effects of hypothermia than humans.¹⁷⁸ Additionally, the maximum decrease in core body temperature in our study (8.5 °C) was much higher than current clinical protocols indicate, corresponding to 28.5 °C in humans. The time of initiation and length of cooling was also different from clinical practice, which was primarily due to feasibility reasons. However, this model still produces brain injury which is very similar to human babies with moderate to severe HIE and our cooling protocol offers comparable neuroprotection to clinical hypothermia.¹⁹¹ The use of isoflurane anesthesia in combination with fentanyl analgesia was necessary in our case, due to animal ethics considerations. Inhalation anesthetics, including isoflurane has been shown to suppress cardiac function¹⁹² and possibly also deliver neuroprotective effects in animal studies.¹⁹³ Thus the potential interaction between isoflurane and hypothermia cannot be ruled out, either beneficial or detrimental. Finally, we used the pH-stat approach for acid-base management, which considers the blood gas values at the patient's temperature instead of standardizing it to 37 °C (alpha-stat strategy). The pH-stat approach has been shown to result in improved neurological outcome compared to the alpha-stat strategy.¹⁹⁴ However, such acid-base management leads to an acidic shift during hypothermia, which has been shown to decrease heart muscle contractility.¹⁹⁵

To summarize, in our neonatal piglet model of HIE, we have demonstrated an abnormal metabolic homeostasis (lactic acidosis, hyperglycemia and hypokalemia), an increased need for volume replacement and inotrope support to maintain MABP, and a higher number of cardiac arrhythmias and fatalities with 30 °C deep hypothermia when compared to 33.5 °C or 35 °C. Subsequent analysis of mRNA responses and neurohistological outcomes in these experiments also showed that 30 °C hypothermia

either provided no additional neuroprotection¹⁶⁷ or even offered less protection than 33.5 °C or 35 °C.¹²² These experiments, in combination with the negative results from recent clinical trials of 32 °C hypothermia¹³⁴ suggest that cooling to lower temperatures than the current clinical guidelines imposes significant risks while offering little or no additional benefit in neuroprotection. These findings have significant implications for neonatal care providers around the world, who are now adopting hypothermia as standard of care.

7.2 Hypothermia and hypoxic hypometabolism

Our experiments using a novel, non-invasive rodent model of HIE found that hypoxic hypometabolism is indeed present in the newborn rat and that it can significantly influence the tolerance to hypoxia. Even at ambient temperatures around thermoneutrality, the magnitude of the hypoxic hypothermia was highly variable and it greatly determined insult tolerance. After investigating the dose-dependence of insult tolerance to ambient temperatures, we found that maintaining a strict control of rectal temperature and adjusting ambient temperature in order to clamp the animal's core temperature within the desired range was an effective strategy to minimize variability in hypoxia tolerance. However, the regulatory response to hypoxia could still be present, even though systemic hypothermia was prevented. Indeed, we should speculate that the animals were attempting to dissipate heat via peripheral vasodilatation (decreasing systemic vascular resistance) and hyperventilation, although these effects could not be directly observed in our setup. Such attempts could possibly impose an additional metabolic 'cost' for the animal and thus decrease its hypoxia tolerance to sub-physiological values. However, this trade-off is unavoidable, since minimizing the effect of hypoxic hypometabolism by definition creates conditions which are different from extra-uterine hypoxia, naturally occurring in rodents. On the other hand, our experimental settings mimic the *in utero* conditions, where neonatal temperature is clamped at approximately 0.5 °C above the mother's and the fetus has no options to dissipate heat.¹⁷⁴ Hence, setting ambient temperature at 37 ± 0.5 °C and ensuring that rectal temperature does not decrease below this range creates a situation which is less

physiological for postnatal rodents and more similar to human *in utero* asphyxia.

Such considerations have been largely missing from a number of animal experiments, potentially creating a significant confounder in these studies.¹⁶³⁻¹⁶⁵ Looking back at clinical and preclinical approaches to temperature management, we can observe a rather converse situation. In the past, term newborns suffering *in utero* asphyxia were actively rewarmed and maintained at temperatures considered normothermic, but for these injured neonates during the first several hours of life, these temperatures could likely have been relatively hyperthermic.¹⁴² At the same time, preclinical models of HIE rarely cited hypoxic hypometabolism and endogenous hypothermia as potential confounders and offered no assurance that these effects were properly controlled.¹⁶³⁻¹⁶⁵ Thus not considering this evolutionary protective mechanism possibly led to working against it in certain clinical situations, as well as failing to compensate for it in preclinical experiments.

