Molecular Mechanisms Of Neuroinflammation
Following Global Cerebral Ischemia: The Role Of Hypothermia Therapy

by

Anh Thi Ngoc Nguyen

A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

© Copyright by Anh Nguyen (2011)
Molecular Mechanisms Of Neuroinflammation Following Global Cerebral Ischemia: The Role Of Hypothermia Therapy

Anh Thi Ngoc Nguyen

Master of Science

Institute of Medical Science
University of Toronto

2011

Abstract

Hypothermia therapy (HT) is used clinically following global cerebral ischemia (GCI) but its therapeutic mechanisms are not completely understood. An elucidation of such mechanisms may lead to novel therapeutic approaches that improve patient outcome. Using a murine model of GCI, we determined the effect of HT on the expression of inflammatory proteins in the hippocampus and serum. We also examined its effect on microglia/macrophage activation and neurodegeneration in the brain at 72 hours following ischemia, and its effect on long-term spatial memory/learning and contextual fear response. GCI led to increased neurodegeneration and microglia/macrophage activation in the hippocampus, and increased IL-1β and KC protein expression in the hippocampus at 72 hours. Hypothermia therapy attenuated these inflammatory responses. It also improved spatial learning/memory at 7 and 21 days, and preserved contextual fear response 21 days post-ischemia. Hypothermia therapy attenuated the post-ischemic inflammatory response, protected hippocampal neurons, and preserved long-term memory and learning.
Acknowledgements

This work was funded by the Heart and Stroke Foundation of Ontario (Grant numbers: NA5013 and NA6006) and the Heart and Stroke Foundation of Canada’s Jump Start Resuscitation Master’s Studentship.

I am especially indebted to my supervisor Dr. James Hutchison for the opportunity to study in his laboratory. His mentorship and support throughout these last two years have been critical for my progress. I would also like to thank my thesis advisory committee, Dr. Sheena Josselyn and Dr. Cynthia Hawkins, as their guidance and help have been invaluable during my graduate career. I am grateful to Hui Shi and Zarko Todorovski for their technical knowledge and training for my histological analysis and behavioural experiments, respectively. I warmly acknowledge Carmen Hiplooley, Samira Riahizadeh, Samantha Snell, and Denis Reynaud for their technical assistance.

I would also like to thank my parents Quang and Thuan, and my sister Hang for supporting me throughout my studies. They have instilled in me a good work ethic and shown me the value of hard work and perseverance, all of which have helped me accomplish this work.
# TABLE OF CONTENTS

**ABSTRACT** .......................................................................................................................... ii
**ACKNOWLEDGEMENTS** ........................................................................................................... iii
**TABLE OF CONTENTS** ............................................................................................................. iv
**LIST OF FIGURES** ..................................................................................................................... vii

## CHAPTER

### Introduction

1.1 Cardiac Arrest .......................................................................................................................... 1
1.2 Cerebral Ischemia/Reperfusion ............................................................................................... 1
1.3 Primary Ischemic Brain Injury ................................................................................................. 2
1.4 Secondary Ischemic (Reperfusion) Brain Injury ................................................................. 3
1.5 Excitotoxicity, Calcium Accumulation and Oxidative Stress ........................................... 5
1.6 Neuroinflammation ............................................................................................................... 7
1.7 Inflammatory Cells ............................................................................................................... 8
1.7.1 Leukocytes ..................................................................................................................... 8
1.7.2 Microglia/Macrophages ............................................................................................... 10
1.7.3 Astrocytes ................................................................................................................... 11
1.8 Leukocyte-Endothelial Interactions .................................................................................... 12
1.8.1 Selectins ...................................................................................................................... 12
1.8.2 Immunoglobulin (Ig) Superfamily ............................................................................. 14
1.8.3 Integrins ...................................................................................................................... 16
1.9 Inflammatory Mediators ..................................................................................................... 17
1.9.1 Cytokines .................................................................................................................... 17
1.9.2 Chemokines ................................................................................................................ 20
1.9.3 Other Molecular Mediators Associated with Neuroinflammation ............................... 22
1.10 Mechanisms of Cell Death after Global Cerebral Ischemia ............................................ 23
1.11 Therapeutic Interventions .................................................................................................... 25
1.11.1 Acute Interventions ..................................................................................................... 25
1.11.2 Anti-inflammatory Therapies ..................................................................................... 26
1.12 Importance of Hypothermia Therapy ................................................................................. 27
1.12.1 Historical Perspective ............................................................................................... 27
1.12.2 Intra-ischemic and Post-ischemic Hypothermia ....................................................... 29
1.13 Potential Mechanisms of Hypothermic Neuroprotection ................................................ 31
1.14 Hypothermia and Inflammation ......................................................................................... 33
1.15 Animal Models of Transient Global Cerebral Ischemia ................................................... 35
1.16 Hippocampal-based Learning and Memory ....................................................................... 36
1.17 Assessing Spatial Memory after GCI using the Morris Water Maze ............................... 37
1.18 Contextual Fear Conditioning ............................................................................................. 39
1.19 Rationale ........................................................................................................................... 40
Hypothesis and Objectives

