Hypothermia and the Evaluation of Combination Therapies for Neonatal Hypoxic-Ischemic Brain Damage

by

Ann-Marie Terese Przyslupski

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

Medical Sciences – Pediatrics

University of Alberta

Abstract

Hypoxic-ischemic brain damage (HIBD) is still of major concern in the neonatal period, resulting in chronic neurological sequelae stemming from damage to the term newborn brain. Full-body and focal-head cooling within 6 hours of birth have proven neuroprotective in human newborns and various animal models of HIBD while independent pharmacotherapies have failed. Hence, post-ischemic cooling is the current standard of care for neonatal HIBD. However, hypothermia offers incomplete protection against the HIBD neurotoxic cascade, meaning that cooled newborns may still exhibit brain injury and neurological deficiencies. To offset this transient neuroprotection, and possibly extend the therapeutic window of opportunity, combining hypothermic cooling with pharmacotherapy has been explored. Using an established neonatal rat model of HIBD, my two studies respectively examined (1) if sulforaphane (SFN), a potent anti-oxidant and anti-inflammatory agent, could enhance hypothermic neuroprotection, and also (2) analysed the effect of hypothermia within the HIBD core and penumbra across time. Our results indicate that SFN did not further reduce gross pathological brain injury when provided with hypothermia, and that high concentrations of the compound (10 mg/kg) may have been deleterious. Our mechanistic results suggest that hypothermia differentially affects the core and penumbra. However, further studies will need to be undertaken to clarify the role played by hypothermia on cell survival/death, in this paradigm. The results of these two studies provide a stepping stone with which researchers can test promising neuroprotective therapies with post-ischemic hypothermia for newborn HIBD. Further studies are required to determine the optimal timing where additional therapy can be administered to the HIBD core and/or penumbra, as well as what type of adjunct therapy to achieve enhanced hypothermic neuroprotection. From this,

treatment paradigms that effectively enhance short and long-term pathological and behavioural outcomes could be developed.

Preface

This thesis is an original work by Ann-Marie Przyslupski. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board: Animal Care and Use Committee Health Sciences 2; 'Neuroprotective Role of Combining Mild Hypothermia and Chemotherapy in Perinatal Hypoxias-Ischemia and Breeding Colony', AUP00000363.

For R.Z.

Acknowledgements

I would like to thank the Yager Lab for their assistance and Dr. Jerome Y. Yager for being an accommodating and patient supervisor. Thank you to Ed Armstrong for assistance with many experimental techniques, and Katherine (Ke Qin) Shen for assisting with the histology for my Study #1.

For their assistance with Western Blotting and troubleshooting, I would like to thank Elizabeth Garcia and Geetha Venkateswaran from Dr. Sujata Persad's laboratory, and Carrie-Lynn Soltys, Jamie Boisvenue, Nikole Byrne, Dr. Miranda Sung, Dr. Shereen Hamza, and Dr. Ian Robertson from Dr. Jason Dyck's laboratory at the University of Alberta (Cardiovascular Research Institute).

Many thanks to my Graduate Committee (Dr. Persad, Dr. Colbourne) for their advice and expertise, and to Trish Kryzanowski for guiding my way to the end.

Most of all, thank you to those who have continually encouraged me and provided support. Yours I cherish the most.

These studies were supported by funding from the Branch Out Neurological Foundation's Masters Scholarship, the University of Alberta's Faculty of Medicine and Dentistry's Medical Sciences Graduate Program Scholarship, the Women's and Children's Research Institute (WCHRI) at the University of Alberta, the NeuroDevNet National Centre of Excellence, and the Alva Foundation.

Table of Contents

List of Figures

List of Abbreviations

Ab - Antibody

AIF – Apoptosis Inducing Factor

AMPK – Adenosine Monophosphate Kinase

AMP – Adenosine Monophosphate

ANOVA – Analysis of Variance

ARE – Antioxidant Response Element

ATP – Adenosine Triphosphate

BCA - Bicinchoninic Acid

BAF - Boc-aspartyl—(OMe)-fluoromethyl-ketone

BME - β-mercaptoethanol

CaMKK-β – Calcium/Calmodulin-dependent Protein Kinase Kinase β

CBF – Cerebral Blood Flow

CCA – Common Carotid Artery

CP – Cerebral Palsy

ECL - Enhanced chemiluminescent

EDTA - Ethylenedianimetetreacetic Acid

EPO – Erythropoietin

EUK-134 - Manganese 3-methoxy N,N'-bis(salicylidene)ethylenediamine chloride

GLUT3 – Glucose Transporter 3

H&E – Hematoxylin & Eosin

HIBD – Hypoxic-Ischemic Brain Damage

HIF-1α – Hypoxia-inducible factor-1α

HYPO – Hypothermia

IGF-1 - Insulin-like grown factor-1

Keap1 - Kelch-like ech-associated protein 1

LC3-II – Light-Chain 3-II

LKB1 – Liver Kinase B1

MCAO - Middle Cerebral Artery Occlusion

MK-801 - Dizocilpine

MMP-9 – Matrix Metalloproteinase-9

mTOR – Mammalian Target of Rapamycin

NAD+ - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

NMDA - N-methyl D-aspartate

NORMO - Normothermia

NOS – Nitric Oxide Synthetase

Nrf2 - Nuclear Related Factor 2

NS – Normal Saline

p-AMPK – Phosphorylated (Activated) Adenosine Monophosphate Kinase

PARP – Poly (ADP ribose) Polymerase

PD – Postnatal-day

PE – Polyethylene

PTEN - Phosphatase and Tensin Homolog

PVDF - Polyvinylidene difluoride

RCT - Randomized-Controlled Trial

ROS – Reactive Oxygen Species

SDS - Sodium dodecyl sulfate

SFN - Sulforaphane

S-PBN - N-tert-butyl-(2-sulfophenyl)-nitrone

SD – Standard Deviation

TAK1 – Transforming Growth Factor- β-Actvated Kinase 1

TBS - Tris-buffered saline

TBST – Tris-buffered saline - 0.1% Tween

TRAIL - Tumor necrosis factor-related apoptosis inducing ligand

TTC - 2,3,5,-triphenyl-2H-tetrazolium chloride

Xe - Xenon

XIAP - X-linked Inhibitor of Apoptosis

Chapter 1: Introduction

Perinatal Asphyxia and Cerebral Palsy

Asphyxiation in the perinatal period is a prominent cause of brain injury in neonates, as oxygen deprivation from impaired blood flow will cause neuronal degradation, prominent behavioural deficits, and/or death.[1–3] Asphyxia affects approximately 3-5/1000 term births,[4] but not all asphyxiated births will result in perinatal brain injury. Those that do are largely as a result of hypoxic-ischemic brain damage (HIBD) which accounts for 1-3/1000 of asphyxiated term births[4–6] with a 15-20% mortality rate.[7] Cortical damage and neuronal death sustained from HIBD may also impair proper cognitive and physiological development depending on the severity and location of injury.[3] Delaying treatment for HIBD may potentially damage deep gray matter brain structures, such as the basal ganglia, which are implicated in movement disorders. The primary outcome parameter is generally referred to as cerebral palsy (CP).[8,9]

CP is a chronic, non-progressive developmental disorder of the brain most predominately characterized by motor afflictions, but also encompassing cognitive and mental health disorders.[10–12] Current management for CP involves physiotherapy and other rehabilitation services.[13–15] Maternal infection has been strongly correlated with CP in the neonate[16,17], with general brain lesions serving a highly predictive role in diagnosis.[18,19] Unfortunately, the epidemiology of CP remains at 1-3/1000 of term births, and has not changed over the past 40 years, despite obstetric and neonatal innovations.[18,20] Developmental disabilities stemming from CP and HIBD are chronic and lifelong with tremendous emotional and financial complications for those affected, their families, and society.[21–23] CP has an expected lifetime cost of nearly $1.2

million per affected individual, and families tending to dependent CP individuals can be burdened in regards to time commitment and emotional frustration.[24,25]

Hypoxic-Ischemic Brain Damage (HIBD)

Brain damage due to hypoxia-ischemia emerges from oxygen deprivation coupled with a loss of blood flow to the brain in the neonatal period.[26,27] HIBD is unique in that it is characterized by both immediate and delayed phases of energy failure within the brain, as well as acute and delayed cell death, making it especially difficult to treat completely and effectively.[28,29] Risk factors such as maternal pyrexia, maternal inflammation, and emergency vaginal and caesarian section deliveries have been reported to increase the chance of HIBD.[5,30] Additionally, pre-eclampsia, maternal thyroid disease, fetal growth restriction, and birth after more than 42 weeks gestation have been noted as potential HIBD risk factors.[30–33] Unfortunately, despite advances in perinatal care, approximately 1-3/1000 term births in developed countries are still affected by HIBD.[5,6] Neonates affected by moderate and severe HIBD have respective mortality rates of relatively 10% and 60% [34,35] with a primary outcome of death and chronic disability by 18 months.[21,22,36] Approximately 25% of affected survivors also suffer lifelong consequences (e.g. permanent brain damage, neurodevelopmental disabilities)[4,34,37,38], also extending to future academic performance.[26,39,40] A more favourable neurodevelopmental outcome is associated with normal brain imaging and/or cortical, watershed, and white matter injury[41], while selective and diffuse gray matter injury, and deep basal ganglia injury are closely indicative of poor prognosis.[42] Selective regional susceptibility of the hippocampus, its dentate gyrus, cerebral cortex, basal ganglia, thalamus, and the striatum (caudate nucleus and putamen) to HIBD has also been characterized.[43–48] A definite correlation between risk factors and the distribution of neonatal brain damage has not yet been concluded due to heterogeneity, but perhaps this will

come to light with a better understanding of the neurobiology of the HIBD cascade and where injury is taking place.[49]

Pathophysiology of HIBD

The Hypoxic-Ischemic Core and Penumbra

HIBD brain injury is an evolving process. From the initial cytotoxic cascade, necrotic and apoptotic damage develops in specific regions of the brain depending on the duration and severity of HIBD, both of which have been shown to be positively correlated.[44] HIBD severity is associated with adverse neurodevelopmental outcome in young children.[50,51] Discerning the phase of injury or recovery being experienced by an individual, as well as where the injury is occurring, are crucial to providing the most optimal treatment.

HIBD injuries are composed of two parts: the Core and its surrounding Penumbra; these areas are central to understanding the evolution of HIBD.[52] The core is the area of the brain where cessation of blood flow and oxygen deprivation first occur, and this depletion of ATP and nutrients is believed to result in necrotic cell death. The penumbra is the hypo-perfused area of brain surrounding the core that contains both living and dying neurons; these characteristics make the penumbra a desirable therapeutic target for HIBD.[52] Theoretically the availability of survival substrates, oxygen, and living cells could be harnessed to prevent neuronal death, prevent expansion of the necrotic core, and strengthen the capacity of penumbral cells to overcome HIBD injury. Hence, penumbral targeting is especially important for functional recovery post-stroke due to this remaining perfusion capacity.[53]

While studies examining the HIBD core and penumbra are limited, the core has been found to contain both necrotic and apoptotic cells after HIBD, with the penumbra mainly composed of

apoptotic cells.[54,55] Apoptosis was also maximally-increased at 24h post-HIBD, with a decline in numbers at 48 and 72 hours post-injury, and 7 days after HIBD, with Cleaved Caspase-3 staining higher in the core as opposed to the penumbra for all time points.[55] Even in adult ischemia-reperfusion injuries, caspase-3 activation has been observed in the penumbra as early as 3h into recovery, with a more pronounced activation occurring in the core at 24h post-ischemia.[56] Moreover, it has been suggested that caspase-independent mechanisms of cell death may be favoured in areas of substantially-reduced perfusion (e.g. the core).[56] These data suggest that apoptotic and non-apoptotic damage occurs differentially between the core and penumbra not long after HIBD/ischemic stroke and may persist for hours, days, or months if left untreated.

The full picture of how the different phases of HIBD and related therapeutic agents differentially affect the core requires further investigation. The notion of the 'expanding core' (i.e. the infarct growing in size due to penumbral neurons becoming necrotic) is detrimental to HIBD and contributes to increased HIBD severity if left untreated.[52] The extent at which each area of the lesion is affected by the multiple phases of immediate and delayed energy failure that characterize HIBD likely depends on the severity of the insult itself.

Primary Energy Failure

When the HIBD insult first occurs, after the loss of blood flow, oxygen, and substrates to the brain, there is an immediate loss of adenosine triphosphate (ATP), phosphocreatine[57–59], and glucose[60] in the areas affected by the loss of oxygen and blood flow.[61] This combination of events results in the production of a neurotoxic cascade of drops in neuronal, glial, and mitochondrial membrane potentials, thus promoting their depolarization and consequently potentiating a burst of mitochondrial reactive oxygen species (ROS) production [i.e. superoxide anions (O_2^-), hydroxyl radicals (*OH), nitric oxide (NO), peroxynitrite ($ONOO^-$), hydrogen

peroxide (H_2O_2)][22,28], thus promoting the Fenton reaction that results in H_2O_2, one of the major players in the destruction of brain tissue post-HIBD, as well as the activity of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and xanthine oxidase that have been shown to exacerbate HIBD; this phenomenon however later recedes with the reduction and loss of mitochondrial membrane potentials.[62] HIBD injury is a highly paradoxical phenomenon, in that ROS and other effectors are only produced in the availability of oxygen, however the immature brain does not have a fully-developed antioxidant system, thus limiting inactivation of ROS. [62] Additionally, the HIBD injury is not a contained event, but rather spreads outwards in a wave of depolarization. Hence, some oxygen presence in areas close to more damaged areas may be more vulnerable and more susceptible to producing ROS.

These events also result in the uncontrolled secretion of potassium (K^+) ions and intracellular influx of calcium (Ca^{2+}) ions.[28,63–65] Excess K^+ and Ca^{2+} have been demonstrated to contribute to enhanced and sustained glutamate excitotoxicity[59,66–68]; this excitotoxic state also occurs due to overall brain vulnerability[69,70], glutamate reuptake prevention[71], and reduced cerebral blood flow (CBF)[7,68] that may last for days or weeks if the injury is severe.[28,72,73] Neurotoxicity in this regard stems from its glutamate's involvement in the activation of lytic enzymes such as cyclooxygenase, lipoxygenase, proteases, and nitric oxide synthetase (NOS)[28,74,75], which have the potential to cause considerable damage to the neonatal brain as it is extremely concentrated in fatty acids and immature cells, thus being highly susceptible to oxidative deterioration.[70] Riikonen et al.[51] and Hagberg et al.[76] found the severity of injury to also be correlated with excitatory glutamate and aspartate levels, shown by Pu et al.[77] to be elevated in the basal ganglia and thalamus in moderate-severe HIBD neonates.

These have been demonstrated to contribute to the primarily necrotic[78], but also apoptotic[46,54,78–81], atrophy of human and animal neonatal brains post-HIBD due to their deleterious effect on neurons, astrocytes, and endothelial tissue[82–84], ultimately resulting in immediate energy failure and cell death at the primary lesion site. Necrosis is accidental cell death characterized by the rupture of cell membranes and the disintegration of nuclei, and isprominent in this immediate neurotoxic period.[72] Apoptosis is a type of programmed cell death evident by the presence of nuclear fragmentation and condensation (karyorrhexis) and intact cellular membranes.[81]

Latent/Reperfusion Phase

When researchers refer to a "window of opportunity," this is in reference to the latent phase of HIBD neuropathology within 2-6h post-HIBD, clinically characterized by seizure absence, reduced cytotoxic edema, and a superficial return to homeostasis.[85] The longer that hypoperfusion post-HIBD occurs without treatment during this time, the more likely it is that secondary energy failure will occur.[86,62] Unfortunately, reperfusion exerts more stress on the cells that have already been damaged[86], leading to further oxidative stress via further free radical, NOS, and ROS generation.[83,87] This leads to outright energy depletion via poly (ADP ribose) polymerase (PARP) enzyme stimulation,[88] consequently inducing a perpetually depreciating cycle of nicotinamide adenine dinucleotide (NAD+) consumption and production to generate more ATP.[89–91] Stimulation of the PARP-1 enzyme has also been discovered to be necessary for translocating apoptosis inducing factor (AIF) in caspase-independent apoptotic cell death.[92,93] Additional biomarkers of reperfusion injury include nitric oxide and matrix metalloproteinase-9 (MMP-9).[94–98]

Post-ischemic inflammation largely characterizes this latent phase of injury.[99] Here, tumor necrosis factor-α, interleukin-6, and interleukin 1β pro-inflammatory cytokines[100,101], and other

pro-inflammatory cytokines, are produced by neurons, microglia, and astrocytes when perfusion is re-introduced.[102–105] Furthermore, evidence has shown interleukin-6 and interleukin-8 concentrations to positively correspond to the degree of HIBD injury, increased white matter injury, and an increased risk for developing CP.[99] Enhanced inflammation during the post-HIBD reperfusion phase has also been demonstrated to result in the increased production of ROS, NOS, and free radicals; these are known to contribute to the deleterious and long-lasting effects of inflammation in the post-HIBD reperfusion period and, as pervious stated, contribute to further brain injury due to the neonatal brain's underdeveloped antioxidant system.[62,105] Inflammation arising in the latent reperfusion phase may last for weeks, months, or years if left untreated, contributing to further brain injury.[57] Hence, reducing the inflammatory processes as soon as possible during the 6-hour window of opportunity is an essential treatment strategy and vital therapeutic target for neonatal HIBD. In hand, inhibiting inflammation has demonstrated clinical and biomedical relevance through the reduction of inflammatory cytokines, activated microglia, and neuronal death.[106–109]

The brain is also capable of producing enzymes such as superoxide dismutase, glutathione peroxidase, thioredoxin, heme oxygenase, and glutathione to endogenously protect itself against the damaging effects of oxidative and nitrative stress caused by ROS, NOS, and free radicals during the latent phase.[83,110–114] Astrocytes resistant to HIBD have been hypothesized to confer neuroprotection via their ability to produce glutathione as well as its glutamine, cysteine, and glycine precursors, which may be utilized by neurons to produce their own glutathione.[83,115–119]

Secondary Energy Failure

In the event that interventions are unable to fully ameliorate the brain injury during the latent phase of injury, delayed oxidative mechanisms stemming from the initial HIBD insult and

energy depletion may resurface at early or late time-points, approximately 6-18h or between 24-48h respectively after reperfusion.[57,120] This phenomenon was first observed in adult HIBD gerbils[121] and later reported in human HIBD neonates in the late 1980s.[122–125] This secondary phase is characterized by seizure reappearance in human neonates[85], brain swelling (edema)[126], mitochondrial morbidity due to ATP exhaustion[127], lack of acidosis,[128] and most importantly, delayed pathological apoptotic cell death[29,46,128,129], the latter of which is the target of many therapies currently under study.[81] Apoptosis may be either caspase-dependent or independent.[130] While the extrinsic and intrinsic divisions of caspase-dependent apoptosis both result in immutable cell death via the activation of the caspase-3 death effector, extrinsic apoptosis is mediated by caspase-8 stimulation, whereas intrinsic apoptosis requires caspase-9 activation.[130] Conversely, caspase-independent apoptosis involves cell death via the release of AIF from mitochondria.[131] Askalan et al. found caspase-dependent apoptosis to prevail in the leading core insult as opposed to the surrounding penumbral infarct in neonatal rat HIBD.[54] Caspase inhibition post-HIBD has been assessed with studies demonstrating reduced apoptosis and neuronal injury.[132] While studies have focused on combating neuronal death in this stage of HIBD, the effect of energy manipulation has not been assessed.

AMPK and Stroke-Related Brain Injury

As previously mentioned, energy metabolism in the brain is dependent on oxygen and glucose[133] and cerebral HIBD emerges from the brain's deprivation from these substrates and the resulting energy failure. Sustained ATP loss may lead to energy dysregulation and possibly infarction in the areas affected by HIBD. Adenosine monophosphate kinase (AMPK) is the master cell energy regulator and modulates energy homeostasis by promoting catabolic activities and turning off anabolic ones.[134,135] AMPK is a trimeric holoenzyme consisting of the α, β, and γ subunits that is

activated (phosphorylated; p-AMPK) by upstream mediators such as the ratio of low ATP:high AMP, liver kinase B-1 (LKB1), calcium/calmodulin-dependent protein kinase kinase β (CaMKK-β), and transforming growth factor – β-activated kinase 1 (TAK1).[134,136,137] AMPK activation, or lack thereof, in the injured brain may be linked to how affected brain tissue responds to HIBD.

While AMPK activation in times of energy failure and nutrient restriction may be cytoprotective[134,137], the role of AMPK in the brain has yet to be fully examined.[138] Several adult stroke models have shown prolonged activation of p-AMPK to be associated with increased neuronal death[136,139–143], with AMPK α -1/2 involved in damage progression.[136] Artificially inhibiting AMPK with Compound-C prior to ischemic stroke has resulted in increased ischemic tolerance in astrocytes[144] and reduced apoptosis in neurons.[145] Hence, researchers have postulated that inhibition of AMPK early after HIBD and activation of AMPK in the late stages of HIBD may be neuroprotective for targeting chronic apoptosis and inflammation.[139]

To date, few studies have examined the role of AMPK activation/inhibition in the neonatal brain after HIBD. Rousset et al. studied AMPK *in vivo* and *in vitro* with time-points during hypoxia (5min, 20min, 50min) and into recovery (10min, 30min, 1h, 3h, 6h, 24h, 72h), finding p-AMPK to peak at the end of hypoxia and return to baseline levels during recovery in the hippocampus, striatum, and cortex.[138] Prolonged artificial AMPK activation during *in vitro* HIBD resulted in more injury, however abolishing AMPK prior to injury was also detrimental.[138,142] This presents a dual role for AMPK activation and downstream activity, in that the neonatal brain requires p-AMPK as a preventative protection.

Currently, knowledge is limited on how therapeutic agents may affect AMPK-mediated activity in the core and penumbra of an HIBD-induced lesion, but this knowledge may be beneficial in developing novel therapeutic regimens for the treatment and/or prevention of neonatal HIBD.

Post-Ischemic Hypothermia

Currently, post-ischemic hypothermia, or cooling of the head or full body, is the only independent intervention to have successfully conferred lasting neuroprotection to both human infants and animals with HIBD as a rescue therapy, with significant reductions in mortality and neurological disability by 18 months of age.[146] Administered within the window-of-opportunity of 6h of HIBD diagnosis after birth, it is the gold-standard rescue therapy for HIBD.[146] While other drug interventions have demonstrated effectiveness in reducing HIBD in animal and cell models, none have been as independently successful in human neonates as hypothermia.[24] Unfortunately, the neuroprotection offered by cooling is incomplete and does not extend to the full extent of injury, nor does it benefit neonates with severe HIBD (>50% brain damage with large HIBD cores that extend into the deep gray matter), with approximately 22% of cooled neonates still presenting with mortality and neurological disabilities.[21,146] These complications enforce why more adequate and effective treatments must be developed for HIBD newborns.

History of Hypothermia for Neonatal HIBD

Therapeutic hypothermia has been utilized in clinical medicine for thousands of years.[147] Cooling as a therapeutic agent has been documented as early as in Hippocrates' time (460-375 BC), where he found benefit in covering wounded soldiers in snow.[148] Hypothermia was first introduced into medical practice by Dominique-Jean Larrey (1766-1842), a surgeon during Napoleon's reign, as a form of emergency clinical therapy.[149] Animal experiments studying the

effects of cooling for various bodily ailments occurred in the 1950s, but ceased in the 1970s-80s due to reports that hypothermia was having harmful repercussions.[148] Studies re-commenced in the 1990s when beneficial evidence surfaced of hypothermia as a protective agent against cardiac arrest and ischemic stroke.[150,151]

While Colbourne et al. sparked interest in the field of cooling as a viable and effective post-ischemic therapy[152,153],Young et al.[154,155] and Busto et al.[156,157] pioneered the use of post-ischemic cooling as a therapy for neonatal HIBD, and hence it is currently the clinical standard of care and only approved therapy for neonatal HIBD.[2,158] The aim of hypothermia, either via selective head cooling or whole body cooling, is to reduce an individual's body temperature by approximately 3-5°C in an effort to reach and protect areas of the brain that are selectively vulnerable to HIBD.[146,159] To be clinically relevant as a rescue therapy, hypothermia in animal models must be administered after HIBD induction and within the 6h window of opportunity; while some studies have shown neuroprotection with delayed cooling administration (e.g. cooling induced 1-3h post-HIBD), treatments provided after this time window are less likely to exert protection.[160] Hence, hypothermia's protection is correlated with its initiation time and duration of maintenance[161–164], thus having minimal-to-no effect on HIBD injury that has been evolving for more than 6 hours.[165] Trescher et al. were the first to discover that hypothermia's neuroprotection is merely transient, with post-HIBD cooled rats showing exacerbated brain damage akin to HIBD-only controls at 4 weeks post-injury.[166] Clinical interest in post-HIBD hypothermia piqued with Gunn et al.'s fetal lamb studies[85,163,167] , showing dramatic injury reduction via selective heard cooling, after which clinical trials began to surface.

Hypothermia in Clinical and Randomized-Controlled Trials

Since its translation from animal to human neonates, mild-moderate hypothermia (32-34°C) has shown to be neuroprotective in term and late preterm HIBD neonates, also improving long-term neurodevelopmental outcomes; this is supported by several recent systematic reviews and meta-analyses[146,168–170] and is now standard treatment for moderate-severe HIBD neonates, even though cooling does not protect against severe HIBD and standardized cooling protocols remain to be developed between and across institutions.[2,146,158,170] Initially, preliminary pilot studies in the HIBD newborn confirmed the safety and efficacy of cooling administration in term neonates[85,171–177], but hypertension, sinus bradycardia, fat necrosis, and increased duration of ventilatory aid were noted as possible side effects.[146,171] Neonates with normal or mild neuroimaging results do not undergo hypothermia, as they have been shown to exhibit relatively normal outcomes in childhood without treatment.[178]

To date, many large-scale randomized-controlled trials (RCTs) have demonstrated consistent safety and efficacy of whole-body[36,179–181] and selective-head cooling[182,183] in human neonates with mild-moderate HIBD. Overall, 48-72h of mild-moderate cooling (33–35°C) within 6h of birth has been demonstrated to reduce seizure occurrence and improve physical and cognitive outcomes, as well as survival rate, in mild-moderate HIBD newborns after follow-up at 18 months of age.[146,168,175] Neuromotor outcome was enriched only with whole-body cooling.[36,179–181] Granted, death or severe neurodevelopmental disability (i.e. CP, cognitive delay[169]) subsisted as the primary outcomes of all these RCTs[170], but were significantly and clinically reduced as compared to neonates not receiving post-ischemic cooling.[146] While post-ischemic cooling remains the most successful rescue therapy for mild-moderate neonatal HIBD, the full range of mechanisms by which neuroprotection is conferred are still being uncovered.

Proposed Mechanisms of Hypothermic Neuroprotection

Post-ischemic hypothermia is believed to exert a general suppressive effect on metabolic activity, glutamate excitotoxicity, and oxidative stress[65,184], however the specific mechanisms underlying hypothermic neuroprotection for mild-moderate HIBD are multifactorial, globally-acting, and still not comprehensively understood.[54,184] Part of the difficulty of developing effective therapies for HIBD arises from how currently available treatments are likely unable to target all of the cellular pathways affected by neurotoxicity. A multi-pronged approach is important in this case to prevent injury form spreading. Reviews examining the potential mechanisms of hypothermic neuroprotection are available.[65,184–188]

Overall, reduced cerebral metabolism[189], improved glucose metabolism[190–192], and delayed brain atrophy[166] contribute to hypothermic neuroprotection and manifest as improved neuronal survival[164,193–199] and behavioural/neurological outcome.[1,200,201] Zhao et al. demonstrated that per every °C lowered, cooling reduced brain oxygen consumption and glucose metabolism by 5%.[202] To complement this, hypothermia has also been demonstrated to limit the passage of Ca^{2+} through AMPA receptor channels by modestly upregulating the glutamate receptor-2 subunit of the AMPA receptor to prevent excitotoxicity.[203] Prevention of lactate accumulation and ATP loss in the HIBD brain[192] , as well as improved integrity of the blood-brain barrier through reduction of matrix metalloproteinases[204] are also evident as neuroprotective mechanisms of hypothermia.

Reduced cerebral blood flow, increased blood viscosity, and extended cerebral hypo-perfusion have also been reported during cooling.[171,189,205–207] Together, these mechanisms may help prevent complete blood flow cessation as induced by ischemia, perhaps functioning to delay cell depolarization during anoxic conditions.[65,208] However, these factors should be monitored, as

sustained cerebral hypo-perfusion[209], as well as hyperthermia[210–214], during ischemic reperfusion have been demonstrated to exacerbate HIBD under normothermic conditions.

The anti-oxidative, anti-inflammatory, and anti-apoptotic effects of post-ischemic hypothermia have been widely deliberated. Research has shown cooling to enhance anti-oxidant production[215] and suppress the release of deleterious excitotoxins (e.g. ROS, free radicals, glutamate, calcium, nitric oxide)[203,216–220] and inflammatory mediators (e.g. NFκB)[188,221–223]. Additionally, hypothermia has been demonstrated to upregulate caspase inhibitors[54,55] and inhibit pro-apoptotic caspases (e.g. caspase-3[193,224,225], caspase-2[224]) and caspase-independent mediators[54,226–228], thus having an overall suppressive effect on apoptotic cell death.

Furthermore, hypothermia has been theorized to only further delay the process of cell death.[54] This may represent and give consideration to the plausibility that damage beyond a proposed injury threshold is unrescuable.[164,229,230] For instance, early cooling has been shown to preserve the inactive phosphorylated form of phosphatase and tensin homolog (PTEN), but not delayed cooling which allowed PTEN activation and the perpetuation of cell death functions.[228]

With regards to the HIBD core and penumbra, while Yuan et al. found cooling to reduce their respective volumes between 24-48h after HIBD[223], and decreased translocation of apoptosis-inducing factor (AIF) has been reported in each area of injury[54], the precise mechanisms of hypothermia during HIBD neuropathology and whether they benefit or harm the core/penumbra are still at large. Current research demonstrates that hypothermia does not upregulate X-linked inhibitor of apoptosis (XIAP) in the penumbra, an aspect that could be harnessed with combination therapies.[55] Also, hypothermia has been found to reduce caspase-3 expression in the infarct core for up to 1 week, also having a transient effect on the penumbra with caspase-3 levels initially reduced, but quantitatively increased after 48 hours.[54] The role of hypothermia has

not been fully elucidated regarding its effects on AMPK energy homeostasis, however some evidence suggests that cooling may suppress AMPK activation after ischemic stroke.[231,232]

A positive correlation between reduced brain damage and decreasing temperature with focal[233] and whole-body cooling[60,234,235] emerged in neonatal animal models, with optimal protection at temperatures below 34°C. Interestingly, evidence points to a correlation between the increasing depth of hypothermia and neuroprotection, with an internal temperature of 31°C protecting better against neurotoxic injury than 34°C in neonatal rats.[192] In newborn pigs, no additional metabolic benefit has been seen with cooling at colder than 18°C[189], and increasing the depth (30°C) and duration of cooling (48h) in newborn rats was not effective against severe HIBD.[236] Thus, progressively decreasing the body's internal temperature may not be a good idea. While different temperature depths and durations may be effective against different HIBD animal species, human clinical care has standardized hypothermia administration to 72h at 33.5 ± 0.5 °C.[85,146,237] Research has shown adequate protection of both the deep and cortical gray matter with this temperature range, whereas other temperatures may affect brain structures differently.[238] Clinical trials examining post-ischemic hypothermia at 32°C for 120h after HIBD found increased brain acidosis along with a risk for increased mortality; the calculated probability for seeing a statistically significant benefit for longer, deeper cooling was 2%.[239,240] However, evidence demonstrates that the neuroprotective effect of cooling is neither complete nor indiscriminate, as moderately-affected neonates appear to benefit most from hypothermia as opposed to those severely-affected.[36,179,182,241]

In any case, the complete gamut of cellular mechanisms affected by hypothermia during HIBD pathogenesis and reperfusion remains elusive. Notwithstanding, the extent of hypothermia's therapeutic effect on the damaged brain likely depends on the severity of HIBD, as well as the

susceptibility of the brain tissue affected.[242] As cooling simultaneously exerts neuroprotective effects so broadly on multiple levels during injury and manipulates drug activity and effectiveness[243,244], supplementing hypothermia with another therapy is a challenging task.[245] Reviews examining hypothermic neuroprotection also note that it is largely incomplete and likely depends on the extent and severity of the initial insult, with protection for moderate brain injury typically being more robust than for severe HIBD.[36,182,241,246] Thus, the concept of additive/synergistic neuroprotective treatments in combination with cooling has been pursued in an effort to further improve outcome and perhaps even benefit neonates with severe damage.[4,24,247,248]

Pharmacotherapy in Combination with Hypothermia for Newborn HIBD

One could postulate that the next step in developing new therapeutics would be in the direction of enhancing hypothermic neuroprotection**,** but this is a challenging task**.** Theoretically, this could be accomplished by pharmacotherapies acting either synergistically or additively with hypothermia. Although a review paper detailing the effects of combination therapies for adult ischemic stroke is available[249], no such all-encompassing review currently exists for neonatal HIBD; Cilio and Ferriero published a review in 2010 detailing only the effects of various anticonvulsants as adjuncts with cooling for HIBD with minimal discussion on other tested treatments.[24] Below, the successful and unsuccessful adjuncts together with hypothermia for HIBD are described.

Experimentally-Successful Combination Therapies

Currently, the noble gas inhalant Xenon (Xe; 20-50%[250]) is the only therapy that has consistently shown additive neuroprotection clinically and experimentally with post-ischemic cooling.[251]

Notably, neuroprotection has been retained with delayed and asynchronous administration of Xe at 1h, 4h, and up to 5h following experimental neonatal HIBD; this was shown by significant neuronal viability, reduced brain lesion volumes, less apoptotic cell death.[252–254] Restoration of functional behaviour[255] and stabilization of blood pressure and cerebrovascular reactivity[256,257] have also been recorded with this combination therapy in the neonatal rat and pig respectively. However, neuroprotection may be limited to mild-moderate HIBD, as no protection with Xe and cooling have been observed for severe HIBD.[258] The mechanisms by which Xe exerts neuroprotection include inhibition of the N-methyl D-aspartate (NMDA) receptor.[259,260] While standard clinical practice has not yet implemented the use of Xe inhalation therapy alongside standard therapeutic hypothermia, recent clinical studies[261,262] and an RCT[263] have confirmed the safety and feasibility of recirculating Xe therapy, although results from the TOBY RCT have shown no effect of this combination therapy on moderate-severe HIBD.[263]

Erythropoietin (EPO), a hematopoietic cytokine that stimulates the oxygen-carrying capacity of red blood cells, is perhaps the next treatment in line with considerable promise for improving HIBD injury. Only two studies to date[230,264] have combined EPO and hypothermia to treat neonatal HIBD, and although both utilized similar animal models and protocols, they offer contradictory results.[265] Fan et al.[264] observed neuroprotection and sensorimotor improvement in female rats treated with 5000 U/g EPO plus hypothermia (33°C for 3h), while Fang et al.[230] implemented cooling for 8h with 3 doses of 1000U/g EPO, showing no significant effect of combination treatment at any point. RCTs assessing EPO for term neonatal HIBD deemed EPO to be safe while also reducing death and disability by 18 months, but showed improvement for moderate and not severe injury.[266,267]

Other studies examining adjunct treatments with post-ischemic cooling have also reported enhanced hypothermic neuroprotection. As phenobarbital, a seizure treatment drug, is frequently administered in the clinic post-HIBD, it was hypothesized that perhaps it would promote further protection with post-ischemic cooling. Laboratory results indicate varied internal temperature profiles[268], no additional effect with phenobarbital pretreatment[269], and long-term contralateral motor improvement even though short-term histological protection was not sustainable after 32-40 days.[270] Additionally, the anticonvulsant topiramate has been examined as a safe potential adjunct therapy[271] and a clinical RCT is currently ongoing.[272,273] Delayed administration of topiramate with hypothermia in a neonatal animal model demonstrated an extended window of opportunity post HIBD, further reduced cortex and gray matter injury, and improved behavioural function.[274] Further studies are required to determine the full range of phenobarbital's and topiramate's neuroprotective efficacies with hypothermia for HIBD.

Several one-off studies have also reported enhanced HIBD protection with cooling. For instance, statistically-significant additive neuroprotection with cannabidiols plus hypothermia combination therapy for neonatal HIBD in piglets.[275] Additionally, the anti-oxidant N-acetylcysteine has been shown to reduce HIBD infarct volumes, white matter damage, and attenuate neonatal reflexes when combined with post-ischemic cooling[204,276], but other studies have reported detrimental or unchanging prognoses N-acetylcysteine which must not be overlooked.[277–279] Another anti-oxidant, N-tert-butyl-(2-sulfophenyl)-nitrone (S-PBN), exerted robust neuroprotection of striatal spiny neurons and contralateral forepaw reaching when delivered every 12h in a pre- and post-treatment paradigm with hypothermia and HIBD.[280] Caspase inhibitors as a method of reducing apoptotic cell death may also be beneficial to counteract the HIBD neurotoxic cascade, but although the caspase inhibitor Boc-aspartyl—(OMe)-fluoromethyl-ketone (BAF) together with

post-ischemic cooling significantly reduced Cleaved Caspase-3 activity and protect hippocampal cells[281], BAF has also not been successful in reducing brain damage volume.[282] Insulin-like grown factor-1 (IGF-1) has also been examined with hypothermia for neonatal HIBD, but the results offer conflicting reports; one study found multiple doses of IGF-1 with cooling to extend the therapeutic time window[283], whereas another study did not find this duo to enhance hypothermic neuroprotection.[284] Notably, the application of mesenchymal stem cells in combination with hypothermia (34°C for 5h) under oxygen-glucose deprivation (OGD) conditions yielded approximately 90% cell viability, also reducing mitochondrial stress much more effectively than with cooling at 25°C.[285]

Unsuccessful Combination Therapies

The potent ROS scavenger Manganese 3-methoxy N,N'-bis(salicylidene)ethylenediamine chloride (EUK-134) with hypothermia has demonstrated enhanced neuroprotection of the striatum and putamen, but in a follow-up study with a similar protocol, this combination therapy was neither additive nor synergistic. [286] These negative results are consistent with previous work,[287,288] warranting caution and further study. Finally, dizocilpine (MK-801) was shown to protect the brain against HIBD[289–293], but these claims were refuted with data indicating that MK-801 acted independently to inherently produce its own neuroprotective cooling, irrespective of additional therapeutic hypothermia.[294–296] Due to its negative side effects and safety concerns in human neonates and children,[24] MK-801 is no longer recommended for use.

Current Gaps in the Literature

All-in-all, neonatal HIBD is a complex pathological process, beginning with a depletion of energy regulation. Hypothermia, while an effective neuroprotectant, complicates this process further. The notion that the HIBD core and penumbra may be differentially-affected during post-

HIBD hypothermia also complicates this paradigm and the approach to future and additive neuroprotective treatments.

A review of the literature has determined that anti-oxidant therapies and nutraceuticals have been sparsely tested as HIBD therapies. Due to the neonatal brain's immature anti-oxidant system[297,298], it may be beneficial to provide an additional boost of anti-oxidants with the intent of reducing brain injury. Furthermore, as previously mentioned, energy failure due to AMPK dysregulation is believed to play a major role in the neurotoxic cascade and delayed neuronal injury in HIBD. Although post-ischemic cooling is standard of care for HIBD, the full extent of hypothermia's effect on AMPK and its downstream regulators has not been completely elucidated. Additionally, neither has been examined in the HIBD core and penumbra, two distinct regions of injury.

The two studies encompassing my Master's Thesis aimed to examine sulforaphane (SFN), a potent anti-oxidative and anti-inflammatory compound derived from the ingestion of cruciferous vegetables, as a potential rescue therapy supplement to hypothermia. Our findings in this first study, that SFN did not potentiate the beneficial effects of hypothermia, in conjunction with that of others failing to enhance the effect of hypothermia, led to the development of my second study. That is, it became clear that a better understanding of the role of hypothermia in protecting the brain from damage was necessary in order to effectively supplement this therapy. In particular, knowing the role of hypothermia in the post-ischemic 'window of opportunity' would lead to a better understanding of the type and timing of supplementary therapeutic intervention. In this regard, we were interested in better understanding the effect of hypothermia on energy regulation, as that initial phase leading to cellular injury, either necrotic or apoptotic in nature. We set out to delineate the evolution of p-AMPK activation during and after post-

ischemic hypothermia in the HIBD core and penumbra to determine when and where biomedical research and clinical practice could possibly provide additional beneficial influence.

Chapter 2: Objectives, Rationales, and Hypotheses

Study #1: Sulforaphane plus Hypothermia Combination Therapy for Neonatal HIBD

Purpose

- To examine if a combination therapy of sulforaphane (SFN) plus moderate post-ischemic hypothermia (HYPO) would further reduce HIBD injury as compared to HYPO alone.

Objectives

- Determine whether HYPO + SFN is more neuroprotective than either therapy alone, after HIBD.
- Test a dose-response paradigm of various SFN doses.
- Determine if additional neuroprotection is afforded by an increase in the dose of SFN.

Rationale

Sulforaphane (SFN) is an isothiocyanate compound produced via the catalyzed reaction of ingested glucoraphanin and the myrosinase enzyme.[299,300] Metabolized via the mercapturic acid pathway and excreted as dithiocarbamates via the urine[301], SFN has been demonstrated to exert neuroprotective and anticarcinogenic influences against disorders such as stroke[83,302–304], and various types of cancer.[305–311] Mechanistically, protection by SFN involves stimulating antioxidant production via the molecular Nrf2-ARE (Nuclear Related Factor 2 – Antioxidant Response Element) pathway.[83,312,313] In this paradigm, SFN binds to the Keap1-Nrf2 (Kelch-like ech-associated protein 1 – Nuclear factor (erythroid-derived 2)-like-2) complex within the cell, resulting in Nrf2's dissociation and translocation to the nucleus, thereby inducing the production of Phase II antioxidants via activated ARE gene expression. Another recently discovered

mechanism of SFN, inflammasome inhibition, may also contribute to its potent effects[314]. Inflammasomes are innate cytosolic sensors that mount inflammatory responses against pathogens. This anti-inflammatory mechanism of SFN has been reported to act independently of the Nrf2-ARE pathway to indirectly inhibit caspase-1 and interleukin-1β secretion and maturation.[314] Collectively, these two mechanisms contribute to the protective and preventative effects of SFN seen in numerous biomedical research studies.[83,299,303,307,315–318] Given the neuroprotective action of SFN discussed, it is conceivable that its combination with HYPO would provide additive benefit in an HIBD model.

Only four HIBD studies to date have examined the effect of SFN therapy on *in-vivo*[319]and *in-vitro*[320–322] neonatal HIBD, all demonstrating neuroprotection as measured by reduced brain damage, increased cell viability, reduced inflammation, and increased antioxidant production. Fundamentally, these studies demonstrate that SFN is capable of protecting neonatal cells and neurons against hypoxic-ischemic damage.

However, the neuroprotection offered by both hypothermic cooling and SFN independently is insufficient to completely protect the HIBD brain. Post-ischemic hypothermia within 6h of birth is only partially-protective against mild-moderate HIBD and not protective at all for severe HIBD.[36,146,179,182,241] Also, SFN therapy has not been observed to completely absolve the neonatal hypoxic-ischemic brain from injury.[319]

Research suggests that combining post-ischemic cooling with another independently-neuroprotective therapy may produce additive neuroprotection of the hypoxic-ischemic brain injury itself. Whether an even further protection of brain tissue from HIBD affords additional behavioural improvement, or is even necessary to improve behavioural impairments, is currently unknown. While a review of the literature has revealed that most pharmacotherapies tried

together with hypothermia have not been successful in enhancing hypothermic neuroprotection, no studies have examined SFN and cooling together on HIBD. Due to SFN's status as a safe-to-use compound with potent anti-oxidant and anti-inflammatory effects[323], we theorized that it may be of advantage to examine these two therapies together. Furthermore, we examined several different SFN doses (1, 5, and 10 mg/kg) to elucidate any dose-dependent neuroprotective effects.

Hypotheses

- Supplementation of HYPO with SFN will have an additive neuroprotective benefit beyond HYPO alone.
- A higher dosage of SFN will be more neuroprotective than a lower dose, both independently and in conjunction with HYPO.
    - i.e.) 10 mg/kg > 5 mg/kg > 1 mg/kg

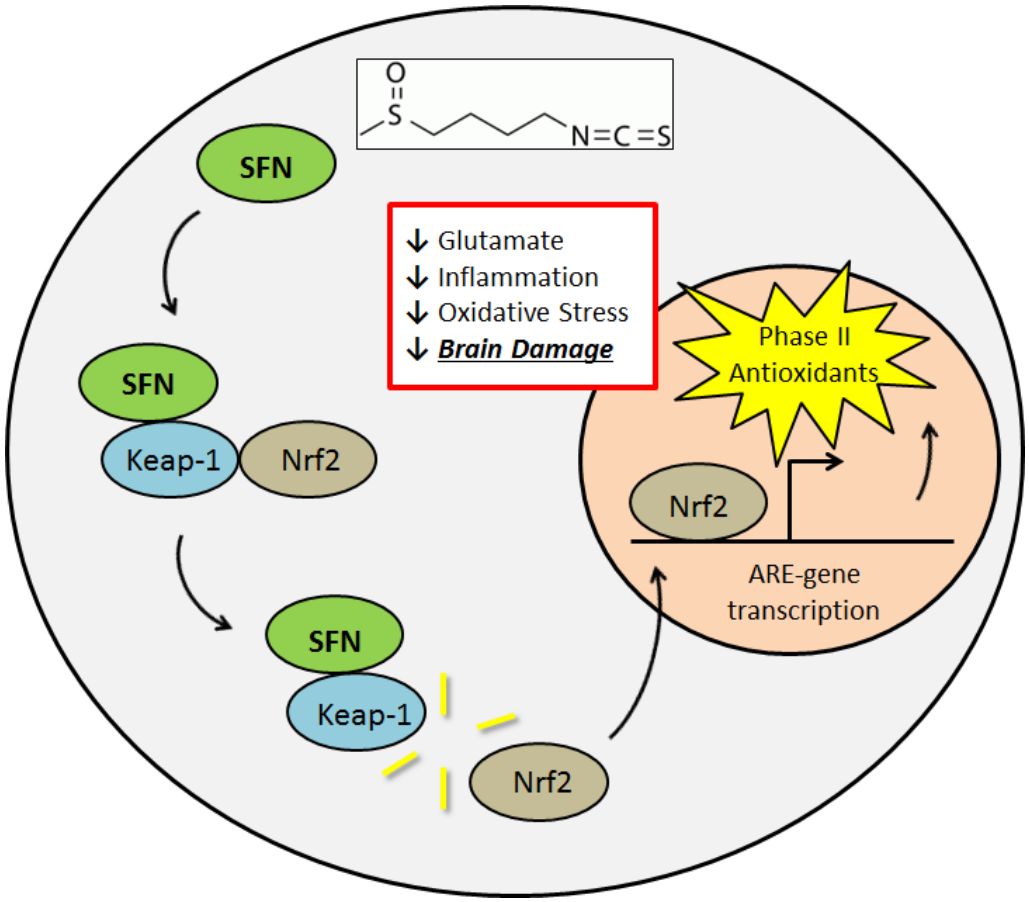


Figure 1: Mechanism of Sulforaphane Action via the Nrf2-ARE Pathway

SFN has been shown to act primarily through the Nuclear factor-erythroid 2-related factor 2 – Antioxidant Response Element (Nrf2-ARE) pathway. Once SFN enters the cell, it binds to the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 complex, dissociating Nrf2 and allowing it to enter the nucleus. Within the nucleus, Nrf2 is free to bind to the promoter of the Antioxidant Response Element gene and induce the transcription of downstream Phase II antioxidants. This in turn has been shown to result in the reduction of glutamate, inflammatory mediators, and oxidative stress, ultimately aiding in the reduction of brain injury.