This bears great significance for both clinicians and scientists working with hypothermia and asphyxia, but also for other aspects of clinical care and research. Adopting such an evolutionary and physiological point of view can illuminate potential pitfalls and inadvertent detrimental effects.

7.3 Creating translational models of HIE

Similar to hypoxic hypometabolism, the fact that *in utero* asphyxia quickly leads to CO₂ retention and acidosis has been generally ignored in preclinical studies of HIE. Except for the models which closely mimic human pathology (such as the umbilical cord occlusion in fetal lambs¹⁹⁶), most of the established experimental paradigms – including most adaptations of the widely used Vannucci-model – only employ exposure to hypoxia.²⁴ Freely breathing animals in hypoxia can prevent CO₂ retention by hyperventilation; and while subsequent hypoventilation and ischemia will increase pCO₂, the dynamics and presumably the mechanism of injury is significantly different from human HIE. This was confirmed in studies by the Vannucci group itself, who used their established rodent model to investigate the effect of additional hypercapnia during hypoxia.⁶⁴ They found a U-shaped response curve in brain damage, ie. increasing

concentrations of CO₂ resulted in decreased neuronal injury up to a certain level, above which hypercapnia become deleterious.¹⁹⁷ Since these results, however, most of the research groups using this model still conduct hypoxia without parallel hypercapnia.

The exclusion of hypercapnia and acidosis from the asphyxic injury is not the only aspect of the Vannucci-model which leads to translational difficulties. As discussed in the Introduction, in order to sensitize the forebrain to subsequent hypoxia, a permanent unilateral carotid artery occlusion is used in this model. Neither its unilateral nature, nor the mechanism of permanent ischemia preceding hypoxia have meaningful clinical parallels. A number of groups have previously attempted to address these issues, for example by extending the ischemia to two or four vessels,¹⁹⁸ or using hypoxia only.¹⁹⁹ However, none of these adaptations included concomitant hypercapnia/acidosis with hypoxia.

We have adopted the model developed by Prof. Kai Kaila's group at the University of Helsinki.¹⁶⁶ We have attempted to collaboratively characterize this novel, non-invasive rodent model of perinatal asphyxia, taking into account human pathophysiology and rodent physiology as well. The use of 4% O₂ combined with 20% CO₂ was also suggested by Prof. Kaila in order to achieve tissue gas concentrations similar to asphyxic neonates (unpublished results). Our preliminary experiments showed that using this gas mixture, 15 minutes of asphyxia at 37 ± 0.5 °C could be tolerated by P7 Wistar rat pups with < 30% overall mortality. Thus we began to characterize this novel model of birth asphyxia.

7.4 A novel rodent model of birth asphyxia

When characterizing a novel experimental model, it is necessary to conduct a large number of preliminary experiments first, using robust biomarkers in order to identify the major pathophysiological processes involved, which in turn should be investigated more thoroughly. This approach will inevitably lead to a large number of negative preliminary results and relatively few positive findings. The goal of these preliminary experiments is to guide further investigations, but they are not designed to confirm or exclude the potential involvement of pathways with statistical certainty. Thus, we have

conducted a number of investigations listed in the Methods section, which are not discussed in detail in the Results. The reason for this is that while we did not find indications about the sensitivity of these markers for our experimental paradigm, we cannot exclude this possibility with certainty.

These preliminary biomarker experiments included the screening of serum and urine samples for laboratory parameters potentially indicating changes in ionic homeostasis, kidney- and liver function. In connection to this we have also analyzed serum and tissue homogenate samples for inflammatory cytokine levels. The fact that neither of these approaches revealed significant effects of the asphyxia paradigm is in accordance with other preclinical studies using non-invasive methods of injury and resuscitation.^{166,199} Rodents in general are remarkably resistant to systemic injury. Multi-organ failure comparable to babies with severe HIE could only be elicited by extreme induction of inflammation.²⁰⁰ Similarly, histological markers did not reveal significant neuronal death or post-hypoxic necrosis in the brain of these animals. This also correlates well with findings from other models employing hypoxia alone¹⁹⁹ as well as the early preclinical experiments in the '60-s and '70-s which showed that hypoxia without ischemia did not lead to significant histological damage.³⁴

While we could not identify regions of major neuronal death, axonal injury, or white matter damage, we found an increased local inflammatory activation in the brain shortly after asphyxia. This activation was primarily localized in the hippocampus and the prefrontal cortex, both known to be sensitive to hypoxic injury.⁶¹ The fact that this inflammatory activation was not associated with immediate structural changes prompted us to investigate more functional consequences of this asphyxia paradigm.