2.1 Hypothesis................................................................. 41
2.2 Objectives................................................................. 41

Material and Methods

3.1 Animals ......................................................................... 43
3.2 Murine Global Cerebral Ischemia Model.............................. 43
3.3 Brain Inflammation Model ................................................. 44
3.4 Postoperative Recovery and Hypothermia Therapy in Global Cerebral
Ischemia ........................................................................ 44
3.5 Assessment of Systemic and Cerebral Inflammatory Mediators Post-GCI ...... 45
Quantification of systemic inflammatory mediators.......................... 45
Serum Sample Preparation ................................................................ 45
Soluble Adhesion Molecule/MMP-9/PAI-1 Microbead Array ................. 46
Quantification of cerebral inflammatory mediators............................ 46
Brain Homogenate Sample Preparation ........................................... 46
Cytokine/Chemokine Microbead Array ............................................. 47
3.6 Immunohistochemistry .......................................................... 47
Tissue Preparation ...................................................................... 47
Citrate Buffer Antigen Retrieval ..................................................... 48
Immunohistochemistry .................................................................. 48
Image acquisition and analysis ....................................................... 49
3.7 Histological Analysis .............................................................. 49
Short-term (72 hours post-surgery): FluoroJade B Histochemistry ...... 49
3.8 Morris Water Maze and Spatial Memory Analysis ..................... 51
Training 1 ........................................................................... 51
Testing 1 ............................................................................. 52
Training 2 ........................................................................... 52
Testing 2 ............................................................................. 52
3.9 Contextual Fear Conditioning .................................................. 53
Training .............................................................................. 53
Testing .............................................................................. 54
Data acquisition and analysis ......................................................... 54
3.10 Statistical Analysis ............................................................... 55

Results ................................................................. 56
4.1 Maintenance of Animals’ Rectal Temperature ......................... 56
4.2 Hypothermia Did Not Affect the Systemic Concentrations of Soluble
Adhesion Molecules 24 hours Following GCI .............................. 56
4.3 Hypothermia Blocks Inflammatory Mediators IL-1β and KC in the
Hippocampus 72 hours Following Cerebral Ischemia ...................... 57
4.4 Mild Hypothermia Reduces the Inflammatory Response 72 hours
Post-ischemia ................................................................. 57
4.5 Hypothermia Improved Survival of Brain Cells 72 hours after Global
Cerebral Ischemia ........................................................... 58
4.6 Hypothermia Therapy Preserved Spatial Memory after Global Cerebral
Ischemia ........................................................................ 59
4.7 Normothermic-ischemic Mice (NT-2VO) Exhibit Lowered Fear
Response 21 days Post-ischemia ......................................... 61

Discussion ........................................................................ 62
5.1 Hypothermia Protects Against Cell Death ................................ 62
5.2 Hypothermia Attenuates Microglial/Macrophage Activation .... 63
5.3 The Systemic Inflammatory Response in the Peripheral Blood .... 66
5.4 The Cerebral Inflammatory Response .................................... 67
5.5 Spatial Memory Assessment .............................................. 68
5.6 Contextual Fear ............................................................. 71
5.7 Relevance to Clinical Trials ............................................... 72
5.8 Limitations ...................................................................... 73
    Bilateral Common Carotid Artery Occlusion Model ................ 73
    Neurodegeneration after global cerebral ischemia ................. 75
    Morris Water Maze ......................................................... 76
    Contextual Fear Conditioning .......................................... 77
5.9 Conclusions .................................................................... 77