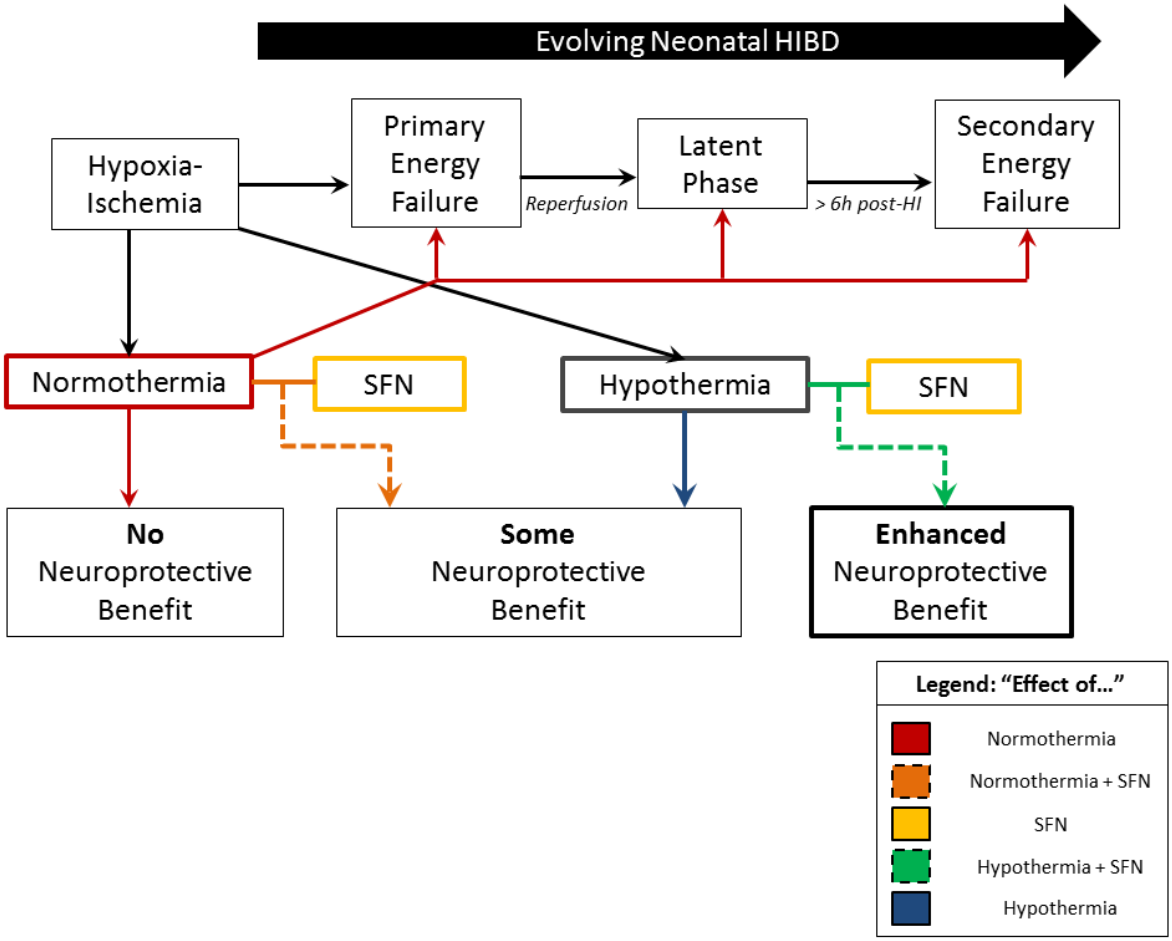


Figure 2: Conceptual Framework of Study #1

This conceptual framework illustrates my hypotheses for Study #1. Through a literature review, I hypothesized that combining both HYPO and SFN therapies would produce an enhanced neuroprotective effect for HIBD. No therapy at all would promote the evolution of injury, while hypothermia would provide only partial neuroprotection. Studies examining SFN alone have also seen some neuroprotective benefit.

Study #2: The Effect of Hypothermia on AMPK/p-AMPK and Cell Survival/Death in the Hypoxic-Ischemic Core/Penumbra

Purpose

To elucidate how HYPO affects energy recovery and cell death/survival pathways in the Core and Penumbra during HIBD in the neonatal rodent.

Objectives

- To determine the differential effects of hypothermia on the evolution of neonatal HIBD in the core and penumbra.

Specific Aims

- To determine the effects of hypothermia on: (1) AMPK activation, (2) pathways leading to cell survival and (3) cell death, in the core and penumbra of the HIBD lesion.
    - We quantified GLUT3 as a marker of cell survival, LC3-II as a marker of starvation-induced autophagic activity, Bim as a mediator of intrinsic apoptosis, and Cleaved Caspase-3 as a final marker of apoptotic cell death.

Rationale

As indicated in my Introduction, while post-ischemic hypothermia has been partially protective following hypoxic-ischemic brain injury in the newborn, and is currently standard of care, supplementing this therapy has been less than successful. My first study, combining SFN and HYPO underscores the challenge in finding supplemental therapies, as do other studies from our laboratory (Creatine [unpublished]), and others.[24] In our laboratory, this had led to a further review of the underlying mechanisms of HYPO, and questioning where supplemental therapies may be beneficial or additive.

Examining hypothermia's effect on the master energy regulator, AMPK, after HIBD is of interest from a therapeutic perspective. However, limited studies have examined how cooling acts on this axis in the brain.[231,324] One study found perinatal stroke to robustly activate AMPK in the brain; this effect was less pronounced in older mice due to the baseline increase of p-AMPK during aging, and may contribute to the differential effects of stroke pathophysiology and effect of treatment between neonates and the elderly.[324] Hypothermia was found to be protective through inhibiting AMPK activity after middle cerebral artery occlusion (MCAO) 24h after MCAO, in adult rodents.[231] In a mouse model of hindlimb ischemia, exposure to post-ischemic hypothermia (32°C and 28°C) suppressed p-AMPK and increased mammalian target of rapamycin (mTOR) expression via increased phosphorylation of ribosomal protein S6.[325] Interestingly, however intra-hypoxic (1% O_2) hypothermia at 32°C and 28°C did not inhibit AMPK-activation.[325]

Currently, it is unknown how hypothermia affects AMPK-mediated activity in the core and penumbra of an HIBD-induced lesion. The penumbra is secondarily affected by waves of stroke-induced neurotoxicity and surrounds the lesion's core, which is primarily necrosed by HIBD. Penumbral targeting is especially important for functional recovery post-stroke due to its remaining perfusion capacity.[53] Administering hypothermia after HIBD reduced the amount of cell death in both areas, but the penumbra most significantly.[54] This suggests that the penumbra is sensitive to signals communicating cell death versus survival. Hence, targeting the penumbra with therapeutic interventions to enhance neuronal survival after HIBD would be of interest. Furthermore, determining the role of hypothermia on the activation/inhibition of AMPK and its downstream effectors is highly valuable in elucidating the mechanisms of hypothermia on HIBD pathophysiology, as well as in determining additional drug treatments or specific adjunct

therapies that could specifically target proteins within the pathways that are either not influenced by hypothermia, or influenced adversely.

To gain an understanding of how p-AMPK and related downstream neuronal survival/death markers are affected by HIBD and cooling, several protein markers were examined. These were deemed as most representative of their effector functions and most directly linked to p-AMPK activity. GLUT3 was chosen as a marker of neuronal survival, LC3-II represented the final autophagosome of the autophagic cell death system, and Bim and Cleaved Caspase-3 exemplified intrinsic apoptosis; Bim was chosen due to its function as a pro-apoptotic mediator and its apoptotic role in the FoxO3a pathway that is directly affected by p-AMPK.[326–329]

Hypotheses

- Hypothermia will **suppress** p-AMPK expression in the Core and Penumbra.
- **Suppressing** p-AMPK in the **Core** → May be **protective.**
    - Due to the **absent** substrate supply in the HIBD core and primary location of energy failure, we hypothesize that **activating** p-AMPK in the HIBD core would be detrimental to neuronal survival. Activating AMPK in the HIBD core, requires some substrate and energy to initiate ATP recovery through uniting ADP and AMP. Stimulating the core may therefore act to further aggravate the injured area by requiring a substrate demand that outstrips supply.
- **Suppressing** p-AMPK in the **Penumbra** → May be **detrimental.**
    - The HIBD penumbra is hypo-perfused, as opposed to having no blood supply, and contains both living and dying cells. Due to the **present** substrate supply in the

HIBD penumbra and the presence of living cells, we hypothesize that rather **activating** p-AMPK in the HIBD penumbra would be beneficial in harnessing the endogenous ability of this injured area to generate ATP to promote neuronal survival and prevent the depolarizing nature of the core (prevent core expansion).

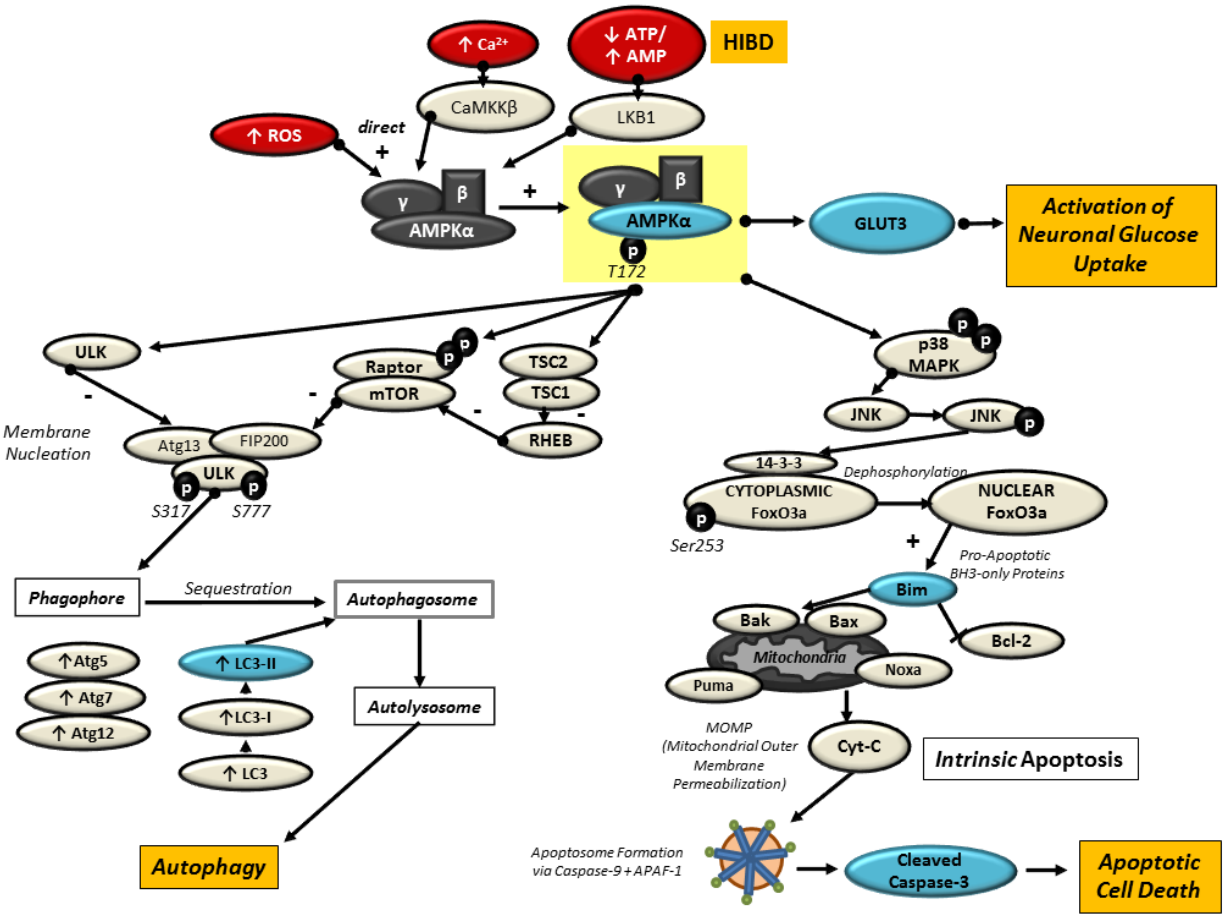


Figure 3: Molecular Pathways and Antibodies Examined in Study #2

This figure depicts the molecular pathways and proteins examined during Western Blotting for the Core/Penumbra study. As energy failure and fluctuations characterize HIBD, we probed for p-AMPKα (Thr172), the activated form of the master energy regulator AMPK, and its downstream effectors. GLUT3 was examined as a marker of neuronal glucose uptake. For autophagy, LC3-II expression was analyzed. Within the intrinsic apoptosis pathway, we examined the mediator, Bim, and the final effector, Cleaved Caspase-3.

Chapter 3: Materials and Methods

Study Population

Studies have demonstrated that the 7-day-old (PD7) neonatal rat brain is developmentally similar to that of the late premature human neonate and is at its peak growth period at this time.[330–332] The Rice-Vannucci model of perinatal HIBD[333], modified after Levine's model of adult anoxic-ischemia[334], is the best-characterized and most utilized model of comparative biomedical HIBD research. Their landmark study is considered the cornerstone of HIBD research. Thus, PD7 neonatal rats used under the Rice-Vannucci protocol permit conceivably translatable research findings. In my studies, both male and female PD7 Long-Evans rats (12-16g) were used, with litters culled to 10 pups to ensure an equal growth rate within and between groups, preventing litter bias.

Pregnancies for these studies were untimed as all work was done postnatally. Two female rats was placed with one male rat for 2 weeks. The female was then placed in a cage by herself until delivery. Rats were checked twice daily for delivery. The day of birth was postnatal day 1. Rat pups were reared with their dam until weaning at PD21. At this point rats were separated from the dam and housed at 2 rats/cage until euthanasia. Rat dams and pups (after PD21) were fed *ad libitum* and maintained on a 12 hour on/off light cycle.

Ethical Considerations

All research was conducted in accordance with the guidelines set by the Canadian Council for Animal Care and approved by the University of Alberta's Health Sciences Institutional Animal Care and Use Committee (Animal Use Protocol #AUP00000363). Rats were maintained by our Health Sciences Laboratory Animal Services and had *ad libitum* access to chow and water.

Study Designs

Study #1: Post-Ischemic Hypothermia and Sulforaphane Combination Therapy

Study #1 aimed to address the effects of hypothermia (HYPO) + sulforaphane (SFN) combination therapy on moderate neonatal HIBD.

All rat pups were randomized for sex, weight, and therapy. On PD7, experimental pups underwent surgery to have their right carotid artery ligated and severed (Rice-Vannucci model of HIBD[333]). After a two-hour recovery period, these pups were exposed to hypoxia at 8% O_2/ 92% N_2 for 90 minutes to induce Moderate HIBD in the right hemisphere of the brain. When possible, 2 of the 10 pups within each litter remained with their dam throughout the study to facilitate milk production. Immediately after hypoxia, experimental pups were randomly assigned to receive either Normothermic (NORMO, 37°C) or Hypothermic (HYPO, 28°C) environmental conditions for 24h, and received either normal saline vehicle (NS) or sulforaphane (SFN) injections, with 2 pups per condition per litter to avoid influences of batch-effects.

All experimental pups were given one subcutaneous injection (100 µl) of either NS (0.9% NaCl; Normal Saline) or SFN (1, 5, or 10 mg/kg) made up in NS within 15 minutes of NORMO/HYPO induction. Hence, four treatment groups were stratified and randomly assigned: NORMO + NS, NORMO + SFN, HYPO + NS, and HYPO + SFN. Injections were administered once every 24 h for a total of 7 days (PD7-13). Pups remained with their dams until PD21 when they were weaned and separated into their own cages based on sex. Euthanization occurred on PD30.

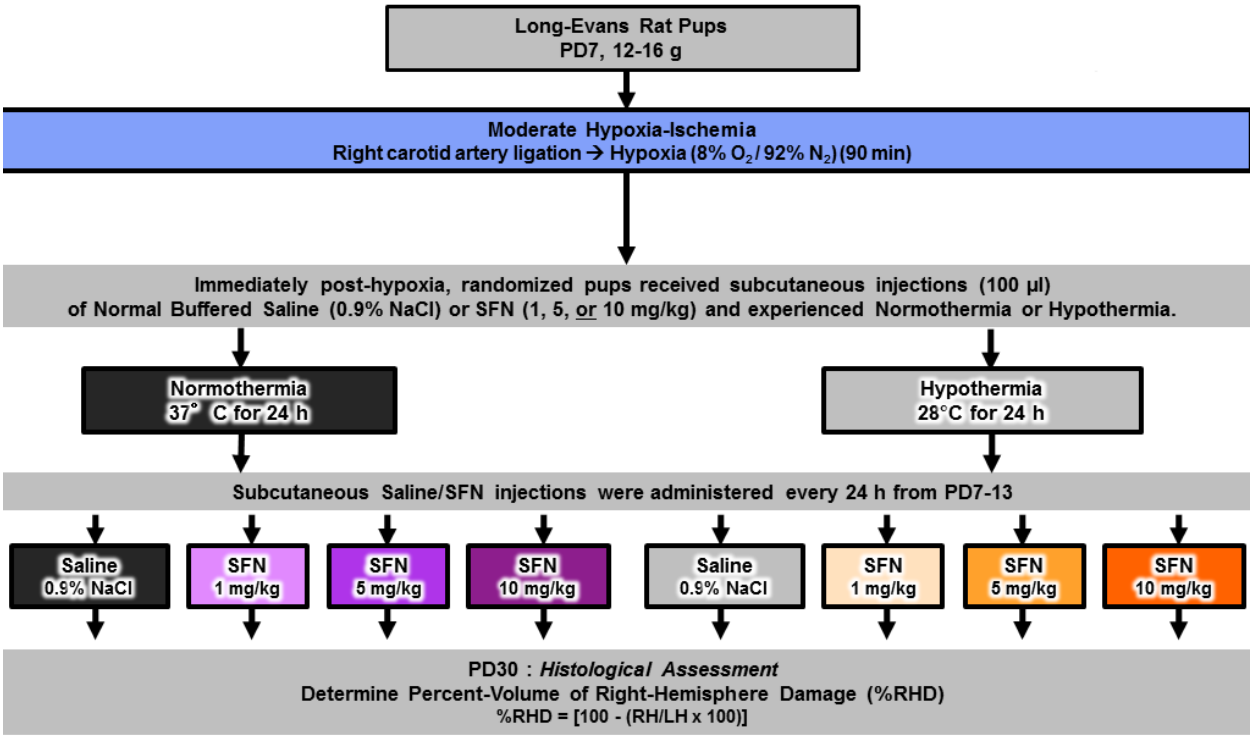


Figure 4: Hypothermia + Sulforaphane Combination Therapy Study Design

This flowchart depicts the study design for the HYPO + SFN combination therapy experiment.

Study #2: AMPK, Cell Death and/or Survival during Hypothermic Recovery in the Core & Penumbra

Study #2 aimed to address the results from Study #1 and provide insight into the mechanisms by which HYPO exerts neuroprotection for HIBD. The information will aid us in determining what supplemental agents in conjunction with HYPO, will be beneficial.

As in Study #1, PD7 experimental pups underwent Moderate HIBD as described within sections in Study #1. NORMO and HYPO conditions were identical to Study #1, however the time-points examined were different.

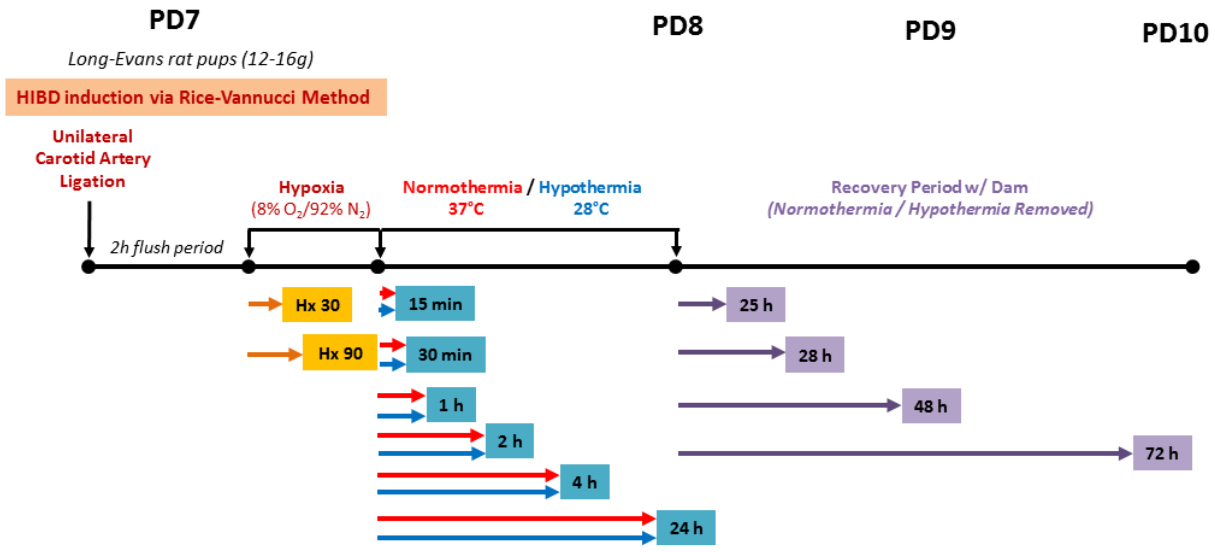


Figure 5: Post-Ichemic Hypothermia in the Core & Penumbra – Study Design & Timeline

The timeline above depicts an overview of the experimental time course and methods for Study #2.

General Methodologies

Induction of Moderate Neonatal HIBD

Rice-Vannucci Model

Unilateral Common Carotid Artery Ligation Surgery

The standard Rice-Vannucci model of neonatal HIBD was performed on PD7 Long-Evans rat pups (12-16 g)[335]. Prior to surgery, all pups were weighed, and sexed. Pre- and post-surgery, pups were held in an infant incubator (Isolette Infant Incubator; Air-Systems Vickers) set at 35°C to maintain normal body temperature. During surgery, pups were laid supine and fitted with a nose cone by which they were anaesthetized with isoflurane anaesthetic (4% induction, 2% maintenance). Each surgical procedure was completed in under 10 minutes, as isoflurane has been reported to exert neuroprotection after more than 10 minutes of exposure.

Once fully anaesthetized, as determined via lack of reflexes after pinching the hands, feet, and tail, a 1cm-long vertical incision was made with straight, fine scissors (Catalogue No. 91460-11; Fine Science Tools) at the midline of the pup's neck. Two sets of forceps, a thick pair (RS-5138 Graefe Micro-Dissecting Forceps [ROBOZ Surgical Store]) and a thin pair (Miltex 18-784 Eye Dressing Forceps [MedPlus Physician Supplies]) were used. Once the trachea was located, the isoflurane was turned down and maintained at 2% throughout surgery.

Afterwards, the thin pair of forceps was used to break through the translucent membrane of tissue underneath the sternohyoid muscles and immediately right of the trachea and the common carotid artery (CCA) was identified. The CCA is attached to the white vagal nerve and is located immediately alongside the trachea. To validate its identity, the thin forceps were manoeuvred

underneath and used to gently scoop and isolate it. If the red colour appeared and disappeared due to fluctuations in blood flow during this process, the CCA had been identified.

The right CCA was subsequently isolated from the vagal nerve and ligated twice with double-knots using 5-0 braided surgical silk (Harvard Apparatus). The right CCA was then permanently cut between the two ligations to stop blood flow. Once the CCA was successfully ligated the incision closed up with 5-0 silk sutures using a curved needle held with a needle driver (Halsey Needle Holder; 13 cm, Catalogue No. 91201-13) and trimmed of excess silk.

Post-surgery, pups were ear-notched for identification according to their assigned number and returned to the incubator to recover. When all surgeries were completed, pups were returned to their dam to recover for 2 hours.

Hypoxia

After 2 hours with their dam, pups were individually placed in covered glass jars (with inlet and exit ports) floating and partially submerged in a pre-heated water bath (36.5-37°C) and exposed to a hypoxic environment of 8% O_2/92% N_2 for 90 min. The combination of cerebral ischemia and hypoxia act together to produce moderate brain damage in the right hemisphere of the brain (ipsilateral to the CCA ligation)[335]. Mortality was approximately 5% for moderate HIBD. Pups were regularly monitored throughout hypoxia. Post-hypoxia, pups to be used for maintaining milk production were removed from their glass jars and held at room temperature for 5 minutes before being returned to their home cage. The remaining experimental pups either remained at 37°C or were transferred to glass jars partially submerged in a cooled 28°C environment. This procedure is further elucidated in the next section.

Normothermia & Moderate Post-Ischemic Hypothermia

Immediately after hypoxia, pups were randomized by sex and matched by weight to either NORMO (36.5-37°C) or HYPO (28°C, core temperature of 31°C[234]) experimental group. NORMO/HYPO was conducted in individual glass jars floating in water baths and lasted for 24 hours. Prior to induction, each experimental pup was fitted with a polyethylene (PE-10) catheter inserted subcutaneously between the scapulae on their back (into a hole created by a 25G needle) which delivered 15% glucose at a flow rate of 40 µl/h for NORMO or 10 µl/h for HYPO during the 24-hour period away from their dam[54] to prevent hypoglycemia.[336–338]

Rats experienced NORMO (37°C) or moderate HYPO (28°C) conditions in glass jars floating in water baths for the entire 24-hour period, which extended into PD8, unless part of Study #2 and allocated to an experimental timepoint occurring during NORMO/HYPO. Water baths were covered with enclosed lids to maintain humidity levels. Following NORMO/HYPO on PD8, PE tubing was removed and pups were held at room temperature for 5 minutes before being weighed and returned to their dam unless their experimental timepoint was at the end of NORMO/HYPO.

Sample Processing & Assessment

Histopathology

Coronal Brain Slice Collection

All HIBD brains were first assessed for neuropathology via histological staining. Whole brains were cut coronally at 14 µm/slice using a cryostat (Cryocut 1800, Reichert-Jung) set at -20°C.

Hematoxylin & Eosin (H&E) Staining

Brain slices was stained with Harris' H&E, using our lab's modified protocol, to visualize the two hemispheres of the brain for a gross physical assessment of brain damage. This immunostaining method allowed for easy determination of HIBD cysts. All sections were photographed (Leica MC170 HD) through a microscope (Leica GZGE) over a lightbox prior to assessment.

Study-Specific Methodologies

Study #1

Subcutaneous Injections

Shortly after initiating NORMO/HYPO, all experimental rats in Study #1 were randomized by sex and weight to receive subcutaneous injections (100 µl) of either normal saline vehicle (NS; 0.9% NaCl) or SFN diluted in normal saline (1, 5, or 10 mg/kg). Injections were administered every 24h for 7 days (PD7-13) via a 30-gauge needle attached to a 1cc syringe. Rats were weighed daily, and the concentration of SFN adjusted according to the average weight of the litter on the day of injection. PD8 injections occurred 2 hours after NORMO/HYPO end to allow for the skin opening on their scapulae (exposed after removal or the PE tubing) to close.

Experimental Time-Points & Animal Euthanization

Rats were given an overdose of 5% isoflurane and euthanized via decapitation 21 days after NORMO/HYPO (PD30). Their brains were subsequently extracted and brains were immediately frozen in ice-cold isopentane (2-methylbutane, 99%) in a dry ice + ethanol bath and stored at -80°C, for later histopathological assessment.

Infarct Volume Measurement

For Study #1 brains, serial sections were cut anterior to posterior and obtained in duplicate every 0.5 cm (36 turns), beginning at the joining of the anterior commissure and ending at the separation of the corpus callosum.

H&E-stained brain sections were then analyzed for percent-volume of right hemisphere brain damage. From the beginning of the anterior commissure to the separation of the corpus callosum, the areas of all right and left hemispheres, of each section and of each brain, were individually traced in ImageJ (Version 1.42q; National Institutes of Health). Due to brain size variability, the number of sections analyzed anterior (2) and posterior to bregma (5) were standardized across brains to provide the most accurate volume determinate. The areas of each hemisphere were then integrated with the distance between sections to determine the total hemisphere volume. The percent-damage of the right hemisphere was taken to be: [100 – ([right hemisphere volume – left hemisphere volume] x 100)].

Study #2

Experimental Time-Points & Animal Euthanization

Randomized pups from different litters were utilized for Study #2 to understand how AMPK and its downstream effectors are expressed during HIBD. Here, subsets of pups were euthanized at 30 and 90 minutes of hypoxia. After HIBD, other pups were euthanized at 15min, 30min, 1h, 2h, 4h, and 24h into NORMO/HYPO to examine how each temperature treatment affected the proteins of interest. Brains were additionally collected at 25h, 28h, 48h, and 72h after the end of hypoxia (1, 4, 24, and 48h after removal from NORMO/HYPO) to examine protein expression after hypothermic conditions ceased.

At each experimental time point, pups were swiftly removed from their glass jars and rapidly decapitated, with brains extracted (in less than 1 minute) and frozen in isopentane in a dry ice + ethanol bath. All brains were stored at -80°C until further processing. This method of euthanasia ensures that proteins are not affected by prolonged exposure to anaesthesia or room temperature.

Determination of Core and Penumbra

To aid in the determination of the core and penumbra within HIBD brains, posterior brain sections from Study #2 were stained with H&E, and a separate subset from an individual litter stained with TTC (2,3,5,-triphenyl-2H-tetrazolium chloride). Comparisons between both these stains showed no difference in the localization of core and penumbra. Thus, the H&E-stained sets were used for core/penumbra determination.

Our goal was to determine the differences in the core and penumbral insults, and how hypothermia affected each pattern of damage. From this information, core and penumbra tissue was collected from each brain for Western Blotting.

TTC Staining

TTC is a rapid metabolic tissue stain that colours metabolically-active tissue red while leaving dead or dying tissue unstained. In the stroke literature, it is a common and reliable stain used to define the location and relative perfusion of cerebral infarction. TTC staining was performed to determine the location, severity, and extent of the HIBD lesion, as well as the location of core and penumbra at each time point.

A preliminary set of PD7 Long-Evans rats were subjected to Moderate or Severe HIBD (90 and 150 minutes of hypoxia, respectively) plus NORMO or HYPO and euthanized 4h, 24h, 48h, or 72h after hypoxia had ended. Immediately after extraction, serial (2 mm) coronal brain sections were obtained using a rat brain cutting matrix and stained with 2% TTC for 10 minutes at 37°C. Brains were then transferred to 10% neutral-buffered formalin, photographed, and subsequently stored at 4°C; TTC brains were not used or analyzed for any other procedure.

While Kramer et al.[339] confirmed that TTC-stained brain tissue is indeed reliably able to be used for Western Blotting, for our purposes we opted to use a different set of animals whose brains had not been stained with TTC.

H&E Staining

All Study #2 posterior brain sections were obtained in duplicate at the first instance of a full hippocampus, but prior to the joining of the corpus callosum (14 μm/slice). This was achieved by mounting the anterior end of the Study #2 brains to the cryostat chuck with CryoMatrix™ (Thermo Scientific) and cutting from the posterior end beginning at the cerebellum and moving anteriorly with each section cut. Slides were stored at -20°C until stained.

All posterior brain sections were stained with H&E as per protocol and photographed, the locations of the HIBD core and penumbra for each brain then determined by investigators blinded to the experimental groups and timepoints. Next, core and penumbra were collected into pre-chilled 2 ml Eppendorf tubes via punch biopsy (2 mm, Miltex by Kai) at -20°C. Any remaining brain tissue was stored at -80°C. Core and penumbra samples were then kept on dry ice and prepared for Western Blotting.

Western Blotting

Sample Preparation

Core and penumbra samples were each respectively homogenized in 200 μl of AMPK lysis buffer (recipe courtesy of Dr. Jason Dyck's lab of the University of Alberta's Cardiovascular Research Institute; resulted in the best visualization of p-AMPKα) including protease/phosphatase inhibitors (ThermoFisher) and sodium orthovanadate and allowed to sit on ice for 10 minutes to permit activation of the inhibitors. Samples were subsequently spun at 10,000 g at 4°C for 10 minutes. Supernatants were extracted and pellets were discarded.

Supernatant lysates were aliquoted, used for protein concentration analysis, and stored at -80°C until further use.

Bicinchoninic Acid (BCA) Assay

BCA assays were performed in 96-well plates using gradients of colour change compared to a standard curve to determine the protein concentration of each core and penumbra sample. For each plate, dilutions of 2000 µg/mL of Bovine Serum Albumin (Product #23210, ThermoScientific) were prepared, according to protocol, to form a standard curve when read by the plate reader. A 1:50 dilution of each homogenized sample lysate was prepared in ddH_2O. Each well was then filled with 125 µl of Reagent A and Reagent B (1:50, Product #23223 and #23224, ThermoScientific). All samples and standard curve determinates were pipetted in triplicate to ensure readout accuracy and incubated at 37°C for 30 min. Protein concentrations within each well were determined using a VersaMax Turntable Plate Reader (Molecular Devices) and SoftMax Pro software (Version 5.2, Molecular Devices); r^2 values of 0.95 or greater were deemed acceptable. Protein concentrations derived from the BCA assays were used to determine stock protein loading amounts for immunoblotting. BCA assays were repeated for dilutions with r^2 values lower than 0.95.

Western Blotting Procedure

Western blotting was performed to quantify the amounts of proteins of interest in each core and penumbra brain sample per experimental condition. Initially, stock sample lysates were each briefly centrifuged at 10,000 g. Diluted stock samples were then separately prepared on ice in a mixture of ddH_2O + 4X SDS (sodium dodecyl sulfate) + BME (β-mercaptoethanol; 40µl per

1000µl 4X SDS) + 0.1 M EDTA (ethylenedianimetetreacetic acid) as per calculation for a total of 35 µg of protein per 10 µl. Diluted samples were briefly vortexed and centrifuged at 10,000 g before being sonicated in a water bath for 2 minutes to further promote protein denaturation; this was to better allow the proteins to run smoothly through the gel. Subsequently, samples were boiled at 95°C for 5 minutes. Boiled samples were then briefly vortexed, centrifuged briefly at 10,000 g, and left at room temperature to prevent SDS precipitation. They were subsequently pipetted into their respective wells in a premade 15-well electrophoresis gel (4-15% Mini-PROTEAN® TGX™ Precast Protein Gels; Bio-Rad; #4561086) preassembled within a Mini-PROTEAN® Electrophoresis Cell filled with 1X running buffer. The samples loaded into each gel were representative of one time point and contained core and penumbra samples from both NORMO and HYPO animals. A combination of 4 right hemispheres of the brain from 4 animals that had not received HIBD was used as a naïve control between gels, and an electrophoresis ladder (Precision Plus Protein™ Dual Color Standard; Bio-Rad) was run in the first and last lanes of each gel. Gels were run at 100V until the sample buffer dye had exited the stacking gel, whereafter the voltage was increased to 130V and stopped when the dye had just reached the bottom of the gel and before the ladder could run off the gel.

Pre-transfer, PVDF (polyvinylidene difluoride) membranes were pre-activated for 10 minutes in 100% methanol and equilibrated in transfer buffer for at least 30 minutes until the membrane no longer floated on the surface of the buffer. When electrophoresis was complete, samples were transferred from the gels to PVDF membranes at 100V for 2 hours on ice at 4°C within a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) containing an ice-pack, stir bar, and filled with transfer buffer. After transferring, membranes were rinsed in 1X tris-buffered saline (TBS) and blocked in 5% milk in 1X TBS at room temperature for 1 hour on a rotator. Afterwards,

membranes were cut horizontally just above the 50 kDa and 25 kDa molecular marker locations to probe for multiple antibodies (Ab) at once and also prevent unnecessary protein stripping. Membranes were individually incubated in primary antibodies (1°Ab) at 4°C overnight, or at room temperature for 1 hour, on a rotator; 1°Ab utilized were: p-AMPKα (1:1000, Cell Signaling, #2535), GLUT3 (1:1000, AbCam, #ab191071), Bim (1:500, AbCam, #ab17003), cleaved caspase-3 (1:1000, Cell Signaling, #9664), and LC3II (1:1000, AbCam, #ab48394). β-actin (1:1000, AbCam, #ab8226) was used as the normalized loading control. All 1°Ab were made-up in 5% milk-TBS. The next day, membranes were washed in 1X TBS-0.1% Tween (TBST) once for 5 minutes and 3 times for 10 minutes and incubated in their respective anti-rabbit (αR-IgG Horseradish Peroxidase-Linked Whole Ab from Donkey; 1:1000; GE Healthcare LTD; #NA934V) or anti-mouse (αM-IgG Horseradish Peroxidase-Linked Whole Ab from Sheep; 1:10,000; GE Healthcare LTD; #NA931V) 2°Ab in 5% TBS-milk at room temperature for 1 hour. Membranes were then washed in 1X TBST once for 5 minutes and 3 times for 10 minutes and incubated in an enhanced chemiluminescent (ECL) detection reagent (Western Lightning Plus ECL; PerkinElmer Inc.) This brand of ECL provided stronger signal readouts than our usual Millipore ECL (Millipore Luminata^{TM} Classic – Western HRP Substrate). In the event that bands were still weak after these steps, a much stronger Femto ECL (SuperSignal^{TM} West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific; #34095) was applied; this method was used sparingly due to the side effect of producing high background distortion. Films were subsequently developed onto X-ray film (Super RX Fuji Medical X-ray Film; 47410 19238) in a darkroom. Films were scanned as Greyscale JPEGs, converted to TIFF format, and analyzed for densitometry using the ImageJ (Version 1.42q, National Institute of Health) software.

In the event that membranes needed to be stripped of the last 1°/2° Ab, they were first washed in 1X TBS for 5 minutes and then incubated in a thin layer of Restore™ Stripping Buffer (Thermo Scientific) at room temperature for 7 minutes on a rotator. Afterwards, membranes were washed in 1X TBS for 5 minutes followed by blocking and 1°/2° Ab procedures, as previously stated. When all probing was complete, membranes were stored in 50 mL Falcon tubes completely filled with 1X TBS at 4°C.

Western Blotting Analysis

For densitometry, scanned images were saved as 8-bit TIFF files and analyzed in ImageJ (Version 1.42q, National Institutes of Health). The density (peak area + darkness) of each band of interest was determined. The raw density value for each Ab was divided over its β-actin value to determine the respective ratio for the sample's protein expression. Ratio values were graphed per time-point.

Statistical Analyses

Sample sizes for both studies were determined in STATA (Version 12.0, StataCorp LP). To calculate these, standard deviations were obtained from a previous unpublished HIBD variability study in our lab for Study #1 and data from previous HIBD immunoblotting literature for Study #2; sample sizes of 8 pups per group and 6 pups per group were respectively required to reach a significance of a = 0.05, with a power of 0.80 and Type II b-error of 0.20.

Statistical analyses were performed in PRISM. Statistical significance was $p < 0.05$ for all analyses. All data are presented as mean ± SD (standard deviation). All photographs are representative samples of their respective experimental groups.

Study #1

Infarct volume analyses for Study #1 were assessed via a 2-way Analysis of Variance (ANOVA) parametric statistics, with 1-way ANOVAs plus Tukey's post-test then conducted to determine the loci of significance. These analyses were chosen to compare the multiple drug doses within and between temperatures with their relevant comparison groups.

Study #2

The ultimate goal of this study was to determine whether hypothermia has a different effect on p-AMPK and its downstream markers of cell death and survival in the core and penumbra during and following HIBD. As a result, comparisons were made between the normothermic and hypothermic core and the normothermic and hypothermic penumbra during a variety of time points during the evolution of HIBD injury. Secondary analyses determined differences between the core and penumbra within treatment groups. [Antibody/β-actin] ratios derived from immunoblotting were analyzed via 2-way ANOVA followed by 1-way ANOVA with Tukey's post-test when comparing between Normothermia and Hypothermia within and between areas. T-tests were performed when comparing between two groups only. Outliers were removed using the box-plot feature of SPSS.

Chapter 4: Results

Study #1

Mortality

Of the 103 experimental pups, 2 pups died during HIBD surgery due to rupture of the CCA and 2 died during hypoxia. 1 pup was euthanized after HYPO due to excessive swelling to one of its hindlimbs that had severely impeded its movement and was causing pain. All other pups survived to their experimental endpoint. No mortality occurred as a result of HYPO or SFN treatment.

Weights

There were no significant differences in weight between any of the groups during the 7 days of injections (PD7-13; Figure 6). However, on PD30, pups administered 10 mg/kg SFN at either temperature were significantly heavier than all other rats (NORMO: $p < 0.001$-0.05; HYPO: $p < 0.0001$-0.001). Data depicted as mean ± SD.

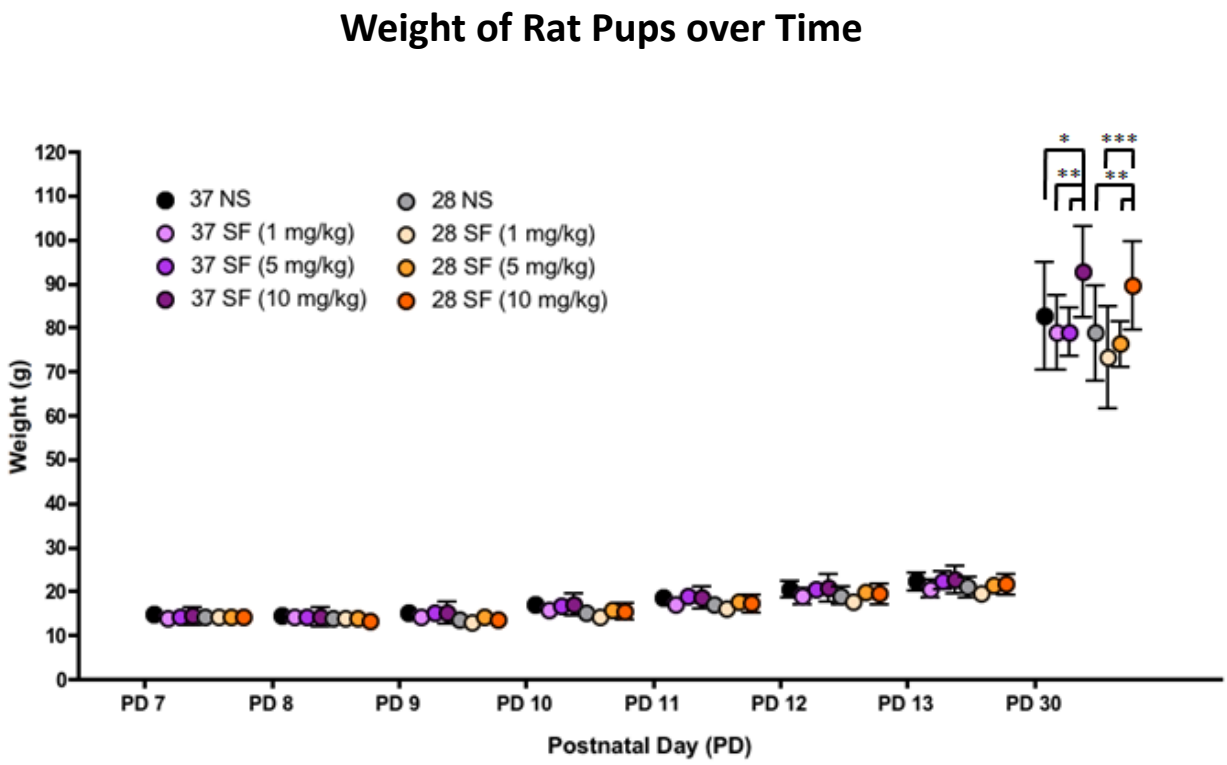


Figure 6: Weight of Rat Pups over Time

Weights between groups only differed on PD30 in regards to the 10 mg/kg SFN dose. On average, these pups were significantly heavier than all other pups (NORMO: $p < 0.001$-0.05; HYPO: $p < 0.0001$-0.001). Data depicted as mean ± SD.

Histology

The images depicted in Figure 7 show H&E-stained HIBD brain sections on PD30 in each of the experimental groups. Noticeable infarcts were present in the Normothermic control and SFN (10 mg/kg) groups.