In accordance with the lack of neuronal necrosis, gross neuro-motor function of these animals was not significantly affected by asphyxia, as evidenced by the Open Field, the Elevated Plus Maze and the Rotarod tests. However, we found an explicit phenotype of anxiety in the asphyxia group in the Elevated Plus Maze test. This result has been reproduced since and appears to be a stable outcome of this asphyxia paradigm.

We have used the Operant Learning – Delayed Discounting paradigm to investigate more sophisticated neurological functions related to learning and impulsivity. The results showed that large reward preference was similar between the

two groups at all stages of the test. This means that both learning during the training phase and re-learning during the delay discounting phase was similar, corresponding to no overt differences in operant learning capabilities. However, when the increasing delay was introduced between the large reward response and the receipt of the reward, the asphyxia group reacted with a higher number of inadequate responses, indicating a phenotype of motor impulsivity.

These results are only the first steps in the characterization of this novel rodent model of perinatal asphyxia. However, together they suggest long-term effects of birth asphyxia on neurodevelopment, which are not due to direct and immediate cell death, but lead to a phenotype of increased anxiety and motor impulsivity.

7.5 Relevance and limitations of these findings

We have begun to characterize a novel rodent model of birth asphyxia in collaboration with Prof Kai Kaila's group at the University of Helsinki, who originally developed this paradigm. This model encompasses many of the pathological features of *in utero* asphyxia, including clinically relevant O₂, CO₂ and Trec ranges. It resulted in acute inflammation in certain well-defined areas of the brain without overt neuronal death or systemic injury. When these animals were followed into adulthood, they showed a phenotype of anxiety and impulsivity, without neuromotor deficiencies.

The combination of anxiety and impulsivity is a characteristic of attention-deficit/hyperactivity disorder (ADHD).²⁰¹ ADHD is a multifactorial developmental disorder which usually presents itself in school-age children with an overall prevalence of approximately 2-3%.²⁰¹ While its pathogenesis is largely unknown, epidemiological studies suggest that genetic as well as pre- and postnatal environmental factors are involved.²⁰¹ Recent studies indicated perinatal hypoxia as a potential risk factor in the development of ADHD.²⁰²

As discussed in the Introduction, approximately 10% of all newborns require some form of assistance at birth and 1% need vigorous resuscitation, while only a small subset of these children will go on to develop neonatal encephalopathy.⁹ Thus, there is a large cohort of children who likely suffer some above-normal level of asphyxia at birth,

but will not require intensive care and thus will not receive neuroprotective interventions such as hypothermia. It has been suggested long ago that if birth asphyxia is a continuous spectrum, then infants who die or suffer moderate to severe HIE must only represent one end of the spectrum and there should be a large group of neonates, who suffered relatively mild asphyxia.²⁰³ This group of infants is not seen in asphyxia trials and their first interaction with healthcare might be much later, when neurodevelopmental problems emerge. Recently there have been attempts to follow such large cohorts into childhood to prospectively determine the effects of perinatal factors. These cohort studies have employed stratification criteria based on Apgar-scores,^{13,14} a need for resuscitation,^{15,16} or acidosis at birth.¹⁷ Their results have been equivocal, a few studies providing some level of confirmation to the theory of a continuous birth asphyxia spectrum,^{14,15} but the effect size of mild asphyxia appears to be rather low and of questionable clinical significance. However, we should note that these stratification criteria, such as the 5-minute Apgar score are generally poor predictors of outcome even in children with moderate to severe HIE, hence their usefulness in more complex and long-term neurodevelopmental disorders are probably rather limited. Unfortunately, we currently have no better means for designing such cohort studies and for stratifying patients. Hence the potential causative role of perinatal hypoxia probably has to be first elucidated in well-controlled animal studies. We propose that this novel rodent model of birth asphyxia originally developed by Prof. Kai Kaila's group might be optimally suited for such work. It offers the possibility to identify neuroanatomical structures and functional connections specifically affected by mild perinatal asphyxia, the role of which could in turn be confirmed in human ADHD neuropathology. Even if these abnormalities can only be identified in later childhood, it would still offer the possibility to study the role of neuroprotective interventions on these deficiencies. These therapies could theoretically be applied around birth or possibly later on. However, without the means of definitive diagnosis at the time of intervention, these therapies will have to be designed as preventive measures, which benefit those in need of them, but does not impose a risk to others. One such therapy might be the restoration of normocapnia after asphyxia in a graded, instead of an abrupt manner.¹⁶⁶