Future Directions ............................................................. 79

References ........................................................................ 83
List of Figures

Figure 1. Ischemia/Reperfusion cascade................................................................. 109

Figure 2. Timeline for molecular and behavioral experiments................................. 111

Figure 3. Timeline for Morris water maze experiments. ......................................... 113

Figure 4. Rectal temperature recordings of experimental animals during 10-mins of surgery and 4 hours post-surgery. .................................................. 115

Figure 5. Hypothermia did not significantly affect the systemic concentrations of inflammatory mediators 24 hours following GCI................................. 117

Figure 6. Hypothermia attenuated the concentrations of pro-inflammatory mediators IL-1β and KC in the hippocampus 72 hours following cerebral ischemia .... 119

Figure 7. Hypothermia reduced microglia and macrophage activation 72 hours after global cerebral ischemia (GCI).......................................................... 121

Figure 8. Mild hypothermia reduced microglia/macrophage staining 72 hours after global cerebral ischemia. ................................................................. 123

Figure 9. Hypothermia decreased microglia and macrophage staining in C57Bl/6 mice 72 hours after global cerebral ischemia.................................................. 125

Figure 10. Hypothermic-ischemic (HT-2VO) mice showed fewer FluoroJade B-positive cells 72 hours post-ischemia............................................................... 127

Figure 11. Mild hypothermia reduced neurodegeneration in the hippocampus CA-1 and CA-2 regions 72 hours following global cerebral ischemia ....................................................... 129

Figure 12. Hypothermia improves spatial memory 7 days following global cerebral ischemia .......................................................... 131

Figure 13. Hypothermia improves spatial memory 21 days following global cerebral ischemia .......................................................... 133

Figure 14. Hypothermia preserves contextual fear response 21 days post-ischemia .............................................................................................................. 135
Chapter 1

Introduction

1.1 Cardiac Arrest

Cardiac arrest is the third leading cause of death in industrialized countries, with an incidence rate of 2-4/1000 and mortality rate of 30% (Lo et al., 2003). It is also the leading cause of permanent disability in adults and children worldwide (Donnan et al., 2008). The etiology of cardiac arrest is different in pediatric patients compared to adults. In children, cardiac arrest is most commonly caused by cardiorespiratory failure or asphyxia. The most common cause of cardiac arrest in adults is ventricular fibrillation or ventricular tachycardia secondary to coronary artery disease. In both children and adults, the effects of cardiac arrest can be devastating. Even with effective resuscitation from cardiac arrest, with more than 60% of pediatric patients having return of circulation, the 1-year survival rate of children after cardiac arrest is less than 35% (Doherty et al., 2009).

Cardiac arrest with widespread cerebral ischemia often leads to severe neurological impairment. The most common cause of morbidity and mortality after resuscitation is hypoxic-ischemic encephalopathy (de Mos et al., 2006), with severity dependent on the cardiac arrest duration and resuscitation effectiveness (Torbey et al., 2004; de Mos et al., 2006). Among survivors, recovery without residual neurological damage is uncommon (Holzer et al., 2002). Previous studies report neurological insult in almost half of all patients after successful resuscitation (Torbey et al., 2004). Survivors often experience substantial and life-long cognitive, sensory and motor disabilities (Northington et al., 2001).
1.2 Cerebral Ischemia/Reperfusion

Cerebral ischemia occurs when the cerebral blood flow (CBF) is insufficient in meeting the brain’s metabolic need. The brain critically depends on a continuous supply of oxygen and glucose, more so than any other organ. While the brain represents only 2% of total body weight, it receives 15% of the total cardiac output. This high oxygen and energy demand is largely due to the necessity for active maintenance of ion gradients (i.e., Na\(^+\)/K\(^+\) ATPase) in excitable neurons. Neuronal discharge and release of neurotransmitters and neuropeptides all require exceptionally large amounts of energy (Atwell et al., 2001). Thus, due to its high-energy demand, coupled with its limited capacity to store energy, the brain is uniquely sensitive to reductions in blood flow. Cerebral ischemia can be further categorized as either focal or global depending on the extent of blood flow reduction.