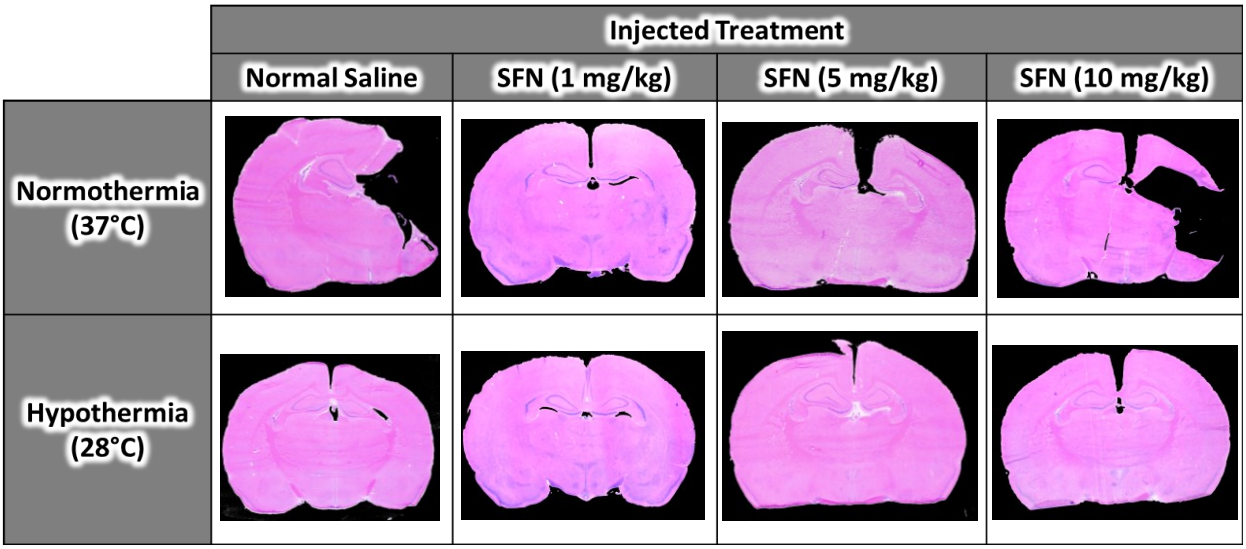


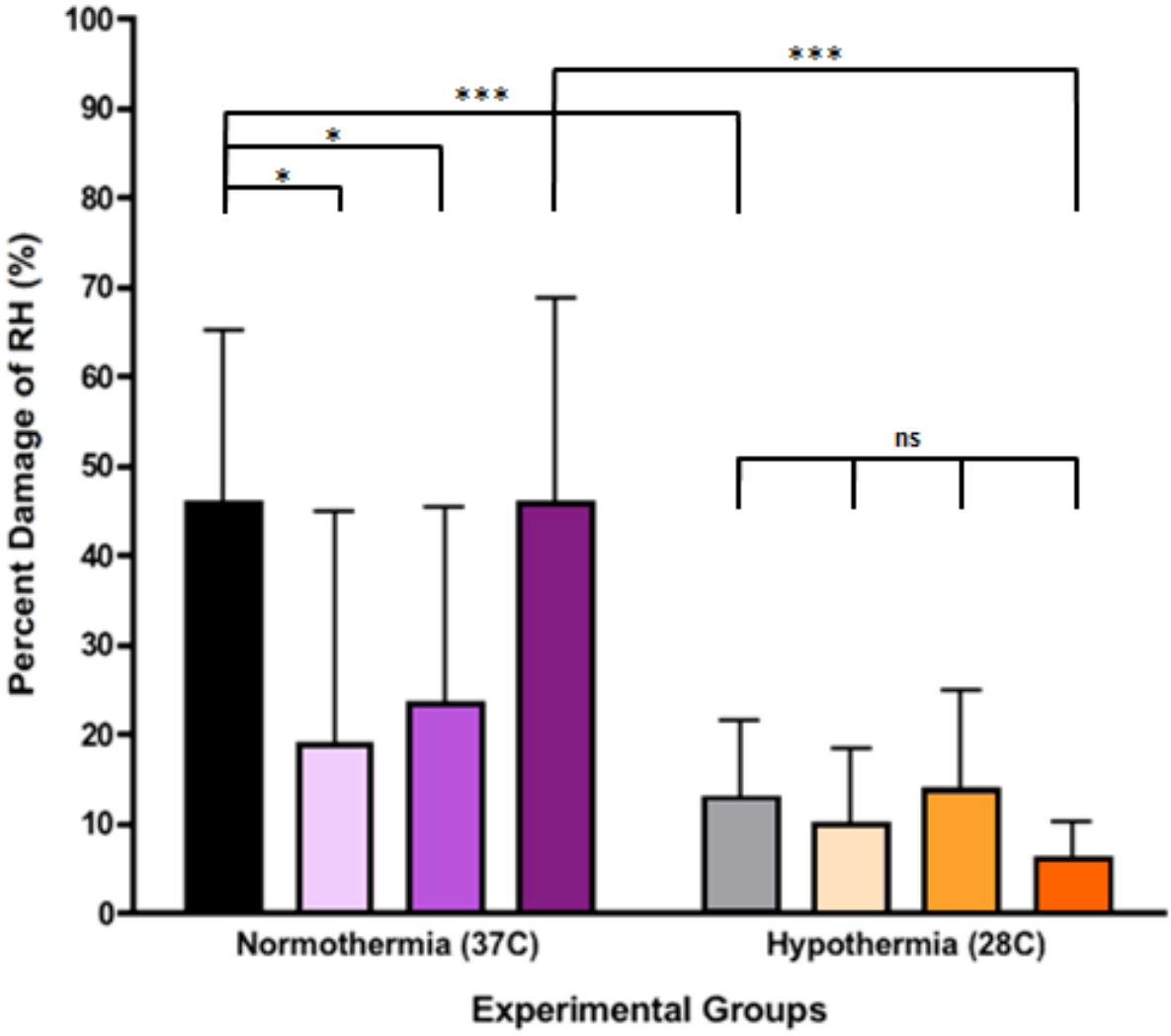
Figure 7: Study #1 – H&E-stained Brains from each Experimental Group

Brain Injury Pathology

Figure 8 A & B depict the percentage of right-hemisphere brain damage as determined by Image-J tracing. Hypothermia (28°C) was significantly neuroprotective alone and regardless of SFN dose ($p < 0.001$). SFN at 1 mg/kg and 5 mg/kg independently reduced injury ($p < 0.05$). However, SFN (10 mg/kg) alone did not provide HIBD neuroprotection. No additive effect was demonstrated when hypothermia (28°C) and SFN were combined at any of the administered doses. Statistics obtained via Two-way and One-way ANOVA with Tukey's post-test [Interaction: $p = 0.0095$ ($F = 4.105$); Drug Dose: $p = 0.0253$ ($F = 3.287$); Temperature: $p < 0.0001$ ($F = 34.9$)]. Data depicted as mean ± SD.

(A)

Temperature vs. Drug Dose: Comparison of %RH Volume Damage



(B)

%RH Brain Damage								
	37°C Saline	37 SFN (1 mg/kg)	37 SFN (5 mg/kg)	37 SFN (10 mg/kg)	28°C Saline	28 SFN (1 mg/kg)	28 SFN (5 mg/kg)	28 SFN (10 mg/kg)
Mean	45.72%	18.81%	23.5%	45.83%	12.69%	9.948%	13.77%	6.162%
SD	±19.32%	±26.02%	±21.85%	±22.93%	±8.805%	±8.44%	±11.05%	±4.074%

Figure 8: Temperature vs. Drug Dose in relation to Percent Right-Hemisphere Brain Damage

SFN at 1 and 5 mg/kg was significantly neuroprotective compared to the Normothermic controls ($p < 0.05$), whereas the 10 mg/kg dose was not protective. Hypothermia significantly conferred neuroprotection alone and also together with SFN ($p < 0.001$). SFN combination therapy did not enhance hypothermic neuroprotection.

Study #2

Mortality

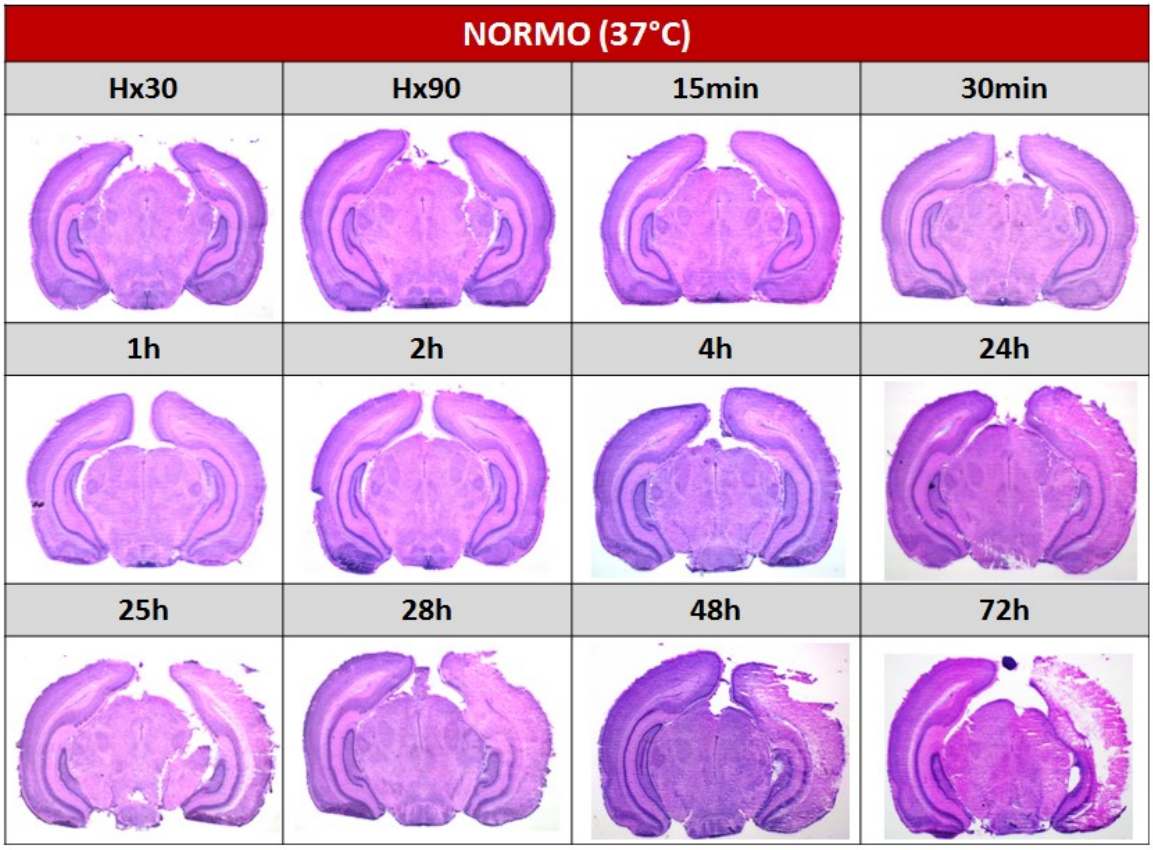
Of the 170 experimental pups, 2 pups died during HIBD surgery due to rupture of the CAA and 1 died during hypoxia. All other pups survived to their experimental time-point.

H&E Staining

Brains of NORMO pups exhibited a progressive degeneration of the right hemisphere (ipsilateral to the CAA ligation). This was evident by H&E starting at 24h post-HIBD and most severe at 72h post-HIBD (Figure 9A).

Pups exposed to HYPO also showed right hemisphere brain injury, appearing restricted to the region of core injury, in the cortex, with minimal expansion to the penumbra (thalamus). Minimal-to-no gross pathologic damage was observed during hypoxia (90 min) and from 15min to 2h of recovery, following HIBD for both temperatures (Figure 9B). All images depicted are representative of their treatment groups and time-point.

(A)



(B)

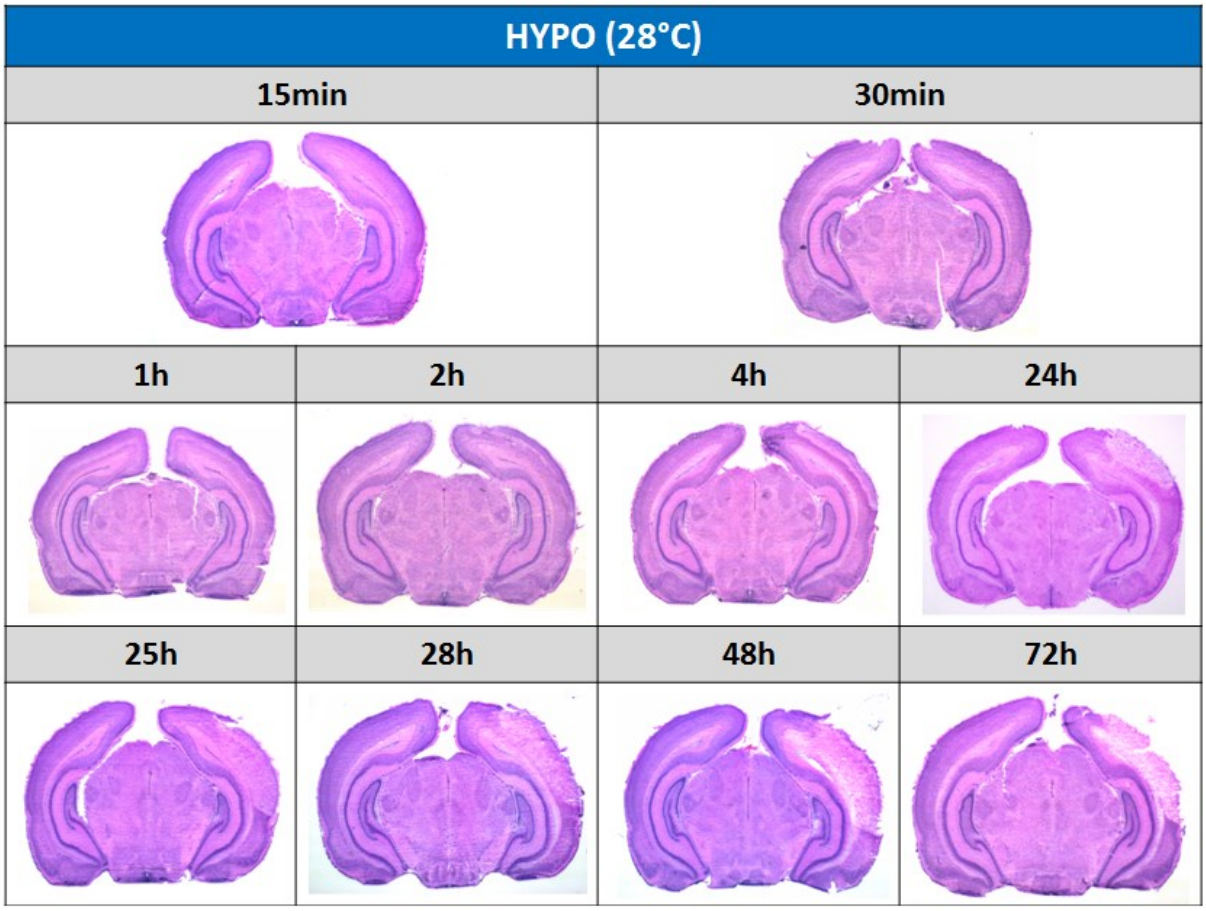


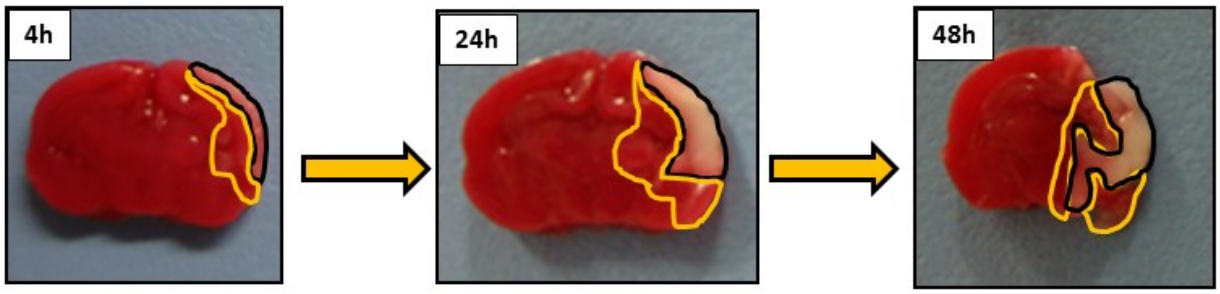
Figure 9: Study #2 – H&E-stained Brain Sections from NORMO and HYPO HIBD Pups

These images depict representative posterior sections from HIBD rat pups exposed to (A) NORMO and (B) HYPO conditions at each time-point. Each section was assessed for the approximate locations of core and penumbra to be later extracted for Western Blot protein analysis.

Comparison of H&E and TTC Staining

Figure 10 photosets A & B depict TTC- and H&E-stained coronal brain slices from six different neonatal Long-Evans rats taken at the level of the full hippocampus at 4h, 24h, and 48h after HIBD. The core of the HIBD lesion is outlined in black, whereas the surrounding penumbra is outlined in yellow. Left untreated, the injury progressively became worse, with the apoptotic and necrotic core increasing in size and reducing the amount of salvageable tissue available. Each stain visualized the damage similarly.

(A) TTC Staining



(B) H&E Staining

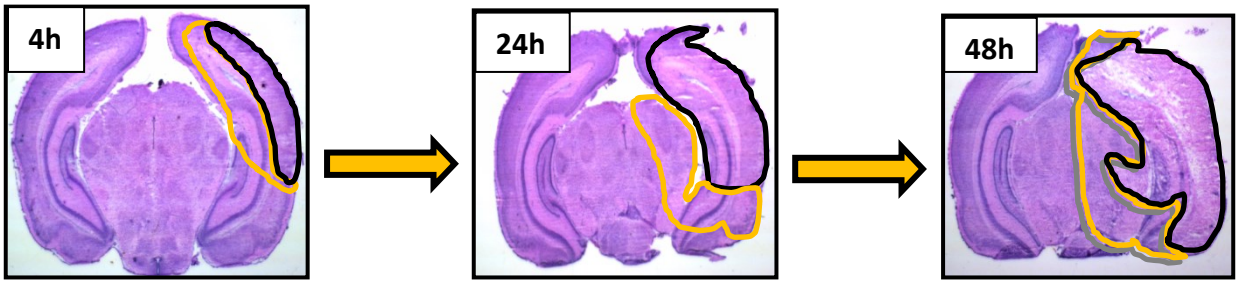


Figure 10: Evolution of HIBD Injury in a Normothermic Rat

(A) TTC-stained brain sections and (B) H&E-stained posterior brain sections at the level of a full hippocampus at 4, 24, and 48h post-HIBD. Left untreated, the HIBD core expanded from the cortex into the deep gray matter tissue. Each pictured section is from a different rat pup.

Western Blot Densitometry

The following graphs and photos depict Western Blot analysis for markers depicting energy regulation, and those along the pathway of cell death or survival (p-AMPK, GLUT3, LC3-II, Bim, and Cleaved Caspase-3).

Expression of p-AMPK

Figure 11 depicts p-AMPK as analyzed on each gel at the representative time-points of (A) 90 minutes of hypoxia, (B) 4h after HIBD (During NORMO/HYPO) and (C) 25h post-HIBD (After NORMO/HYPO).

By the end of hypoxia, penumbral p-AMPK was significantly higher than in the HIBD core. No differences between the two areas were seen at 30min into hypoxia (Figure 12 A-B).

Figure 13 (A-F) depict p-AMPK expression during the NORMO/HYPO period. At 1h into recovery, p-AMPK was significantly elevated in the HYPO core compared to the HYPO penumbra ($F(1,16) = 4.925$ $p = 0.01$). This phenomenon in the HYPO group was also observed after the removal of NORMO/HYPO (Figure 14 A-D) at 25h ($F(1,17) = 16.10$, $p = 0.01$), 28h ($F(1,17) = 7.097$, $p = 0.0164$; Interaction: $F(1,17) = 14.885$, $p = 0.0417$), and 72h ($F(1,18) = 10.87$, $p = 0.004$; Interaction: $F(1,18) = 11.98$, $p = 0.028$) post-HIBD, after HYPO was removed. Differences in regional p-AMPK exposure in the NORMO group were only observed at 4h post-HIBD, with activity higher in the penumbra ($F(1,20) = 8.810$, $p = 0.0076$; Interaction: ($F(1,20) = 8.180$, $p = 0.0097$). This elevation of NORMO penumbral p-AMPK was significantly higher than in the HYPO penumbra ($p < 0.05$). No differences within or between the core and penumbra were observed at any of the other time-points.

Figure 15 (A-B) shows p-AMPK activity over time within the (A) core and (B) penumbra comparing between the NORMO and HYPO groups. Within group differences were seen only at 4h in the penumbra ($p < 0.05$). All data depicted as mean ± SD.

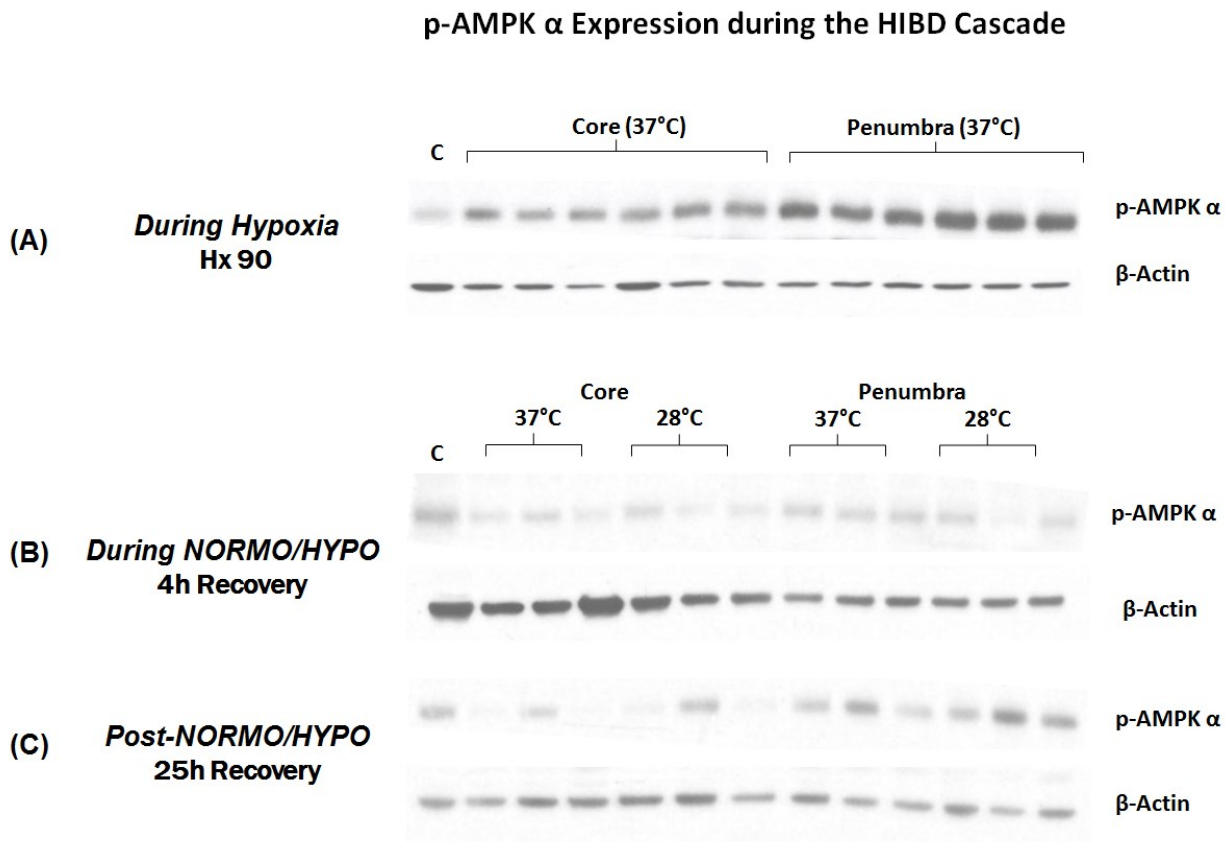


Figure 11: p-AMPK Western Blots – During Hypoxia, During Treatment, and Post-Treatment

These sets of Western Blot images depict p-AMPK α (62 kDa) as expressed at (A) the end of 90 minutes of hypoxia (Hx90), (B) 4h during the NORMO/HYPO normoxic temperature exposure, and (C) 25h after HIBD, 1h after removal from NORMO/HYPO. NORMO and HYPO core and penumbra samples from the same time-point were run per gel. A homogenate of undamaged right hemispheres from 4 naïve pups was used as a control between gels and pipetted into the first lane (denoted 'C'). β-actin (42 kDa) was used as a loading control.

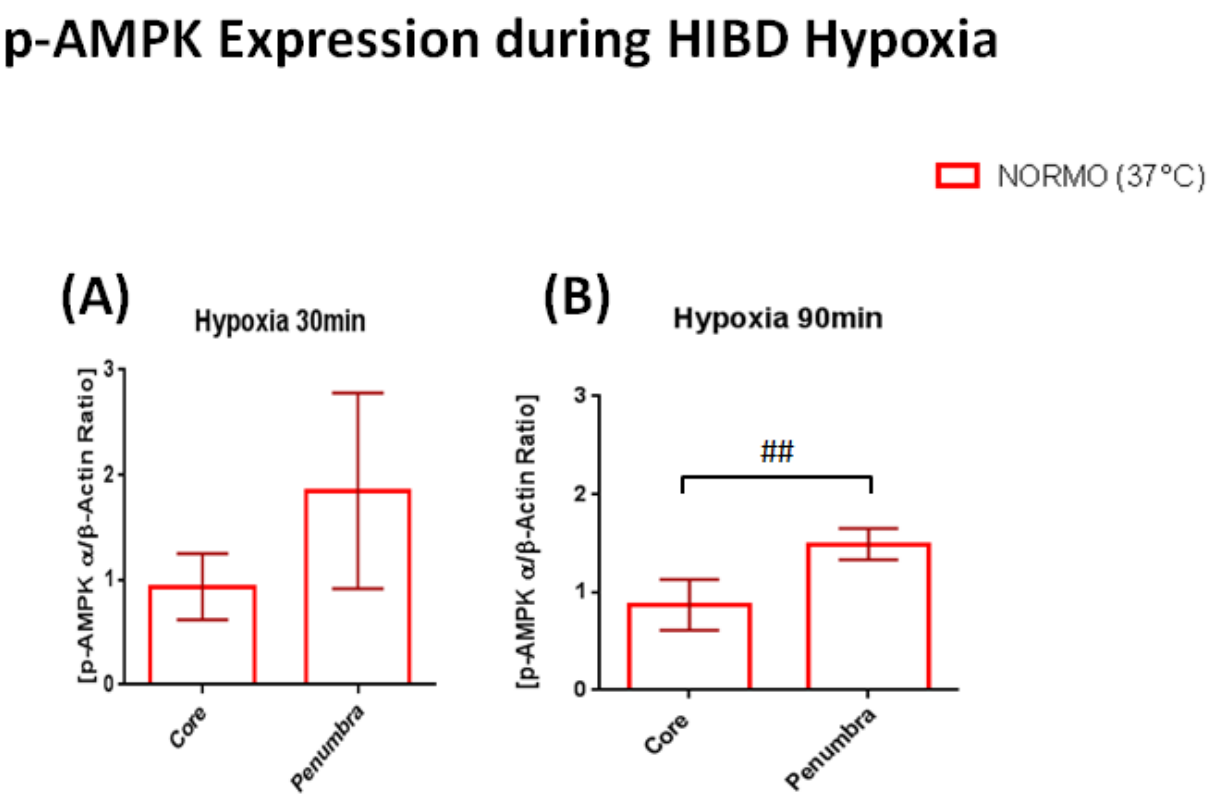


Figure 12: p-AMPK Expression during Hypoxia

Expression of p-AMPK α at (A) 30min and (B) 90min of hypoxia in the core and penumbra at 37°C. No differences were observed at 30min of hypoxia. Penumbra p-AMPK α was significantly increased at the end of 90min of hypoxia (## $p < 0.01$). Mean ± SD.

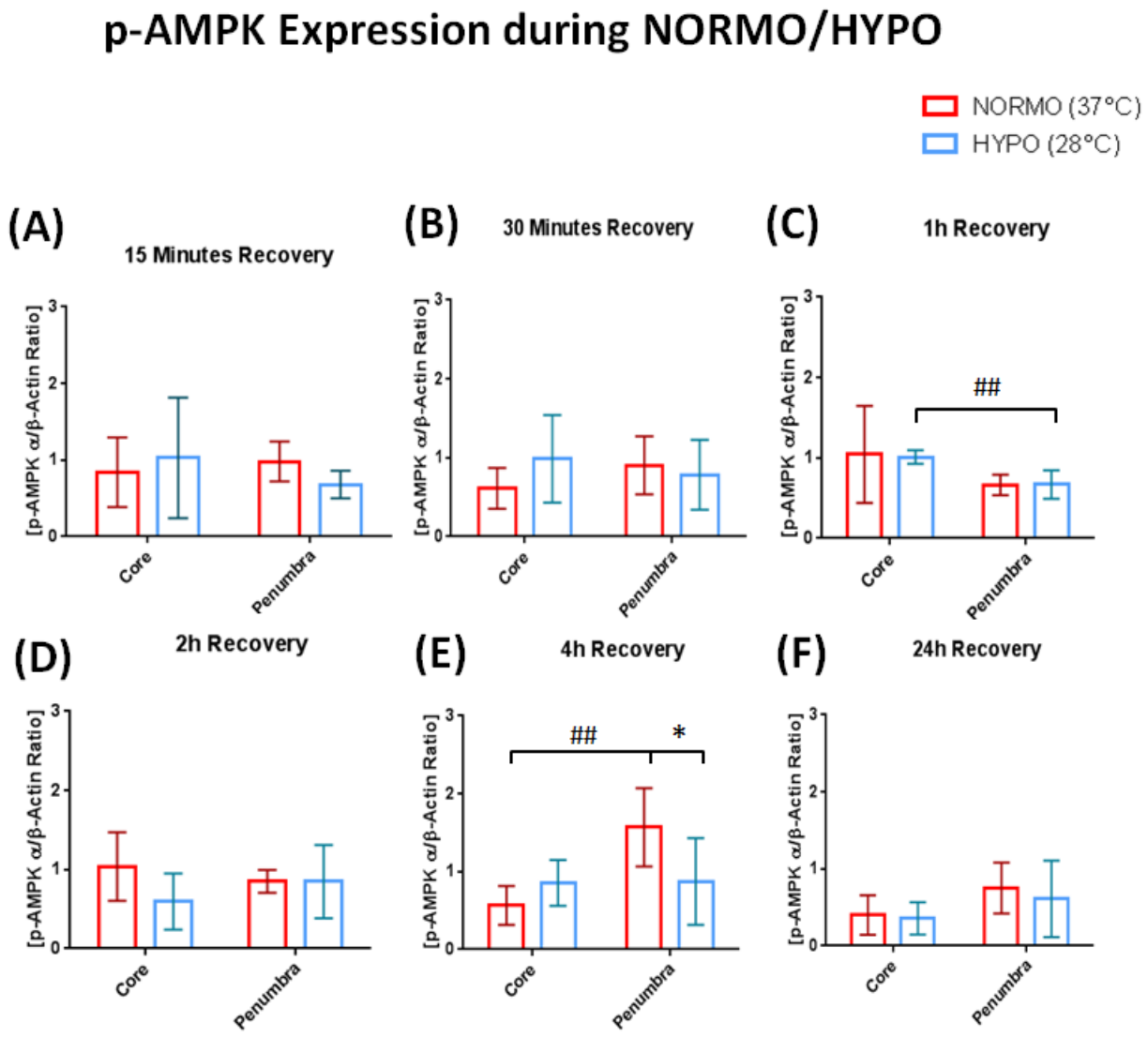


Figure 13: p-AMPK Expression During NORMO/HYPO

Expression of p-AMPK α at (A) 15min, (B) 30min, (C) 1h, (D) 2h, (E) 4h, and (F) 24h after HIBD during the NORMO and HYPO temperature exposures. At (C) 1h post-HIBD, penumbral p-AMPK α in the HYPO penumbra was significantly lower than in the HYPO core (## $p < 0.01$). At (E) 4h post-HIBD, HYPO suppressed p-AMPK α expression in the penumbra (* $p < 0.05$). Mean ± SD.

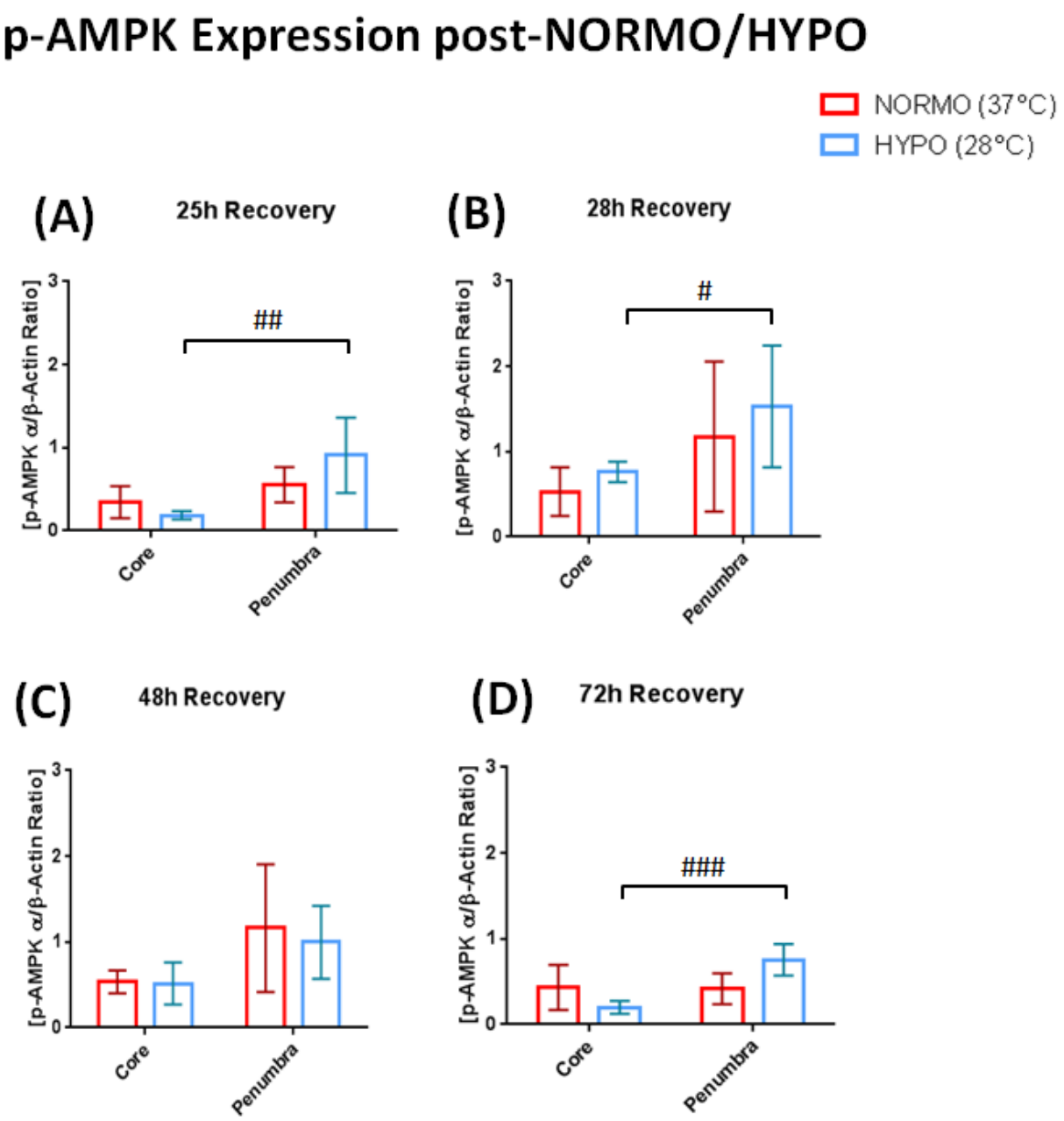
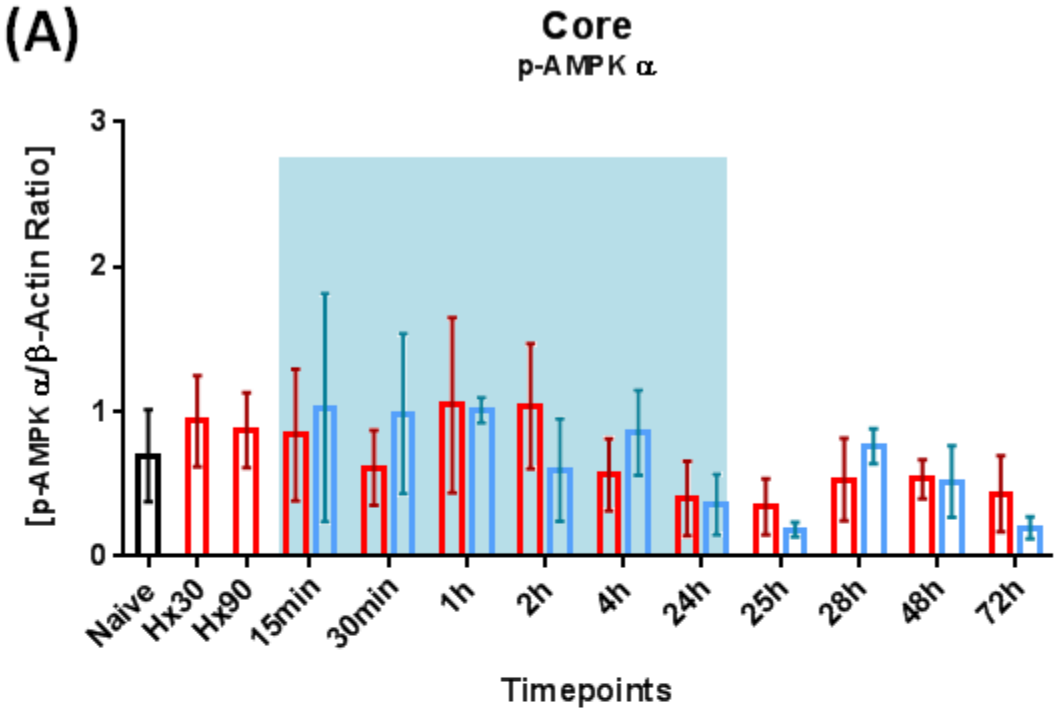


Figure 14: p-AMPK Expression post-NORMO/HYPO

Expression of p-AMPK α at (A) 25h (B) 28h, (C) 48h, and (D) 72h post-HIBD, after removal from the NORMO and HYPO environments. HYPO p-AMPK α was significantly elevated compared to the HYPO core at (A) 25h (## $p < 0.01$), (B) 28h (# $p < 0.05$), and (D) 72h (### $p < 0.001$). Mean ± SD.


(A)
Core
p-AMPK α
[p-AMPK α/β-Actin Ratio]
3
2
1
0
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

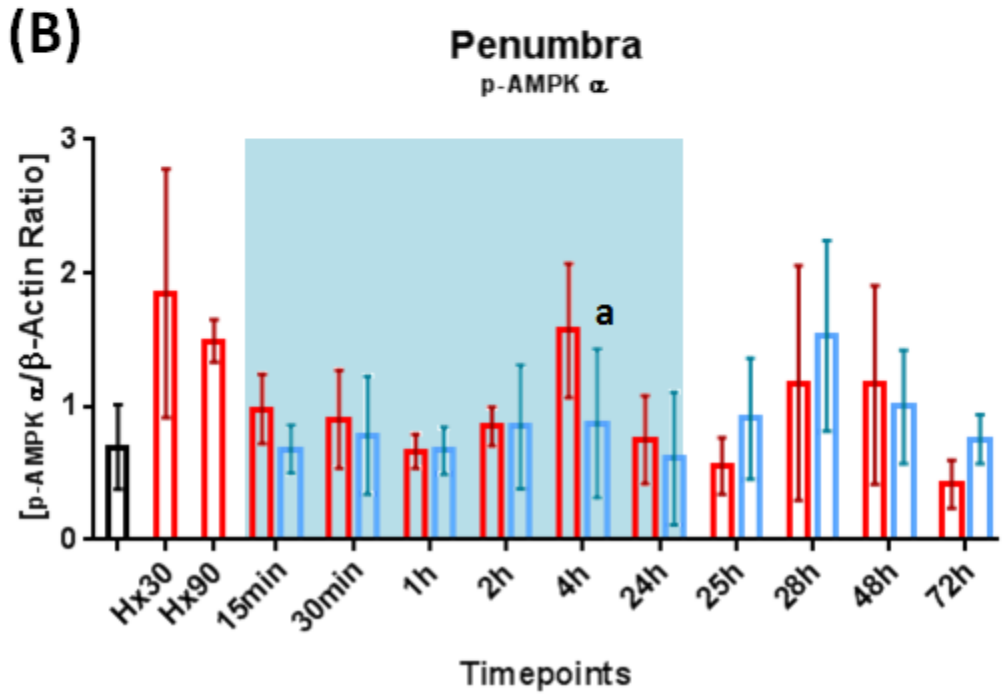

(B)
Penumbra
p-AMPK α
[p-AMPK α/β-Actin Ratio]
3
2
1
0
a
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

Figure 15: Temporal p-AMPK Expression in the NORMO and HYPO Core and Penumbra

Expression levels of p-AMPK α with NORMO and HYPO at each of the examined time-points within the (A) core and (B) penumbra. Solid blue box indicates the 24 hour NORMO/HYPO normoxic exposure period. (a) $p < 0.05$ vs. NORMO. Mean ± SD.

Expression of GLUT3

Figure 16 depicts GLUT3 as analyzed on each gel at the representative time-points of (A) 90 minutes of hypoxia, (B) 4h after HIBD (During NORMO/HYPO) and (C) 25h post-HIBD (After NORMO/HYPO).

GLUT3 expression was significantly higher in the penumbra throughout hypoxia (Hx30: $p < 0.05$; Hx90: $p < 0.00l$ Figure 17 A-B).

During NORMO/HYPO (Figure 18 A-F), this elevation in penumbral GLUT3 remained significant 15min ($F(1,19) = 9.025$, $p < 0.01$.), 30min ($F(1,18) = 71.25$, $p < 0.0001$) , 2h, and 4h into NORMO recovery, with the exception of 1h and 24h post-HIBD, where levels returned to baseline. The same was true within the HYPO group (15min: $p < 0.01$; 30min: $p < 0.0001$; 2h: $p < 0.01$; 4h: $p < 0.01$).

After NORMO/HYPO were removed (Figure 19 A-D), the NORMO penumbra again displayed significantly higher levels of GLUT3 at 28h post-HIBD ($F(1,18) = 7.097$, $p < 0.05$). However in the HYPO group, penumbral GLUT3 activity rose significantly compared to the HYPO core at 25h ($F(1,20) = 15.96$, $p < 0.01$), 28h ($p < 0.01$), and 72h ($F(1,20) = 16.59$, $p < 0.05$). Within the HIBD core, HYPO consistently suppressed GLUT3 activity at 25h ($p < 0.05$), 28h ($p < 0.001$), and 72h ($p < 0.05$) compared to NORMO after HIBD. Values between regions and treatment groups did not differ at 48h post-HIBD.

GLUT3 was significantly higher in the NORMO core than the HYPO core at 30min ($p < 0.05$), 25h ($p < 0.05$), 28h ($p < 0.001$), and 72h ($p < 0.05$; Figure 20 A-B). GLUT3 values between the temperatures in the penumbra did not statistically differ at any of the examined time-points. All data depicted as mean ± SD.

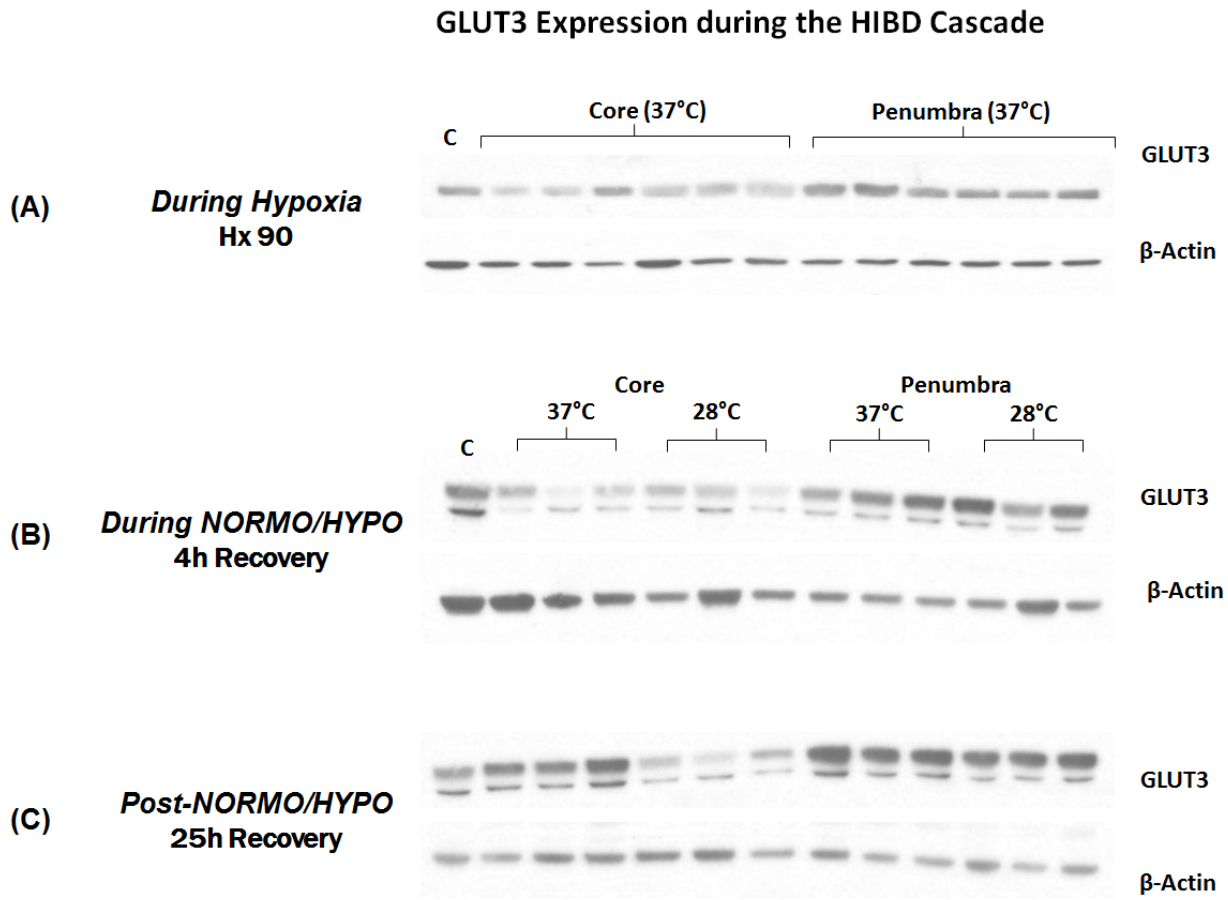


Figure 16: GLUT3 Expression – During Hypoxia, During Treatment, and Post-Treatment

These sets of Western Blot images depict GLUT3 (54 kDa) as expressed at (A) the end of 90 minutes of hypoxia (Hx90), (B) 4h during the NORMO/HYPO normoxic temperature exposure, and (C) 25h after HIBD, 1h after removal from NORMO/HYPO. NORMO and HYPO core and penumbra samples from the same time-point were run per gel. A homogenate of undamaged right hemispheres from 4 naïve pups was used as a control between gels and pipetted into the first lane (denoted 'C'). β-actin (42 kDa) was used as a loading control.

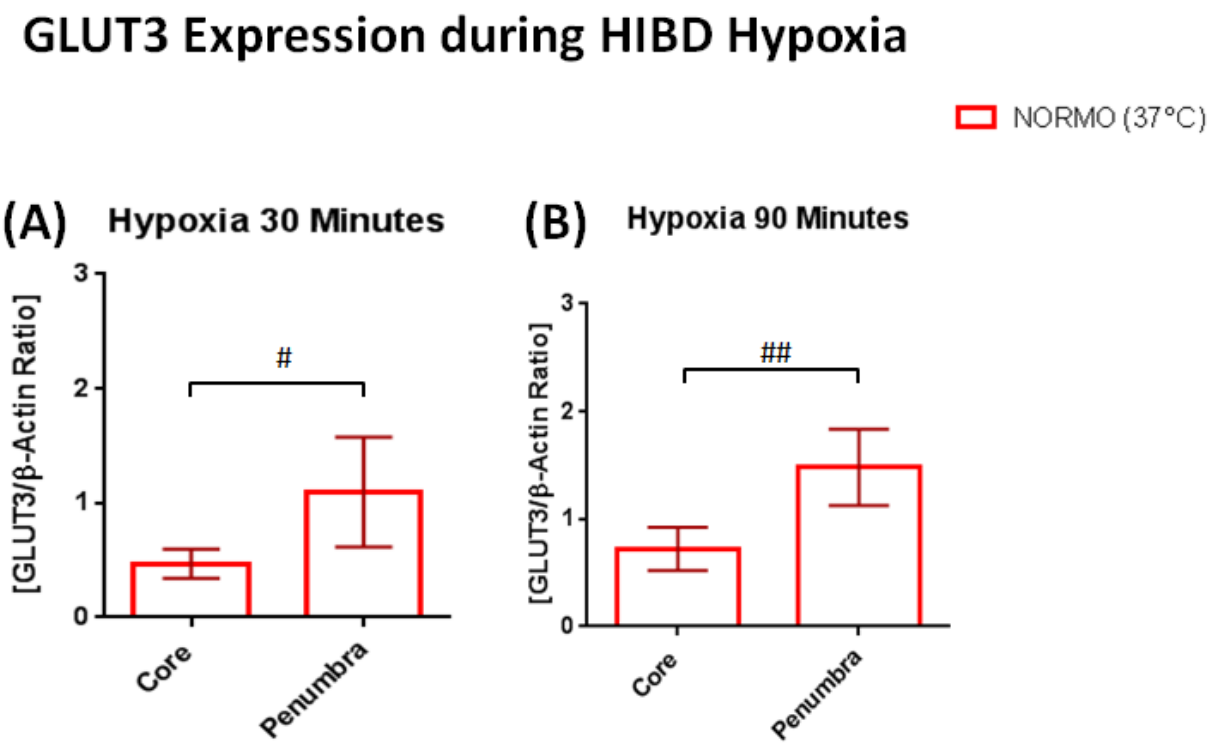


Figure 17: GLUT3 Expression during Hypoxia

Expression of GLUT3 at (A) 30min and (B) 90min of hypoxia in the core and penumbra at 37°C. GLUT was consistently and significantly elevated in the HIBD penumbra (30min: # $p < 0.05$; 90min: ## $p < 0.01$). Mean ± SD.