Certain limitations to our study should be noted here. The rodent brain has an

inherently different structure than the human CNS. Additionally, as discussed in the Introduction, the optimal postnatal age for developmental comparison is unclear. We have chosen P7 animals largely to obtain results comparable to the most widely-used rodent models and to be able to identify model- and not age-specific differences. There are convincing arguments that a P10-12 rat might be more comparable to the term human newborn.²⁰⁴ Another important difference from human perinatal care is the lack of resuscitative efforts in this model. While our goal was to use minimal intervention, and intensive care would have required a number of significantly more stressful actions, there are other groups who are exploring these opportunities for translational preclinical studies with good results.²⁰⁵ Finally, extrapolating from rodent behavioral studies to humans is of course never straightforward, therefore our findings in this model will have to be confirmed in human patients. This is both the most challenging and the most promising aspect of our future work.

In summary, we have begun to characterize a novel, non-invasive rodent model of mild perinatal asphyxia. The animals surviving this insult showed localized inflammation in the brain without overt neuronal death, axonal injury or white matter damage. In adulthood, these animals displayed increased anxiety and motor impulsivity. This correlates well with the human phenotype of ADHD. These findings support previous epidemiological data suggesting a correlation between mild perinatal hypoxic events and neurodevelopmental impairments in later childhood, such as ADHD. This novel preclinical model might be instrumental in the future investigation of ADHD and childhood neurodevelopmental deficiencies.

8. CONCLUSIONS

Based on the work described in this thesis, the following conclusions can be drawn:

- 1. Deep hypothermia to 30 °C poses a significant risk for systemic side-effects and offers no additional benefit in neuroprotection in neonatal hypoxic-ischemic encephalopathy. Our results confirm that physicians now adopting therapeutic hypothermia should follow established treatment guidelines strictly, and carefully avoid inadvertent overcooling.**
- 2. Hypoxic hypometabolism and endogenous hypothermia are important confounders in a preclinical rodent model of birth asphyxia. In order to maximize translational value, researchers should take specific measures to avoid unintended hypothermia during hypoxia.**
- 3. This novel, non-invasive rodent model of mild perinatal asphyxia presents a neurodevelopmental and behavioral phenotype consistent with human ADHD-like symptoms. This paradigm could be valuable for developing a causal understanding between perinatal environmental effects and psychodevelopmental impairments in later childhood.**

9. SUMMARY

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of the most severe diseases in the perinatal period. Establishing therapeutic hypothermia as standard of care for infants with moderate to severe HIE has been arguably the most important recent development in the field. As many clinical centers are now adopting this practice, questions about the optimal administration of cooling are increasingly pressing. While hypothermia is often viewed by clinicians as an artificial intervention, hypometabolism and endogenous hypothermia during and after hypoxia are well-researched evolutionary protective mechanisms in newborn mammals. Preclinical studies of HIE have also often failed to address these mechanisms, potentially incorporating a significant confounder.

Our first objective was to investigate the effects of deep hypothermia in a piglet model of HIE. We have found that 30 °C deep hypothermia led to significant metabolic derangement, circulatory compromise and cardiac arrhythmias, while offering little or no additional protection. Our results together with recent clinical trials suggest that current cooling protocols should be strictly followed and inadvertent overcooling avoided.

Our second objective was to observe the prevalence of endogenous hypothermia in a rodent model of HIE. We found that strict control of ambient temperature and direct monitoring of rectal temperature are both necessary to minimize the confounding effect of endogenous hypothermia in our rodent model.

Finally, we have begun to characterize a novel non-invasive rodent model of HIE, which incorporates many aspects of the human clinical condition, including hypoxia, hypercapnia, acidosis and normo- or mild hyperthermia. This model produced local inflammation in certain brain areas without overt neuronal necrosis, axonal injury or white matter damage. In adulthood these animals displayed anxiety and motor impulsivity. These phenotypes are consistent with the symptoms of human attention deficit/hyperactivity disorder (ADHD). Thus, our results support the potential causative role of perinatal asphyxia in the development of certain neuropsychiatric disorders in later life. We suggest that this novel translational rodent model of mild HIE might prove useful in future investigations of these effects.