Focal cerebral ischemia (FCI), commonly referred to as stroke, is the result of a highly localized reduction in CBF to a discrete area of the brain. It is most often caused by a thrombosis or embolism. In contrast, global cerebral ischemia (GCI) occurs when there is a widespread reduction in blood flow to all or most parts of the brain. Global ischemia and reperfusion is relevant to cardiac arrest, hypoxic-ischemic encephalopathy of the newborn (birth asphyxia), and occurs during severe intracranial hypertension caused by trauma or cerebral edema from infectious and metabolic causes.

The most successful treatment strategy for rescuing ischemic tissue and improving functional outcome after cardiac arrest is reperfusion, which is defined as the state of reflow following transient reduction of blood flow. While prompt reperfusion of cerebral ischemic brain tissue is critical for restoring normal function, and the ultimate survival of
the tissue, it may also exacerbate cerebral injury and thus represents a treatment paradox (Safar et al., 2002). In general, the reperfusion injury is likely caused by extended reactions from the primary insult, and often deteriorates the brain metabolism by increasing oxidative stress damage. Particular roles for increase in extracellular glutamate, excessive activation of glutamate receptors (excitotoxicity), increase in cytosolic calcium (Ca\(^{2+}\)), and the generation of free radicals are emphasized, and further described in section 1.4.

### 1.3 Primary Ischemic Brain Injury

Cerebral ischemia and reperfusion initiates a complex cascade of pathological events, encompassing many different pathways that ultimately leads to irreversible tissue injury, i.e., infarction (Lo et al., 2003). The acute injury phase begins within minutes of the ischemic event and may continue for days to weeks following the onset of ischemia (Block, 1999), during which time the decrease in blood flow disrupts ionic homeostasis. The majority of the injury may not occur immediately following insult but rather develop gradually over many hours and days (Back et al., 2004). The extent and temporal evolution of ischemic injury are affected by many factors, including the adequacy of collateral blood flow, temperature, glucose levels, and other metabolic factors. The variability of these factors determines the therapeutic window of opportunity, whose optimization may minimize the size of the ultimate infarction among individual patients (Martini and Kent, 2007; Bang et al., 2008).

The cellular changes caused by a reduction in blood flow (i.e., the primary injury) include a reduction in oxygen delivery, a switch to anaerobic glycolysis, a progressive
fall in high-energy phosphate compounds [i.e., adenosine triphosphate (ATP) and phosphocreatine], intracellular acidosis, and intracellular accumulation of sodium and calcium.

1.4 Secondary Ischemic (Reperfusion) Brain Injury

Secondary consequences of these alterations include free radical formation, an increase in extracellular glutamate, intracellular calcium accumulation, and nitric oxide production with eventual mitochondrial cell death. Cells swell as water shifts to the intracellular space due to osmotic gradients. The resulting vasogenic edema can influence reperfusion negatively and cause intracranial pressure, vascular compression and herniation (Barone et al., 1999). Furthermore, the generation of reactive oxygen species (ROS), especially if reperfusion takes place, can damage membranes, mitochondria and DNA, leading to the misfolding of proteins and enzyme dysfunctions.

Secondary brain injury is a significant cause of impairment and death and is an important area of research. Not surprisingly, it is the focus of many therapeutic interventions that aim to lessen or prevent its pathophysiological effects leading to cell death. An increased understanding of the mechanism of cerebral ischemia/reperfusion injury may allow for the optimization of current therapies, or the emergence of new treatments that could reduce morbidity and mortality rates. Indeed much of the current research on brain ischemia concentrates on the ischemia/reperfusion injury after focal cerebral ischemia. There is not much known about the mechanisms of secondary brain injury after global cerebral ischemia, which is the focus of this thesis.
1.5 Excitotoxicity, Calcium Accumulation and Oxidative Stress