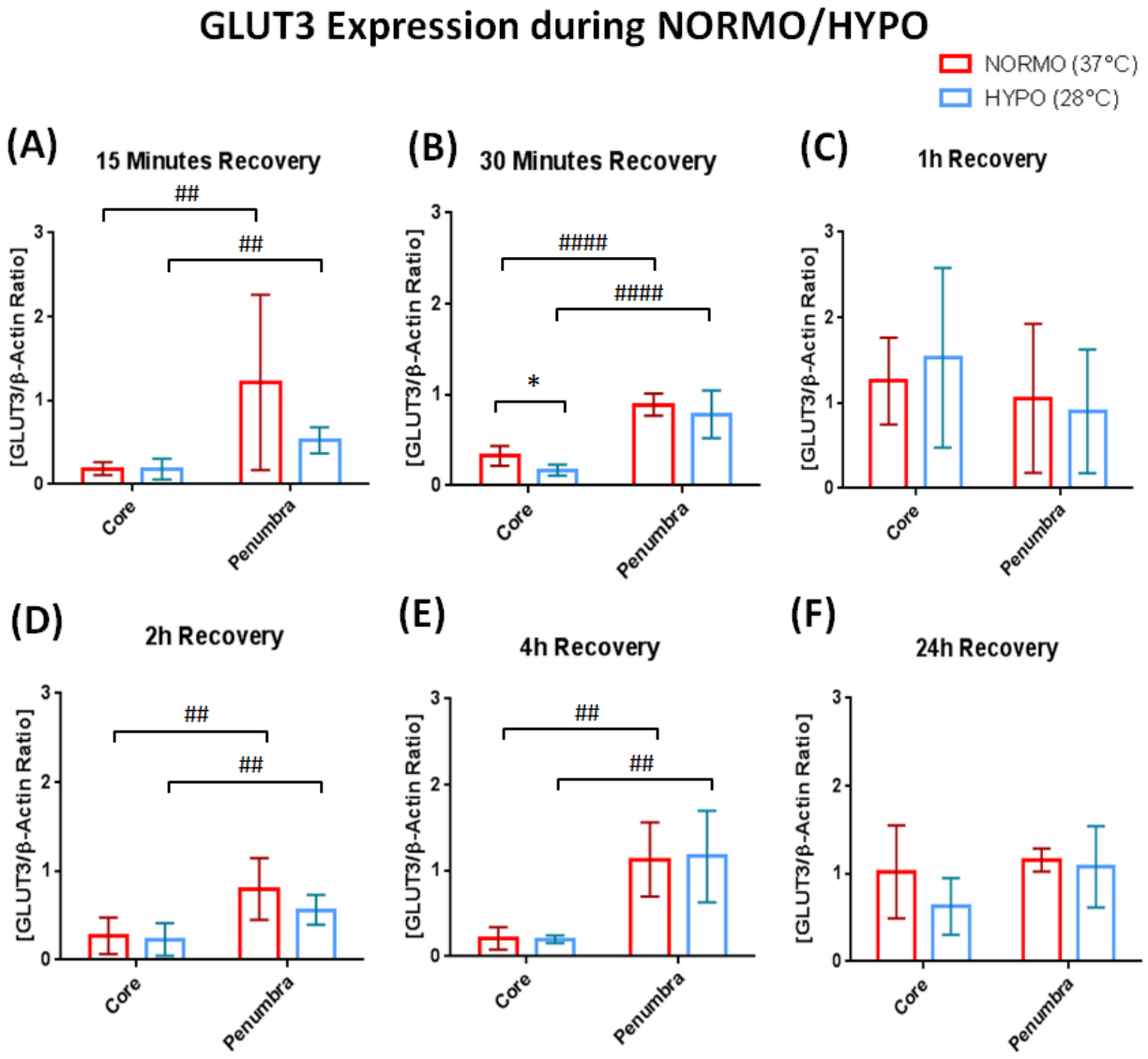


Figure 18: GLUT3 Expression During NORMO/HYPO

Expression of GLUT3 at (A) 15min, (B) 30min, (C) 1h, (D) 2h, (E) 4h, and (F) 24h after HIBD during the NORMO and HYPO temperature exposures. Within the HIBD core, compared to NORMO, HYPO suppressed GLUT3 at 30min of recovery (B; * $p < 0.05$). At (A) 15min (## $p < 0.01$), (B) 30min (### $p < 0.001$), (D) 2h (## $p < 0.01$), and (E) 4h (## $p < 0.01$) of recovery, GLUT3 was significantly suppressed in the NORMO and HYPO core compared to their corresponding penumbra. Mean ± SD.

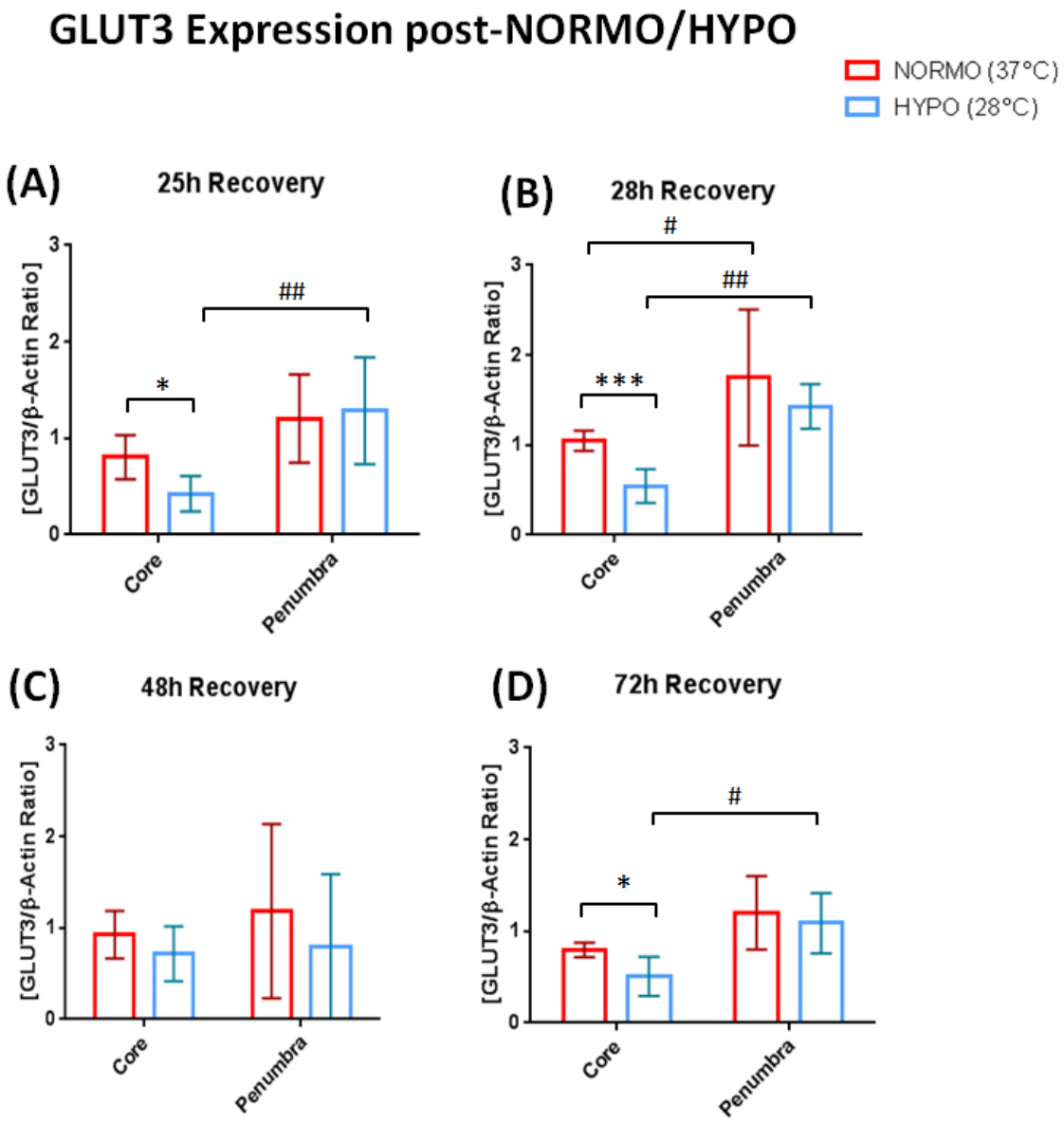
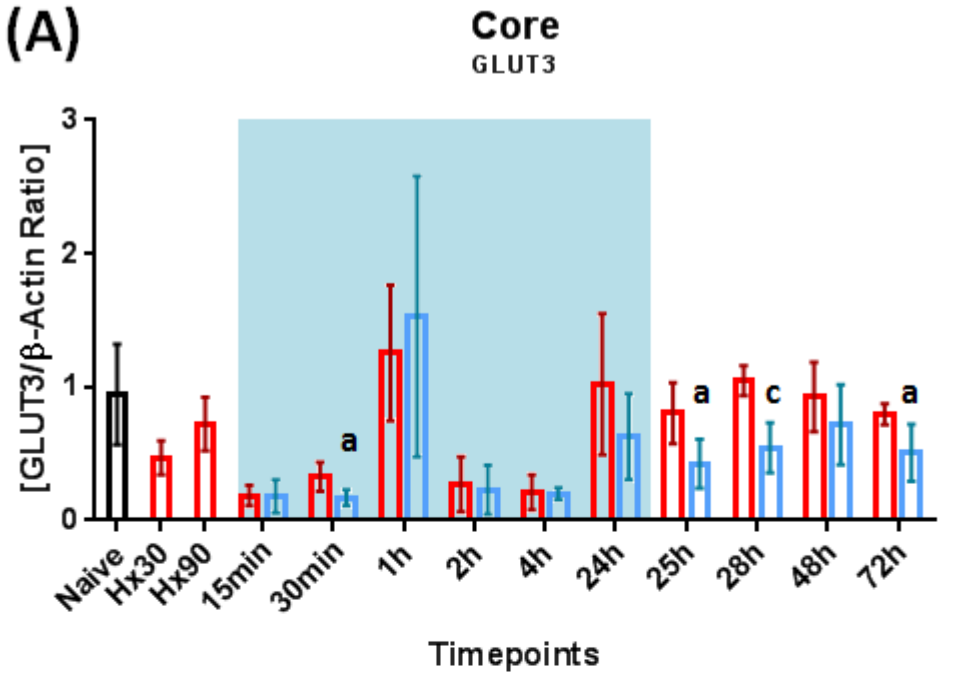


Figure 19: GLUT3 Expression post-NORMO/HYPO

Expression of GLUT3 at (A) 25h (B) 28h, (C) 48h, and (D) 72h post-HIBD, after removal from the NORMO and HYPO environments. Within the HIBD core, HYPO significantly suppressed GLUT3 at (A) 25h (* $p < 0.05$), (B) 28h (*** $p < 0.001$), and (C) 72h (* $p < 0.05$). GLUT3 was also significantly suppressed in the HYPO core compared to the penumbra within temperatures at these time-points. Mean ± SD.


(A)
Core
GLUT3
[GLUT3/β-Actin Ratio]
3
2
1
0
a
a
c
a
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

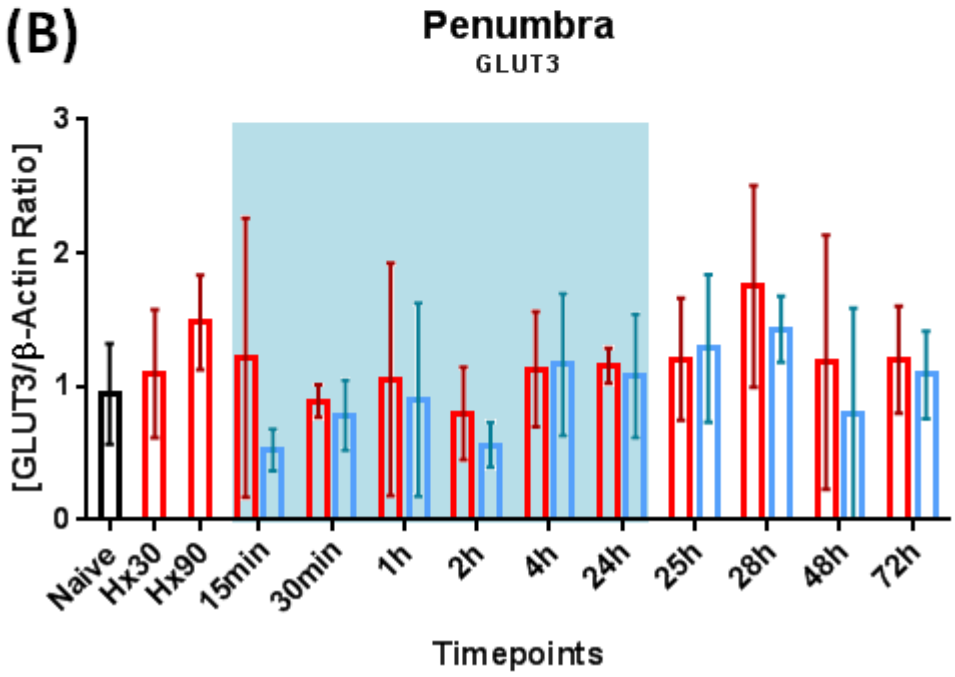

(B)
Penumbra
GLUT3
[GLUT3/β-Actin Ratio]
3
2
1
0
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

Figure 20: Temporal GLUT3 Expression in the NORMO and HYPO Core and Penumbra

Expression levels of GLUT3 with NORMO and HYPO at each of the examined time-points within the (A) core and (B) penumbra. Solid blue box indicates the 24 hour NORMO/HYPO normoxic exposure period. (a) $p < 0.05$ vs. NORMO, (c) $p < 0.001$ vs. NORMO. Mean ± SD.

Expression of LC3-II

Figure 21 (A-B) depicts LC3-II as analyzed on each gel at the representative time-points of (A) 90 minutes of hypoxia, (B) 4h after HIBD (During NORMO/HYPO) and (C) 25h post-HIBD (After NORMO/HYPO).

LC3-II expression did not differ between core and penumbra during hypoxia (Figure 22 A-B). During NORMO/HYPO, LC3-II levels did not differ significantly between and within regions (Figure 23 A-F), apart from 1h post-HIBD where the HYPO penumbra expressed significantly higher levels of LC3-II than the NORMO penumbra ($p < 0.05$; Figure 22 C).

After removal of NORMO/HYPO (Figure 24 A-D), HYPO suppressed LC3-II levels in the HIBD penumbra at 25h ($p < 0.05$) and 48h ($p < 0.05$). At 28h post-HIBD, LC3-II was significantly upregulated in the NORMO core as compared to the NORMO penumbra ($F(1,19) = 20.06$, $p < 0.01$) and the HYPO core ($p < 0.05$); this shows that HYPO significantly suppressed autophagy in the core.

Figure 25 (A-B) depict LC3-II levels within their respective time points and treatments in the (A) core and (B) penumbra. In the HIBD core, no differences in expression were observed within each temperature, apart from 28h post-HIBD where HYPO exerted a suppressive effect ($p < 0.05$). In the HIBD penumbra, HYPO enhanced LC3-II expression at 1h post-HIBD only and suppressed its expression at 25h and 48h post-HIBD. No other significant differences were observed within each time-point. All data depicted as mean ± SD.

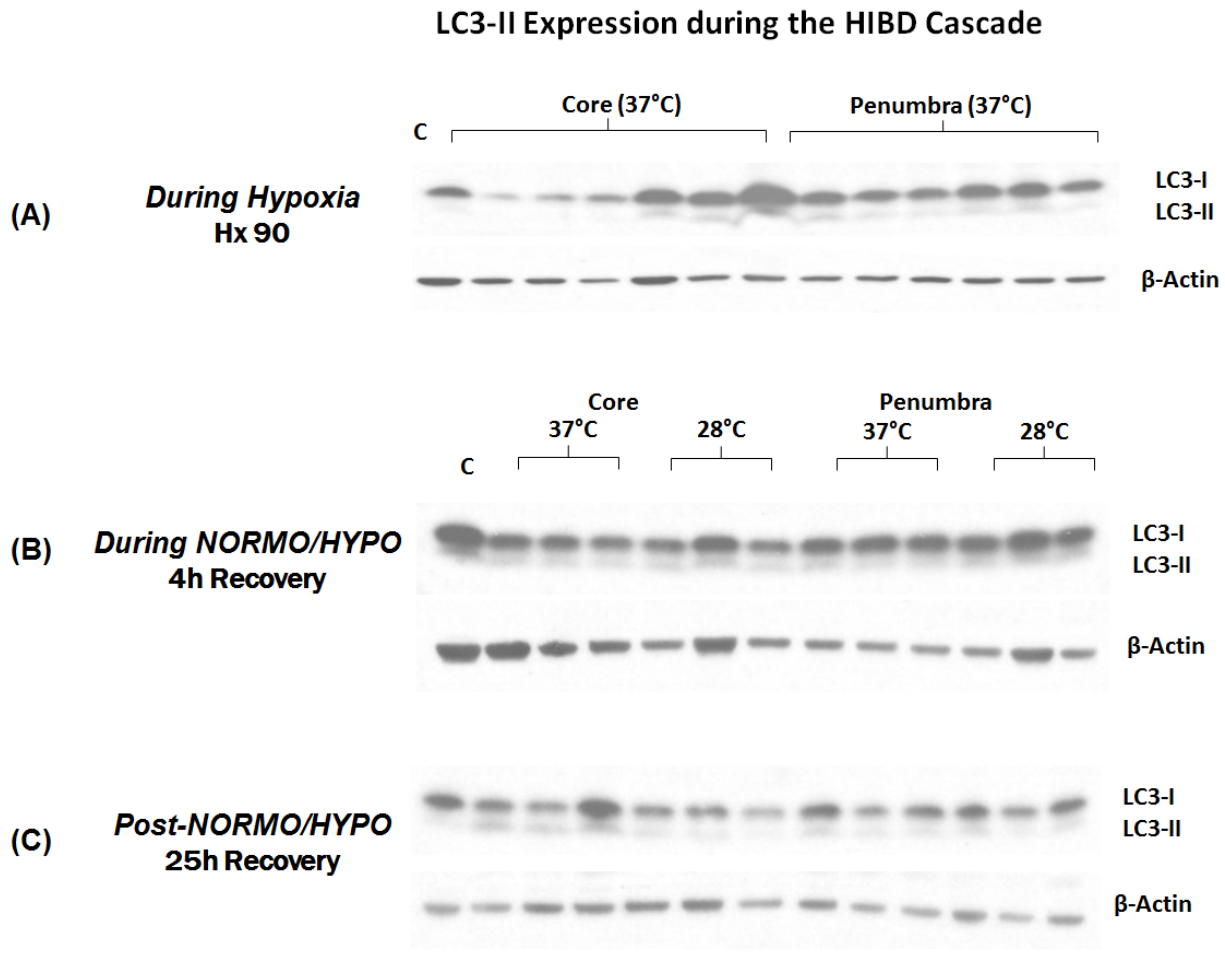


Figure 21: LC3-II Expression – During Hypoxia, During Treatment, and Post-Treatment

These sets of Western Blot images depict LC3-II (17-19 kDa) as expressed at (A) the end of 90 minutes of hypoxia (Hx90), (B) 4h during the NORMO/HYPO normoxic temperature exposure, and (C) 25h after HIBD, 1h after removal from NORMO/HYPO. NORMO and HYPO core and penumbra samples from the same time-point were run per gel. A homogenate of undamaged right hemispheres from 4 naïve pups was used as a control between gels and pipetted into the first lane (denoted 'C'). β-actin (42 kDa) was used as a loading control.

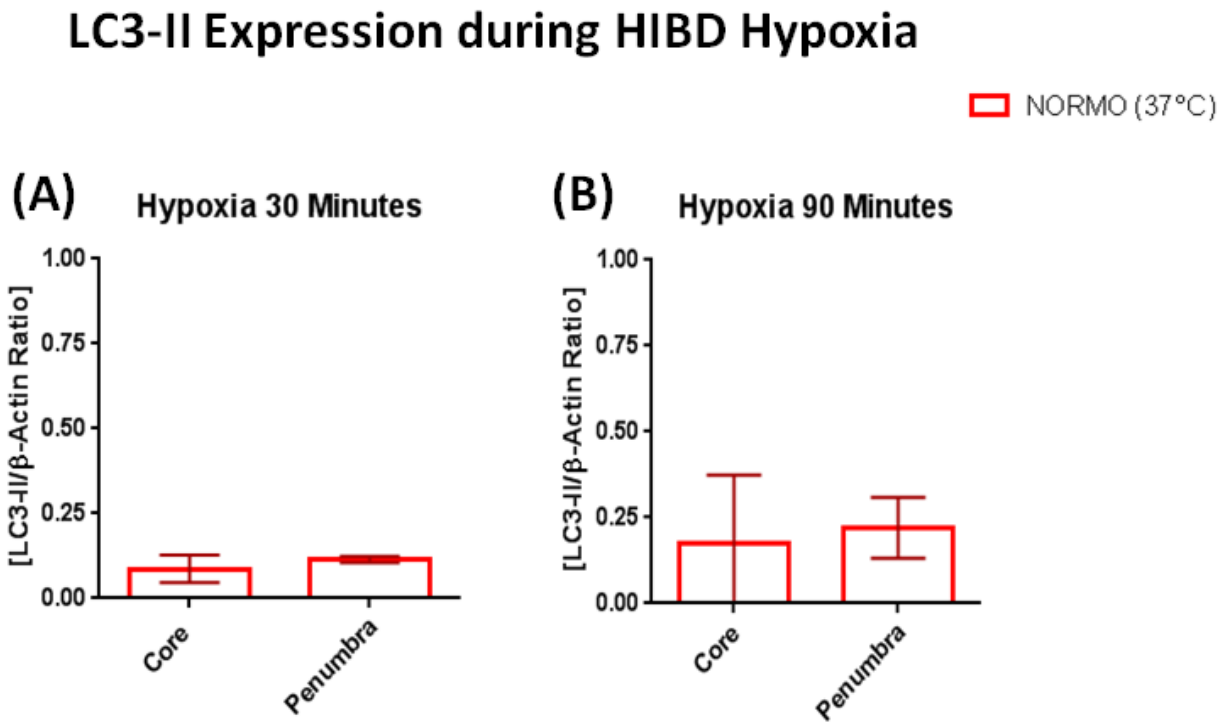


Figure 22: LC3-II Expression during Hypoxia

Expression of LC3-II at (A) 30min and (B) 90min of hypoxia in the core and penumbra at 37°C.

No differences in expression were observed at these time-points. Mean ± SD.

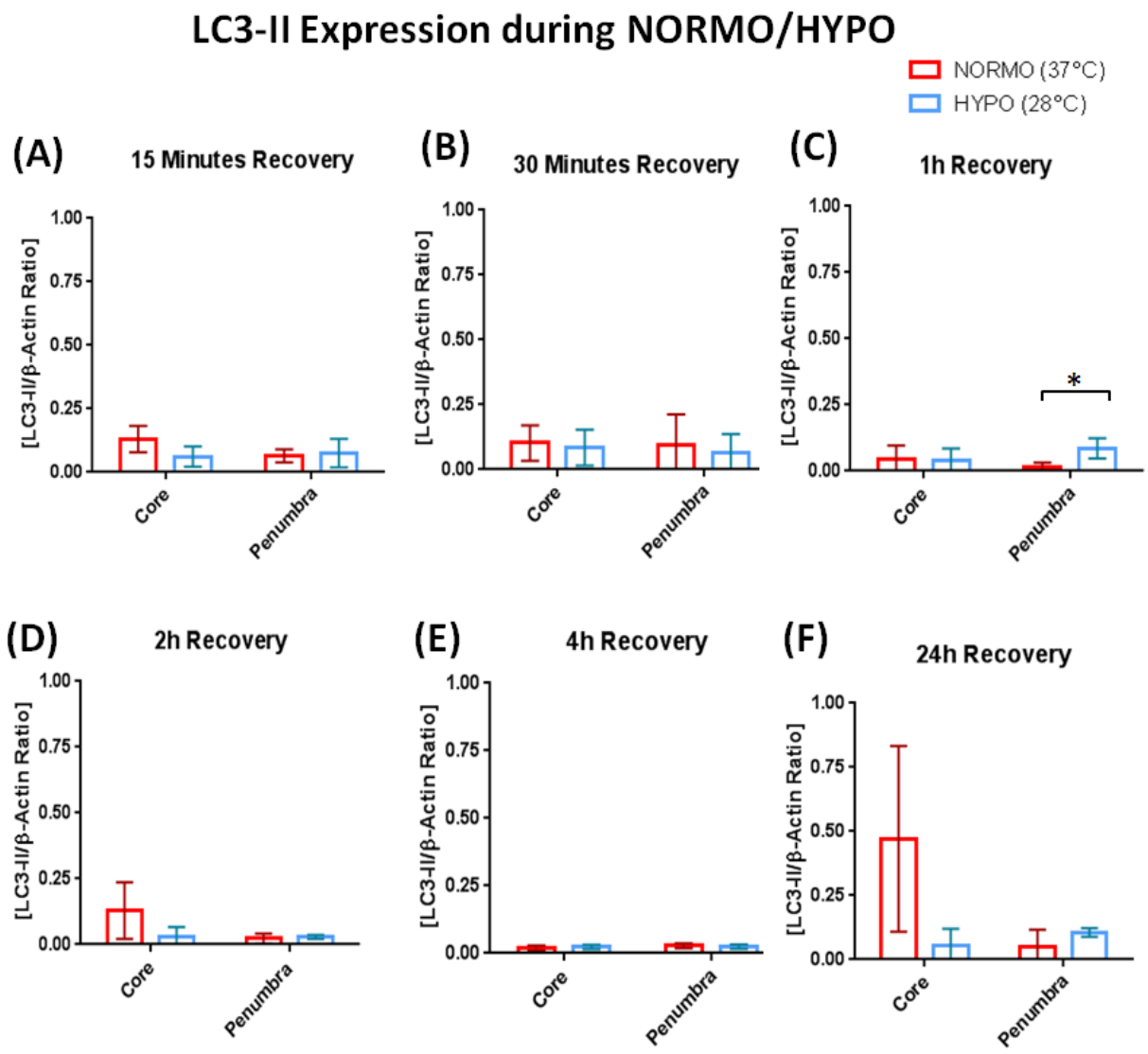


Figure 23: LC3-II Expression During NORMO/HYPO

Expression of LC3-II at (A) 15min, (B) 30min, (C) 1h, (D) 2h, (E) 4h, and (F) 24h after HIBD during the NORMO and HYPO temperature exposures. In the HIBD core, no differences between temperatures were observed. In the HIBD penumbra, a significant increase in LC3-II expression was seen at (C) 1h post-HIBD in the HYPO treatment (* $p < 0.05$). Mean ± SD.

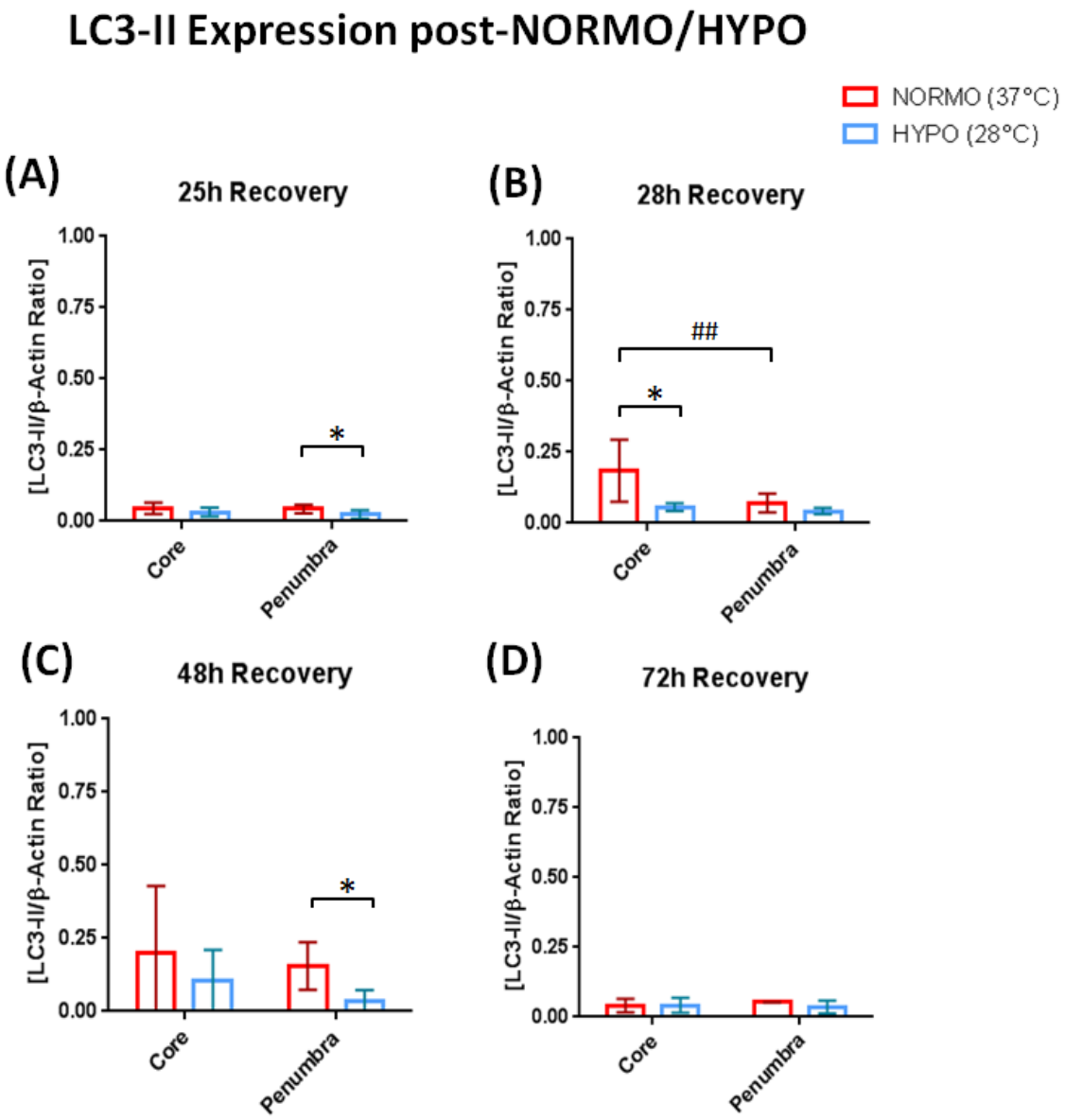
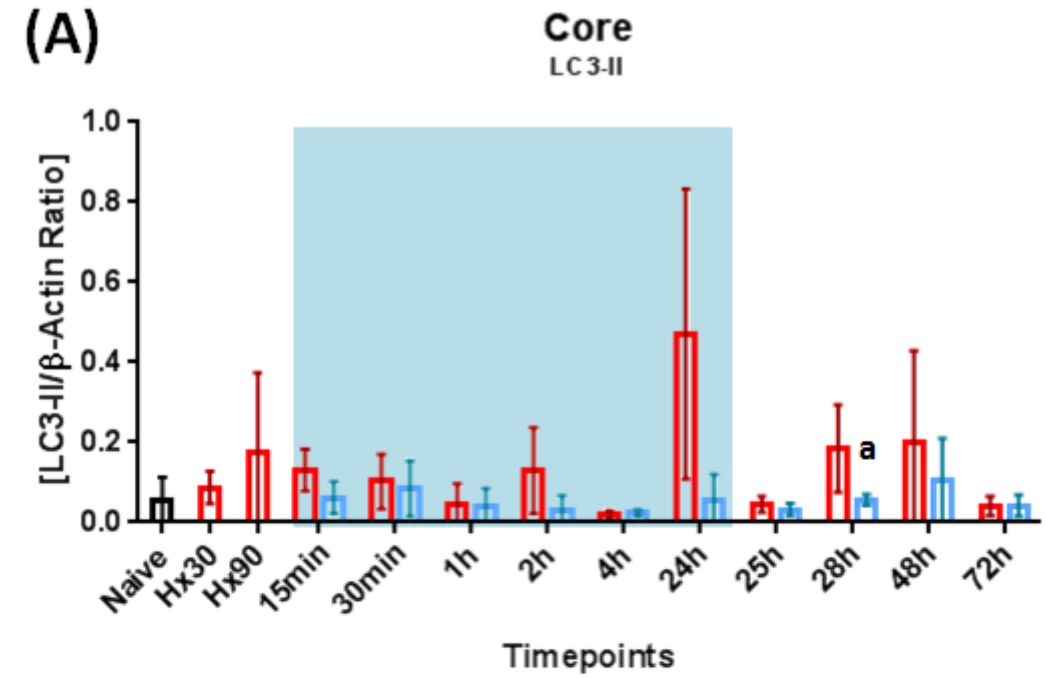


Figure 24: LC3-II Expression post-NORMO/HYPO

Expression of LC3-II at (A) 25h (B) 28h, (C) 48h, and (D) 72h post-HIBD, after removal from the NORMO and HYPO environments. LC3-II was significantly suppressed in the HYPO penumbra at (A) 25h and (C) 48h of recovery (* $p < 0.05$). HYPO suppressed LC3-II in the HIBD core only at (B) 28h of recovery (* $p < 0.05$). Mean ± SD.


(A)
Core
LC3-II
[LC3-II/β-Actin Ratio]
1.0
0.8
0.6
0.4
0.2
0.0
a
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

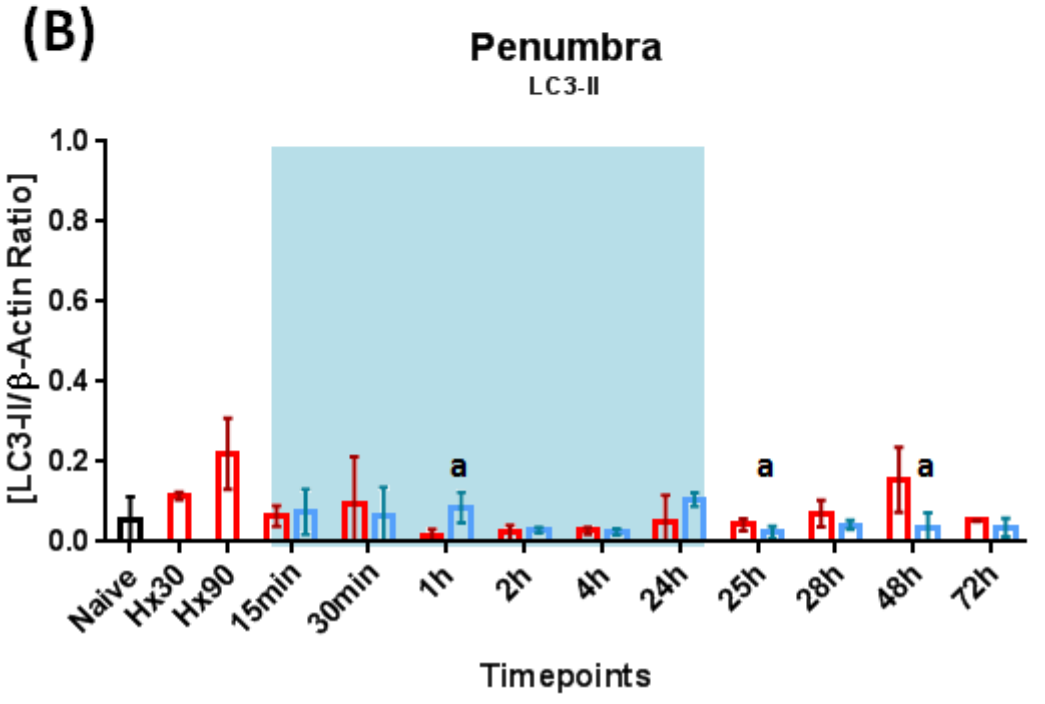

(B)
Penumbra
LC3-II
[LC3-II/β-Actin Ratio]
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

Figure 25: Temporal LC3-II Expression in the NORMO and HYPO Core and Penumbra

Expression levels of LC3-II with NORMO and HYPO at each of the examined time-points within the (A) core and (B) penumbra. Solid blue box indicates the 24 hour NORMO/HYPO normoxic exposure period. (a) $p < 0.05$ vs. NORMO. Mean ± SD.

Expression of Bim

Figure 26 (A-B) depicts Bim as analyzed on each gel at the representative time-points of (A) 90 minutes of hypoxia, (B) 4h after HIBD (During NORMO/HYPO) and (C) 25h post-HIBD (After NORMO/HYPO).

Bim activity was significantly higher in the penumbra compared to the core at 30min into hypoxia ($p < 0.001$), but this difference was not maintained to the end of hypoxia (Figure 27 A-B).

During NORMO/HYPO (Figure 28 A-F), Bim expression was significantly increased in the NORMO penumbra as compared to the core ($F(1,19) = 9.361$, $p < 0.01$) at 4h post-HIBD; this activity was suppressed by HYPO in the penumbra at this time ($p < 0.001$). At 24h post-HIBD, HYPO significantly upregulated Bim expression in the core as compared to the NORMO core ($p > 0.05$) and the HYPO penumbra ($F(1,19) = 8.750$, $p < 0.0001$).

After removal of NORMO/HYPO (Figure 29 A-D), suppression of Bim was observed in the HYPO core at 25h ($p < 0.05$) and 48h ($p < 0.05$) post-HIBD. At these times ($F(1,18) = 10.14$, $p < 0.05$), and also at 28h post-HIBD ($F(1,18) = 30.74$, $p < 0.01$), Bim was also significantly lower than in the HYPO penumbra. 28h post-HIBD, BIm expression in the NORMO penumbra was significantly higher than the NORMO core ($p < 0.05$).

In the HIBD core, HYPO enhanced Bim at 24h ($p < 0.05$) and suppressed it at 25h ($p < 0.05$) and 48h ($p < 0.05$) compared to NORMO (Figure 30 A). In the HIBD penumbra, HYPO only suppressed Bim activity at 4h post-HIBD ($p < 0.001$; Figure 30 B). No other significant differences were observed within each time-point. All data depicted as mean ± SD.

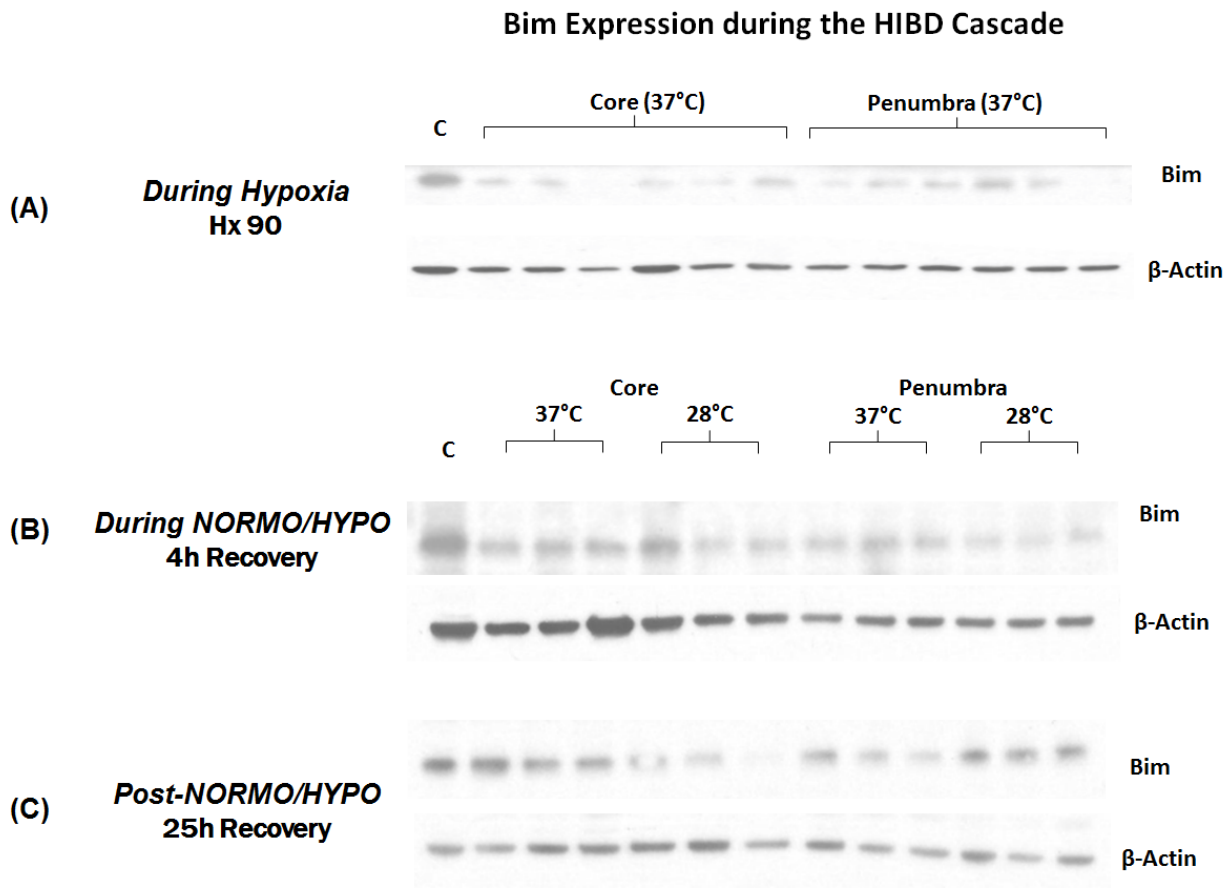


Figure 26: Bim Expression – During Hypoxia, During Treatment, and Post-Treatment

These sets of Western Blot images depict Bim (23 kDa) as expressed at (A) the end of 90 minutes of hypoxia (Hx90), (B) 4h during the NORMO/HYPO normoxic temperature exposure, and (C) 25h after HIBD, 1h after removal from NORMO/HYPO. NORMO and HYPO core and penumbra samples from the same time-point were run per gel. A homogenate of undamaged right hemispheres from 4 naïve pups was used as a control between gels and pipetted into the first lane (denoted 'C'). β-actin (42 kDa) was used as a loading control.

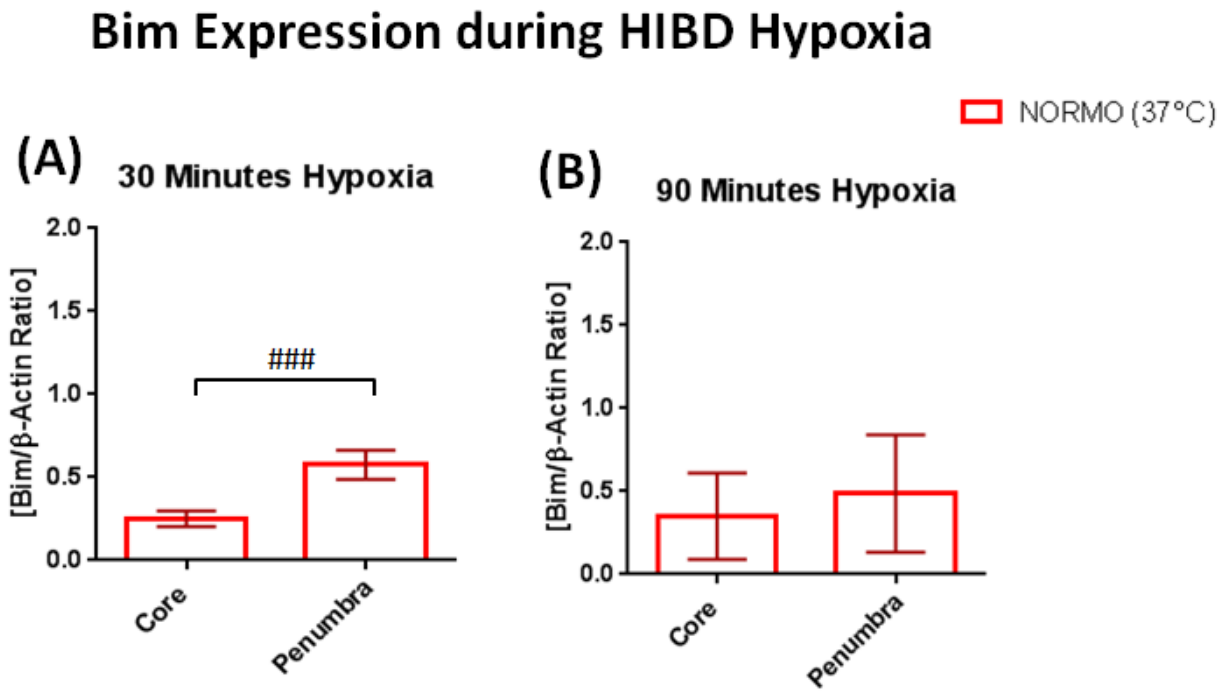


Figure 27: Bim Expression during Hypoxia

Expression of Bim at (A) 30min and (B) 90min of hypoxia in the core and penumbra at 37°C. Bim was significantly elevated in the penumbra at (A) 30min of hypoxia (### $p < 0.001$), but this difference was not observed at the end of hypoxia. Mean ± SD.

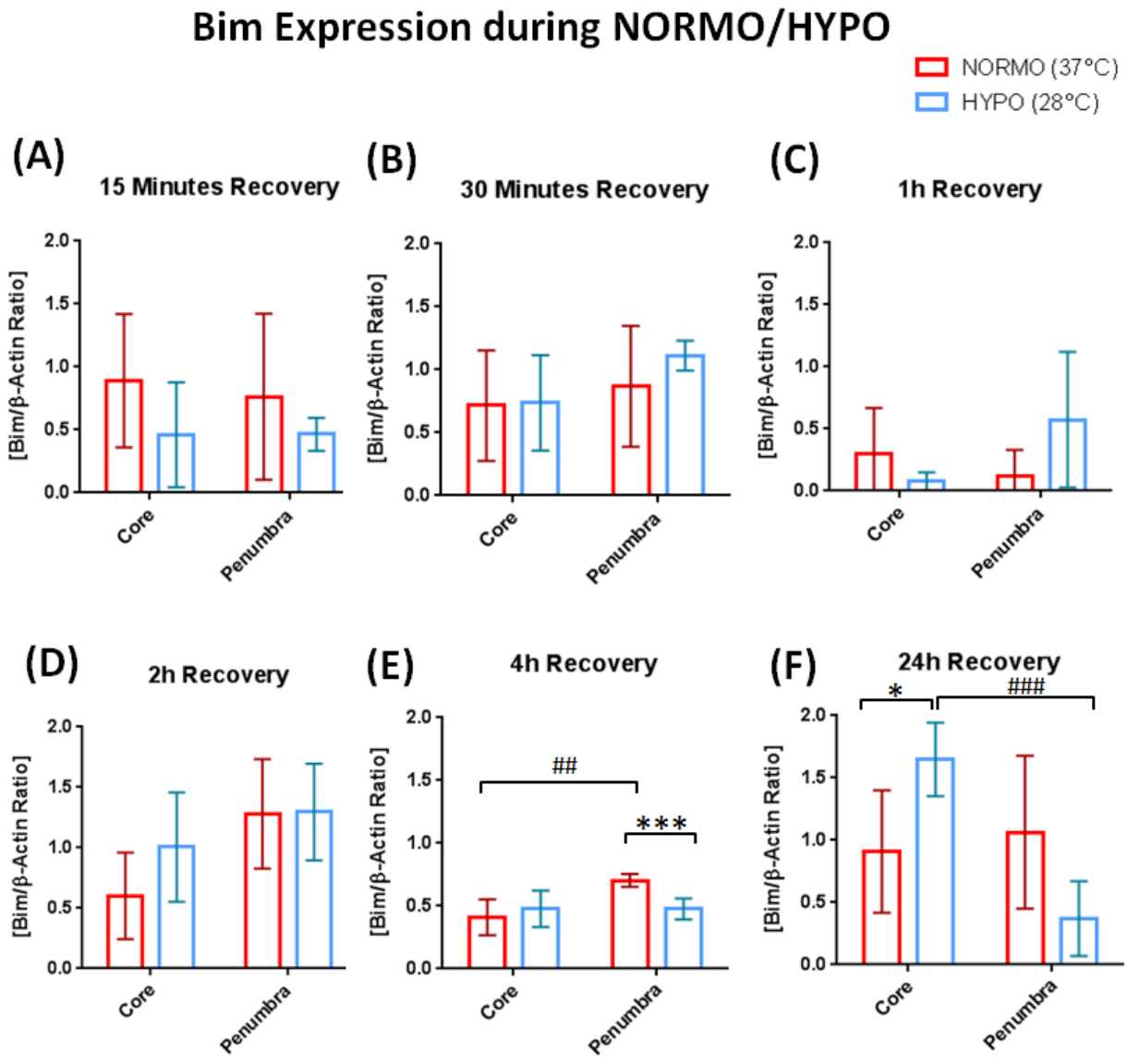


Figure 28: Bim Expression During NORMO/HYPO

Expression of Bim at (A) 15min, (B) 30min, (C) 1h, (D) 2h, (E) 4h, and (F) 24h after HIBD during the NORMO and HYPO temperature exposures. HYPO significantly suppressed Bim in the HIBD penumbra at (E) 4h post-HIBD (*** $p < 0.001$). In the HIBD core, Bim was significantly elevated with HYPO at 24h post-HIBD (* $p < 0.05$). Mean ± SD.