10. ÖSSZEFOGLALÁS

Az újszülöttkori hipoxiás-iszkémiás enkefalopátia (HIE) a perinatális életkor egyik legsúlyosabb betegsége. A közelmúlt legfontosabb eredményeinek egyike volt ezen a területen a hipotermiás terápia hatékonyságának igazolása és a kezelés beépítése a klinikai protokollokba világszerte. Ahogy napjainkban egyre több ellátóhely alkalmazza ezt a kezelést, a hűtés optimális módjával kapcsolatos kérdések is egyre inkább az érdeklődés középpontjába kerülnek. Ugyanakkor a legtöbb klinikus jelenleg is mesterséges beavatkozásként tekint a hűtésre, miközben újszülött emlősökben a hipometabolizmus és az endogén hipotermia jól ismert és evolúciósan konzervált protektív mechanizmusok. Hasonlóképp, a HIE-vel foglalkozó preklinikai vizsgálatok általában figyelmen kívül hagyták ezeket a protektív mechanizmusokat, amelyek ugyanakkor komoly zavaró tényezőt jelenthetnek az eredmények értelmezésekor.

Kutatásunk első célkitűzése a mély hipotermia hatásainak vizsgálata volt a HIE újszülött malac modelljében. Eredményeink azt mutatták, hogy a 30 °C-os mély hipotermia jelentős metabolikus zavarokat, keringési instabilitást és kardiális aritmiákat okozott, miközben legfeljebb minimális additív hatása volt a neuroprotekción terén. Ezen eredmények a közelmúltbeli klinikai vizsgálatokkal együtt alátámasztják a jelenlegi hűtési protokollok szigorú követésének és a véletlen hipotermia elkerülésének jelentőségét.

Második célkitűzésünk az endogén hipotermia előfordulásának vizsgálata volt a HIE rágszáló modelljében. Eredményeink azt mutatták, hogy mind a környezeti hőmérséklet szigorú szabályozása, mind a rektális hőmérséklet szoros követése fontos az endogén hipotermia hatásának minimalizálásához.

Mindezek fényében kutatócsoportunk megkezdte a HIE egy új, non-invazív rágszáló modelljének jellemzését, amely tükrözi a humán kórkép számos aspektusát, úgy, mint a hipoxia, a hiperkapnia, az acidózis és a relatív normo- vagy hipertermia. Ebben a modellben az aszfixia rövidtávon lokalizált agyi gyulladáshoz vezetett, amelyet nem kísért jelentős neuronhalál. Felnőtt korukban azonban az aszfixiás állatok szorongás és motoros impulzivitás fenotípusait mutatták, neuro-motoros diszfunkció nélkül. Ezen fenotípus-jegyek karakterisztikusak az emberi figyelemhiányos

hiperaktivitás-zavar klinikai tünettanában. Eredményeink tehát alátámasztják a feltételezést, miszerint perinatális hipoxiás ártalmak oki szerepet játszhatnak bizonyos gyermekkori neuro-pszichiátriai kórképek kialakulásában. Az enyhe HIE ezen rácsáló modellje a jövőben hasznosnak bizonyulhat ezeknek a klinikai összefüggéseknek a vizsgálatában.

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12. PUBLICATION LIST

List of publications:

Alonso-Alconada D, Broad KD, Bainbridge A, Chandrasekaran M, Faulkner SD, **Kerenyi A**, Hassell J, Rocha-Ferreira E, Hristova M, Fleiss B, Bennett K, Kelen D, Cady E, Gressens P, Golay X, Robertson NJ

Brain cell death is reduced with cooling by 3.5 degrees C to 5 degrees C but increased with cooling by 8.5 degrees C in a piglet asphyxia model.

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Olson L, Faulkner S, Lundstromer K, **Kerenyi A**, Kelen D, Chandrasekaran M, Aden U, Olson L, Golay X, Lagercrantz H, Robertson NJ, Galter D

Comparison of three hypothermic target temperatures for the treatment of hypoxic ischemia: mRNA level responses of eight genes in the piglet brain.

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Kerenyi A, Kelen D, Faulkner SD, Bainbridge A, Chandrasekaran M, Cady EB, Golay X, Robertson NJ

Systemic effects of whole-body cooling to 35 degrees C, 33.5 degrees C, and 30 degrees C in a piglet model of perinatal asphyxia: implications for therapeutic hypothermia.

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