Disruption of the central nervous system homeostasis occurs rapidly following the onset of ischemia. Within 4 minutes of complete cessation of blood flow, the ATP supply is depleted (Krause et al., 1988), which then causes the failure of the ATP-dependent Na\(^{+}\)-K\(^{+}\) pumps. Transcellular ion pump failure leads to the intracellular accumulation of Na\(^{+}\), Ca\(^{2+}\), and water (cytotoxic edema) followed by membrane depolarization and an excessive release of excitatory neurotransmitters, especially glutamate. Glutamate is the brain’s predominant excitatory amino acid neurotransmitter, and has 3 major types of ionotropic receptors, NMDA, AMPA, and KA, as well as a group of G-protein-linked metabotropic receptors. Under normal conditions, glutamate stimulates its receptors only transiently. These receptors, in turn, work cooperatively to stabilize synapses. Under ischemic conditions, the extracellular glutamate concentrations increase manifold (Jantzie et al., 2010). Thus glutamate activity is sustained, causing prolonged depolarization of presynaptic vesicles (Krause et al., 1988), which is further exacerbated by the inhibition of neurotransmitter reuptake and energy failure (Siesjo and Siesjo, 1996). The increase in extracellular glutamate concentration and activation of glutamate receptors trigger an excitotoxic environment in the cell.

Glutamate can also bind to its receptors on the post-synaptic membrane, thereby creating an increase in cytosolic Ca\(^{2+}\) by influx via open NMDA and Ca\(^{2+}\) permeable AMPA receptor channels and other voltage-dependent Ca\(^{2+}\) channels, and the release of calcium from intracellular stores. The deleterious effects of increased cytosolic calcium include a) the activation of neuronal nitric oxide synthase (nNOS) to form nitric oxide, b) generation of free radicals, disruption of mitochondrial function, c) degradation of
cellular lipids by activation of phospholipases and proteases, and d) deterioration of DNA by activation of nucleases (Ankarcrona et al., 1995; Gilland et al., 1998). It is known that mitochondrial dysfunction, specifically the permeabilization of the mitochondrial outer membrane elicits the release of cytochrome C, activation of caspases 9 and 3, and apoptosis-inducing factor (AIF), which leads to apoptosis (Hagberg et al., 2009). The combined effects of cellular energy failure, acidosis, glutamate release, intracellular Ca\(^{2+}\) accumulation, lipid peroxidation, and nitric oxide neurotoxicity serve to disrupt essential cellular functions.

There is also evidence that excitotoxicity and the increased intracellular Ca\(^{2+}\) concentration are associated with the generation of reactive oxygen species (ROS) and free radicals (Kristian and Siesjo, 1998). ROS and reactive nitrogen intermediates (i.e., nitric oxide) produced by phagocytes are important for defense against the inflammatory response. Although free radicals are usually a component of the immune reaction and defense, toxic effects of these substances also cause cellular injury and death in neurons and other cells, and therefore may promote complications and long-term deficits. Generally released by granulocytes, microglia and endothelial cells, oxygen and nitrogen radicals promote the formation of the relatively stable peroxynitrite, which has been considered a central mediator of cellular damage. These molecules mediate cell death by membrane peroxidation, breakdown of protein structure, DNA damage, and subsequent activation of poly(ADP)-ribose polymerase (PARP) leading to energy depletion and cell death.
1.6 Neuro-inflammation

Although for many years, the CNS was considered an immune-privileged organ, it is now well accepted that the immune and the nervous system communicate extensively. Moreover, mounting data suggest that like in other peripheral organs, the inflammatory cells of brain participate in tissue remodeling after injury. Brain inflammation induced by cerebral ischemia and reperfusion was initially thought to be primarily a repair process in response to the primary injury. Inflammatory mediators, such as cytokines and chemokines, are not directly neurotoxic to neurons in a healthy, uninjured brain (Piani et al., 1992). An inflammatory environment after brain injury has been shown to promote plasticity and functional recovery (Johansson and Grabowski, 1994; Kawamata et al., 1997). However in contrast to this beneficial role, there is growing evidence indicating that inflammatory pathways can contribute to cell death following ischemia/reperfusion, i.e., secondary brain injury (Becker et al., 1998). This hypothesis is supported by multiple experiments demonstrating neuroprotection through the inhibition of inflammatory mediators following ischemia/reperfusion injury (Betz et al., 1995; Relton et al., 1996; Nawashiro et al., 1997; Barone et al., 1998). Current evidence suggests that the acute inflammatory response is involved in furthering neuronal damage while the later delayed inflammation may be responsible for facilitating remodeling and neurogenesis (Barone and Feuerstein, 1999). Because of the complexity of the inflammatory response following ischemia, spatial and temporal parameters must be taken into consideration when evaluating the neuroprotective and neuropathogenic effects of neuro-inflammation.