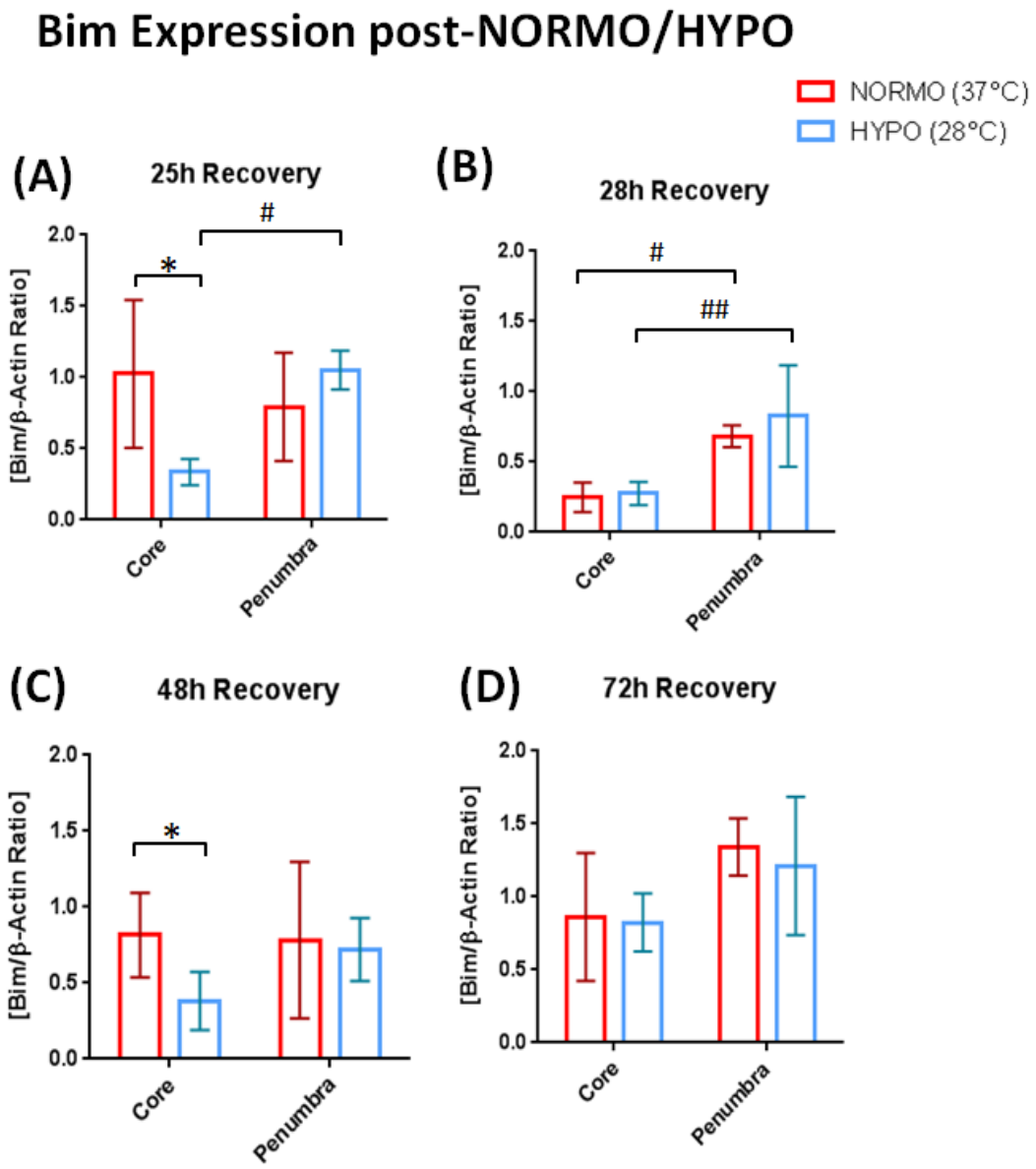
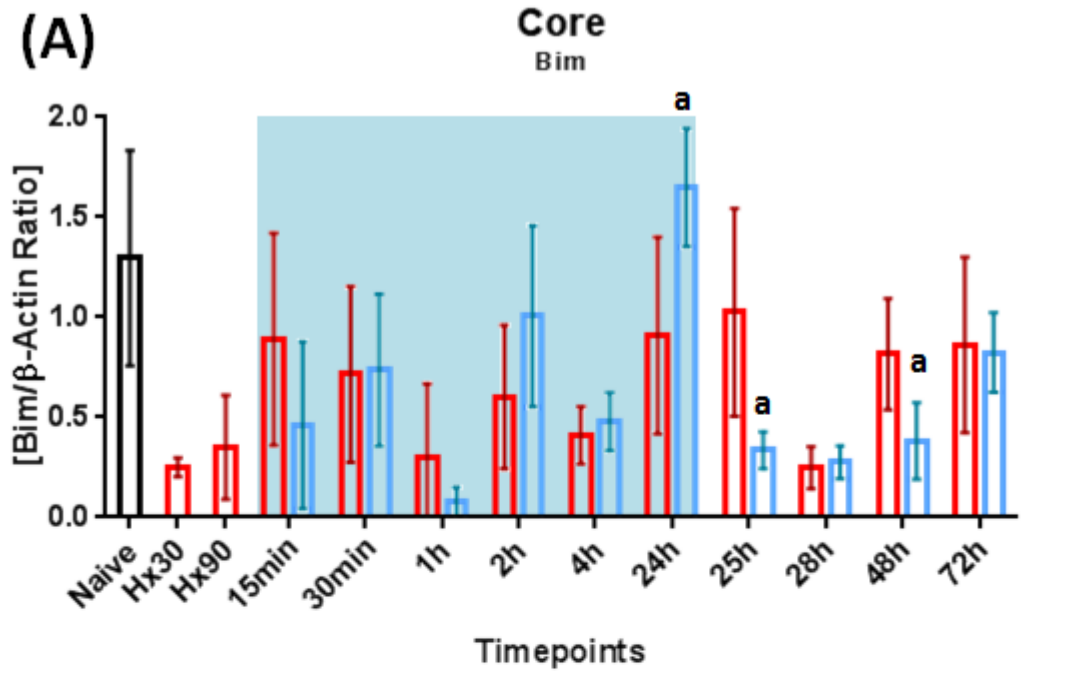


Figure 29: Bim Expression post-NORMO/HYPO

Expression of Bim at (A) 25h (B) 28h, (C) 48h, and (D) 72h post-HIBD, after removal from the NORMO and HYPO environments. HYPO significantly suppressed Bim in the HIBD core at (A) 25h and (C) 48h of recovery (* $p < 0.05$). The HYPO penumbra expressed significantly more Bim than its core at (A) 25h (# $p < 0.05$) and (B) 28h (## $p < 0.01$) of recovery. Mean ± SD.


(A)
Core
Bim
[Bim/β-Actin Ratio]
2.0
1.5
1.0
0.5
0.0
a
a
a
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

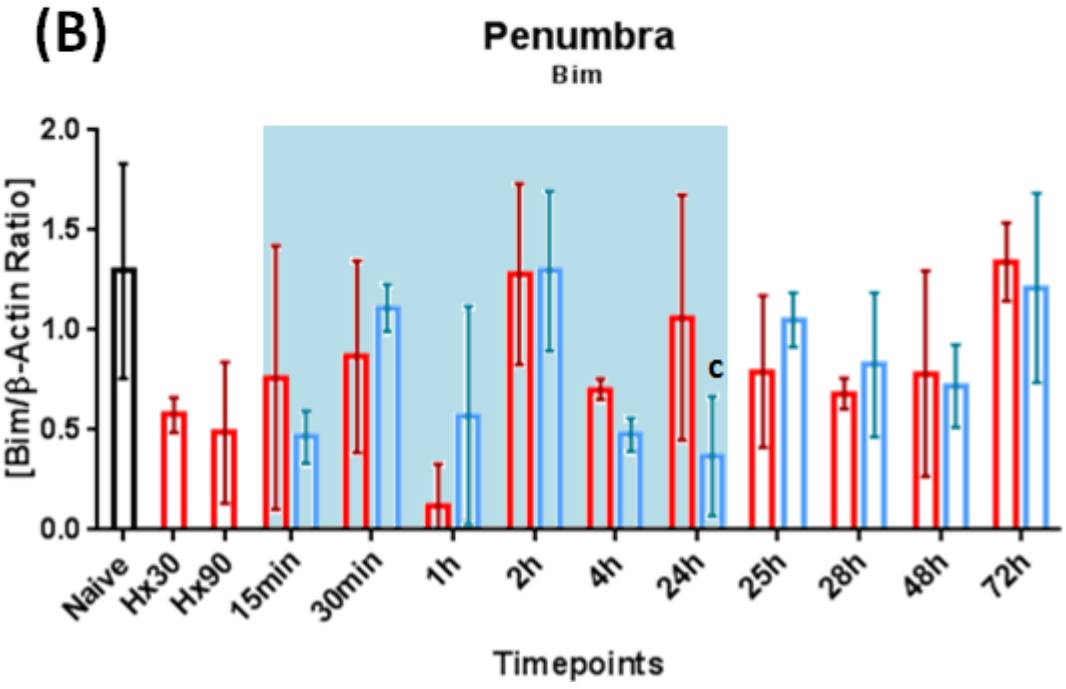

(B)
Penumbra
Bim
[Bim/β-Actin Ratio]
2.0
1.5
1.0
0.5
0.0
c
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

Figure 30: Temporal Bim Expression in the NORMO and HYPO Core and Penumbra

Expression levels of Bim with NORMO and HYPO at each of the examined time-points within the (A) core and (B) penumbra. Solid blue box indicates the 24 hour NORMO/HYPO normoxic exposure period. (a) $p < 0.05$ vs. NORMO. (C) $p < 0.001$ vs. NORMO. Mean ± SD.

Expression of Cleaved Caspase-3

Figure 31 (A-B) depicts Bim as analyzed on each gel at the representative time-points of (A) 90 minutes of hypoxia, (B) 4h after HIBD (During NORMO/HYPO) and (C) 25h post-HIBD (After NORMO/HYPO).

Activity of Cleaved Caspase-3 did not differ between the core and penumbra during hypoxia (Figure 32 A-B).

During NORMO/HYPO (Figure 33 A-F), the only differences observed in expression of Cleaved Caspase-3 were at 4h post-HIBD (Figure 33 E); expression was significantly higher in the NORMO penumbra ($F(1,16) = 57.30$, $p < 0.00001$). HYPO suppressed this penumbra increase in Cleaved Caspase-3 at this time ($p < 0.01$). No differences in apoptosis were observed between the core and penumbra and between NORMO and HYPO after removal of NORMO/HYPO (Figure 34 A-D).

Figure 35 (A-B) depicts Cleaved Caspase-3 expression within the (A) core and (B) penumbra between each temperature treatment at each time-point. HYPO did not significantly affect expression in the HIBD core. HYPO suppressed Cleaved Caspase-3 at 4h-post HIBD in the penumbra ($p < 0.01$). No other significant differences were observed within each time-point. All data depicted as mean ± SD.

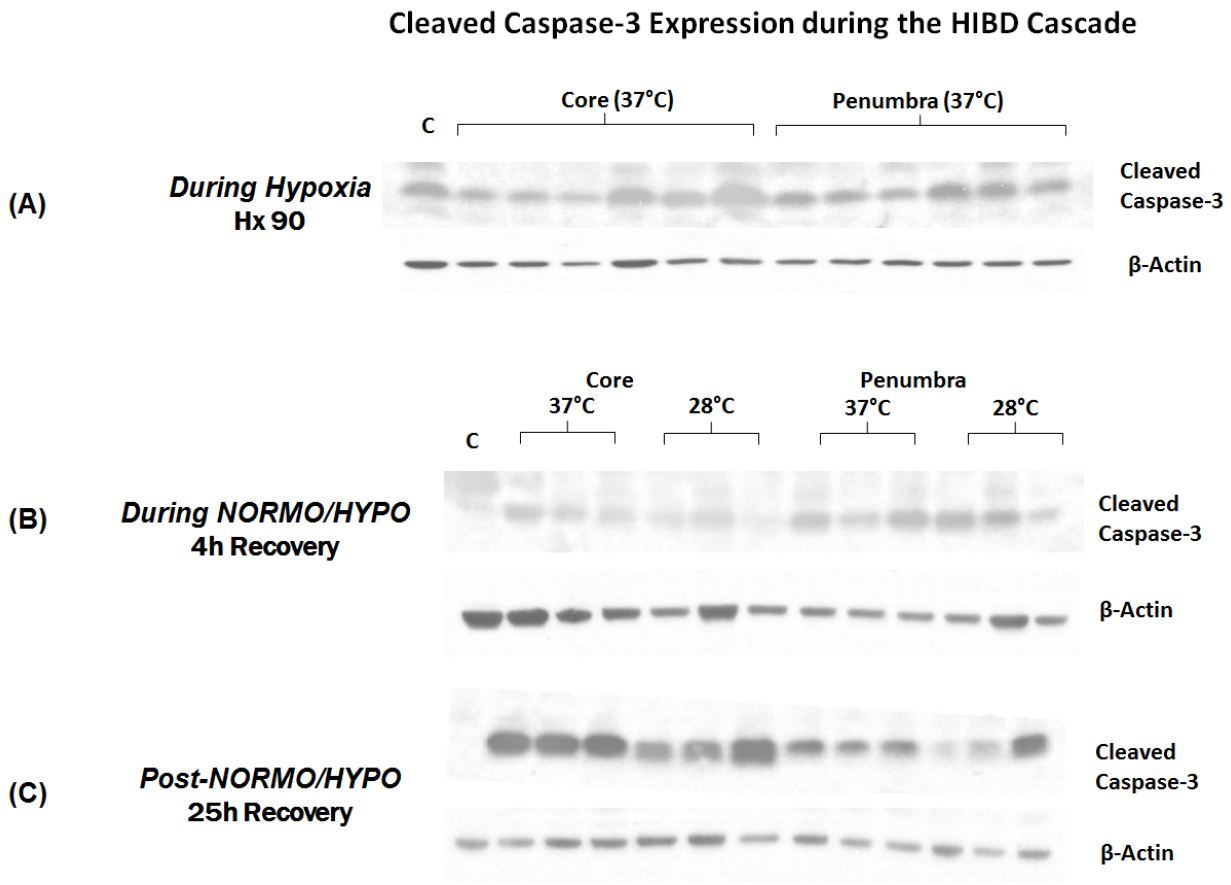


Figure 31: Cleaved Caspase-3 Expression – During Hypoxia, During Treatment, and Post-Treatment

These sets of Western Blot images depict Cleaved Caspase-3 (17-19 kDa) as expressed at (A) the end of 90 minutes of hypoxia (Hx90), (B) 4h during the NORMO/HYPO normoxic temperature exposure, and (C) 25h after HIBD, 1h after removal from NORMO/HYPO. NORMO and HYPO core and penumbra samples from the same time-point were run per gel. A homogenate of undamaged right hemispheres from 4 naïve pups was used as a control between gels and pipetted into the first lane (denoted 'C'). β-actin (42 kDa) was used as a loading control.

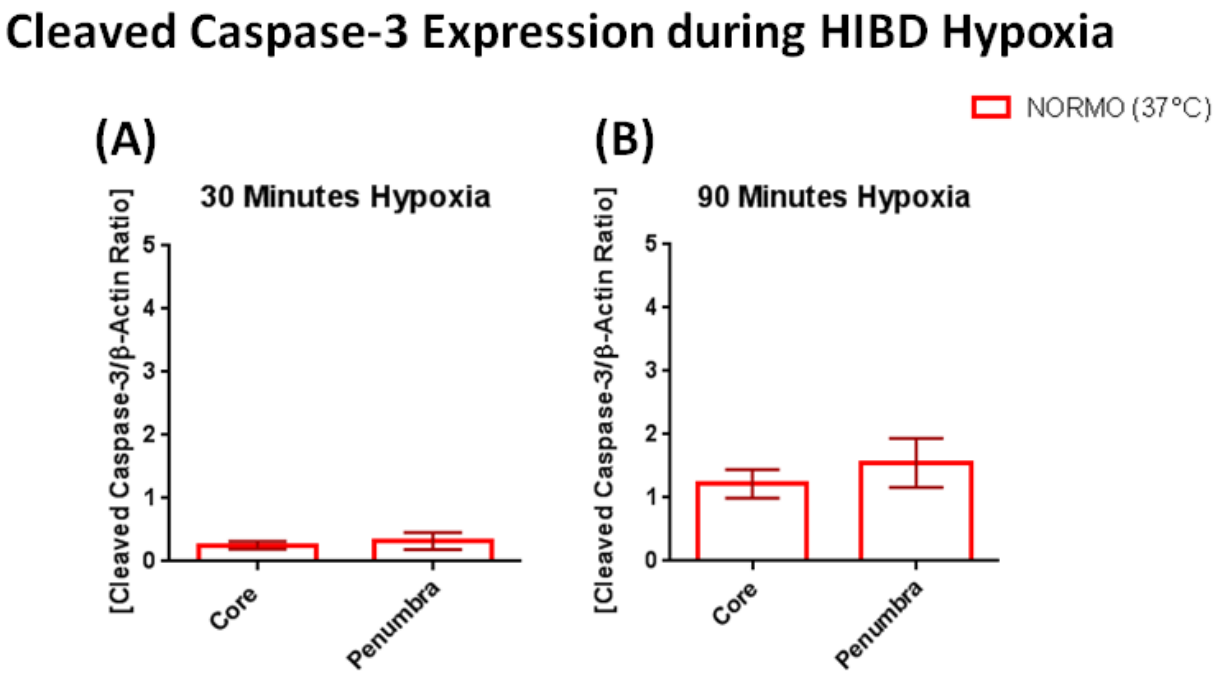


Figure 32: Cleaved Caspase-3 Expression during Hypoxia

Expression of Cleaved Caspase-3 at (A) 30min and (B) 90min of hypoxia in the core and penumbra at 37°C. No differences in expression between the two regions were observed at each examined time-point. Mean ± SD.

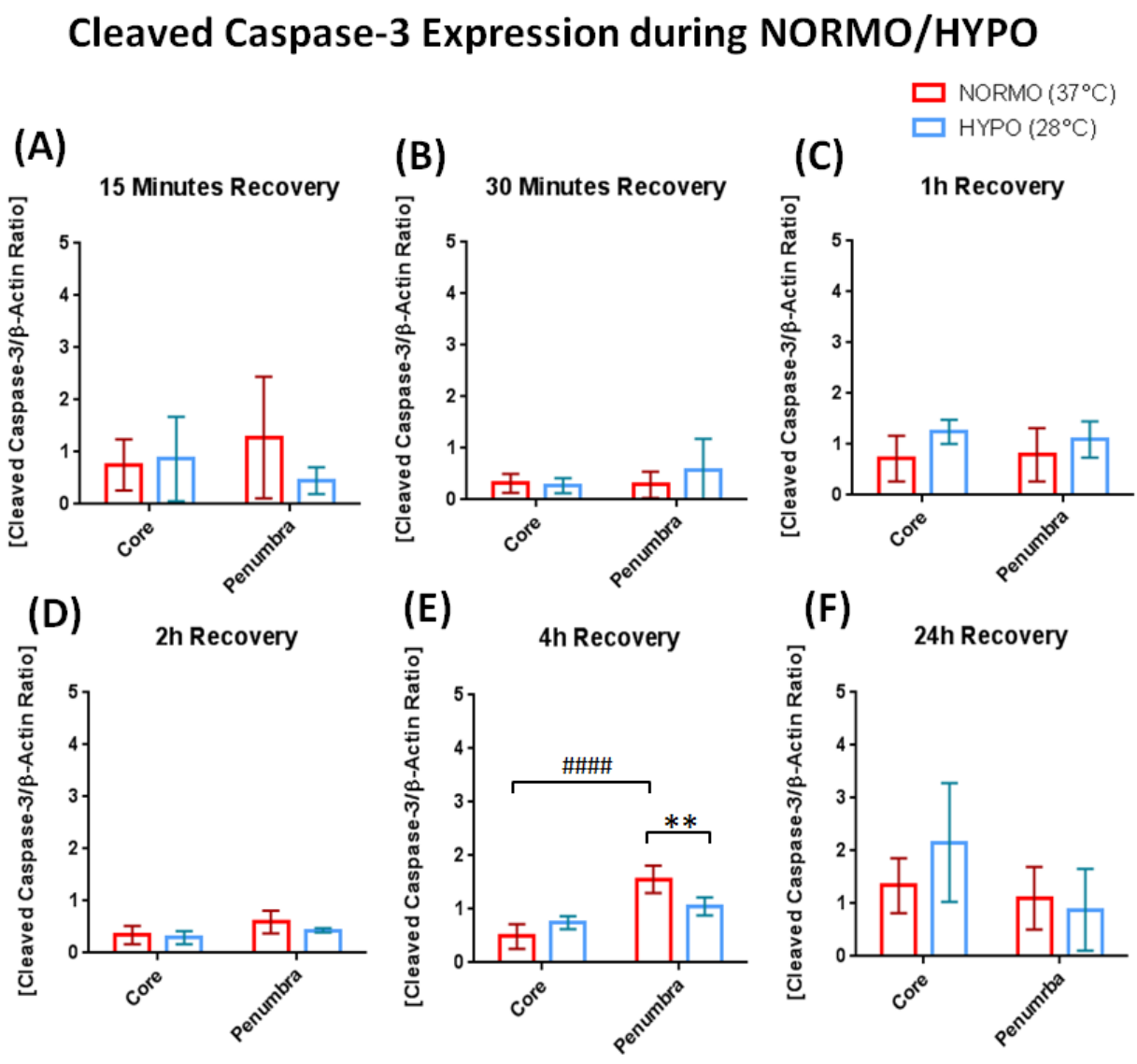


Figure 33: Cleaved Caspase-3 Expression During NORMO/HYPO

Expression of Cleaved Caspase-3 at (A) 15min, (B) 30min, (C) 1h, (D) 2h, (E) 4h, and (F) 24h after HIBD during the NORMO and HYPO temperature exposures. HYPO suppressed expression of Cleaved Caspase-3 at (E) 4h post-HIBD (** $p < 0.01$). No other differences in expression were observed between treatment groups. Mean ± SD.

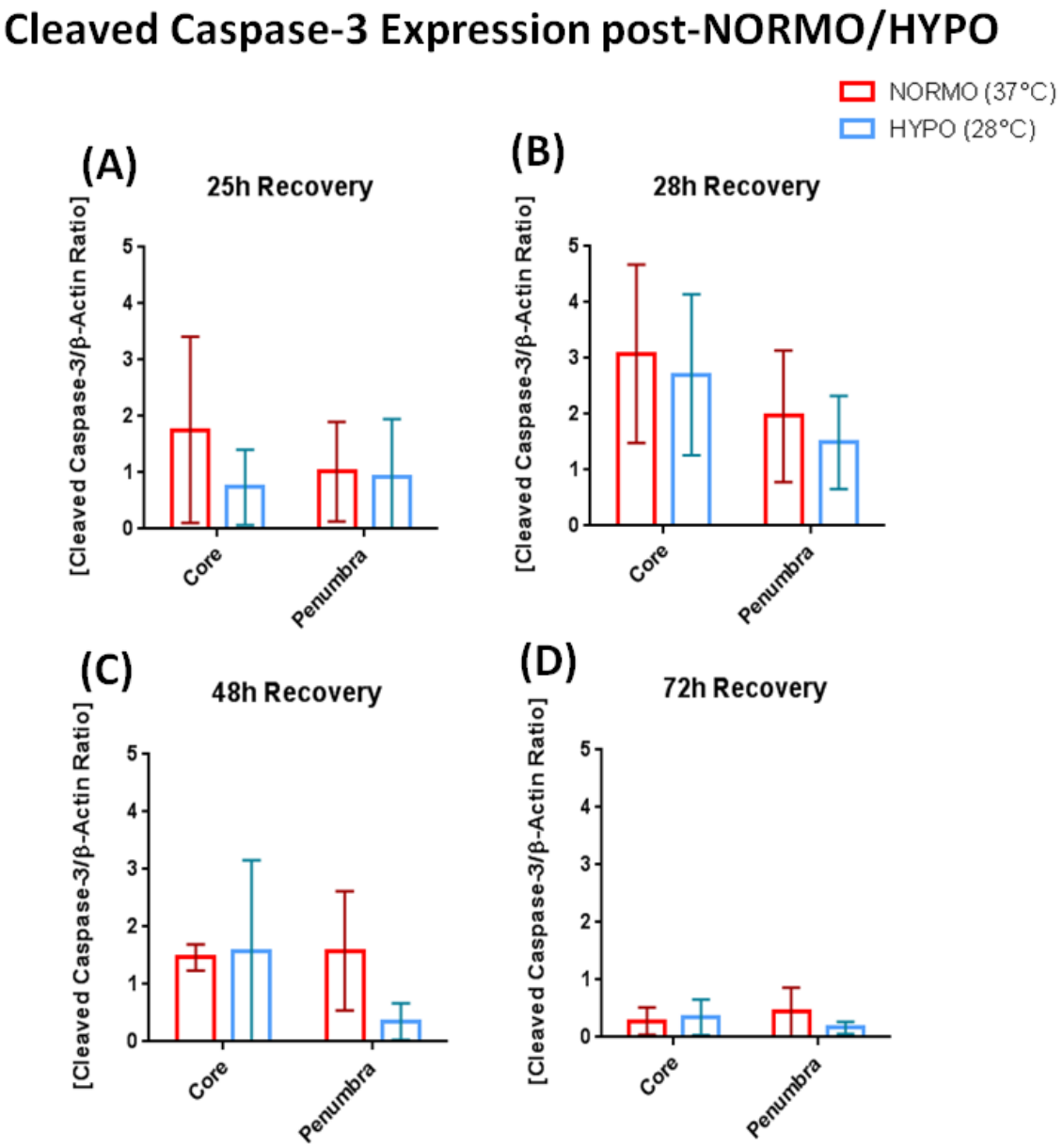
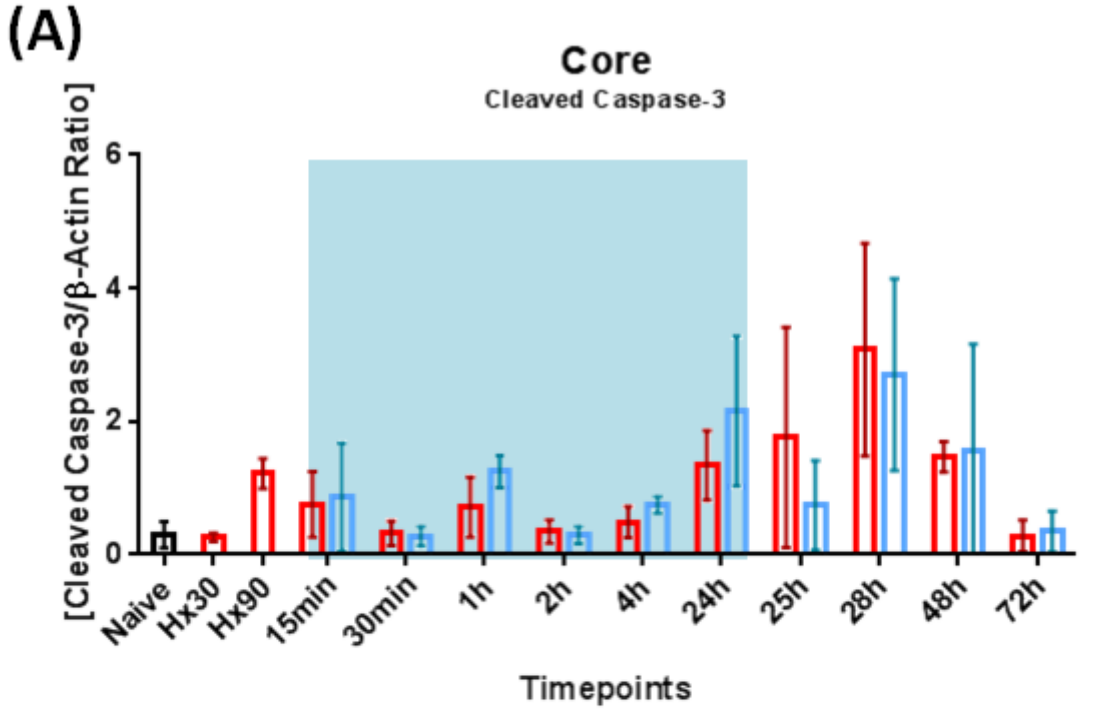


Figure 34: Cleaved Caspase-3 Expression post-NORMO/HYPO

Expression of Cleaved Caspase-3 at (A) 25h (B) 28h, (C) 48h, and (D) 72h post-HIBD, after removal from the NORMO and HYPO environments. No differences in expression were observed between regions or within treatment groups. Mean ± SD.


(A)
Core
Cleaved Caspase-3
[Cleaved Caspase-3/β-Actin Ratio]
0
2
4
6
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

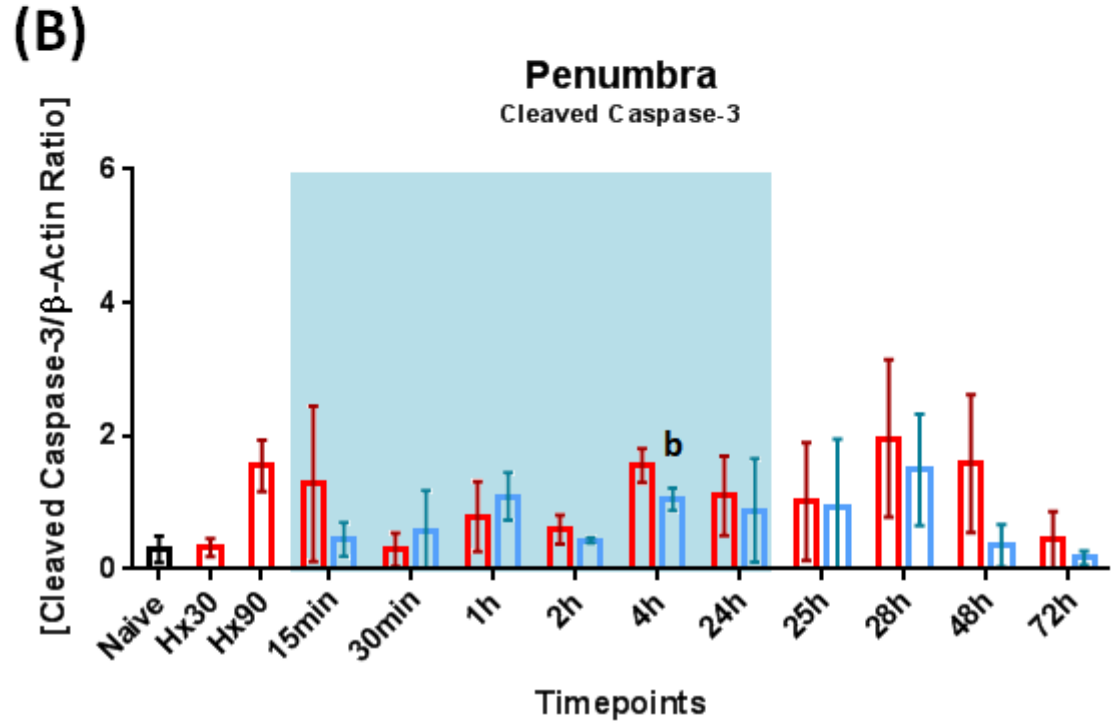

(B)
Penumbra
Cleaved Caspase-3
[Cleaved Caspase-3/β-Actin Ratio]
0
2
4
6
b
Naive
Hx30
Hx90
15min
30min
1h
2h
4h
24h
25h
28h
48h
72h
Timepoints

Figure 35: Temporal Cleaved Caspase-3 Expression in the NORMO and HYPO Core and Penumbra

Expression levels of Cleaved Caspase-3 with NORMO and HYPO at each of the examined time-points within the (A) core and (B) penumbra. Solid blue box indicates the 24 hour NORMO/HYPO normoxic exposure period. (b) $p < 0.01$ vs. NORMO. Mean ± SD.

Chapter 5: Conclusions

Study #1

- SFN (1 & 5 mg/kg) and Hypothermia (28°C) independently provide significant neuroprotection following an HIBD insult.
- Our highest dose of SFN (10 mg/kg) alone was not neuroprotective. Whether it was detrimental or not is unknown.
- Our results do not show additive neuroprotection in HIBD pups treated with a combination of both hypothermia and SFN.
- These results are consistent with several other laboratories that have studied combination therapies for neonatal HIBD.
- Our results further suggest that hypothermic neuroprotection, and the effect of SFN on HIBD, could be targeting similar mechanisms, and therefore may be competitive rather than complementary.
- A better understanding of the mechanisms underlying hypothermic neuroprotection is required to determine the best timing and therapeutic modality for an additive and/or synergistic effect with hypothermia.

Study #2

- **Energy regulation** through **p-AMPK** examination:
    - During HIBD and recovery there were no differences in the HYPO vs NORMO p-AMPK expression in the HIBD core.
    - HYPO suppressed penumbral p-AMPK activity at 4h compared to normothermia during the first 24 hours of recovery. There were no other differences between the normothermic and hypothermic groups.
    - Following hypothermic recovery at 25 – 72 hours, p-AMPK activity was higher in the penumbra of the hypothermic group compared to its homologous core.

- **Neuronal survival** as expressed by **GLUT3:**
    - Within the HIBD core, HYPO had a suppressive effect on GLUT3 at 30min of recovery from HIBD, during Hypothermia, and following Hypothermia at 25h, 28h, and 72h after HIBD, compared to the Normothermic group.
    - Penumbral GLUT3 activity did not differ between the HYPO and NORMO groups at any of the examined time-points.
    - Between the HYPO core and penumbra, GLUT3 expression in the core was consistently and significantly lower than in the penumbra at 15min, 30min, 2h, 4h, 25h, 28h, and 72h post-HIBD.

- **Autophagy** as expressed by **LC3-II**:
    - HYPO suppressed autophagy in the HIBD core at 15min and 28h, as well as at 25h and 48h in the penumbra after HIBD. Upregulation of autophagy in HYPO was observed only at 1h of recovery in the penumbra.
- **Intracellular apoptosis** as examined through **Bim** and **Cleaved Caspase-3**:
    - Administration of HYPO enhanced Bim activity in the core at 24h post-HIBD, during Hypothermia, whereas it was suppressed at 25h and 48h after HIBD and temperature normalization, in the hypothermic group.
    - HYPO suppressed Bim expression in the penumbra at 4h post-HIBD
    - Elevated Cleaved Caspase-3 activity in the NORMO penumbra was observed at 4h into recovery, but this was significantly suppressed by HYPO at this time-point.
    - HYPO did not significantly affect core or penumbral expression of Cleaved Caspase-3 and penumbral activity of Bim.
    - HYPO generally did not have a suppressive effect against apoptosis in this examined paradigm.
- **Post-ischemic hypothermia** overall did not have a sustained suppressive or enhancing effect on all of the proteins examined.
    - The only major effect of HYPO recorded was a sustained suppression in the HYPO core of GLUT3 at 30min, 25h, 28h, and 72h into recovery, and Bim at 25h and 48h into HYPO.

- HYPO did not have an effect on p-AMPK in the HIBD core. It only exerted significant suppressive influence at 4h post-HIBD in the penumbra.
- While differences between NORMO and HYPO, as well as between the core and penumbra, were seen at several time-points, many of these effects were variable between time points and regions.

Chapter 6: Discussion

There is increasing awareness about the problems of neonatal encephalopathy and how to effectively treat it. While some successes have been made in the field of HIBD, such as post-ischemic hypothermia therapy, the growing understanding of HIBD mechanisms, as well as therapeutic timing, there is still much work to be done in improving HIBD neuroprotection and long-term recovery.

Study #1

The purpose of my Study #1 was to examine whether SFN combination therapy at various doses would enhance hypothermic neuroprotection in a neonatal rat model of HIBD. As SFN is an anti-oxidative and anti-inflammatory compound that has been demonstrated to reduce brain injury, it was hypothesized that these mechanisms would either complement or add to hypothermic neuroprotection.

SFN Dosages

Here, SFN at 1 and 5 mg/kg independently conferred neuroprotection against neonatal HIBD when administered once every 24 hours for 7 days following HIBD induction (Figure 8). Many SFN studies have utilized the dosage of 5 mg/kg, and this particular dosage has demonstrated neuroprotective properties against spinal cord injuries[318,340–344], neurodegenerative brain disorders such as Parkinson's disease[345,346], and adult acute ischemic stroke[302–304,317,322,347,348]. Cardiovascular[349] and cancer[305,307,308,311,312,350–353] studies have also shown 5 mg/kg of SFN to induce protection. Notably, Ping et al.'s 2010 study evaluating SFN for neonatal HIBD demonstrated reductions in infarct size, neuroinflammation, and oxidative stress with this SFN dosage[319]. While I did not directly examine neuroinflammation and oxidative stress, HIBD rats treated alone with 5 mg/kg once every 24h for 7 days exhibited significantly reduced brain injury

than those left untreated (Figure 8). From this, we can infer that SFN at a dose of 5 mg/kg is independently neuroprotective against gross neonatal HIBD pathology.

The independent SFN dose of 1 mg/kg also significantly improved brain pathology when administered to HIBD rats. This dose was chosen to complement other studies, as well as the projects in our lab, that have consistently observed long-term improvement in behaviour and brain connectivity with the dietary supplementation of 200 mg of broccoli sprouts during gestation and weaning[354] and for hypertension[355,356]; This amount of broccoli sprouts has been deemed to be equivalent to 500μg of SFN in fetuses after their dams ingested 200 mg of broccoli sprouts.[354] As similar HIBD neuroprotection was afforded with the SFN doses of 1 mg/kg and 5 mg/kg, it could be postulated that SFN at these doses may induce neuroprotection to an analogous extent, meaning that although SFN is a safe-to-use compound, perhaps the lower dose of SFN would be favoured as opposed to the higher dose of 5 mg/kg in a clinical or research setting. However, it is unknown if either SFN dose affords differential behavioural improvement after neonatal HIBD. Since the 5 mg/kg SFN dose has been used consistently in the SFN literature, more studies would be required to further evaluate the neuroprotective efficacy of the 1 mg/kg dose.

Additionally, we found that 10 mg/kg of SFN did not independently reduce brain injury (Figure 8). This is a notable finding, as cortical neuronal oxygen-glucose deprivation cell culture results from a collaborating lab have demonstrated neuronal cytotoxicity when SFN was administered at 50μM under normoxic conditions in premature neurons [unpublished data]. This may indicate that SFN at high concentrations may be, in fact, detrimental to the immature brain. However, this SFN dose together with HYPO still resulted in HIBD neuroprotection (Figure 8). It is unknown whether the potentially harmful effects of this high SFN dose were counter-acted by hypothermia.

Weights

Pups injected with 10 mg/kg of SFN weighed on average significantly more than their experimental counterparts (Figure 6). This was evident regardless if pups received 10 mg/kg SFN under NORMO or if they received the combination of HYPO + SFN (10 mg/kg) treatment. As all SFN doses were administered subcutaneously, this weight gain effect could have been due to various global systemic effects caused by an over-concentration of phytochemicals (e.g. vitamins E, C, K, iron, zinc, etc.); these are all associated with SFN-related health effects. [357] Alternatively, the weight gain in this group of animals could have been simply due to an increase in appetite, but this is in contrast to research that has demonstrated SFN to attenuate obesity; this has been demonstrated with both the upregulation and suppression of AMPK activation, hence further studies are required in this field.[358,359] While the 10 mg/kg SFN may have resulted in weight gain regardless of treatment, the overall health effects of this dose have not been fully elucidated on the brain, behaviour, and other systemic systems throughout the pups' bodies. Independently and combined, the weights of pups that received SFN at 1 or 5 mg/kg and HYPO did not significantly differ from their grouped controls (Figure 6), and may be an indicator of treatment safety. More research into the safety of SFN dose is recommended.

Post-Ischemic Hypothermia

As expected, post-ischemic hypothermia for 24 hours after moderate HIBD was consistently and significantly neuroprotective, including when combined with SFN at all three doses utilized (Figure 8). Our results are thus consistent with the hypothermia literature for mild-moderate HIBD, and clinically confirmed through multiple RCTs and its gold-standard status as a rescue therapy.[146,170] While no statistically-significant differences were observed in pathology between

rats treated with SFN at 1 and 5 mg/kg and all the HYPO and HYPO + SFN groups, I would recommend that therapeutic cooling always be administered as opposed to only providing low doses of SFN to infants affected by mild-moderate HIBD. This is due to hypothermia's consistency and reliability as a neuroprotective intervention.

Hypothermia + SFN Combination Therapy

No additive histological neuroprotection (i.e. reduction in brain damage) was observed when SFN supplementation was combined with hypothermia. While a trend of reduced HIBD injury was observed with 10 mg/kg of SFN plus HYPO, the number needed to treat to observe a statistically-significant difference numbered in the thousands, indicating that this combination therapy would still not be a successful neuroprotective candidate. These negative results are also consistent with other combination therapy studies for HIBD.

Pharmacokinetics and Pharmacodynamics with Hypothermia

Furthermore, HYPO's ability to modulate pharmacokinetics and pharmacodynamics must not be overlooked. Reviews by Van den Broek et al.[360], Totrorici et al.[244], and Zhou & Poloyac[361] illustrate these well. However, they raise caution that while TH may extend a drug's half-life, its efficacy may also be reduced. Generally, HYPO functions to reduce cerebral metabolism, but in the process, also reduces drug clearance and absorption.[360] Hence, cooling may be preventing the complete and effective metabolism of some combination drug treatments. Importantly, raising a drug's dose to counterbalance this situation could produce systemic toxicity due to reduced clearance of the drug[322,362–368]; this has also been observed to be the case for drugs metabolized

by the cytochrome-P450 pathway.[244,368] As post-ischemic HYPO has been demonstrated to slow cerebral blood flow[171,205,206,369], this may restrict optimal delivery of pharmacotherapies delivered via the bloodstream. Drug-binding capabilities to their respective enzymes may also be compromised.[244] Thus, the safety, efficacy, and dosage of drugs administered during hypothermia must be fully evaluated before administering to patients in need.

Limitations

Perhaps the biggest limitation of Study #1 was not including a behavioural analysis component. Many studies in the HIBD field unfortunately do not include this portion as well, focusing more on the short-term effects of HIBD and potential treatments. To effectively see if long-term improvement is afforded, behavioural studies are required. Behavioural studies for HIBD in neonatal rats are exceedingly rare, but some research has demonstrated favourable long-term outcomes of animals and human infants with HIBD when exposed to hypothermic cooling[146,170,370–372], although any long-term benefits of SFN administration for neonatal HIBD have not yet been elucidated. While the examination of gross pathology provided a clear image of how HYPO and each SFN dose affect overall brain injury, it is unknown whether the combination would have provided behavioural improvement. Additionally in this Rice-Vannucci model, rats only received a unilateral brain injury, leaving the other hemisphere intact to HIBD cystic pathology. Due to the time-period in which HIBD occurs, the brain is still malleable and responsive to cortical reorganization and plasticity.[373] Perhaps the administration of SFN + HYPO combination therapy may have resulted in the reorganization of neural circuitry in the ipsilateral brain hemisphere, thus improvement of hemiplegic motor impairment; unfortunately we did not test these parameters. In my study, to research these ideas and examine any cognitive

or motor changes, behavioural tests such as skilled reaching (depending on paw preference), beam walking, the cylinder test, and the Morris Water maze could have been conducted.

The temporality in which drug therapies are administered relative to HYPO onset must also be considered and evaluated prior to clinical trial. Hence, the administration paradigm of SFN together with HYPO may have contributed to the final results. In this study, SFN was first supplied at the onset of HYPO and was re-injected into the subcutaneous space once every 24h for a period of 7 days. Due to SFN's anti-oxidative and anti-apoptotic nature, this rescue therapy dosing regime was chosen to theoretically further protect against oxidative stress in the immediate post-hypoxic period and provide a lasting protective base against inflammatory mechanisms in the delayed period of secondary energy failure.[57] From our results, HYPO consistently and significantly protected the brain until the final endpoint of 21 days after HIBD, but SFN treatment in combination with HYPO did not improve the gross neuroprotection seen in the damaged hemisphere. Perhaps altering the administration schedule could have made a neuroprotective difference. For instance, delayed administration of SFN in a traumatic brain injury model at 1h post-injury, but no longer than 6h, attenuated brain damage and improved behavioural function.[374] Studies examining other therapies with HYPO for HIBD have also utilized delayed administration models and seen additional benefit.[375–377] Even delayed administration of HYPO itself for up to 6h has shown to be neuroprotective for HIBD.[201,376,378,379] Thus, hypothermia would still be considered the gold-standard therapy for neonatal HIBD, but the optimal timing of SFN administration, HYPO, or any other adjunct therapy after newborn HIBD is still unclear.

To address these concerns, Study #2 was undertaken to better understand how HYPO modulates energy regulation and cell death/survival at various time-points during and after HIBD.

Study #2

Study #2 was focused on examining how hypothermia affected the HIBD core and penumbra at various time-points during recovery. Reviewing the literature, it was hypothesized that downstream effectors of AMPK would be differentially affected by hypothermic cooling in the core and penumbra. This is due to AMPK's critical involvement in energy failure and recovery that define HIBD cell death, and how cooling has been consistently shown to protect the mild-moderate HIBD brain against injury. Our results show differential expression profiles within the NORMO and HYPO HIBD core and penumbra. These are interesting and may point to where and when additional neuroprotective therapies could be introduced.

Energy Regulation by p-AMPK

As previously elaborated, p-AMPK was the main protein of interest in this study. McCullough et al.'s 2005 study initially pioneered research examining AMPK activation and inhibition in adult mice and rats after MCAO stroke, also studying how the core and penumbra were differentially-affected. They discovered that from 2-6h post-stroke, p-AMPK activity is significantly higher in the adult cortical penumbra than in the ischemic core.[142] In newborn rats, the temporal activation course of AMPK after HIBD in several brain regions has been recently detailed by Rousset et al.[138] Here, p-AMPK levels peaked during hypoxia/HIBD, returning to baseline levels once normoxia was restored; this pattern was evident in the cortex, striatum and hippocampus. Comparison of core and penumbra were not evaluated. In our study, we examined the core and penumbra of the HIBD lesion and the influence that hypothermia demonstrated, post-ischemia, to: (1) Determine whether hypothermia might influence energy regulation differentially, and (2) Examine a time frame during which supplemental therapy may be beneficial.

In concordance with recent work by Rousset et al.[138], p-AMPK levels rose significantly in the penumbra by the end of HIBD (Figure 12 B). As expected, p-AMPK in the immediate post-hypoxic recovery period returned to baseline levels as has been observed in the literature (Figure 13).[138] Due to the potential for cystic infarction and necrotic death in the immediate primary energy failure period, we hypothesized that p-AMPK levels would be nearly absent in the HIBD core. However, in contrast to our hypotheses, p-AMPK expression did not significantly decrease in the NORMO HIBD core at any of the time-points examined, instead remaining at baseline. It is possible that while p-AMPK levels, as measured, did not change, the requirements for p-AMPK increase; demand outstripping supply. Additionally, though dying, cells and their intracellular components have not yet been removed in the early post-ischemic period. As such their diminished effective concentrations may not be reflected. Nonetheless, cell death continues from other sources including glutamate excitotoxicity and the production of reactive oxygen species,.[28] In this regard, the unchanging temporal profile of p-AMPK in the core during HIBD may be a sign of the core's inability to generate additional energy supplies to overcome the neurotoxic cascade, hence irreversible cell death in 'core' cells.

Similar to adult MCAO work performed by McCullough et al.[142], our results did show significant differences in p-AMPK expression between the NORMO HIBD core and penumbra. Their results depicted increases in penumbral p-AMPK, theorizing this to represent the activation of compensatory protective pathways in viable neurons to retain a survivable energy supply. We found p-AMPK to be significantly increased in the penumbra compared to the core in the Normothermic group. Though only seen at the terminus of HIBD, and at 4h into HIBD recovery (Figure 13 E), we did not measure this marker again until 24 hours of recovery. How long penumbral p-AMPK remained elevated is therefore unclear, and hence may well represent an

attempt of penumbral cells, during normothermic, normoxic recovery, to replenish their energy supply, leading to survival. Alternatively, this may represent the differences and challenges regarding age and sensitivity to brain injury.[380] This increase was not shown by Rousset et al. in their neonatal HIBD p-AMPK time course findings.[138]

Furthermore, core p-AMPK was not affected by HYPO at any point when compared with NORMO (Figure 13). We hypothesized that p-AMPK activation in the core would be detrimental, due to the need for a supply of nutrients to replenish energy stores. However, given that activation of p-AMPK requires energy, its activation is likely detrimental in attempting to maintain constant energy stores, in the core. Although p-AMPK activity was significantly higher in the HYPO core than the HYPO penumbra at 1h of recovery, neither of the NORMO values differed from each other, nor the HYPO values within each respective region of injury (Figure 13). The increased core value of p-AMPK at 1 hour of recovery, in hypothermia, compared to the penumbra, may therefore be a detrimental effect of hypothermia on the core, and be partially responsible for the incomplete effect of hypothermia as a neuroprotectant. Moreover this may partially explain the complete lack of effect of hypothermia on severe injury, where the core consumes the majority of the lesion. Alternatively, the findings may be a statistical error, given the wide SD of the normothermic core group.

At 4h of recovery, p-AMPK was significantly increased in the NORMO penumbra, while hypothermia led to a significant suppression, compared to the normothermic group (Figure 13 E). No further differences were found between the normothermic and hypothermic groups. This may again, be of interest regarding treatment. We would assume that p-AMPK suppression 4h post-HIBD in the penumbra would be detrimental.

Following hypothermia, there was an increase in p-AMPK activity from 25-28h after HIBD, and again at 72h (Figure 14). This phenomenon is in keeping with our hypotheses that p-AMPK upregulation in the penumbra could be beneficial to neuroprotection. These results may suggest that the lasting protective effects of cooling may act on and influence the HIBD penumbra rather than the core at various time-points, and may contribute to why the HYPO penumbra was diminished in this group and did not expand into the thalamus. Additionally, as HYPO is an established and consistent therapeutic modality, this sustained enhancement in energy metabolism in the penumbra after the initial phase of primary energy failure may serve as a preconditioning and preventative measure to preclude further damage as a result of secondary energy failure.[381] This could be further verified by testing p-AMPK-enhancing and suppressing agents in the HIBD penumbra after cooling is removed. [146,382]

Interpretation of our results, particularly with respect to p-AMPK is challenging. The findings are somewhat inconsistent through the course of injury, recovery and post-hypothermic recovery. This may simply be a reflection of wide standard deviations, challenges in sampling core vs penumbral tissue and the variability that this model presents, or technical difficulties.

Our findings at 4 hours, whereby hypothermic brains showed a decrease compared to the normothermic group, might, on the other hand, reflect the total reduction of metabolism during hypothermia. Hence the reduced p-AMPK may in fact not be detrimental, but rather beneficial or simply neutral. Its increase in the post-hypothermic phase, may be a phase during which a rebound of p-AMPK in the hypothermic group provides if protection.

There is currently a debate regarding whether activation or inhibition of AMPK in the damaged HIBD brain is beneficial. While some preventative stroke research has implied AMPK activation to exert neuroprotection, its sustained activation into the neurotoxic and recovery

periods has been deemed detrimental to brain recovery.[138,139,383–388] Recently, studies have strongly pointed to AMPK inhibition as a neuroprotective strategy.[138,383,389–391] Brain cells require ATP to function, meaning that sustained suppression of AMPK may not be a viable route either. This duality of survival and death must be considered when exposing injured tissue to agents that affect AMPK activity.[136,139,387]

GLUT3 and Neuronal Survival

Neurons require high amounts of glucose, but are poor at storing it.[392] GLUT3 is a high-affinity extracellular glucose transporter that mediates glucose influx into neurons, and astrocytes to a lesser extent, and is hypoxia-responsive.[393] In addition to being directly upregulated by p-AMPK[394], GLUT3 is a downstream target of HIF-1α[133,395] which is also activated in response to HIBD.[393,396] Surface expression of GLUT3 due to p-AMPK activation has been shown to promote neuronal and astrocytic tolerance to HIBD energy failure[397], with glycolysis and glucose/glycogen uptake/storage as a pro-survival adaptation to post-HIBD glutamate excitotoxicity.[394,396–399] Dornbos et al. found GLUT3 levels to be enhanced acutely post-stroke in adult rats receiving chronic exercise, suggesting exercise to stimulate glucose metabolism post-stroke that may have contributed to neuroprotection.[400] Additionally, Li et al. demonstrated increased neuronal GLUT3 levels 24h post-HIBD in neonatal rats pre-treated with progesterone.[399] Vannucci et al. conducted a temporal analysis of glucose transporter expression in neonatal HIBD rat brains and found enhanced GLUT3 levels in the penumbral piriform cortex and amygdala during HIBD.[396] In the cortex, GLUT3 expression was reduced after 1, 3, and 5h of recovery after HIBD, and was absent after 24 and 72h; absence of GLUT3 expression was correlated with emergence of necrosis in the HIBD hemisphere. At the 3h and 5h time-points,

GLUT3 reduction spread to include the cortex and hippocampus, while positive expression was still detected in the amygdala. My study is the first to examine how hypothermia affects the GLUT3 pathway in the HIBD core and penumbra.

Our results show an increase in GLUT3 expression in the HIBD penumbra compared to the core during hypoxia ischemia (Figure 18). This would reflect enhanced utilization of glucose in penumbral cells during ischemia, which makes sense, given this is the region of injury with better circulation and therefore substrate supply.

During recovery, in both the hypothermic and normothermic groups, this pattern continued. Penumbral GLUT3 was significantly greater in the penumbra then the core up to 4 hours of recovery (Figure 18). No differences were found in GLUT3 concentrations between the hypothermic and normothermic recovery groups. Though not significant, the hypothermic penumbral GLUT3 was lower than the normothermic group, perhaps suggesting suppression in its activity. This alteration in neuronal glucose uptake is similar to what has been observed in the literature.[396]

Following hypothermic recovery at 25-72h, penumbral GLUT3 was consistently more elevated than the core in both the normothermic and hypothermic groups, though not always statistically (Figure 19). Interestingly, core GLUT3 was significantly lower in the hypothermic animals compared to the normothermic ones. Whether this was protective, given the lack of blood flow to this region, is frankly difficult to know. One might think this to be the case, given previous findings from our group[55], that the hypothermic core shows a decrease in apoptotic death. However, to be sure, further analysis may be required in measuring the volume of core injury in the hypothermic vs normothermic groups. GLUT3 expression in the NORMO and HYPO penumbra did not significantly differ from each other at any of the examined time-points. This

may indicate that HYPO neuroprotection in the penumbra may not be dependent on the significant upregulation of glucose uptake.

In adults, glucose accumulation during periods of metabolic failure may lead to post-stroke hyperglycemia and increased brain damage.[143] Overexpression of GLUT3 post-HIBD has been linked to neuronal death.[396] While therapeutic targeting of GLUT3 may be beneficial to reducing stroke pathophysiology in neonates, consideration regarding the time, duration, and extent of activation must be taken if applying GLUT3 activators in the immediate period following HIBD.[401]

Autophagy and LC3-II

Autophagy is an increasingly important non-apoptotic mode of cell death/survival that contributes to programmed cell turnover and quality control.[402] The role of autophagic cell death is still not fully understood in the brain, with conflicting studies showing both protective[403–406] and damaging effects of autophagy.[402,407–411] While autophagy enhancement in the penumbra of adult brains affected by ischemic stroke has been shown to provide protection[412], other studies paint a less favourable picture.[409,413] Within the autophagy pathway, evidence linking deletion of the Atg7 autophagy mediator gene to neuroprotection after severe HIBD provides the most compelling evidence for autophagic cell death as detrimental to the neonatal HIBD brain.[411] Studies have also shown the extent of autophagy to be influenced by the severity of excitotoxic stress and the presence of reactive oxygen species[402,410], which may be a factor in why autophagy has been reported to be a debilitating factor in severe HIBD.[410,411]

HYPO has largely demonstrated neuroprotection by suppressing autophagy in focal ischemic stroke[414] and cardiovascular dysfunction.[415] In traumatic brain injury, however, research indicates that the activation of autophagy by cooling has been protective. [416–418]

Overall, we did not find many differences in the LC3-II activity between the core and penumbra, or between the hypothermic and normothermic animals (Figures 32-34). Significantly higher levels of LC3-II were recorded at a single time point during the recovery period. One hour into recovery the HYPO penumbra showed significantly greater concentrations of LC3-II compared to the normothermic penumbra. In conjunction with the alterations of p-AMPK at this time point, where there is no difference in the penumbra, but is significantly lower than the hypothermic core, might this suggest autophagy to be in a 'holding pattern'; deciding or as yet determining the path to take. In other words, does hypothermia at this stage simply delay the decision making of the cell?

LC3-II was significantly reduced in the HYPO core at only 28h into recovery (Figure 19 B), and in the HYPO penumbra at 25h and 48h post-HIBD (Figure 19 A & C), compared to the equivalent time frame normothermic group. Again, together with the changes in GLUT3, this action may represent neuroprotection. By suppressing autophagic cell death in the period after hypothermic cooling is removed, perhaps allowing for sustained neuroprotection. This mechanism may also serve in preventing the expansion of the HIBD core into the penumbra. It should be noted that at no point did the HYPO core and penumbra differ in regards to their LC3-II expression profiles. Hence, neuroprotection against autophagic cell death could be as simple as inhibiting or suppressing LC3-II expression via HYPO administration in both the core and penumbra at different times.

Intrinsic Apoptosis via Bim & Cleaved Caspase-3

Delayed neuronal cell death is the most characteristic feature of secondary phase of energy failure in HIBD neuropathology.[28,29] This may occur hours, days, or weeks after the onset of HIBD. Study #2 assessed how post-ischemic cooling affected apoptotic cell death via the internal mitochondrial pathway. Bim was chosen as a pro-apoptotic intermediate marker of this pathway as its immediate upstream regulator, FoxO3a, is directly manipulated by activated AMPK. Nuclear p-FoxO3a is an important regulator of cell survival and death related to energy failure, and acts directly on Bim.[419–421] Ischemic stroke studies have shown that prolonged activation of AMPK results in apoptosis stemming from Bim activation.[136,139,141,383,385,422] Activation of Bim also suppresses anti-apoptotic Bcl-2 proteins, thus resulting in the formation of the Bak/Bax pro-apoptosis/mitochondrial permeability pore initiation complex[139,420], and accordingly, it has been shown to be involved in neuronal apoptosis after neonatal HIBD.[326,423] This is the first study to examine the effect of hypothermia on the expression of Bim in the HIBD core and penumbra. Cleaved Caspase-3 was measured as the final effector of apoptosis.

Our results showed that during hypoxia, Bim was significantly upregulated in the HIBD penumbra, during the early phases (Figure 27).[424,425] During the recovery period, no significant differences between NORMO and HYPO within and between the core and penumbra were recorded until 4h post-HIBD (Figure 28 E). At this time, there was a significant upregulation of Bim and Cleaved Caspase-3 in the NORMO penumbra compared to the HYPO group (Figure 28 E, Figure 33 E). We would theorize that this shows that intrinsic apoptosis was evident in the HIBD penumbra at this time, and that HYPO's neuroprotection extended to its suppression.

No other differences in Cleaved Caspase-3 were recorded between the experimental groups and regions at any other time. Bim expression varied until 72h post-HIBD. At the end of

Hypothermic recovery at 24h, Bim activity was significantly elevated in the HYPO core compared to the NORMO core, as well as the HYPO penumbra (Figure 28 F). This finding is difficult to justify, given the known protective effect of hypothermia. However, it does reflect only the intrinsic death pathway, and it is therefore possible to affect this mitochondrial pathway differently than the extrinsic cytoplasmic death pathway, which was not measured in our study.

Once hypothermia ended, Bim activity in the HYPO core became significantly reduced compared to the normothermic group (Figure 29). Perhaps this latter result indicates a neuroprotective mechanism of HYPO within the core at these times.

This suppression of Bim by HYPO after HIBD, but no differences in total levels of the final Cleaved Caspase-3 effector, suggests that cooling has a selective, yet incomplete, suppressive effect on the intrinsic apoptotic pathway but may not be exerting complete protection against extrinsic death receptor-mediated apoptosis, or even necroptosis in this model.[426] HIBD-induced neurotoxicity has been demonstrated to induce extrinsic apoptosis via TRAIL (tumor necrosis factor-related apoptosis inducing ligand), tumor necrosis factor-α, and the Fas ligand through inflammation.[426,427] HYPO has been shown to suppress inflammatory responses[428,429], but has not been successful in a two-hit inflammation-sensitized model of neonatal HIBD.[430,431] Additionally, Caspase-8 inhibition has been demonstrated to significantly reduce HIBD.[432] Adult rat MCAO studies have shown reduced Fas ligand and Caspase-8 levels with HYPO[433,434], but the effect of HYPO on these modes of apoptosis in the newborn HIBD brain has not yet been fully elucidated and these types of cell death were not examined in Study #2. While administering anti-inflammatory SFN in Study #1 proved protective on its own at low doses when provided every 24h starting at the onset of HYPO, this paradigm was not sufficient to additively enhance hypothermic neuroprotection (Figure 8). It is reasonable that this indicates that additionally

suppressing inflammation during HYPO may not be a viable method of improving neuroprotection. Unfortunately molecular pathway analyses were not conducted on these brains to determine the extent of HYPO + SFN combination therapy on the different types of programmed cell death. If it is the case that post-ischemic hypothermia does not fully protect against extrinsic cell death or necroptosis, then combination therapies targeting inflammation, TRAIL, Caspase-8, and/or the RIP1-RIP3 necrosome complex may be beneficial.[426]

Limitations

Some limitations arising from Study #2 include the (1) time-points sampled, (2) the limited number of samples per group, (3) the lack of cell death markers other than for intrinsic apoptosis, (4) possible technical issues, and (5) the variability of the Rice-Vannucci HIBD model itself.

In this study, many times along the evolutionary course of the HIBD neuropathologic cascade were examined. While this allowed for a more in-depth examination of the various stages of brain injury in a temporal manner, the many different level of groups and subgroups introduced within each time-point increased the number of comparisons to be made, thus increasing the likelihood of false positive statistical errors in significance in the results. Hence, some of our results may be falsely significant. This issue goes hand-in-hand with our limitation regarding group sample sizes; while sample-size calculations were run to optimize the number of samples per group/subgroup, 6 samples per group is still relatively small; however this study is more powered than others that only have group sizes of 2-3. Further increasing the power of each group would permit more confidence in the results obtained, and would likely reduce the large variations in SDs observed. Additionally, with a larger group sample size, comparisons between

male and female pups would have been able to be conducted; the importance of these analyses is discussed in Chapter 7.

Ideally, one time-point during hypoxia, during NORMO/HYPO, and post-NORMO/HYPO could have been evaluated, resulting in increased numbers per group and significantly reduced the statistical type-1 errors of false positives. To remedy this using the current results, the data could be lumped into the 3 groups as previously suggested; (e.g.) (1) During Hypoxia = Hypoxia at 30min and 90min, (2) During NORMO/HYPO = all time-points from 15min – 24h post-HIBD, and (3) Post-NORMO/HYPO = all time-points from 25h – 72h post-HIBD. Next, the Levene Test of equal variance and heterogeneity could be conducted. In the event of unequal variances or not normally-distributed data, log transformations of each data point could be performed in an attempt to mathematically transform and normalize any inequalities. Multi-factor ANOVAs could then be performed to more correctly determine any areas of significance and examine the temporal changes of each protein over time within each group and region.

As discussed in the previous section, in Study #2, only markers of intrinsic apoptosis were examined. However, extrinsic apoptosis, necrosis, and necroptotic modes of cell death also exist. Unfortunately, these were not assessed and could have provided valuable insight into where HYPO could have had effect during and after HIBD.

Possible variability regarding technical issues (e.g. sampling the core and penumbra) and the HIBD model itself also could have lent to the overall lack of differences seen within and between treatment groups and regions. Furthermore, inherent model variability resulted in difficulties obtaining consistent regions of injury. Allowing for increased sample power could have alleviated the statistical ramifications of these issues.

Summary

Interpretation of our current results is challenging. There are several areas of potential deficits in our data and findings. Given that this was a first attempt at analyzing metabolic alterations in the core and penumbra of a newborn rat brain, and that the injury itself is known to be variable, it is not surprising that our standard deviation errors are wide, and may contribute to the variability in significance found. Moreover, sampling of the core and/or penumbra is technically difficult, also perhaps contributing to the variability in the results.

Nonetheless, it may be possible to see some patterns. Overall there were alterations in p-AMPK activity as a result of post-ischemic hypothermia, which may suggest a beneficial role of hypothermia during the time frame after post-ischemic hypothermia is lifted. Whether the opposite is true (its effect, or lack of it, on p-AMPK during post-ischemic hypothermia is detrimental) is unclear, and requires further, and perhaps more specific evaluation. Similarly, our results for GLUT3, as a reflection of cell survival, and Bim and Cleaved Caspase-3 for cell death, may have suffered similar technical challenges.

Improvements to the current work would stem from an examination of: (1) the core and penumbral volume of damage, (2) markers reflecting the extrinsic death pathway, to correlate findings within the intrinsic pathway, and (3) perhaps working with an injury that is less variable in nature, such that representation of the core and penumbra are technically less challenging.

Chapter 7: Future Directions

Possible Combination Therapies Arising from the Current Thesis

To achieve additive neuroprotection for neonatal HIBD, combining the gold standard of post-ischemic hypothermia with another neuroprotective therapy appears to be the best bet. While many such treatments have failed to achieve this, perhaps examining AMPK activators and inhibitors together with post-ischemic cooling would be advantageous.

Currently, the drug metformin is showing promise. Metformin is the first line approved hypoglycemic therapy for type 2 diabetes[435] and is also prescribed for cardiovascular dysfunction.[436] For these diseases, metformin functions to indirectly upregulate AMPK and p-AMPK activity, via increasing AMP:ATP ratios, thereby increasing ATP production and promoting glycolysis to improve energy supply in impoverished areas.[437,438] Long-term metformin treatment has been reported to significantly reduce the incidence and severity of stroke in diabetic adults[435,439], prompting its investigation in stroke research.

Current studies illustrate a more complex nature of metformin post-treatment for stroke, showing both beneficial and detrimental results.[438] While exacerbated brain injury has been demonstrated with cerebral AMPK activation[142,440], long-term AMPK activation beginning in the secondary phase of energy failure has demonstrated neuroprotective effects.[143,436] These outcomes may be influenced by the timing and duration of metformin treatment, as well as cell type; neuronal activation of AMPK by metformin in the acute injury period was not protective, whereas glial AMPK activation was neuroprotective.[438,441]

It must be noted that it is still unknown how AMPK upregulation by metformin would affect a neonatal HIBD-affected brain in the immediate injury period. As previously mentioned, AMPK

upregulation in the neonatal HIBD brain may be detrimental due to its potential to upregulate AMPK activity which may exacerbate brain injury.[141,143,442] However, from our Study #2 data and hypotheses, we can propose that supplying metformin as a supplement to hypothermia, in the HIBD penumbra starting as early as 4h post-injury and continuing into the phase of secondary HIBD energy failure could provide neuroprotection. Another potential biomedical test would be to pre-treat pregnant animals with metformin and examine how their offspring fare when induced with HIBD. Theoretically, this may be neuroprotective with regards to stimulating the prenatal brain to produce additional energy stores.[443,444] While pretreatment with AMPK-stimulating compounds may be effective in this regard, the safety, efficacy, and other various clinical implications within the pregnant mother's body under these circumstances have not been fully assessed.[445] Additionally, metformin's pharmacokinetics and neuroprotective potential have still not been assessed under hypothermic conditions. With regard to AMPK inhibitors, Compound-C has been used extensively in the experimental literature, but this agent is likely not viable for clinical use due to its global suppressive effects. More studies into developing AMPK inhibitors that are safe for human use are required, as well as when/for how long would be most appropriate to administer them during the HIBD neurotoxic cascade. Further safety and efficacy studies are required in these areas before attempting clinical translation.

Patient-Specific Therapies

In this age of technology and knowledge, patient-specific therapies are the future of medical treatments. In the instance of neonatal HIBD, several technologies could be harnessed to deliver individualized therapy to patients. For instance, the progression of magnetic resonance neuroimaging (i.e. Diffusion-Weighed Imaging) is a technique that could be used to identify and delineate possible HIBD core and penumbral areas within the brain. Therapeutic treatments

could then be chosen specifically for the patient(s) at hand in a targeted approach to treating the core and penumbral areas of injury to prevent further infarction. Furthermore, developing core- and penumbral-targeted therapies using biologically-tagged nano-scaffolds and nanoparticles is also a possible therapeutic route. Nano-scaffolds and nanoparticles as therapeutic vessels are up-and-coming in the field of medical technology and medicine.[446–448] Recent experimental studies have shown that these nanotechnologies are safe for brain tissue and able to deliver therapeutic agents to the brain[448,449], including neonatal rat brain tissue.[450] While the implementation of these therapies in clinical medicine is still in the distant future, the first step to promoting patient-specific therapies for neonatal HIBD is delineating the underlying HIBD mechanisms. Additionally, these technologies have not yet been tested or trialed in HIBD individuals. Hence, further studies are required to determine the safety and efficacy of such specific treatments, and whether these therapies are effective at differentially-targeting the HIBD core and penumbra.

Sex Differences

Research now shows that sex differences play a significant role in individuals' susceptibility to and ability to recover from neonatal HIBD. Overall, HIBD males consistently present with more severe damage and are neuroprotected to a lesser degree than their female counterparts.[451–456] This male vulnerability is also evident in reduced neurogenesis early after injury, and also presents with impaired motor and cognitive functioning.[457] Interestingly, neuroprotection has been achieved with exposure to the estrogen hormone, which may partially explain why term females typically present with better outcomes[458–460], however enhanced estrogen has been reported to be detrimental for HIBD in premature infants.[461] In developing novel treatment methods, the two sexes appear to stimulate different main apoptotic pathways in response to HIBD.[462,463] Data demonstrating increased caspase-3 expression in females and AIF

translocation in males, as well as more enhanced neuroprotection overall in females than males, has provided evidence for a possible sex difference in apoptotic response to neonatal HIBD.[451,453,455,462,463] This could serve as an additional target for future HIBD therapies. As few studies have examined caspase-independent pathways of neuronal death with regards to neuroprotection, even with HYPO suppressing this type of apoptosis to an extent[54,226–228], this avenue of study may prove fruitful for improving male neuropathological and behavioural outcome after HIBD.

Severity of Brain Damage

No biomedically- and clinically-successful therapies for mild-moderate HIBD have been translatable to those with severe HIBD. This includes post-ischemic hypothermia[146,258,464] and Xe plus HYPO combination therapy.[258] Clinically, infants with severe HIBD are more likely to present with death and significant cognitive and behavioural impairments at 18 months.[36,146] The duration of exposure to the hypoxic-ischemic environment is possibly the most vital factor in the development of severe injury, as to experimentally induce severe HIBD, animals/cells are exposed to hypoxia for longer than the duration necessary to produce a moderate injury.[165] Within this extended duration, there is likely a point of no return at which rescue therapies just may not work at all regardless of the timing, type, and duration of treatment.[465] This may be due to overwhelming glutamate excitotoxicity that irreversibly destroys brain tissue, something which is unlikely to be cured by hypothermia and another drug and is probably best remedied with preventative treatments. Perhaps the core and penumbra of severely-affected hypoxic-ischemic brains are different from those with moderate HIBD and may require different types and dosages of therapeutics. Examining how AMPK and its downstream effectors expressed in

the severe HIBD core and penumbra may lead to possible strategies to treat severe HIBD with rescue therapies and prevent lasting neurological disorders in newborns with severe HIBD.

Prevention of HIBD

Finally, a major clue into how we can approach the treatment of HIBD may actually lie in its early prevention. My experiments have approached HIBD treatment from the perspective of a rescue therapy, meaning that the interventions being applied are to brain damage that has already occurred or is in the process of occurring. Due to the accumulating destructive nature of this type of formative injury, many protective agents that show potential for other neural or systemic disorders may not be protective against neonatal HIBD as rescue therapies in the post-injury period. However, approaching this issue from a preventative perspective, if we start early by treating fetuses through pregnant mothers, the issue of neonatal HIBD could become less of a problem. With ongoing prevention, the theory is that preventative therapies may be more effective in reducing the risk of newborn HIBD and/or preparing the brain tissue against severe HIBD to result in an injury that is less severe and more likely to be salvageable.

Pretreatment in neonatal animals has been examined with regards to neonatal HIBD, showing significant neuroprotection with agents such as resveratrol[466], carnosine[467], and hesperidin[468], showing reduced infarct volume, ROS, inflammation, apoptosis, and also behavioural improvement. Pretreatment experiments in pregnant animals have yet to be conducted for neonatal HIBD, however research has shown increased progesterone to be protective against HIBD.[469] The mechanism by which preventative treatments offer protection has been hypothesized to lie in the maintenance of mitochondrial integrity and the reduction of ROS[319,466], as well as preparing the brain tissue with survival substrates to cushion the blow against potential forthcoming neurotoxicity. Also, metformin ,as previously mentioned, has been documented to

reduce the incidence of stroke in diabetic adults.[435] While the effectiveness of neonatal HIBD prevention or reduction has not yet been evaluated in mothers using metformin, this is an interesting avenue of study.

While prevention of neonatal HIBD is a viable strategy, implementing effective preventative strategies may be clinically difficult during pregnancy. Our laboratory (Yager) has been focusing on supplementing pregnant rats with 200 mg of dried broccoli sprouts every day during the final trimester of their pregnancy, and during the first 3 weeks of the pups' lives, with successful neuropathological and neurodevelopmental outcomes.[470,471] Broccoli sprouts have been determined to contain the highest concentration of SFN precursors, glucoraphanin[300], and their safe and efficacious properties have been confirmed.[323] Additionally, pretreating newborn rats with SFN prior to HIBD protected against injury.[319] As there is heightened interest in complementary and alternative therapies, including natural foods, pregnant women may be more willing to consume such cruciferous plants.[472]

In summary, together with these ideas, we are closer to determining in which direction to embark in studying therapeutic interventions that may be effective in enhancing hypothermic neuroprotection for neonatal HIBD, and also determining the best time(s) to administer them during the neurotoxic cascade. Although post-ischemic hypothermia is the most effective neuroprotectant available for mild-moderate HIBD, there is still a ways until we uncover the optimal equilibrium of adjunct therapy type, timing, and duration of administration to complement post-ischemic hypothermia and improve HIBD outcome.

References

1. Hoeger H, Engidawork E, Stolzlechner D, Bubna-Littitz H, Lubec B. Long-term effect of moderate and profound hypothermia on morphology, neurological, cognitive and behavioural functions in a rat model of perinatal asphyxia. *Amino Acids*. 2006;31(4):385-396.

2. Perlman JM. Summary proceedings from the neurology group on hypoxic-ischemic encephalopathy. *Pediatrics*. 2006;117(3):S28-S33. doi:10.1542/peds.2005-0620E.

3. van Handel M, Swaab H, de Vries LS, Jongmans MJ. Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. *Eur J Pediatr*. 2007;166(7):645-654. doi:10.1007/s00431-007-0437-8.

4. Perrone S, Stazzoni G, Tataranno ML, Buonocore G. New pharmacologic and therapeutic approaches for hypoxic-ischemic encephalopathy in the newborn. *J Matern Fetal Neonatal Med*. 2012;25:83-88. doi:10.3109/14767058.2012.663168.

5. Badawi N, Kurinczuk JJ, Keogh JM, et al. Intrapartum risk factors for newborn encephalopathy: the Western Australian case-control study. *Br Med J*. 1998;317(7172):1554-1558.

6. Kurinczuk JJ, White-Koning M, Badawi N. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum Dev*. 2010;86(6):329-338. doi:10.1016/j.earlhumdev.2010.05.010.

7. Vannucci RC, Perlman JM. Interventions for perinatal hypoxic-ischemic encephalopathy. *Pediatrics*. 1997;100(6). doi:10.1542/peds.100.6.1004.

8. Johnston MV, Hoon AH Jr. Cerebral palsy. *Neuromolecular Med*. 2006;8(4). doi:10.1385/NMM:8:4:435.

9. Bennet L, Tan S, Van den Heuij L, et al. Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann Neurol*. 2012;71(5):589-600. doi:10.1002/ana.22670.

10. Rosenbaum P, Paneth N, Leviton A, et al. A report: the definition and classification of cerebral palsy April 2006. *Dev Med Child Neurol*. 2007;109:8-14.

11. Bax MC. Terminology and Classification of Cerebral Palsy. *Dev Med Child Neurol*. 1964;6:295-297.

12. Bax M, Goldstein M, Rosenbaum P, Leviton A, Paneth N. Proposed definition and classification of cerebral palsy, April 2005 - Introduction. *Dev Med Child Neurol*. 2005;47(8):571-576. doi:10.1017/S001216220500112X.

13. Boyd RN, Morris ME, Graham HK. Management of upper limb dysfunction in children with cerebral palsy: a systematic review. *Eur J Neurol*. 2001;8:150-166. doi:10.1046/j.1468-1331.2001.00048.x.

14. Boyd RN, Hays RM. Current evidence for the use of botulinum toxin type A in the management of children with cerebral palsy: a systematic review. *Eur J Neurol*. 2001;8:1-20. doi:10.1046/j.1468-1331.2001.00034.x.

15. Gage JR, Novacheck TF. An update on the treatment of gait problems in cerebral palsy. *J Pediatr Orthop-Part B*. 2001;10(4):265-274. doi:10.1097/00009957-200110000-00001.

16. Grether JK, Nelson KB. Maternal infection and cerebral palsy in infants of normal birth weight. *Jama-J Am Med Assoc*. 1997;278(3):207-211. doi:10.1001/jama.278.3.207.

17. Gaudet LM, Smith GN. Cerebral palsy and chorioamnionitis: The inflammatory cytokine link. *Obstet Gynecol Surv*. 2001;56(7):433-436. doi:10.1097/00006254-200107000-00023.

18. Nelson KB. The epidemiology of cerebral palsy interm infants. *Ment Retard Dev Disabil Res Rev*. 2002;8(3):146-150. doi:10.1002/mrdd.10037.

19. Shevell MI, Majnemer A, Rosenbaum P, Abrahamowicz M. Etiologic determination of childhood developmental delay. *Brain Dev*. 2001;23(4):228-235. doi:10.1016/S0387-7604(01)00212-1.

20. Hankins GDV, Speer M. Defining the pathogenesis and pathophysiology of neonatal encephalopathy and cerebral palsy. *Obstet Gynecol*. 2003;102(3):628-636. doi:10.1016/S0029-7844(03)00574-X.

21. Edwards AD, Brocklehurst P, Gunn AJ, et al. Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ*. 2010;340:363. doi:10.1136/bmj.c363.

22. Calvert JW, Zhang JH. Pathophysiology of an hypoxic-ischemic insult during the perinatal period. *Neurol Res*. 2005;27(3):246-260. doi:10.1179/016164105X25216.

23. Nassef SK, Blennow M, Jirwe M. Experiences of Parents Whose Newborns Undergo Hypothermia Treatment Following Perinatal Asphyxia. *Jognn-J Obstet Gynecol Neonatal Nurs*. 2013;42(1):38-47. doi:10.1111/j.1552-6909.2012.01429.x.

24. Cilio MR, Ferriero DM. Synergistic neuroprotective therapies with hypothermia. *Semin Fetal Neonatal Med*. 2010;15(5):293-298. doi:10.1016/j.siny.2010.02.002.

25. Kruse M, Michelsen SI, Flachs EM, Bronnum-Hansen H, Madsen M, Uldall P. Lifetime costs of cerebral palsy. *Dev Med Child Neurol*. 2009;51(8):622-628. doi:10.1111/j.1469-8749.2008.03190.x.

26. Dilenge ME, Majnemer A, Shevell MI. Long-term developmental outcome of asphyxiated term neonates. *J Child Neurol*. 2001;16(11):781-792. doi:10.1177/088307380101601102O1.

27. MacLennan A, Int Cerebral Palsy Task F. A template for defining a causal relation between acute intrapartum events and cerebral palsy: international consensus statement. *Br Med J*. 1999;319(7216):1054-1059.

28. Johnston MV, Trescher WH, Ishida A, Nakajima W. Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatr Res*. 2001;49(6):735-741. doi:10.1203/00006450-200106000-00003.

29. Nakajima W, Ishida A, Lange MS, et al. Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. *J Neurosci*. 2000;20(21):7994-8004.

30. Badawi N, Kurinczuk JJ, Keogh JM, et al. Antepartum risk factors for newborn encephalopathy: the Western Australian case-control study. *Br Med J*. 1998;317(7172):1549-1553.

31. Finer NN, Robertson CM, Richards RT, Pinnell LE, Peters KL. Hypoxic-ischemic encephalopathy in term neonates: perinatal factors and outcome. *J Pediatr*. 1981;98(1):112-117.

32. Devisme L, Merlot B, Ego A, Houfflin-Debarge V, Deruelle P, Subtil D. A case-control study of placental lesions associated with pre-eclampsia. *Int J Gynecol Obstet*. 2013;120(2):165-168. doi:10.1016/j.ijgo.2012.08.023.

33. Adamson SJ, Alessandri LM, Badawi N, Burton PR, Pemberton PJ, Stanley F. Predictors of Neonatal Encephalopathy in Full-Term Infants. *Br Med J*. 1995;311(7005):598-602.

34. Vannucci RC. Experimental Biology of Cerebral Hypoxia-Ischemia - Relation to Perinatal Brain-Damage. *Pediatr Res*. 1990;27(4):317-326. doi:10.1203/00006450-199004000-00001.

35. Lai MC, Yang SN. Perinatal hypoxic-ischemic encephalopathy. *J Biomed Biotechnol*. 2011;2011:609813.

36. Shankaran S, Laptook AR, Ehrenkranz RA, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med*. 2005;353(15). doi:10.1056/NEJMcps050929.

37. Geva A, Gray J. A Quantitative Analysis of Optimal Treatment Capacity for Perinatal Asphyxia. *Med Decis Making*. 2012;32(2):266-272. doi:10.1177/0272989X11421527.

38. Boichot C, Walker PM, Durand C, et al. Term neonate prognoses after perinatal asphyxia: Contributions of MR imaging, MR spectroscopy, relaxation times, and apparent diffusion coefficients. *Radiology*. 2006;239(3):839-848. doi:10.1148/radiol.2393050027.

39. Simon NP. Long-term neurodevelopmental outcome of asphyxiated newborns. *Clin Perinatol*. 1999;26(3):767-+.

40. Stelmach T, Kallas E, Pisarev H, Talvik T. Antenatal risk factors associated with unfavorable neurologic status in newborns and at 2 years of age. *J Child Neurol*. 2004;19(2):116-122.

41. Rutherford M, Pennock J, Schwieso J, Cowan F, Dubowitz L. Hypoxic-ischaemic encephalopathy: Early and late magnetic resonance imaging findings in relation to outcome. *Arch Dis Child*. 1996;75(3):F145-F151. doi:10.1136/fn.75.3.F145.

42. Belet N, Belet U, Incesu L, et al. Hypoxic-ischemic encephalopathy: Correlation of serial MRI and outcome. *Pediatr Neurol*. 2004;31(4):267-274. doi:10.1016/j.pediatrneurol.2004.04.011.

43. Pulera MR, Adams LM, Liu HT, et al. Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. *Stroke*. 1998;29(12):2622-2629.

44. Towfighi J, Yager JY, Housman C, Vannucci RC. Neuropathology of Remote Hypoxic-Ischemic Damage in the Immature Rat. *Acta Neuropathol (Berl)*. 1991;81(5):578-587.

45. Towfighi J, Zec N, Yager J, Housman C, Vannucci RC. Temporal Evolution of Neuropathologic Changes in an Immature Rat Model of Cerebral Hypoxia - a Light-Microscopic Study. *Acta Neuropathol (Berl)*. 1995;90(4):375-386.

46. Northington FJ, Ferriero DM, Flock DL, Martin LJ. Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. *J Neurosci*. 2001;21(6):1931-1938.

47. Miller SP, Ramaswamy V, Michelson D, et al. Patterns of brain injury in term neonatal encephalopathy. *J Pediatr*. 2005;146(4):453-460. doi:10.1016/j.jpeds.2004.12.026.

48. Rutherford M, Counsell S, Allsop J, et al. Diffusion-weighted magnetic resonance imaging in term perinatal brain injury: A comparison with site of lesion and time from birth. *Pediatrics*. 2004;114(4):1004-1014. doi:10.1542/peds.2004-0222.

49. O'Shea TM. Diagnosis, Treatment, and Prevention of Cerebral Palsy. *Clin Obstet Gynecol*. 2008;51(4):816-828.

50. Roth SC, Baudin J, Cady E, et al. Relation of deranged neonatal cerebral oxidative metabolism with neurodevelopmental outcome and head circumference at 4 years. *Dev Med Child Neurol*. 1997;39(11):718-725.

51. Riikonen RS, Kero PO, Simell OG. Excitatory Amino-Acids in Cerebrospinal-Fluid in Neonatal Asphyxia. *Pediatr Neurol*. 1992;8(1):37-40. doi:10.1016/0887-8994(92)90050-9.

52. Sweeney MI, Yager JY, Walz W, Juurlink BH. Cellular mechanisms involved in brain ischemia. *Can J Physiol Pharmacol*. 1995;73(11):1525-1535.

53. Zhang F, Wang SP, Luo YM, Ji XM, Nemoto EM, Chen J. When hypothermia meets hypotension and hyperglycemia: the diverse effects of adenosine 5 ’-monophosphate on cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 2009;29(5):1022-1034. doi:10.1038/jcbfm.2009.28.

54. Askalan R, Wang C, Shi H, Armstrong E, Yager JY. The Effect of Postischemic Hypothermia on Apoptotic Cell Death in the Neonatal Rat Brain. *Dev Neurosci*. 2011;33(3-4):320-329. doi:10.1159/000329924.

55. Askalan R, Salweski R, Tuor UI, Hutchison J, Hawkins C. X-linked inhibitor of apoptosis protein expression after ischemic injury in the human and rat developing brain. *Pediatr Res*. 2009;65(1):21–26.

56. Manabat C, Han BH, Wendland M, et al. Reperfusion differentially induces caspase-3 activation in ischemic core and penumbra after stroke in immature brain. *Stroke J Cereb Circ*. 2003;34(1):207-213.

57. Vannucci RC, Towfighi J, Vannucci SJ. Secondary energy failure after cerebral hypoxia-ischemia in the immature rat. *J Cereb Blood Flow Metab*. 2004;24(10):1090-1097. doi:10.1097/01.WCB.0000133250.03953.63.

58. Penrice J, Lorek A, Cady EB, et al. Proton magnetic resonance spectroscopy of the brain during acute hypoxia-ischemia and delayed cerebral energy failure in the newborn piglet. *Pediatr Res*. 1997;41(6):795-802. doi:10.1203/00006450-199706000-00001.

59. Andine P, Sandberg M, Bagenholm R, Lehmann A, Hagberg H. Intracellular and Extracellular Changes of Amino-Acids in the Cerebral-Cortex of the Neonatal Rat during Hypoxic-Ischemia. *Dev Brain Res*. 1991;64(1-2):115-120. doi:10.1016/0165-3806(91)90214-4.

60. Yager JY, Brucklacher RM, Vannucci RC. Cerebral oxidative metabolism and redox state during hypoxia-ischemia and early recovery in immature rats. *Am J Physiol*. 1991;261(4 Pt 2):H1102-8.

61. Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. Neuroscience - Energy on demand. *Science*. 1999;283(5401):496-497. doi:10.1126/science.283.5401.496.

62. Ten VS, Starkov A. Hypoxic-Ischemic Injury in the Developing Brain: The Role of Reactive Oxygen Species Originating in Mitochondria. *Neurol Res Int*. 2012;2012:e542976. doi:10.1155/2012/542976.

63. Schanne FA, Kane AB, Young EE, Farber JL. Calcium dependence of toxic cell death: a final common pathway. *Science*. 1979;206(4419):700-702.

64. Lee JM, Zipfel GJ, Choi DW. The changing landscape of ischaemic brain injury mechanisms. *Nature*. 1999;399(6738 Suppl):A7-14.

65. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. *Nat Rev Neurosci*. 2012;13(4):267-278. doi:10.1038/nrn3174.

66. Rothman S. Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J Neurosci*. 1984;4(7):1884-1891.

67. Andine P, Jacobson I, Hagberg H. Enhanced Calcium-Uptake by Ca1 Pyramidal Cell Dendrites in the Postischemic Phase Despite Subnormal Evoked Field Potentials - Excitatory Amino-Acid Receptor Dependency and Relationship to Neuronal Damage. *J Cereb Blood Flow Metab*. 1992;12(5):773-783.

68. Delivoria-Papadopoulos M, Mishra OP. Mechanisms of cerebral injury in perinatal asphyxia and strategies for prevention. *J Pediatr*. 1998;132(3 Pt 2):S30-4.

69. McDonald JW, Johnston MV. Physiological and Pathophysiological Roles of Excitatory Amino-Acids during Central-Nervous-System Development. *Brain Res Rev*. 1990;15(1):41-70. doi:10.1016/0165-0173(90)90011-C.

70. Ikonomidou C, Kaindl AM. Neuronal Death and Oxidative Stress in the Developing Brain. *Antioxid Redox Signal*. 2011;14(8):1535-1550. doi:10.1089/ars.2010.3581.

71. Silverstein FS, Buchanan K, Johnston MV. Perinatal Hypoxia-Ischemia Disrupts Striatal High-Affinity [H-3] Glutamate Uptake into Synaptosomes. *J Neurochem*. 1986;47(5):1614-1619. doi:10.1111/j.1471-4159.1986.tb00803.x.

72. Johnston MV. Excitotoxicity in perinatal brain injury. *Brain Pathol*. 2005;15(3):234-240.

73. Yamaguchi S, Endo K, Kitajima T, Ogata H, Hori Y. Involvement of the glutamate transporter and the sodium-calcium exchanger in the hypoxia-induced increase in intracellular Ca2+ in rat hippocampal slices. *Brain Res*. 1998;813(2):351-358. doi:10.1016/S0006-8993(98)01037-3.

74. Kure S, Tominaga T, Yoshimoto T, Tada K, Narisawa K. Glutamate Triggers Internucleosomal Dna Cleavage in Neuronal Cells. *Biochem Biophys Res Commun*. 1991;179(1):39-45. doi:10.1016/0006-291X(91)91330-F.

75. Higuchi Y, Hattori H, Hattori R, Furusho K. Increased neurons containing neuronal nitric oxide synthase in the brain of a hypoxic-ischemic neonatal rat model. *Brain Dev*. 1996;18(5):369-375. doi:10.1016/0387-7604(96)00019-8.

76. Hagberg H, Thornberg E, Blennow M, et al. Excitatory Amino-Acids in the Cerebrospinal-Fluid of Asphyxiated Infants - Relationship to Hypoxic-Ischemic Encephalopathy. *Acta Paediatr*. 1993;82(11):925-929. doi:10.1111/j.1651-2227.1993.tb12601.x.

77. Pu YL, Li QF, Zeng CM, et al. Increased detectability of alpha brain glutamate/glutamine in neonatal hypoxic-ischemic encephalopathy. *Am J Neuroradiol*. 2000;21(1):203-212.

78. Yuan J. Neuroprotective strategies targeting apoptotic and necrotic cell death for stroke. *Apoptosis*. 2009;14(4):469-477. doi:10.1007/s10495-008-0304-8.

79. Tamir S, Tannenbaum SR. The role of nitric oxide (NO center dot) in the carcinogenic process. *Biochim Biophys Acta-Rev Cancer*. 1996;1288(2):F31-F36. doi:10.1016/0304-419X(96)00021-2.

80. Dawson VL, Dawson TM. Free radicals and neuronal cell death. *Cell Death Differ*. 1996;3(1):71-78.

81. Northington FJ, Graham EM, Martin LJ. Apoptosis in perinatal hypoxic-ischemic brain injury: How important is it and should it be inhibited? *Brain Res Rev*. 2005;50(2):244-257. doi:10.1016/j.brainreserv.2005.07.003.

82. Niizuma K, Endo H, Chan PH. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J Neurochem*. 2009;109:133-138. doi:10.1111/j.1471-4159.2009.05897.x.

83. Alfieri A, Srivastava S, Siow RCM, Modo M, Fraser PA, Mann GE. Targeting the Nrf2-Keap1 antioxidant defence pathway for neurovascular protection in stroke. *J Physiol-Lond*. 2011;589(17):4125-4136. doi:10.1113/jphysiol.2011.210294.

84. Juurlink BHJ. Response of glial cells to ischemia: Roles of reactive oxygen species and glutathione. *Neurosci Biobehav Rev*. 1997;21(2):151-166. doi:10.1016/S0149-7634(96)00005-X.

85. Gunn AJ, Gunn TR, Gunning MI, Williams CE, Gluckman PD. Neuroprotection with prolonged head cooling started before postischemic seizures in fetal sheep. *Pediatrics*. 1998;102(5):1098-1106.

86. Hossmann KA. Reperfusion of the brain after global ischemia: Hemodynamic disturbances. *Shock*. 1997;8(2):95-101. doi:10.1097/00024382-199708000-00004.

87. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. *Int J Stroke*. 2009;4(6):461-470.

88. Schmura Martin SM, Perez-Polo JR, Grafe MR. Increased PARP expression and cleavage are early events in neuronal death following hypoxic/ischemic injury in the immature brain. *Soc Neurosci Abstr*. 2000;26(1-2):Abstract No.-88.13.

89. Joashi UC, Greenwood K, Taylor DL, et al. Poly(ADP ribose) polymerase cleavage precedes neuronal death in the hippocampus and cerebellum following injury to the developing rat forebrain. *Eur J Neurosci*. 1999;11(1):91-100. doi:10.1046/j.1460-9568.1999.00409.x.

90. Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA. Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab*. 1997;17(11):1143-1151. doi:10.1097/00004647-199711000-00002.

91. Lenzser G, Snipes JA, Kis B, et al. Contribution of poly(ADP-ribose) polymerase (PAR-P) to postischemic blood-brain barrier (BBB) damage in rats. *Faseb J*. 2006;20(5):A1157-A1157.

92. Yu SW, Wang HM, Poitras MF, et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science*. 2002;297(5579):259-263. doi:10.1126/science.1072221.

93. Cregan SP, Fortin A, MacLaurin JG, et al. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J Cell Biol*. 2002;158(3):507-517. doi:10.1083/jcb.200202130.

94. Bednarek N, Svedin P, Garnotel R, et al. Increased MMP-9 and TIMP-1 in mouse neonatal brain and plasma and in human neonatal plasma after hypoxia-ischemia: a potential marker of neonatal encephalopathy. *Pediatr Res*. 2012;71(1):63-70. doi:10.1038/pr.2011.3.

95. Douglas-Escobar M, Weiss MD. Biomarkers of brain injury in the premature infant. *Front Neurol*. 2012;3:185-185. doi:10.3389/fneur.2012.00185.

96. Gu Z, Cui J, Lipton SA. *Matrix Metalloproteinases in Cerebral Hypoxia-Ischemia*.; 2009.

97. Li Y, Yu L, Li YJ, Yu LJ. Dynamic changes of levels of serum MMP-9 in patients with hypoxic-ischemic encephalopathy and its clinical significance. *Prog Mod Biomed*. 2008;8(6):1122-1123.

98. Wang R, Pang G, Shi Z, Wang RH, Pang GX, Shi ZZ. Changes in serum levels of MMP-9 and TIMP-1 and its clinical significance in HIE. *J Trop Med Guangzhou*. 2011;11(4):454-461.

99. McAdams RM, Juul SE. The role of cytokines and inflammatory cells in perinatal brain injury. *Neurol Res Int*. 2012;2012:561494. doi:10.1155/2012/561494.

100. Hagberg H, Gilland E, Bona E, et al. Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr Res*. 1996;40(4):603-609. doi:10.1203/00006450-199610000-00015.

101. Saliba E, Henrot A. Inflammatory mediators and neonatal brain damage. *Biol Neonate*. 2001;79(3-4):224-227.

102. Hudome S, Palmer C, Roberts RL, Mauger D, Housman C, Towfighi J. The role of neutrophils in the production of hypoxic-ischemic brain injury in the neonatal rat. *Pediatr Res*. 1997;41(5):607-616. doi:10.1203/00006450-199705000-00002.

103. Benjelloun N, Renolleau S, Represa A, Ben-Ari Y, Charriaut-Marlangue C. Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal rat. *Stroke*. 1999;30(9):1916-1923.

104. Bona E, Andersson AL, Blomgren K, et al. Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. *Pediatr Res*. 1999;45(4):500-509. doi:10.1203/00006450-199904010-00008.

105. Mukandala G, Tynan R, Lanigan S, O'Connor JJ. The Effects of Hypoxia and Inflammation on Synaptic Signaling in the CNS. *Brain Sci*. 2016;6(1). doi:10.3390/brainsci6010006.

106. Hamada Y, Hayakawa T, Hattori H, Mikawa H. Inhibitor of Nitric-Oxide Synthesis Reduces Hypoxic-Ischemic Brain-Damage in the Neonatal Rat. *Pediatr Res*. 1994;35(1):10-14.

107. Chen W, Hartman R, Ayer R, et al. Matrix metalloproteinases inhibition provides neuroprotection against hypoxia-ischemia in the developing brain. *J Neurochem*. 2009;111(3):726-736. doi:10.1111/j.1471-4159.2009.06362.x.

108. Ranasinghe HS, Scheepens A, Sirimanne E, Mitchell MD, Williams CE, Fraser M. Inhibition of MMP-9 Activity following Hypoxic Ischemia in the Developing Brain Using a Highly Specific Inhibitor. *Dev Neurosci*. 2012;34(5):417-427. doi:10.1159/000343257.

109. Yang YF, Chen Z, Hu SL, et al. Interleukin-1 Receptor Associated Kinases-1/4 Inhibition Protects Against Acute Hypoxia/ischemia-Induced Neuronal Injury in Vivo and in Vitro. *Neuroscience*. 2011;196:25-34. doi:10.1016/j.neuroscience.2011.08.059.

110. Halliwell B. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. *Drugs Aging*. 2001;18(9):685-716. doi:10.2165/00002512-200118090-00004.

111. Choi AMK, Alam J. Heme oxygenase-1: Function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am J Respir Cell Mol Biol*. 1996;15(1):9-19.

112. Ryter SW, Choi AMK. Heme oxygenase-1: Molecular mechanisms of gene expression in oxygen-related stress. *Antioxid Redox Signal*. 2002;4(4):625-632. doi:10.1089/15230860260220120.

113. Gozzelino R, Jeney V, Soares MP. Mechanisms of Cell Protection by Heme Oxygenase-1. *Annu Rev Pharmacol Toxicol*. 2010;50:323-354. doi:10.1146/annurev.pharmtox.010909.105600.

114. Mizuno K, Kume T, Muto C, et al. Glutathione Biosynthesis via Activation of the Nuclear Factor E2-Related Factor 2 (Nrf2) - Antioxidant-Response Element (ARE) Pathway Is Essential for Neuroprotective Effects of Sulforaphane and 6-(Methylsulfinyl) Hexyl Isothiocyanate. *J Pharmacol Sci*. 2011;115(3):320-328. doi:10.1254/jphs.10257FP.

115. Sukumari-Ramesh S, Laird MD, Singh N, Vender JR, Alleyne CH Jr, Dhandapani KM. Astrocyte-Derived Glutathione Attenuates Hemin-Induced Apoptosis in Cerebral Microvascular Cells. *Glia*. 2010;58(15):1858-1870. doi:10.1002/glia.21055.

116. Desagher S, Glowinski J, Premont J. Astrocytes protect neurons from hydrogen peroxide toxicity. *J Neurosci*. 1996;16(8):2553-2562.

117. Raps SP, Lai JCK, Hertz L, Cooper AJL. Glutathione is Present in High-Concentrations in Cultured Astrocytes but Not in Cultured Neurons. *Brain Res*. 1989;493(2):398-401. doi:10.1016/0006-8993(89)91178-5.

118. Schroeter ML, Mertsch K, Giese H, et al. Astrocytes enhance radical defence in capillary endothelial cells constituting the blood-brain barrier. *FEBS Lett*. 1999;449(2-3):241-244. doi:10.1016/S0014-5793(99)00451-2.

119. Vargas MR, Johnson JA. The Nrf2-ARE cytoprotective pathway in astrocytes. *Expert Rev Mol Med*. 2009;11:e17-e17. doi:10.1017/S1462399409001094.

120. Thoresen M, Penrice U, Lorek A, et al. Mild Hypothermia After Severe Transient Hypoxia-Ischemia Ameliorates Delayed Cerebral Energy Failure in the Newborn Piglet. *Pediatr Res*. 1995;37(5):667-670.

121. Ito U, Spatz M, Walker JT, Klatzo I. Experimental Cerebral Ischemia in Mongolian Gerbils .1. Light Microscopic Observations. *Acta Neuropathol (Berl)*. 1975;32(3):209-223.

122. Azzopardi D, Wyatt JS, Cady EB, et al. Prognosis of Newborn-Infants with Hypoxic-Ischemic Brain Injury Assessed by Phosphorus Magnetic-Resonance Spectroscopy. *Pediatr Res*. 1989;25(5):445-451. doi:10.1203/00006450-198905000-00004.

123. Hamilton PA, Cady EB, Wyatt JS, Hope PL, Delpy DT, Reynolds EOR. Impaired Energy-Metabolism in Brains of Newborn-Infants with Increased Cerebral Echodensities. *Lancet*. 1986;1(8492):1242-1246.

124. Hope PL, Cady EB, Tofts PS, et al. Cerebral Energy-Metabolism Studied with Phosphorus Nmr-Spectroscopy in Normal and Birth-Asphyxiated Infants. *Lancet*. 1984;2(8399):366-370.

125. Wyatt JS, Edwards AD, Azzopardi D, Reynolds EOR. Magnetic-Resonance and Near-Infrared Spectroscopy for Investigation of Perinatal Hypoxic-Ischemic Brain Injury. *Arch Dis Child*. 1989;64(7):953-963.

126. Williams CE, Gunn A, Gluckman PD. Time Course of Intracellular Edema and Epileptiform Activity Following Prenatal Cerebral-Ischemia in Sheep. *Stroke*. 1991;22(4):516-521.

127. Beilharz EJ, Williams CE, Dragunow M, Sirimanne ES, Gluckman PD. Mechanisms of Delayed Cell-Death Following Hypoxic-Ischemic Injury in the Immature Rat - Evidence for Apoptosis during Selective Neuronal Loss. *Mol Brain Res*. 1995;29(1):1-14. doi:10.1016/0169-328X(94)00217-3.

128. Edwards AD, Mehmet H. Apoptosis in perinatal hypoxic-ischaemic cerebral damage. *Neuropathol Appl Neurobiol*. 1996;22(6):494-498.

129. Eastman A. Apoptosis - a Product of Programmed and Unprogrammed Cell-Death. *Toxicol Appl Pharmacol*. 1993;121(1):160-164. doi:10.1006/taap.1993.1141.

130. Reed JC. Mechanisms of apoptosis. *Am J Pathol*. 2000;157(5):1415-1430. doi:10.1016/S0002-9440(10)64779-7.

131. Zhu C, Qiu L, Wang X, et al. Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. *J Neurochem*. 2003;86(2):306-317.

132. Gottron FJ, Ying HS, Choi DW. Caspase inhibition selectively reduces the apoptotic component of oxygen-glucose deprivation-induced cortical neuronal cell death. *Mol Cell Neurosci*. 1997;9(3):159-169. doi:10.1006/mcne.1997.0618.

133. Mobasheri A, Richardson S, Mobasheri R, Shakibaei M, Hoyland JA. Hypoxia inducible factor-1 and facilitative glucose transporters GLUT1 and GLUT3: Putative molecular components of the oxygen and glucose sensing apparatus in articular chondrocytes. *Histol Histopathol*. 2005;20(4):1327-1338.

134. Hardie DG. The AMP-activated protein kinase pathway - new players upstream and downstream. *J Cell Sci*. 2004;117(23):5479-5487. doi:10.1242/jcs.01540.

135. Hardie DG, Alessi DR. LKB1 and AMPK and the cancer-metabolism link - ten years after. *BMC Biol*. 2013;11:36. doi:10.1186/1741-7007-11-36.

136. Manwani B, McCullough LD. Function of the master energy regulator adenosine monophosphate-activated protein kinase in stroke: AMPK and Stroke. *J Neurosci Res*. 2013;91(8):1018-1029. doi:10.1002/jnr.23207.

137. Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev*. 2011;25(18):1895-1908. doi:10.1101/gad.17420111.

138. Rousset CI, Leiper FC, Kichev A, et al. A dual role for AMP-activated protein kinase (AMPK) during neonatal hypoxic-ischaemic brain injury in mice. *J Neurochem*. 2015;133(2):242-252. doi:10.1111/jnc.13034.

139. Weisova P, Davila D, Tuffy LP, Ward MW, Concannon CG, Prehn JHM. Role of 5 '-Adenosine Monophosphate-Activated Protein Kinase in Cell Survival and Death Responses in Neurons. *Antioxid Redox Signal*. 2011;14(10):1863-1876. doi:10.1089/ars.2010.3544.

140. Concannon CG, Tuffy LP, Weisova P, et al. AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis. *J Cell Biol*. 2010;189(1):83-94. doi:10.1083/jcb.200909166.

141. Spasic MR, Callaerts P, Norga KK. AMP-Activated Protein Kinase (AMPK) Molecular Crossroad for Metabolic Control and Survival of Neurons. *The Neuroscientist*. 2009;15(4):309-316. doi:10.1177/1073858408327805.

142. McCullough LD, Zeng Z, Li H, Landree LE, McFadden J, Ronnett GV. Pharmacological Inhibition of AMP-activated Protein Kinase Provides Neuroprotection in Stroke. *J Biol Chem*. 2005;280(21):20493-20502. doi:10.1074/jbc.M409985200.

143. Li J, McCullough LD. Effects of AMP-activated protein kinase in cerebral ischemia. *J Cereb Blood Flow Metab*. 2010;30(3):480-492. doi:10.1038/jcbfm.2009.255.

144. Iwabuchi S, Kawahara K, Harata NC. Effects of pharmacological inhibition of AMP-activated protein kinase on GLUT3 expression and the development of ischemic tolerance in astrocytes. *Neurosci Res*. 2014;84:68-71. doi:10.1016/j.neures.2014.04.007.

145. Nakatsu Y, Kotake Y, Hino A, Ohta S. Activation of AMP-activated protein kinase by tributyltin induces neuronal cell death. *Toxicol Appl Pharmacol*. 2008;230(3):358-363. doi:10.1016/j.taap.2008.03.021.

146. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev*. 2013;(1):CD003311-CD003311. doi:10.1002/14651858.CD003311.pub3.

147. Karnatovskaia LV, Wartenberg KE, Freeman WD. Therapeutic hypothermia for neuroprotection: history, mechanisms, risks, and clinical applications. *The Neurohospitalist*. 2014;4(3):153-163. doi:10.1177/1941874413519802.

148. Rivera-Lara L, Zhang J, Muehlschlegel S. Therapeutic Hypothermia for Acute Neurological Injuries. *Neurotherapeutics*. 2012;9(1):73-86. doi:10.1007/s13311-011-0092-7.

149. Jasqui Remba S, Varon J, Rivera A, Sternbach GL. Dominique-Jean Larrey: The effects of therapeutic hypothermia and the first ambulance. *Resuscitation*. 2010;81(3):268-271. doi:10.1016/j.resuscitation.2009.11.010.

150. Ginsberg M, Sternau L, Globus M, Dietrich W, Busto R. Therapeutic Modulation of Brain Temperature - Relevance to Ischemic Brain Injury. *Cerebrovasc Brain Metab Rev*. 1992;4(3):189-225.

151. Weinrauch V, Safar P, Tisherman S, Kuboyama K, Radovsky A. Beneficial effect of mild hypothermia and detrimental effect of deep hypothermia after cardiac arrest in dogs. *Stroke J Cereb Circ*. 1992;23(10):1454-1462.

152. Colbourne F, Corbett D. Delayed and Prolonged Postischemic Hypothermia is Neuroprotective in the Gerbil. *Brain Res*. 1994;654(2). doi:10.1016/0006-8993(94)90488-X.

153. Colbourne F, Sutherland G, Corbett D. Postischemic hypothermia - A critical appraisal with implications for clinical treatment. *Mol Neurobiol*. 1997;14(3):171-201. doi:10.1007/BF02740655.

154. Young RS, Olenginski TP, Yagel SK, Towfighi J. The effect of graded hypothermia on hypoxic-ischemic brain damage: a neuropathologic study in the neonatal rat. *Stroke*. 1983;14(6):929-934.

155. Young RS, Kolonich J, Woods CL, Yagel SK. Behavioral performance of rats following neonatal hypoxia-ischemia. *Stroke*. 1986;17(6):1313-1316.

156. Busto R, Dietrich WD, Globus MY, Valdes I, Scheinberg P, Ginsberg MD. Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. *J Cereb Blood Flow Metab*. 1987;7(6):729-738.

157. Busto R, Globus MY, Dietrich WD, Martinez E, Valdes I, Ginsberg MD. Effect of Mild Hypothermia on Ischemia-Induced Release of Neurotransmitters and Free Fatty-Acids in Rat-Brain. *Stroke*. 1989;20(7):904-910.

158. Higgins RD, Raju TNK, Perlman J, et al. Hypothermia and perinatal asphyxia: Executive summary of the National Institute of Child Health and Human Development workshop. *J Pediatr*. 2006;148(2):170-175. doi:10.1016/j.peds.2005.12.009.

159. Van Leeuwen GMJ, Hand JW, Lagendijk JJW, Azzopardi DV, Edwards AD. Numerical modeling of temperature distributions within the neonatal head. *Pediatr Res*. 2000;48(3):351-356. doi:10.1203/00006450-200009000-00015.

160. Gunn AJ, Thoresen M. Hypothermic neuroprotection. *NeuroRx J Am Soc Exp Neurother*. 2006;3(2):154-169. doi:10.1016/j.nurx.2006.01.007.

161. Coimbra C, Wieloch T. Moderate Hypothermia Mitigates Neuronal Damage in the Rat-Brain when Initiated several Hours Following Transient Cerebral-Ischemia. *Acta Neuropathol (Berl)*. 1994;87(4):325-331.

162. Laptook AR, Corbett RJT, Sterett R, Burns DK, Garcia D, Tollefsbol G. Modest hypothermia provides partial neuroprotection when used for immediate resuscitation after brain ischemia. *Pediatr Res*. 1997;42(1):17-23. doi:10.1203/00006450-199707000-00004.

163. Gunn AJ, Bennet L, Gunning MI, Gluckman PD, Gunn TR. Cerebral hypothermia is not neuroprotective when started after postischemic seizures in fetal sheep. *Pediatr Res*. 1999;46(3):274-280. doi:10.1203/00006450-199909000-00005.

164. Nedelcu J, Klein MA, Aguzzi A, Martin E. Resuscitative hypothermia protects the neonatal rat brain from hypoxic-ischemic injury. *Brain Pathol*. 2000;10(1):61-71.

165. Iwata O, Iwata S, Thornton JS, et al. "Therapeutic time window" duration decreases with increasing severity of cerebral hypoxia-ischaemia under normothermia and delayed hypothermia in newborn piglets. *Brain Res*. 2007;1154:173-180. doi:10.1016/j.brainres.2007.03.083.

166. Trescher WH, Ishiwa S, Johnston MV. Brief post-hypoxic-ischemic hypothermia markedly delays neonatal brain injury. *Brain Dev*. 1997;19(5):326-338.

167. Gunn AJ, Gunn TR, deHaan HH, Williams CE, Gluckman PD. Dramatic neuronal rescue with prolonged selective heed cooling after ischemia in fetal lambs. *J Clin Invest*. 1997;99(2). doi:10.1172/JCI119153.

168. Tagin MA, Woolcott CG, Vincer MJ, Whyte RK, Stinson DA. Hypothermia for Neonatal Hypoxic Ischemic Encephalopathy An Updated Systematic Review and Meta-analysis. *Arch Pediatr Adolesc Med*. 2012;166(6):558-566. doi:10.1001/archpediatrics.2011.1772.

169. Shah PS. Hypothermia: a systematic review and meta-analysis of clinical trials. *Semin Fetal Neonatal Med*. 2010;15(5):238-246. doi:10.1016/j.siny.2010.02.003.

170. Alkharfy TM. Induced hypothermia to treat neonatal hypoxic-ischemic encephalopathy. Review of literature with meta-analysis and development of national protocol. *Neurosci Riyadh Saudi Arab*. 2013;18(1):18-26.

171. Thoresen M, Whitelaw A. Cardiovascular changes during mild therapeutic hypothermia and rewarming in infants with hypoxic-ischemic encephalopathy. *Pediatrics*. 2000;106(1):92-99. doi:10.1542/peds.106.1.92.

172. Azzopardi D, Robertson NJ, Cowan FM, Rutherford MA, Rampling M, Edwards AD. Pilot study of treatment with whole body hypothermia for neonatal encephalopathy. *Pediatrics*. 2000;106(4):684-694. doi:10.1542/peds.106.4.684.

173. Shankaran S. The postnatal management of the asphyxiated term infant. *Clin Perinatol*. 2002;29(4):675-+. doi:10.1016/S0095-5108(02)00056-8.

174. Akisu M, Huseyinov A, Yalaz M, Cetin H, Kultursay N. Selective head cooling with hypothermia suppresses the generation of platelet-activating factor in cerebrospinal fluid of newborn infants with perinatal asphyxia. *Prostaglandins Leukot Essent Fatty Acids*. 2003;69(1):45-50. doi:10.1016/S0952-3278(03)00055-3.

175. Eicher DJ, Wagner CL, Katikaneni LP, et al. Moderate hypothermia in neonatal encephalopathy: Efficacy outcomes. *Pediatr Neurol*. 2005;32(1). doi:10.1016/j.pediatrneurol.2004.06.014.

176. Eicher DJ, Wagner CL, Katikaneni LP, et al. Moderate hypothermia in neonatal encephalopathy: Safety outcomes. *Pediatr Neurol*. 2005;32(1). doi:10.1016/j.pediatrneurol.2004.06.015.

177. Lin ZL, Yu HM, Lin J, Chen SQ, Liang ZQ, Zhang ZY. Mild hypothermia via selective head cooling as neuroprotective therapy in term neonates with perinatal asphyxia: an experience from a single neonatal intensive care unit. *J Perinatol*. 2006;26(3):180-184. doi:10.1038/sj.jp.7211412.

178. DuPont TL, Chalak LF, Morriss MC, Burchfield PJ, Christie L, Sanchez PJ. Short-Term Outcomes of Newborns with Perinatal Acidemia Who are Not Eligible for Systemic Hypothermia Therapy. *J Pediatr*. 2013;162(1):35-41. doi:10.1016/j.jpeds.2012.06.042.

179. Azzopardi DV, Strohm B, Edwards AD, et al. Moderate Hypothermia to Treat Perinatal Asphyxial Encephalopathy. *N Engl J Med*. 2009;361(14):1349-1358. doi:10.1056/NEJMoa0900854.

180. Simbruner G, Mittal RA, Rohlmann F, Muche R, Neo n ENT. Systemic Hypothermia After Neonatal Encephalopathy: Outcomes of neo.nEURO.network RCT. *Pediatrics*. 2010;126(4). doi:10.1542/peds.2009-2441.

181. Jacobs SE, Morley CJ, Inder TE, et al. Whole-Body Hypothermia for Term and Near-Term Newborns With Hypoxic-Ischemic Encephalopathy A Randomized Controlled Trial. *Arch Pediatr Adolesc Med*. 2011;165(8):692-700. doi:10.1001/archpediatrics.2011.43.

182. Gluckman PD, Wyatt JS, Azzopardi D, et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet*. 2005;365(9460). doi:10.1016/S0140-6736(05)70932-6.

183. Zhou W, Cheng G, Shao X, et al. Selective Head Cooling with Mild Systemic Hypothermia after Neonatal Hypoxic-Ischemic Encephalopathy: A Multicenter Randomized Controlled Trial in China. *J Pediatr*. 2010;157(3):367-372. doi:10.1016/j.jpeds.2010.03.030.

184. Drury PP, Gunn ER, Bennet L, Gunn AJ. Mechanisms of hypothermic neuroprotection. *Clin Perinatol*. 2014;41(1):161-175. doi:10.1016/j.clp.2013.10.005.

185. Han HS, Park J, Kim J-H, Suk K. Molecular and Cellular Pathways as a Target of Therapeutic Hypothermia: Pharmacological Aspect. *Curr Neuropharmacol*. 2012;10(1):80-87.

186. Polderman KH. Mechanisms of action, physiological effects, and complications of hypothermia. *Crit Care Med*. 2009;37(7 Suppl):S186-202. doi:10.1097/CCM.0b013e3181aa5241.

187. Shintani Y, Terao Y, Ohta H. Molecular mechanisms underlying hypothermia-induced neuroprotection. *Stroke Res Treat*. 2010;2011. http://www.ncbi.nlm.nih.gov/pubmed/21151700 http://downloads.hindawi.com/journals/srt/2011/809874.pdf. Accessed December 1, 2010.

188. Chen F, Qi Z, Luo Y, et al. Non-pharmaceutical therapies for stroke: Mechanisms and clinical implications. *Prog Neurobiol*. 2014;115:246-269. doi:10.1016/j.pneurobio.2013.12.007.

189. Ehrlich MP, McCullough JN, Zhang N, et al. Effect of hypothermia on cerebral blood flow and metabolism in the pig. *Ann Thorac Surg*. 2002;73(1):191-197.

190. Shi Y, Zhao J-N, Liu L, et al. Changes of Positron Emission Tomography in Newborn Infants at Different Gestational Ages, and Neonatal Hypoxic-Ischemic Encephalopathy. *Pediatr Neurol*. 2012;46(2):116-123. doi:10.1016/j.pediatrneurol.2011.11.005.

191. Luo M, Li Q, Dong W, Zhai X, Kang L. Evaluation of mild hypothermia therapy for neonatal hypoxic‐ischaemic encephalopathy on brain energy metabolism using 18F‐fluorodeoxyglucose positron emission computed tomography. *Exp Ther Med*. August 2014. doi:10.3892/etm.2014.1884.

192. Yager JY, Asselin J. Effect of mild hypothermia on cerebral energy metabolism during the evolution of hypoxic-ischemic brain damage in the immature rat. *Stroke*. 1996;27(5):919-925.

193. Fukuda H, Tomimatsu T, Watanabe N, et al. Post-ischemic hypothermia blocks caspase-3 activation in the newborn rat brain after hypoxia-ischemia. *Brain Res*. 2001;910(1-2):187-191.

194. Thoresen M, Bagenholm R, Loberg EM, Apricena F, Kjellmer I. Posthypoxic cooling of neonatal rats provides protection against brain injury. *Arch Dis Child Fetal Neonatal Ed*. 1996;74(1):F3-9. doi:10.1136/fn.74.1.F3.

195. Sirimanne ES, Blumberg RM, Bossano D, et al. The effect of prolonged modification of cerebral temperature on outcome after hypoxic-ischemic brain injury in the infant rat. *Pediatr Res*. 1996;39(4 Pt 1):591-597.

196. Thoresen M, Simmonds M, Satas S, Tooley J, Silver IA. Effective selective head cooling during posthypoxic hypothermia in newborn piglets. *Pediatr Res*. 2001;49(4):594-599. doi:10.1203/00006450-200104000-00024.

197. Tooley JR, Satas S, Porter H, Silver IA, Thoresen M. Head cooling with mild systemic hypothermia in anesthetized piglets is neuroprotective. *Ann Neurol*. 2003;53(1):65-72. doi:10.1002/ana.10402.

198. Edwards AD, Yue X, Squier MV, et al. Specific inhibition of apoptosis after cerebral hypoxia-ischaemia by moderate post-insult hypothermia. *Biochem Biophys Res Commun*. 1995;217(3):1193-1199.

199. Zhu CL, Wang XY, Cheng XY, et al. [Neuroprotective effect and mechanisms of hypothermia in neonatal rat cerebral hypoxic-ischemic damages]. *Zhonghua Erke Zazhi*. 2003;41(12):911-915.

200. Bona E, Hagberg H, Loberg EM, Bagenholm R, Thoresen M. Protective effects of moderate hypothermia after neonatal hypoxia-ischemia: short- and long-term outcome. *Pediatr Res*. 1998;43(6):738-745.

201. Wagner BP, Nedelcu J, Martin E. Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. *Pediatr Res*. 2002;51(3):354-360.

202. Zhao H, Steinberg GK, Sapolsky RM. General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. *J Cereb Blood Flow Metab*. 2007;27(12):1879-1894.

203. Colbourne F, Grooms SY, Zukin RS, Buchan AM, Bennett MVL. Hypothermia rescues hippocampal CA1 neurons and attenuates down-regulation of the AMPA receptor GluR2 subunit after forebrain ischemia. *Proc Natl Acad Sci U S A*. 2003;100(5):2906-2910. doi:10.1073/pnas.2628027100.

204. Nie X, Lowe DW, Rollins LG, et al. Sex-specific effects of N-acetylcysteine in neonatal rats treated with hypothermia after severe hypoxia-ischemia. *Neurosci Res*. 2016;108:24-33. doi:10.1016/j.neures.2016.01.008.

205. Laptook AR, Corbett RJT. The effects of temperature on hypoxic-ischemic brain injury. *Clin Perinatol*. 2002;29(4):623-+. doi:10.1016/S0095-5108(02)00057-X.

206. Bernard SA, Buist M. Induced hypothermia in critical care medicine: A review. *Crit Care Med*. 2003;31(7):2041-2051. doi:10.1097/01.CCM.0000069731.18472.61.

207. Schubert S, Brandl U, Brodhun M, et al. Neuroprotective effects of topiramate after hypoxia-ischemia in newborn piglets. *Brain Res*. 2005;1058(1-2):129-136. doi:10.1016/j.brainres.2005.07.061.

208. Yenari MA, Wijman C, Steinberg GK. In: Mayer S, Sessler D, eds. *Hypothermia in Neurocritical Care*. New York: Marcel Dekker; 2004:141-178.

209. Ohshima M, Tsuji M, Taguchi A, Kasahara Y, Ikeda T. Cerebral blood flow during reperfusion predicts later brain damage in a mouse and a rat model of neonatal hypoxic-ischemic encephalopathy. *Exp Neurol*. 2012;233(1):481-489. doi:10.1016/j.expneurol.2011.11.025.

210. Laptook A, Tyson J, Shankaran S, et al. Elevated temperature after hypoxic-ischemic encephalopathy: Risk factor for adverse outcomes. *Pediatrics*. 2008;122(3):491-499. doi:10.1542/peds.2007-1673.

211. Yager JY, Armstrong EA, Jaharus C, Saucier DM, Wirrell EC. Preventing hyperthermia decreases brain damage following neonatal hypoxic-ischemic seizures. *Brain Res*. 2004;1011(1):48-57.

212. Tomimatsu T, Fukuda H, Kanagawa T, Mu J, Kanzaki T, Murata Y. Effects of hyperthermia on hypoxic-ischemic brain-damage in the immature rat: Its influence on caspase-3-like protease. *Am J Obstet Gynecol*. 2003;188(3). doi:10.1067/mob.2003.163.

213. Fukuda H, Tomimatsu T, Kanagawa T, et al. Postischemic hyperthermia induced caspase-3 activation in the newborn rat brain after hypoxia-ischemia and exacerbated the brain damage. *Biol Neonate*. 2003;84(2). doi:10.1159/000071952.

214. Yager JY, Asselin J. The effect of pre hypoxic-ischemic (HI) hypo and hyperthermia on brain damage in the immature rat. *Brain Res Brain Res*. 1999;117(2):139-143.

215. Tomimatsu T, Fukuda H, Endo M, et al. Effects of hypothermia on neonatal hypoxic-ischemic brain injury in the rat: phosphorylation of Akt, activation of caspase-3-like protease. *Neurosci Lett*. 2001;312(1):21-24.

216. Kil HY, Zhang J, Piantadosi CA. Brain temperature alters hydroxyl radical production during cerebral ischemia reperfusion in rats. *J Cereb Blood Flow Metab*. 1996;16(1):100-106.

217. Perrone S, Szabo M, Bellieni CV, et al. Whole Body Hypothermia and Oxidative Stress in Babies With Hypoxic-Ischemic Brain Injury. *Pediatr Neurol*. 2010;43(4):236-240. doi:10.1016/j.pediatrneurol.2010.05.009.

218. Thoresen M, Satas S, PukaSundvall M, et al. Post-hypoxic hypothermia reduces cerebrocortical release of NO and excitotoxins. *Neuroreport*. 1997;8(15):3359-3362. doi:10.1097/00001756-199710200-00033.

219. Hashimoto T, Yonetani M, Nakamura H. Selective brain hypothermia protects against hypoxic-ischemic injury in newborn rats by reducing hydroxyl radical production. *Kobe J Med Sci*. 2003;49(3-4):83-91.

220. Kvrivishvili G. Glycine and neuroprotective effect of hypothermia in hypoxic-ischemic brain damage. *Neuroreport*. 2002;13(16):1995-2000.

221. Han HS, Karabiyikoglu M, Kelly S, Sobel RA, Yenari MA. Mild hypothermia inhibits nuclear factor-kappaB translocation in experimental stroke. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab*. 2003;23(5):589-598. doi:10.1097/01.WCB.0000059566.39780.8D.

222. Yenari MA, Han HS. Influence of hypothermia on post-ischemic inflammation: role of nuclear factor kappa B (NFkappaB). *Neurochem Int*. 2006;49(2):164-169. doi:10.1016/j.neuint.2006.03.016.

223. Yuan X, Ghosh N, McFadden B, et al. Hypothermia modulates cytokine responses after neonatal rat hypoxic-ischemic injury and reduces brain damage. *ASN Neuro*. 2014;6(6). doi:10.1177/1759091414558418.

224. Zhu C, Wang X, Cheng X, et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia-ischemia. *Brain Res*. 2004;996(1):67-75.

225. Ohmura A, Nakajima W, Ishida A, et al. Prolonged hypothermia protects neonatal rat brain against hypoxic-ischemia by reducing both apoptosis and necrosis. *Brain Dev*. 2005;27(7):517-526.

226. Silke J, Ekert PG, Day CL, et al. Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP. *EMBO J*. 2001;20(12):3114-3123.

227. Silke J, Hawkins CJ, Ekert PG, et al. The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. *J Cell Biol*. 2002;157(1):115-124.

228. Lee SM, Zhao H, Maier CM, Steinberg GK. The protective effect of early hypothermia on PTEN phosphorylation correlates with free radical inhibition in rat stroke. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab*. 2009;29(9):1589-1600. doi:10.1038/jcbfm.2009.81.

229. Demers EJ, McPherson RJ, Juul SE. Erythropoietin protects dopaminergic neurons and improves neurobehavioral outcomes in juvenile rats after neonatal hypoxia-ischemia. *Pediatr Res*. 2005;58(2):297-301. doi:10.1203/01.PDR.0000169971.64558.5A.

230. Fang AY, Gonzalez FF, Sheldon RA, Ferriero DM. Effects of combination therapy using hypothermia and erythropoietin in a rat model of neonatal hypoxia-ischemia. *Pediatr Res*. 2013;73(1):12-17. doi:10.1038/pr.2012.138.

231. Li J, Benashski S, McCullough LD. Post-stroke hypothermia provides neuroprotection through inhibition of AMP-activated protein kinase. *J Neurotrauma*. 2011;28(7):1281-1288. doi:10.1089/neu.2011.1751.

232. Tanaka T, Wakamatsu T, Daijo H, et al. Persisting mild hypothermia suppresses hypoxia-inducible factor-1 protein synthesis and hypoxia-inducible factor-1-mediated gene expression. *AJP Regul Integr Comp Physiol*. 2010;298(3):R661-R671. doi:10.1152/ajpregu.00732.2009.

233. Towfighi J, Housman C, Heitjan DF, Vannucci RC, Yager JY. The effect of focal cerebral cooling on perinatal hypoxic-ischemic brain damage. *Acta Neuropathol (Berl)*. 1994;87(6):598-604.

234. Yager J, Towfighi J, Vannucci RC. Influence of mild hypothermia on hypoxic-ischemic brain damage in the immature rat. *Pediatr Res*. 1993;34(4):525-529.

235. Williams GD, Dardzinski BJ, Buckalew AR, Smith MB. Modest hypothermia preserves cerebral energy metabolism during hypoxia-ischemia and correlates with brain damage: a 31P nuclear magnetic resonance study in unanesthetized neonatal rats. *Pediatr Res*. 1997;42(5):700-708.

236. Lee BS, Woo CW, Kim ST, Kim KS. Long-term neuroprotective effect of postischemic hypothermia in a neonatal rat model of severe hypoxic ischemic encephalopathy: a comparative study on the duration and depth of hypothermia. *Pediatr Res*. 2010;68(4):303-308. doi:10.1203/00006450-201011001-00592.

237. Edwards AD. The discovery of hypothermic neural rescue therapy for perinatal hypoxic-ischemic encephalopathy. *Semin Pediatr Neurol*. 2009;16(4):200-206. doi:10.1016/j.spen.2009.09.007.

238. Iwata O, Thornton JS, Sellwood MW, et al. Depth of delayed cooling alters neuroprotection pattern after hypoxia-ischemia. *Ann Neurol*. 2005;58(1):75-87. doi:10.1002/ana.20528.

239. Robertson NJ, Marlow N. Depth and duration of cooling for perinatal asphyxial encephalopathy. *JAMA*. 2014;312(24):2623-2624. doi:10.1001/jama.2014.15959.

240. Shankaran S, Laptook AR, Pappas A, et al. Effect of depth and duration of cooling on deaths in the NICU among neonates with hypoxic ischemic encephalopathy: a randomized clinical trial. *JAMA*. 2014;312(24):2629-2639. doi:10.1001/jama.2014.16058.

241. Haaland K, Loberg EM, Steen PA, Thoresen M. Posthypoxic hypothermia in newborn piglets. *Pediatr Res*. 1997;41(4):505-512. doi:10.1203/00006450-199704000-00009.

242. Covey MV, Oorschot DE. Effect of hypothermic post-treatment on hypoxic-ischemic striatal injury, and normal striatal development, in neonatal rats: a stereological study. *Pediatr Res*. 2007;62(6):646-651.

243. van den Broek MPH, Groenendaal F, Egberts ACG, Rademaker CMA. Effects of Hypothermia on Pharmacokinetics and Pharmacodynamics A Systematic Review of Preclinical and Clinical Studies. *Clin Pharmacokinet*. 2010;49(5):277-294.

244. Tortorici MA, Kochanek PM, Poloyac SM. Effects of hypothermia on drug disposition, metabolism, and response: A focus of hypothermia-mediated alterations on the cytochrome P450 enzyme system. *Crit Care Med*. 2007;35(9):2196-2204. doi:10.1097/01.ccm.0000281517.97507.6e.

245. Drury PP, Bennet L, Gunn AJ. Mechanisms of hypothermic neuroprotection. *Semin Fetal Neonatal Med*. 2010;15(5):287-292. doi:10.1016/j.siny.2010.05.005.

246. Azzopardi D, Brocklehurst P, Edwards D, et al. The TOBY Study. Whole body hypothermia for the treatment of perinatal asphyxial encephalopathy: A randomised controlled trial. *Bmc Pediatr*. 2008;8:17-17. doi:10.1186/1471-2431-8-17.

247. Fan X, van Bel F. Pharmacological neuroprotection after perinatal asphyxia. *J Matern Fetal Neonatal Med*. 2010;23(Suppl 3):17-19.

248. Robertson NJ, Tan S, Groenendaal F, et al. Which Neuroprotective Agents are Ready for Bench to Bedside Translation in the Newborn Infant? *J Pediatr*. 2012;160(4):544-+. doi:10.1016/j.jpeds.2011.12.052.

249. O'Collins VE, Macleod MR, Donnan GA, Howells DW. Evaluation of combination therapy in animal models of cerebral ischemia. *J Cereb Blood Flow Metab*. 2012;32(4):585-597. doi:10.1038/jcbfm.2011.203.

250. Chakkarapani E, Dingley J, Liu X, et al. Xenon Enhances Hypothermic Neuroprotection in Asphyxiated Newborn Pigs. *Ann Neurol*. 2010;68(3):330-341. doi:10.1002/ana.22016.

251. Lobo N, Yang B, Rizvi M, Ma D. Hypothermia and xenon: Novel noble guardians in hypoxic-ischemic encephalopathy? *J Neurosci Res*. 2013;91(4):473-478. doi:10.1002/jnr.23178.

252. Ma D, Hossain M, Chow A, et al. Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. *Ann Neurol*. 2005;58(2):182-193.

253. Martin JL, Ma D, Hossain M, et al. Asynchronous administration of xenon and hypothermia significantly reduces brain infarction in the neonatal rat. *Br J Anaesth*. 2007;98(2):236-240.

254. Liu X, Dingley J, Scull-Brown E, Thoresen M. Adding 5 h delayed xenon to delayed hypothermia treatment improves long-term function in neonatal rats surviving to adulthood. *Pediatr Res*. 2015;77(6):779-783. doi:10.1038/pr.2015.49.

255. Faulkner S, Bainbridge A, Kato T, et al. Xenon Augmented Hypothermia Reduces Early Lactate/N-Acetylaspartate and Cell Death in Perinatal Asphyxia. *Ann Neurol*. 2011;70(1):133-150. doi:10.1002/ana.22387.

256. Chakkarapani E, Thoresen M, Liu X, Walloe L, Dingley J. Xenon offers stable haemodynamics independent of induced hypothermia after hypoxia-ischaemia in newborn pigs. *Intensive Care Med*. 2012;38(2):316-323. doi:10.1007/s00134-011-2442-7.

257. Chakkarapani E, Dingley J, Aquilina K, Osredkar D, Liu X, Thoresen M. Effects of xenon and hypothermia on cerebrovascular pressure reactivity in newborn global hypoxic-ischemic pig model. *J Cereb Blood Flow Metab*. 2013;33(11):1752-1760. doi:10.1038/jcbfm.2013.123.

258. Sabir H, Osredkar D, Maes E, Wood T, Thoresen M. Xenon Combined with Therapeutic Hypothermia Is Not Neuroprotective after Severe Hypoxia-Ischemia in Neonatal Rats. *PLOS ONE*. 2016;11(6):e0156759. doi:10.1371/journal.pone.0156759.

259. Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR. How does xenon produce anaesthesia? *Nature*. 1998;396(6709):324. doi:10.1038/24525.

260. Dickinson R, Peterson BK, Banks P, et al. Competitive inhibition at the glycine site of the N-methyl-D-aspartate receptor by the anesthetics xenon and isoflurane: evidence from molecular modeling and electrophysiology. *Anesthesiology*. 2007;107(5):756-767. doi:10.1097/01.anes.0000287061.77674.71.

261. Dingley J, Tooley J, Liu X, et al. Xenon Ventilation During Therapeutic Hypothermia in Neonatal Encephalopathy: A Feasibility Study. *Pediatrics*. 2014;133(5):809-818. doi:10.1542/peds.2013-0787.

262. Dingley J, Liu X, Gill H, et al. The Feasibility of Using a Portable Xenon Delivery Device to Permit Earlier Xenon Ventilation with Therapeutic Cooling of Neonates During Ambulance Retrieval: *Anesth Analg*. 2015;120(6):1331-1336. doi:10.1213/ANE.0000000000000693.

263. Azzopardi D, Robertson NJ, Bainbridge A, et al. Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomised controlled trial. *Lancet Neurol*. December 2015. doi:10.1016/S1474-4422(15)00347-6.

264. Fan X, van Bel F, van der Kooij MA, Heijnen CJ, Groenendaal F. Hypothermia and erythropoietin for neuroprotection after neonatal brain damage. *Pediatr Res*. 2013;73(1):18-23. doi:10.1038/pr.2012.139.

265. Juul SE. Hypothermia plus erythropoietin for neonatal neuroprotection? Commentary on Fan et al. and Fang et al. *Pediatr Res*. 2013;73(1):10-11. doi:10.1038/pr.2012.148.

266. McPherson RJ, Juul SE. Erythropoietin for infants with hypoxic-ischemic encephalopathy. *Curr Opin Pediatr*. 2010;22(2):139-145.

267. Zhu C, Kang W, Xu F, et al. Erythropoietin Improved Neurologic Outcomes in Newborns With Hypoxic-Ischemic Encephalopathy. *Pediatrics*. 2009;124(2):E218-E226. doi:10.1542/peds.2008-3553.

268. Sant'Anna G, Laptook AR, Shankaran S, et al. Phenobarbital and Temperature Profile During Hypothermia for Hypoxic-Ischemic Encephalopathy. *J Child Neurol*. 2012;27(4):451-457. doi:10.1177/0883073811419317.

269. Sarkar S, Barks JD, Bapuraj JR, et al. Does phenobarbital improve the effectiveness of therapeutic hypothermia in infants with hypoxic-ischemic encephalopathy? *J Perinatol*. 2012;32(1):15-20. doi:10.1038/jp.2011.41.

270. Barks JD, Liu YQ, Shangguan Y, Silverstein FS. Phenobarbital augments hypothermic neuroprotection. *Pediatr Res*. 2010;67(5):532-537. doi:10.1203/PDR.0b013e3181d4ff4d.

271. Filippi L, Poggi C, Ia Marca G, et al. Oral Topiramate in Neonates with Hypoxic Ischemic Encephalopathy Treated with Hypothermia: A Safety Study. *J Pediatr*. 2010;157(3):361-366. doi:10.1016/j.jpeds.2010.04.019.

272. Filippi L, Fiorini P, Daniotti M, et al. Safety and efficacy of topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia (NeoNATI). *Bmc Pediatr*. 2012;12:144-144. doi:10.1186/1471-2431-12-144.

273. Filippi L. Safety and Efficacy of Topiramate in Neonates With Hypoxic Ischemic Encephalopathy Treated With Hypothermia. 2012.

274. Liu Y, Barks JD, Xu G, Silverstein FS. Topiramate extends the therapeutic window for hypothermia-mediated neuroprotection after stroke in neonatal rats. *Stroke*. 2004;35(6):1460-1465.

275. Lafuente H, Pazos MR, Alvarez A, et al. Effects of Cannabidiol and Hypothermia on Short-Term Brain Damage in New-Born Piglets after Acute Hypoxia-Ischemia. *Front Neurosci*. 2016;10:323. doi:10.3389/fnins.2016.00323.

276. Jatana M, Singh I, Singh AK, Jenkins D. Combination of systemic hypothermia and N-acetylcysteine attenuates hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res*. 2006;59(5):684-689.

277. Probyn ME, Cock ML, Duncan JR, et al. The Anti-Inflammatory Agent N-Acetyl Cysteine Exacerbates Endotoxin-Induced Hypoxemia and Hypotension and Induces Polycythemia in the Ovine Fetus. *Neonatology*. 2010;98(2):118-127. doi:10.1159/000280385.

278. Silbergleit R, Haywood Y, Fiskum G, Rosenthal RE. Lack of a neuroprotective effect from N-acetylcysteine after cardiac arrest and resuscitation in a canine model. *Resuscitation*. 1999;40(3):181-186. doi:10.1016/S0300-9572(99)00027-1.

279. Ahola T, Lapatto R, Raivio KO, et al. N-acetylcysteine does not prevent bronchopulmonary dysplasia in immature infants: A randomized controlled trial. *J Pediatr*. 2003;143(6):713-719. doi:10.1067/S0022-3476(03)00419-0.

280. Hobbs CE, Oorschot DE. Neonatal rat hypoxia-ischemia: Long-term rescue of striatal neurons and motor skills by combined antioxidant-hypothermia treatment. *Brain Pathol*. 2008;18(3):443-454. doi:10.1111/j.1750-3639.2008.00146.x.

281. Adachi M, Sohma O, Tsuneishi S, Takada S, Nakamura H. Combination effect of systemic hypothermia and caspase inhibitor administration against hypoxic-ischemic brain damage in neonatal rats. *Pediatr Res*. 2001;50(5):590-595. doi:10.1203/00006450-200111000-00010.

282. Joly LM, Mucignat V, Mariani J, Plotkine M, Charriaut-Marlangue C. Caspase inhibition after neonatal ischemia in the rat brain. *J Cereb Blood Flow Metab*. 2004;24(1):124-131. doi:10.1097/01.WCB.00000100061.36077.5F.

283. Lin S, Rhodes PG, Cai Z. Whole body hypothermia broadens the therapeutic window of intranasally administered IGF-1 in a neonatal rat model of cerebral hypoxia-ischemia. *Brain Res*. 2011;1385:246-256. doi:10.1016/j.brainres.2011.02.013.

284. George S, Bennet L, Weaver-Mikaere L, et al. White Matter Protection with Insulin-Like Growth Factor 1 and Hypothermia Is Not Additive after Severe Reversible Cerebral Ischemia in Term Fetal Sheep. *Dev Neurosci*. 2011;33(3-4):280-287. doi:10.1159/000329923.

285. Kaneko Y, Tajiri N, Su T-P, Wang Y, Borlongan CV. Combination Treatment of Hypothermia and Mesenchymal Stromal Cells Amplifies Neuroprotection in Primary Rat Neurons Exposed to Hypoxic-Ischemic-Like Injury In Vitro: Role of the Opioid System. *Plos One*. 2012;7(10):e47583-e47583. doi:10.1371/journal.pone.0047583.

286. Ni X, Yang Z-J, Carter EL, Martin LJ, Koehler RC. Striatal Neuroprotection from Neonatal Hypoxia-Ischemia in Piglets by Antioxidant Treatment with EUK-134 or Edaravone. *Dev Neurosci*. 2011;33(3-4):299-311. doi:10.1159/000327243.

287. Ni X, Yang Z, Carter EL, Martin LJ, Koehler RC. Lack of additive protection with combined early antioxidant treatment and delayed hypothermia after hypoxia-ischemia in newborn pigs. *Soc Neurosci Abstr Viewer Itiner Plan*. 2008;38.

288. Yang Z, Ni X, Carter EL, Martin LJ, Koehler RC. Early Antioxidant Treatment Does Not Provide Additive Neuroprotection with Delayed Hypothermia after Asphyxic Cardiac Arrest in Newborn Pigs. *Circulation*. 2008;118(18):S1451-S1451.

289. Alkan T, Kahveci N, Buyukuysal L, Korfali E, Ozluk K. Neuroprotective effects of MK 801 and hypothermia used alone and in combination in hypoxic-ischemic brain injury in neonatal rats. *Arch Physiol Biochem*. 2001;109(2):135-144.

290. Hattori H, Morin AM, Schwartz PH, Fujikawa DG, Wasterlain CG. Posthypoxic Treatment with Mk-801 Reduces Hypoxic-Ischemic Damage in the Neonatal Rat. *Neurology*. 1989;39(5):713-718.

291. Ford LM, Sanberg PR, Norman AB, Fogelson MH. Mk-801 Prevents Hippocampal Neurodegeneration in Neonatal Hypoxic-Ischemic Rats. *Arch Neurol*. 1989;46(10):1090-1096.

292. Albensi BC, Schweizer MP, Rarick TM, Filloux F. Unilateral hypoxic-ischemic injury in the neonatal rat brain evaluated by in vivo MRI - Correlation with histopathology and neuroprotection by MK-801. *Invest Radiol*. 1999;34(4):249-261. doi:10.1097/00004424-199904000-00001.

293. Ikonomidou C, Mosinger JL, Olney JW. Hypothermia enhances protective effect of MK-801 against hypoxic/ischemic brain damage in infant rats. *Brain Res*. 1989;487(1):184-187.

294. Buchan A, Pulsinelli WA. Hypothermia but Not the N-Methyl-D-Aspartate Antagonist, Mk-801, Attenuates Neuronal Damage in Gerbils Subjected to Transient Global-Ischemia. *J Neurosci*. 1990;10(1):311-316.

295. Hattori H, Wasterlain CG. Hypothermia does not explain MK-801 neuroprotection in a rat model of neonatal hypoxic-ischemic encephalopathy. *Neurology*. 1991;41(2 (Pt 1)):330.

296. Gilland E, Hagberg H. Is MK-801 neuroprotection mediated by systemic hypothermia in the immature rat? *Neuroreport*. 1997;8(7):1603-1605.

297. Shim S-Y, Kim H-S. Oxidative stress and the antioxidant enzyme system in the developing brain. *Korean J Pediatr*. 2013;56(3):107-111. doi:10.3345/kjp.2013.56.3.107.

298. Tataranno ML, Perrone S, Longini M, Buonocore G. New Antioxidant Drugs for Neonatal Brain Injury. *Oxid Med Cell Longev*. 2015;2015. doi:10.1155/2015/108251.

299. Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. *Food Chem Toxicol*. 1999;37(9-10):973-979. doi:10.1016/S0278-6915(99)00082-4.

300. Fahey JW, Zhang YS, Talalay P. Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci U S A*. 1997;94(19):10367-10372. doi:10.1073/pnas.94.19.10367.

301. Egner PA, Kensler TW, Chen J-G, Gange SJ, Groopman JD, Friesen MD. Quantification of sulforaphane mercapturic acid pathway conjugates in human urine by high-performance liquid chromatography and isotope-dilution tandem mass spectrometry. *Chem Res Toxicol*. 2008;21(10):1991-1996. doi:10.1021/tx800210k.

302. Ma L-L, Xing G-P, Yu Y, et al. Sulforaphane exerts neuroprotective effects via suppression of the inflammatory response in a rat model of focal cerebral ischemia. *Int J Clin Exp Med*. 2015;8(10):17811-17817.

303. Alfieri A, Srivastava S, Siow RCM, et al. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. *Free Radic Biol Med*. 2013;65:1012-1022. doi:10.1016/j.freeradbiomed.2013.08.190.

304. Zhao J, Kobori N, Aronowski J, Dash PK. Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. *Neurosci Lett*. 2006;393(2-3):108-112. doi:10.1016/j.neulet.2005.09.065.

305. Rudolf E, Cervinka M. Sulforaphane induces cytotoxicity and lysosome- and mitochondria-dependent cell death in colon cancer cells with deleted p53. *Toxicol In Vitro*. 2011;25(7):1302-1309. doi:10.1016/j.tiv.2011.04.019.

306. Kaminski BM, Weigert A, Bruene B, et al. Sulforaphane potentiates oxaliplatin-induced cell growth inhibition in colorectal cancer cells via induction of different modes of cell death. *Cancer Chemother Pharmacol*. 2011;67(5):1167-1178. doi:10.1007/s00280-010-1413-y.

307. Clarke JD, Dashwood RH, Ho E. Multi-targeted prevention of cancer by sulforaphane. *Cancer Lett*. 2008;269(2):291-304. doi:10.1016/j.canlet.2008.04.018.

308. Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell Mol Life Sci*. 2007;64(9):1105-1127. doi:10.1007/s00018-007-6484-5.

309. Shinkai Y, Sumi D, Fukami I, Ishii T, Kumagai Y. Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. *FEBS Lett*. 2006;580(7):1771-1774. doi:10.1016/j.febslet.2006.02.031.

310. Tang L, Zhang YS. Isothiocyanates in the chemoprevention of bladder cancer. *Curr Drug Metab*. 2004;5(2):193-201. doi:10.2174/1389200043489027.

311. Hecht SS. Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism. *J Nutr*. 1999;129(3):768S-774S.

312. Zhang YS. Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mutat Res-Fundam Mol Mech Mutagen*. 2004;555(1-2):173-190. doi:10.1016/j.mrfmmm.2004.04.017.

313. Bergstrom P, Andersson HC, Gao Y, et al. Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage. *Neuropharmacology*. 2011;60(2-3):343-353. doi:10.1016/j.neuropharm.2010.09.023.

314. Greaney AJ, Maier NK, Leppla SH, Moayeri M. Sulforaphane inhibits multiple inflammasomes through an Nrf2-independent mechanism. *J Leukoc Biol*. 2016;99(1):189-199. doi:10.1189/jlb.3A0415-155RR.

315. Enrique Guerrero-Beltran C, Calderon-Oliver M, Pedraza-Chaverri J, Irasema Chirino Y. Protective effect of sulforaphane against oxidative stress: Recent advances. *Exp Toxicol Pathol*. 2012;64(5):503-508. doi:10.1016/j.etp.2010.11.005.

316. Deng C, Tao R, Yu S-Z, Jin H. Sulforaphane protects against 6-hydroxydopamine-induced cytotoxicity by increasing expression of heme oxygenase-1 in a PI3K/Akt-dependent manner. *Mol Med Rep*. 2012;5(3):847-851. doi:10.3892/mmr.2011.731.

317. Vauzour D, Buonfiglio M, Corona G, et al. Sulforaphane protects cortical neurons against 5-S-cysteinyl-dopamine-induced toxicity through the activation of ERK1/2, Nrf-2 and the upregulation of detoxification enzymes. *Mol Nutr Food Res*. 2010;54(4):532-542. doi:10.1002/mnfr.200900197.

318. Chang G, Guo Y, Jia Y, et al. Protective Effect of Combination of Sulforaphane and Riluzole on Glutamate-Mediated Excitotoxicity. *Biol Pharm Bull*. 2010;33(9):1477-1483.

319. Ping Z, Liu W, Kang Z, et al. Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme. *Brain Res*. 2010;1343:178-185. doi:10.1016/j.brainres.2010.04.036.

320. Soane L, Dai WL, Fiskum G, Bambrick LL. Sulforaphane Protects Immature Hippocampal Neurons Against Death Caused by Exposure to Hemin or to Oxygen and Glucose Deprivation. *J Neurosci Res*. 2010;88(6):1355-1363. doi:10.1002/jnr.22307.

321. Danilov CA, Chandirasekaran K, Racz J, Soane L, Zielke C, Fiskum G. Sulforaphane Protects Astrocytes Against Oxidative Stress and Delayed Death Caused by Oxygen and Glucose Deprivation. *Glia*. 2009;57(6):645-656. doi:10.1002/glia.20793.

322. Wu X, Zhao J, Yu S, Chen Y, Wu J, Zhao Y. Sulforaphane protects primary cultures of cortical neurons against injury induced by oxygen-glucose deprivation/reoxygenation via antiapoptosis. *Neurosci Bull*. 2012;28(5):509-516. doi:10.1007/s12264-012-1273-z.

323. Shapiro TA, Fahey JW, Dinkova-Kostova AT, et al. Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: A clinical phase I study. *Nutr Cancer- Int J*. 2006;55(1):53-62. doi:10.1207/s15327914nc5501_7.

324. Liu F, Benashski SE, Persky R, Xu Y, Li J, McCullough LD. Age-related changes in AMP-activated protein kinase after stroke. *AGE*. 2012;34(1):157-168. doi:10.1007/s11357-011-9214-8.

325. Tanaka T, Wakamatsu T, Daijo H, et al. Persisting mild hypothermia suppresses hypoxia-inducible factor-1alpha protein synthesis and hypoxia-inducible factor-1-mediated gene expression. *Am J Physiol - Regul Integr Comp Physiol*. 2010;298(3):R661-71. doi:10.1152/ajpregu.00732.2009.

326. Li D, Qu Y, Mao M, et al. Involvement of the PTEN–AKT–FOXO3a pathway in neuronal apoptosis in developing rat brain after hypoxia–ischemia. *J Cereb Blood Flow Metab*. 2009;29(12):1903–1913.

327. Davila D, Connolly NMC, Bonner H, et al. Two-step activation of FOXO3 by AMPK generates a coherent feed-forward loop determining excitotoxic cell fate. *Cell Death Differ*. 2012;19(10):1677-1688. doi:10.1038/cdd.2012.49.

328. Li DY, Qu Y, Li JH, Zhang L, Xiong T, Mu DZ. [Role of the FOXO3a transcription factor in neuronal apoptosis in neonatal rats with hypoxic-ischemic brain damage]. *Zhongguo Dang Dai Er Ke Za Zhi*. 2013;15(11):1023-1027.

329. Gilley J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol*. 2003;162(4):613-622. doi:10.1083/jcb.200303026.

330. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev*. 1979;3(1):79-83.

331. Romijn HJ, Hofman MA, Gramsbergen A. At what age is the developing cerebral cortex of the rat comparable to the full-term human newborn baby? *Early Hum Dev*. 1991;26(1):61-67.

332. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol*. 2013:In Press.

333. Rice Iii JE, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol*. 1981;9(2):131-141. doi:10.1002/ana.410090206.

334. Levine S. Anoxic-Ischemic Encephalopathy in Rats. *Am J Pathol*. 1960;36(1):1-17.

335. Nguyen A, Armstrong EA, Yager JY. Unilateral Common Carotid Artery Ligation as a Model of Perinatal Asphyxia: The Original Rice–Vannucci Model. In: Yager JY, ed. *Animal Models of Neurodevelopmental Disorders*. 1st ed. Springer Protocols. New York, NY: Humana Press; 2015:1-13.

336. Vannucci RC, Mujsce DJ. Effect of Glucose on Perinatal Hypoxic-Ischemic Brain-Damage. *Biol Neonate*. 1992;62(4).

337. Vannucci RC, Yager JY. Glucose, Lactic-Acid, and Perinatal Hypoxic-Ischemic Brain-Damage. *Pediatr Neurol*. 1992;8(1):3-12. doi:10.1016/0887-8994(92)90045-Z.

338. Voorhies TM, Rawlinson D, Vannucci RC. Glucose and Perinatal Hypoxic-Ischemic Brain-Damage in the Rat. *Neurology*. 1986;36(8):1115-1118.

339. Kramer M, Dang J, Baertling F, et al. TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses. *J Neurosci Methods*. 2010;187(1):84-89. doi:10.1016/j.jneumeth.2009.12.020.

340. Wang X, Vaccari JP de R, Wang H, et al. Activation of the Nuclear Factor E2-Related Factor 2/Antioxidant Response Element Pathway Is Neuroprotective after Spinal Cord Injury. *J Neurotrauma*. 2012;29(5):936-+. doi:10.1089/neu.2011.1922.

341. Benedict AL, Mountney A, Hurtado A, et al. Neuroprotective Effects of Sulforaphane after Contusive Spinal Cord Injury. *J Neurotrauma*. 2012;29(16):2576-2586. doi:10.1089/neu.2012.2474.

342. Vargas MR, Johnson DA, Sirkis DW, Messing A, Johnson JA. Nrf2 Activation in Astrocytes Protects against Neurodegeneration in Mouse Models of Familial Amyotrophic Lateral Sclerosis. *J Neurosci*. 2008;28(50):13574-13581. doi:10.1523/JNEUROSCI.4099-08.2008.

343. Liu X-Y, Li C-Y, Bu H, et al. The neuroprotective potential of phase II enzyme inducer on motor neuron survival in traumatic spinal cord injury in vitro. *Cell Mol Neurobiol*. 2008;28(5):769-779. doi:10.1007/s10571-007-9219-0.

344. Mao L, Wang H, Wang X, Liao H, Zhao X. Transcription Factor Nrf2 Protects the Spinal Cord from Inflammation Produced by Spinal Cord Injury. *J Surg Res*. 2011;170(1):E105-E115. doi:10.1016/j.jss.2011.05.049.

345. Morroni F, Tarozzi A, Sita G, et al. Neuroprotective effect of sulforaphane in 6-hydroxydopamine-lesioned mouse model of Parkinson's disease. *Neurotoxicology*. 2013;36:63-71. doi:10.1016/j.neuro.2013.03.004.

346. Han JM, Lee YJ, Lee SY, et al. Protective effect of sulforaphane against dopaminergic cell death. *J Pharmacol Exp Ther*. 2007;321(1):249-256. doi:10.1124/jpet.106.110866.

347. Porritt MJ, Andersson HC, Hou L, et al. Photothrombosis-Induced Infarction of the Mouse Cerebral Cortex Is Not Affected by the Nrf2-Activator Sulforaphane. *Plos One*. 2012;7(7):e41090-e41090. doi:10.1371/journal.pone.0041090.

348. Wang X, Vaccari JP de R, Wang H, Keane RW. Sulforaphane Suppresses Atp-Induced Inflammation Via the Nrf2/are Pathway in Primary Rat Neurons. *J Neurotrauma*. 2011;28(5):A4-A5.

349. Fernandes RO, De Castro AL, Bonetto JHP, et al. Sulforaphane effects on postinfarction cardiac remodeling in rats: modulation of redox-sensitive prosurvival and proapoptotic proteins. *J Nutr Biochem*. 2016;34:106-117. doi:10.1016/j.jnutbio.2016.05.004.

350. Talalay P, Fahey JW, Holtzclaw WD, Prestera T, Zhang YS. Chemoprotection against cancer by Phase 2 enzyme induction. *Toxicol Lett*. 1995;82-3:173-179. doi:10.1016/0378-4274(95)03553-2.

351. Cornblatt BS, Ye L, Dinkova-Kostova AT, et al. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis*. 2007;28(7):1485-1490. doi:10.1093/carcin/bgm049.

352. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome c and apoptosis in human prostate cancer cells. *Cancer Res*. 2006;66(11). doi:10.1158/0008-5472.CAN-06-0139.

353. Gamet-Payrastre L, Li PF, Lumeau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res*. 2000;60(5):1426-1433.

354. Black AM. Broccoli sprout supplementation during placental insufficiency confers structural and functional neuroprotection to the fetal rat. 2010.

355. Wu LY, Juurlink BHJ. The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats. *J Hypertens*. 2001;19(10):1819-1825. doi:10.1097/00004872-200110000-00016.

356. Wu LY, Ashraf MHN, Facci M, et al. Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. *Proc Natl Acad Sci U S A*. 2004;101(18):7094-7099. doi:10.1073/pnas.0402004101.

357. Vasanthi HR, Mukherjee S, Das DK. Potential Health Benefits of Broccoli- A Chemico-Biological Overview. *Mini-Rev Med Chem*. 2009;9(6):749-759.

358. Choi KM, Lee YS, Kim W, et al. Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. *J Nutr Biochem*. 2014;25(2):201-207. doi:10.1016/j.jnutbio.2013.10.007.

359. Lee J-H, Moon M-H, Jeong J-K, et al. Sulforaphane induced adipolysis via hormone sensitive lipase activation, regulated by AMPK signaling pathway. *Biochem Biophys Res Commun*. 2012;426(4):492-497. doi:10.1016/j.bbrc.2012.08.107.

360. van den Broek MP, Groenendaal F, Egberts AC, Rademaker CM. Effects of hypothermia on pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet*. 2010;49(5):277–294.

361. Zhou J, Poloyac SM. The effect of therapeutic hypothermia on drug metabolism and response: cellular mechanisms to organ function. *Expert Opin Drug Metab Toxicol*. 2011;7(7):803-816. doi:10.1517/17425255.2011.574127.

362. Shellhaas RA, Ng CM, Dillon CH, Barks JDE, Bhatt-Mehta V. Population Pharmacokinetics of Phenobarbital in Infants With Neonatal Encephalopathy Treated With Therapeutic Hypothermia. *Pediatr Crit Care Med*. 2013;14(2):194-202. doi:10.1097/PCC.0b013e31825bbbc2.

363. Ezzati M, Broad K, Kawano G, et al. Pharmacokinetics of dexmedetomidine combined with therapeutic hypothermia in a piglet asphyxia model. *Acta Anaesthesiol Scand*. 2014;58(6):733-742. doi:10.1111/aas.12318.

364. Sampson MR, Frymoyer A, Rattray B, et al. Predictive performance of a gentamicin population pharmacokinetic model in neonates receiving full-body hypothermia. *Ther Drug Monit*. 2014;36(5):584-589. doi:10.1097/ftd.0000000000000056.

365. Roberts JK, Stockmann C, Ward RM, et al. Population Pharmacokinetics of Darbepoetin Alfa in Conjunction with Hypothermia for the Treatment of Neonatal Hypoxic-Ischemic Encephalopathy. *Clin Pharmacokinet*. 2015;54(12):1237-1244. doi:10.1007/s40262-015-0286-y.

366. van den Broek MPH, Groenendaal F, Toet MC, et al. Pharmacokinetics and Clinical Efficacy of Phenobarbital in Asphyxiated Newborns Treated with Hypothermia A Thermopharmacological Approach. *Clin Pharmacokinet*. 2012;51(10):671-679. doi:10.1007/s40262-012-0004-y.

367. Li X, Ji Z, Gu Y, Hu Y, Huang K, Pan S. Mild hypothermia decreases the total clearance of glibenclamide after low dose administration in rats. *Neurosci Lett*. 2016;614:55-59. doi:10.1016/j.neulet.2015.12.035.

368. Zhou J, Empey PE, Bies RR, Kochanek PM, Poloyac SM. Cardiac arrest and therapeutic hypothermia decrease isoform-specific cytochrome P450 drug metabolism. *Drug Metab Dispos Biol Fate Chem*. 2011;39(12):2209-2218. doi:10.1124/dmd.111.040642.

369. Schubert A. Side-Effects of Mild Hypothermia. *J Neurosurg Anesthesiol*. 1995;7(2):139-147. doi:10.1097/00008506-199504000-00021.

370. Hosono T, Kamo A, Hakotani S, et al. Effect of hypothermia on motor function of adult rats after neonatal hyperthermic hypoxic-ischemic brain insult. *Eur J Appl Physiol*. 2010;109(1):35-39. doi:10.1007/s00421-009-1156-9.

371. Wu D, Xiong W, Jia X, Geocadin RG, Thakor NV. Short- and long-latency somatosensory neuronal responses reveal selective brain injury and effect of hypothermia in global hypoxic ischemia. *J Neurophysiol*. 2012;107(4):1164-1171. doi:10.1152/jn.00681.2011.

372. Bellot B, Peyronnet-Roux J, Gire C, Simeoni U, Vinay L, Viemari J-C. Deficits of brainstem and spinal cord functions after neonatal hypoxia-ischemia in mice. *Pediatr Res*. 2014;75(6):723-730. doi:10.1038/pr.2014.42.

373. Jung W-B, Im GH, Chung JJ, et al. Neuroplasticity for spontaneous functional recovery after neonatal hypoxic ischemic brain injury in rats observed by functional MRI and diffusion tensor imaging. *Neuroimage*. 2016;126:140-150. doi:10.1016/j.neuroimage.2015.11.032.

374. Dash PK, Zhao J, Orsi SA, Zhang M, Moore AN. Sulforaphane improves cognitive function administered following traumatic brain injury. *Neurosci Lett*. 2009;460(2):103-107. doi:10.1016/j.neulet.2009.04.028.

375. Thoresen M, Hobbs CE, Wood T, Chakkarapani E, Dingley J. Cooling combined with immediate or delayed xenon inhalation provides equivalent long-term neuroprotection after neonatal hypoxia-ischemia. *J Cereb Blood Flow Metab*. 2009;29(4):707-714. doi:10.1038/jcbfm.2008.163.

376. Liu X, Dingley J, Scull-Brown E, Thoresen M. Adding 5 h delayed xenon to delayed hypothermia treatment improves long-term function in neonatal rats surviving to adulthood. *Pediatr Res*. 2015;77(6):779-783. doi:10.1038/pr.2015.49.

377. Wood TL, Loladze V, Altieri S, et al. Delayed IGF-1 administration rescues oligodendrocyte progenitors from glutamate-induced cell death and hypoxic-ischemic brain damage. *Dev Neurosci*. 2007;29(4-5):302-310. doi:10.1159/000105471.

378. Taylor DL, Mehmet H, Cady EB, Edwards AD. Improved neuroprotection with hypothermia delayed by 6 hours following cerebral hypoxia-ischemia in the 14-day-old rat. *Pediatr Res*. 2002;51(1):13-19.

379. Colbourne F, Corbett D, Zhao ZH, Yang J, Buchan AM. Prolonged but delayed postischemic hypothermia: A long-term outcome study in the rat middle cerebral artery occlusion model. *J Cereb Blood Flow Metab*. 2000;20(12):1702-1708.

380. Yager JY, Thornhill JA. The effect of age on susceptibility to hypoxic-ischemic brain damage. *Neurosci Biobehav Rev*. 1997;21(2). doi:10.1016/S0149-7634(96)00006-1.

381. Jiang T, Yu J-T, Zhu X-C, et al. Acute metformin preconditioning confers neuroprotection against focal cerebral ischaemia by pre-activation of AMPK-dependent autophagy. *Br J Pharmacol*. 2014;171(13):3146-3157. doi:10.1111/bph.12655.

382. Roelfsema V, Bennet L, George S, et al. Window of opportunity of cerebral hypothermia for postischemic white matter injury in the near-term fetal sheep. *J Cereb Blood Flow Metab*. 2004;24(8):877-886.

383. McCullough LD, Zeng ZY, Li H, Landree LE, McFadden J, Ronnett GV. Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *J Biol Chem*. 2005;280(21):20493-20502. doi:10.1074/jbc.M409985200.

384. Viollet B, Horman S, Leclerc J, et al. AMPK inhibition in health and disease. *Crit Rev Biochem Mol Biol*. 2010;45(4):276-295. doi:10.3109/10409238.2010.488215.

385. Concannon CG, Tuffy LP, Weisova P, et al. AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis. *J Cell Biol*. 2010;189(1):83-U122. doi:10.1083/jcb.200909166.

386. Eom J-W, Lee J-M, Koh J-Y, Kim Y-H. AMP-activated protein kinase contributes to zinc-induced neuronal death via activation by LKB1 and induction of Bim in mouse cortical cultures. *Mol Brain*. 2016;9:14. doi:10.1186/s13041-016-0194-6.

387. Rousset CI, Leiper F, Thornton C, Gressens P, Carling D, Hagberg H. Is AMP-activated kinase (AMPK) beneficial or detrimental after perinatal HI? *Soc Neurosci Abstr Viewer Itiner Plan*. 2011;41. ://BCI:BCI201200099370.

388. Jiang PZ, Gan M, Ebrahim AS, Castanedes-Casey M, Dickson DW, Yen SHC. Adenosine monophosphate-activated protein kinase overactivation leads to accumulation of alpha-synuclein oligomers and decrease of neurites. *Neurobiol Aging*. 2013;34(5):1504-1515. doi:10.1016/j.neurobiolaging.2012.11.001.

389. Li J, Zeng ZY, Viollet B, Ronnett GV, McCullough LD. Neuroprotective effects of adenosine monophosphate - Activated protein kinase inhibition and gene deletion in stroke. *Stroke*. 2007;38(11):2992-2999. doi:10.1161/strokeaha.107.490904.

390. Li J, Benashski SE, Siegel C, Liu FD, McCullough LD. Adenosine monophosphate activated protein kinase inhibition is protective in both sexes after experimental stroke. *Neurosci Lett*. 2010;482(1):62-65. doi:10.1016/j.neulet.2010.07.007.

391. Zuo XL, Wu P, Liu XX, Liu L, Xu Q, Ji AM. Neuroprotective effect of acute ethanol administration in cerebral ischemia through inhibiting adenosine phosphate-activated protein kinase (AMPK) pathway. *Med Hypotheses*. 2012;79(5):595-597. doi:10.1016/j.mehy.2012.07.028.

392. Bélanger M, Allaman I, Magistretti PJ. Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. *Cell Metab*. 2011;14(6):724-738. doi:10.1016/j.cmet.2011.08.016.

393. Vannucci SJ, Seaman LB, Vannucci RC. Effects of hypoxia-ischemia on GLUT1 and GLUT3 glucose transporters in immature rat brain. *J Cereb Blood Flow Metab*. 1996;16(1):77-81.

394. Weisova P, Concannon CG, Devocelle M, Prehn JHM, Ward MW. Regulation of Glucose Transporter 3 Surface Expression by the AMP-Activated Protein Kinase Mediates Tolerance to Glutamate Excitation in Neurons. *J Neurosci*. 2009;29(9):2997-3008. doi:10.1523/jneurosci.0354-09.2009.

395. Yu J, Li JL, Zhang SY, et al. IGF-1 induces hypoxia-inducible factor 1 alpha-mediated GLUT3 expression through PI3K/Akt/mTOR dependent pathways in PC12 cells. *Brain Res*. 2012;1430:18-24. doi:10.1016/j.brainres.2011.10.046.

396. Vannucci SJ, Reinhart R, Maher F, et al. Alterations in GLUT1 and GLUT3 glucose transporter gene expression following unilateral hypoxia-ischemia in the immature rat brain. *Dev Brain Res*. 1998;107(2):255-264. doi:10.1016/s0165-3806(98)00021-2.

397. Iwabuchi S, Kawahara K. Inducible astrocytic glucose transporter-3 contributes to the enhanced storage of intracellular glycogen during reperfusion after ischemia. *Neurochem Int*. 2011;59(2):319-325. doi:10.1016/j.neuint.2011.06.006.

398. Vannucci SJ, Simpson IA. Glucose transporter proteins in brain: Developmental expression of GLUT1 and GLUT3. *J Neurochem*. 1997;69:S262-S262.

399. Li X, Han H, Hou R, et al. Progesterone treatment before experimental hypoxia-ischemia enhances the expression of glucose transporter proteins GLUT1 and GLUT3 in neonatal rats. *Neurosci Bull*. 2013;29(3):287-294. doi:10.1007/s12264-013-1298-y.

400. Dornbos D, Zwagerman N, Guo M, et al. Preischemic exercise reduces brain damage by ameliorating metabolic disorder in ischemia/reperfusion injury: Exercise Induces Glycolysis and Enhances Cerebral Metabolism. *J Neurosci Res*. 2013;91(6):818-827. doi:10.1002/jnr.23203.

401. Espinoza-Rojo M, Ivonne Iturralde-Rodriguez K, Elena Chanez-Cardenas M, Eugenia Ruiz-Tachiquin M, Aguilera P. Glucose transporters regulation on ischemic brain: possible role as therapeutic target. *Cent Nerv Syst Agents Med Chem Former Curr Med Chem-Cent Nerv Syst Agents*. 2010;10(4):317–325.

402. Lu Q, Harris VA, Kumar S, Mansour HM, Black SM. Autophagy in neonatal hypoxia ischemic brain is associated with oxidative stress. *Redox Biol*. 2015;6:516-523. doi:10.1016/j.redox.2015.06.016.

403. Carloni S, Albertini MC, Galluzzi L, Buonocore G, Proietti F, Balduini W. Increased autophagy reduces endoplasmic reticulum stress after neonatal hypoxia-ischemia: role of protein synthesis and autophagic pathways. *Exp Neurol*. 2014;255:103-112. doi:10.1016/j.expneurol.2014.03.002.

404. Carloni S, Buonocore G, Balduini W. Protective role of autophagy in neonatal hypoxia–ischemia induced brain injury. *Neurobiol Dis*. 2008;32(3):329-339. doi:10.1016/j.nbd.2008.07.022.

405. Carloni S, Girelli S, Scopa C, Buonocore G, Longini M, Balduini W. Activation of autophagy and Akt/CREB signaling play an equivalent role in the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia. *Autophagy*. 2010;6(3):366-377.

406. Gabryel B, Kost A, Kasprowska D, et al. AMP-activated protein kinase is involved in induction of protective autophagy in astrocytes exposed to oxygen-glucose deprivation. *Cell Biol Int*. 2014;38(10):1086-1097. doi:10.1002/cbin.10299.

407. Chu CT. Eaten Alive: Autophagy and Neuronal Cell Death after Hypoxia-Ischemia. *Am J Pathol*. 2008;172(2):284-287. doi:10.2353/ajpath.2008.071064.

408. Descloux C, Ginet V, Clarke PGH, Puyal J, Truttmann AC. Neuronal death after perinatal cerebral hypoxia-ischemia: Focus on autophagy-mediated cell death. *Int J Dev Neurosci Off J Int Soc Dev Neurosci*. 2015;45:75-85. doi:10.1016/j.ijdevneu.2015.06.008.

409. Ginet V, Puyal J, Clarke PGH, Truttmann AC. Enhancement of autophagic flux after neonatal cerebral hypoxia-ischemia and its region-specific relationship to apoptotic mechanisms. *Am J Pathol*. 2009;175(5):1962-1974. doi:10.2353/ajpath.2009.090463.

410. Ginet V, Spiehlmann A, Rummel C, et al. Involvement of autophagy in hypoxic-excitotoxic neuronal death. *Autophagy*. 2014;10(5):846-860. doi:10.4161/auto.28264.

411. Xie C, Ginet V, Sun Y, et al. Neuroprotection by selective neuronal deletion of Atg7 in neonatal brain injury. *Autophagy*. 2016;12(2):410-423. doi:10.1080/15548627.2015.1132134.

412. Jiang Z, Watts LT, Huang S, et al. The Effects of Methylene Blue on Autophagy and Apoptosis in MRI-Defined Normal Tissue, Ischemic Penumbra and Ischemic Core. *PloS One*. 2015;10(6):e0131929. doi:10.1371/journal.pone.0131929.

413. Ginet V, Pittet MP, Rummel C, et al. Dying neurons in thalamus of asphyxiated term newborns and rats are autophagic. *Ann Neurol*. 2014;76(5):695-711. doi:10.1002/ana.24257.

414. Choi K-E, Hall CL, Sun J-M, et al. A novel stroke therapy of pharmacologically induced hypothermia after focal cerebral ischemia in mice. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2012;26(7):2799-2810. doi:10.1096/fj.11-201822.

415. Lu J, Qian H-Y, Liu L-J, et al. Mild hypothermia alleviates excessive autophagy and mitophagy in a rat model of asphyxial cardiac arrest. *Neurol Sci Off J Ital Neurol Soc Ital Soc Clin Neurophysiol*. 2014;35(11):1691-1699. doi:10.1007/s10072-014-1813-6.

416. Jin Y, Lin Y, Feng J, Jia F, Gao G, Jiang J. Moderate Hypothermia Significantly Decreases Hippocampal Cell Death Involving Autophagy Pathway after Moderate Traumatic Brain Injury. *J Neurotrauma*. 2015;32(14):1090-1100. doi:10.1089/neu.2014.3649.

417. Jin Y, Lin Y, Feng J, Jia F, Gao G, Jiang J. Attenuation of Cell Death in Injured Cortex After Post-Traumatic Brain Injury Moderate Hypothermia: Possible Involvement of Autophagy Pathway. *World Neurosurg*. 2015;84(2):420-430. doi:10.1016/j.wneu.2015.03.039.

418. Jin Y, Lei J, Lin Y, Gao G, Jiang J. Autophagy Inhibitor 3-MA Weakens Neuroprotective Effects of Posttraumatic Brain Injury Moderate Hypothermia. *World Neurosurg*. 2016;88:433-446. doi:10.1016/j.wneu.2015.10.055.

419. Gomes AR, Brosens JJ, Lam EW-F. Resist or die: FOXO transcription factors determine the cellular response to chemotherapy. *Cell Cycle*. 2008;7(20):3133–3136.

420. Davila D, Connolly NMC, Bonner H, et al. Two-step activation of FOXO3 by AMPK generates a coherent feed-forward loop determining excitotoxic cell fate. *Cell Death Differ*. 2012;19(10):1677–1688.

421. Zhao XC, Cao XC, Liu F, Quan MF, Ren KQ, Cao JG. Regulation of the FOXO3a/Bim signaling pathway by 5,7-dihydroxy-8-nitrochrysin in MDA-MB-453 breast cancer cells. *Oncol Lett*. 2013;5(3):929-934. doi:10.3892/ol.2012.1077.

422. Li J, McCullough LD. Effects of AMP-activated protein kinase in cerebral ischemia. *J Cereb Blood Flow Metab*. 2010;30(3):480–492.

423. Ness JM, Harvey CA, Strasser A, Bouillet P, Klocke BJ, Roth KA. Selective involvement of BH3-only Bcl-2 family members Bim and Bad in neonatal hypoxia-ischemia. *Brain Res*. 2006;1099(1):150-159. doi:10.1016/j.brainres.2006.04.132.

424. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol*. 2000;88(4):1474-1480.

425. Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann Neurol*. 2000;48(3):285-296. doi:10.1002/1531-8249(200009)48:3<285::AID-ANA2>3.0.CO;2-8.

426. Thornton C, Hagberg H. Role of mitochondria in apoptotic and necroptotic cell death in the developing brain. *Clin Chim Acta Int J Clin Chem*. 2015;451(Pt A):35-38. doi:10.1016/j.cca.2015.01.026.

427. Kichev A, Rousset CI, Baburamani AA, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling and cell death in the immature central nervous system after hypoxia-ischemia and inflammation. *J Biol Chem*. 2014;289(13):9430-9439. doi:10.1074/jbc.M113.512350.

428. Matsui T, Kakeda T. IL-10 production is reduced by hypothermia but augmented by hyperthermia in rat microglia. *J Neurotrauma*. 2008;25(6):709-715. doi:10.1089/neu.2007.0482.

429. Leon LR. Hypothermia in systemic inflammation: Role of cytokines. *Front Biosci*. 2004;9:1877-1888.

430. Osredkar D, Sabir H, Falck M, et al. Hypothermia Does Not Reverse Cellular Responses Caused by Lipopolysaccharide in Neonatal Hypoxic-Ischaemic Brain Injury. *Dev Neurosci*. 2015;37(4-5):390-397. doi:10.1159/000430860.

431. Osredkar D, Thoresen M, Maes E, Flatebø T, Elstad M, Sabir H. Hypothermia is not neuroprotective after infection-sensitized neonatal hypoxic-ischemic brain injury. *Resuscitation*. 2014;85(4):567-572. doi:10.1016/j.resuscitation.2013.12.006.

432. Feng Y, Fratkin JD, LeBlanc MH. Inhibiting caspase-8 after injury reduces hypoxic-ischemic brain injury in the newborn rat. *Eur J Pharmacol*. 2003;481(2-3):169-173.

433. Zhao J, Duan S, Zhou J, Sun R, Zhang L, Wang D. Mild hypothermia reduces expression of Fas/FasL and MMP-3 after cerebral ischemia-reperfusion in rats. *Iran J Basic Med Sci*. 2014;17(6):454-459.

434. Liu L, Kim JY, Koike MA, et al. FasL shedding is reduced by hypothermia in experimental stroke. *J Neurochem*. 2008;106(2):541-550. doi:10.1111/j.1471-4159.2008.05411.x.

435. Cheng Y-Y, Leu H-B, Chen T-J, et al. Metformin-inclusive therapy reduces the risk of stroke in patients with diabetes: a 4-year follow-up study. *J Stroke Cerebrovasc Dis Off J Natl Stroke Assoc*. 2014;23(2):e99-105. doi:10.1016/j.jstrokecerebrovasdis.2013.09.001.

436. Venna VR, Li J, Hammond MD, Mancini NS, McCullough LD. Chronic metformin treatment improves post-stroke angiogenesis and recovery after experimental stroke. *Eur J Neurosci*. 2014;39(12):2129-2138. doi:10.1111/ejn.12556.

437. Zhou GC, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001;108(8):1167-1174. doi:10.1172/JCI13505.

438. Jia J, Cheng J, Ni J, Zhen X. Neuropharmacological Actions of Metformin in Stroke. *Curr Neuropharmacol*. 2015;13(3):389-394. doi:10.2174/1570159X13666150205143555.

439. Turner RC, Holman RR, Stratton IM, et al. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*. 1998;352(9131):854-865.

440. Harada S, Fujita-Hamabe W, Tokuyama S. The importance of regulation of blood glucose levels through activation of peripheral 5 ’-AMP-activated protein kinase on ischemic neuronal damage. *Brain Res*. 2010;1351:254-263. doi:10.1016/j.brainres.2010.06.052.

441. Jin Q, Cheng J, Liu Y, et al. Improvement of functional recovery by chronic metformin treatment is associated with enhanced alternative activation of microglia/macrophages and increased angiogenesis and neurogenesis following experimental stroke. *Brain Behav Immun*. 2014;40:131-142. doi:10.1016/j.bbi.2014.03.003.

442. Manwani B, McCullough LD. Function of the master energy regulator adenosine monophosphate-activated protein kinase in stroke. *J Neurosci Res*. 2013;91(8):1018-1029. doi:10.1002/jnr.23207.

443. Jiang T, Yu J-T, Zhu X-C, et al. Acute metformin preconditioning confers neuroprotection against focal cerebral ischaemia by pre-activation of AMPK-dependent autophagy: Metformin preconditioning confers neuroprotection. *Br J Pharmacol*. 2014;171(13):3146-3157. doi:10.1111/bph.12655.

444. Deng T, Zheng Y-R, Hou W-W, et al. Pre-stroke Metformin Treatment is Neuroprotective Involving AMPK Reduction. *Neurochem Res*. June 2016. doi:10.1007/s11064-016-1988-8.

445. Fantus IG. Is metformin ready for prime time in pregnancy? Probably not yet. *Diabetes Metab Res Rev*. 2015;31(1):36-38. doi:10.1002/dmrr.2587.

446. Saraiva C, Praca C, Ferreira R, Santos T, Ferreira L, Bernardino L. Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases. *J Controlled Release*. 2016;235:34-47. doi:10.1016/j.jconrel.2016.05.044.

447. Weinstein JS, Varallyay CG, Dosa E, et al. Superparamagnetic iron oxide nanoparticles: diagnostic magnetic resonance imaging and potential therapeutic applications in neurooncology

and central nervous system inflammatory pathologies, a review. *J Cereb Blood Flow Metab*. 2010;30(1):15-35. doi:10.1038/jcbfm.2009.192.

448. Kabanov AV, Gendelman HE. Nanomedicine in the diagnosis and therapy of neuro degenerative disorders. *Prog Polym Sci*. 2007;32(8-9):1054-1082. doi:10.1016/j.progpolymsei.2007.05.014.

449. Reddy MK, Labhasetwar V. Nanoparticle-mediated delivery of superoxide dismutase to the brain: an effective strategy to reduce ischemia-reperfusion injury. *Faseb J*. 2009;23(5):1384-1395. doi:10.1096/fj.08-116947.

450. Koss KM, Churchward MA, Nguyen AT, Yager JY, Todd KG, Unsworth LD. Brain biocompatibility and microglia response towards engineered self-assembling (RADA)(4) nanoscaffolds. *Acta Biomater*. 2016;35:127-137. doi:10.1016/j.actbio.2016.02.001.

451. Smith AL, Alexander M, Rosenkrantz TS, Sadek ML, Fitch RH. Sex differences in behavioral outcome following neonatal hypoxia ischemia: insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury. *Exp Neurol*. 2014;254:54-67. doi:10.1016/j.expneurol.2014.01.003.

452. Hill CA, Threlkeld SW, Fitch RH. Early testosterone modulated sex differences in behavioral outcome following neonatal hypoxia ischemia in rats. *Int J Dev Neurosci Off J Int Soc Dev Neurosci*. 2011;29(4):381-388. doi:10.1016/j.ijdevneu.2011.03.005.

453. Mage DT, Donner M. Female resistance to hypoxia: Does it explain the sex difference in mortality rates? *J Womens Health*. 2006;15(6):786-794. doi:10.1089/jwh.2006.15.786.

454. Smith AL, Rosenkrantz TS, Fitch RH. Effects of Sex and Mild Intrainsult Hypothermia on Neuropathology and Neural Reorganization following Neonatal Hypoxic Ischemic Brain Injury in Rats. *Neural Plast*. 2016;2016:2585230. doi:10.1155/2016/2585230.

455. Johnston MV, Hagberg H. Sex and the pathogenesis of cerebral palsy. *Dev Med Child Neurol*. 2007;49(1):74-78. doi:10.1017/S0012162207000199.

456. Mirza MA, Ritzel R, Xu Y, McCullough LD, Liu F. Sexually dimorphic outcomes and inflammatory responses in hypoxic-ischemic encephalopathy. *J Neuroinflammation*. 2015;12(1):32. doi:10.1186/s12974-015-0251-6.

457. Waddell J, Hanscom M, Shalon Edwards N, McKenna MC, McCarthy MM. Sex differences in cell genesis, hippocampal volume and behavioral outcomes in a rat model of neonatal HI. *Exp Neurol*. 2016;275 Pt 2:285-295. doi:10.1016/j.expneurol.2015.09.003.

458. Abdelhamid R, Luo J, VandeVrede L, et al. Benzothiophene Selective Estrogen Receptor Modulators Provide Neuroprotection by a Novel GPR30-Dependent Mechanism. *Acs Chem Neurosci*. 2011;2(5):256-268. doi:10.1021/cn100106a.

459. Feng YZ, LeBlanc MH. Estrogen attenuates hypoxic-ischemic brain injury but does not prevent the increase in caspase-3 activity in neonatal rats. *Stroke*. 2004;35(1).

460. Gerstner B, Lee J, DeSilva TM, Jensen FE, Volpe JJ, Rosenberg PA. 17beta-estradiol protects against hypoxic/ischemic white matter damage in the neonatal rat brain. *J Neurosci Res*. 2009;87(9):2078-2086. doi:10.1002/jnr.22023.

461. Nuñez JL, McCarthy MM. Estradiol exacerbates hippocampal damage in a model of preterm infant brain injury. *Endocrinology*. 2003;144(6):2350-2359. doi:10.1210/en.2002-220840.

462. Renolleau S, Fau S, Charriaut-Marlangue C. Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. *Neuroscientist*. 2008;14(1):46-52. doi:10.1177/1073858407308889.

463. Zhu CL, Xu FL, Wang XY, et al. Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. *J Neurochem*. 2006;96(4):1016-1027. doi:10.1111/j.1471-4159.2005.03639.x.

464. Sabir H, Scull-Brown E, Liu X, Thoresen M. Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. *Stroke*. 2012;43(12):3364-3370. doi:10.1161/STROKEAHA.112.674481.

465. Nichol AD, Cooper DJ. Can we improve neurological outcomes in severe traumatic brain injury? Something old (early prophylactic hypothermia) and something new (erythropoietin). *Inj-Int J Care Inj*. 2009;40(5):471-478. doi:10.1016/j.injury.2009.01.002.

466. Arteaga O, Revuelta M, Urigüen L, Álvarez A, Montalvo H, Hilario E. Pretreatment with Resveratrol Prevents Neuronal Injury and Cognitive Deficits Induced by Perinatal Hypoxia-Ischemia in Rats. *PloS One*. 2015;10(11):e0142424. doi:10.1371/journal.pone.0142424.

467. Zhang X, Song L, Cheng X, et al. Carnosine pretreatment protects against hypoxia-ischemia brain damage in the neonatal rat model. *Eur J Pharmacol*. 2011;667(1-3):202-207. doi:10.1016/j.ejphar.2011.06.003.

468. Rong Z, Pan R, Xu Y, Zhang C, Cao Y, Liu D. Hesperidin pretreatment protects hypoxia-ischemic brain injury in neonatal rat. *Neuroscience*. 2013;255:292-299. doi:10.1016/j.neuroscience.2013.09.030.

469. Li XJ, Han H, Hou RL, et al. Progesterone treatment before experimental hypoxia-ischemia enhances the expression of glucose transporter proteins GLUT1 and GLUT3 in neonatal rats. *Neurosci Bull*. 2013;29(3):287-294. doi:10.1007/s12264-013-1298-y.

470. Black AM, Armstrong EA, Scott O, Juurlink BJH, Yager JY. Broccoli sprout supplementation during pregnancy prevents brain injury in the newborn rat following placental insufficiency. *Behav Brain Res*. 2015;291:289-298. doi:10.1016/j.bbr.2015.05.033.

471. Nguyen AT, Bahry AMA, Shen KQ, Armstrong EA, Yager JY. Consumption of broccoli sprouts during late gestation and lactation confers protection against developmental delay induced by maternal inflammation. *Behav Brain Res*. 2016;307:239-249. doi:10.1016/j.bbr.2016.03.017.

472. Scott O, Galicia-Connolly E, Adams D, Surette S, Vohra S, Yager JY. The safety of cruciferous plants in humans: a systematic review. *J Biomed Biotechnol*. 2012;2012:503241. doi:10.1155/2012/503241.