Brain inflammation following cerebral ischemia is believed to develop as a consequence of microglia and astrocyte activation, and the mobilization and infiltration
of peripheral inflammatory cells into the brain (Feuerstein et al., 1994). The development of post-ischemic brain inflammation is coordinated by activation, expression and secretion of numerous pro-inflammatory genes/mediators from the brain parenchyma and vascular cells, including cytokines, leukotrienes, and adhesion molecules (Giulian et al., 1993; del Zoppo et al., 1994). These post-ischemic neuroinflammatory changes then disrupt the blood-brain barrier (BBB), increase neuronal vulnerability, and further stimulate gliosis. Consequently, more cell damage and death occur. Therefore, therapeutic targeting of the neuroinflammatory pathways in post-ischemia has become an important area of research in translational medicine.

1.7 Inflammatory Cells

1.7.1 Leukocytes

The recruitment of leukocytes from the circulation into the extravascular space is a central feature of inflammation. The leukocyte population primarily consists of neutrophils, monocytes and lymphocytes, each of which can contribute to inflammation following ischemia. Intra-parenchyma infiltration of neutrophils can be seen as early as 6-24 h after the restoration of blood flow, while monocytes and macrophages invade 2-3 days later (Kochanek and Hallenbaek, 1992).

Using intravital microscopy our laboratory has determined that leukocytes, primarily neutrophils, are recruited to sites of injury and interact with the endothelium within minutes of the restoration of blood flow following global cerebral ischemia in our mouse model (unpublished data Hutchison JS et al.). The infiltration of leukocytes into the brain tissue occurs early on following transient ischemia as compared to after
permanent cerebral ischemia (Zhang et al., 1994). Moreover, a finding by Lee and colleagues (1999) suggests that both the number of infiltrating leukocytes and the peripheral leukocyte count are closely related to delayed neuronal damage in global cerebral ischemia. Together, these results support a direct role of leukocytes in the pathogenesis of cerebral ischemic injury.

In general, neutrophils are the first exogenous cells to enter ischemic tissue, and are known to release injurious mediators including cytokines, and lipid-derived mediators (Tomita and Fukuuchi, 1996; Siesjo and Katsura, 1992; Hallenbeck et al., 1996). Other neutrophil-mediated effects following cerebral ischemia include: disruption of the BBB, secretion of vasogenic mediators (prostaglandin H2, thromboxane A2), proteases (elastase, collagenase), generation of ROS (O2 and H2O2) via NADPH oxidase (Ellis et al., 1989), and production of arachidonic acid (Ellis et al., 1989; Akopov et al., 1996; Hallenbeck and Dutka, 1990). Furthermore, it has been suggested that neutrophils hinder reperfusion and exacerbate post-ischemic hypoperfusion (Ames et al., 1968; del Zoppo et al., 1991). Neutrophil activation can alter cerebral artery vasoreactivity (Pantoni et al., 1998). Moreover, due to their adherence onto the vessel wall, they create a secondary obstruction in the cerebral microvasculature, called the no-reflow phenomenon (Huang et al., 2006).

Conflicting evidence exists in the literature regarding the roles of the other leukocyte subtypes, such as monocytes and lymphocytes, with regards to ischemia induced neuro-inflammation (Becker et al., 2001; Nadareishvili et al., 2004). Monocytes migrate into the ischemic tissue after neutrophils and eventually adhere to the vessel walls to replace the neutrophils (Sughrue et al., 2004). When activated, monocytes
transform into blood-borne macrophages, which serve to remove necrotic cell debris, and to secrete pro-inflammatory cytokines.

### 1.7.2 Microglia/Macrophages

Microglia represent 5-50% of the total glial population, and are key modulators of the immune response in the brain (Zhang et al., 1997; Schubert et al., 2000). Quiescent resting microglia have small cell bodies and extensive branches projecting out of the soma. Their activation is characterized by morphologic and phenotypic changes including the expression of specific surface antigens. Once activated these cells are indistinguishable from circulating macrophages (Graeber et al., 1988). Activated microglia eliminate foreign organisms using phagocytosis. After cerebral ischemia, this immune response can often be directed towards otherwise healthy cells, and can lead to further neuronal damage.

How microglia activation occurs after cerebral ischemia is still unclear. A possible mechanism is the rupture of necrotic neurons leading to the release of their contents, which are later recognized as foreign by the microglia (Kaushal et al., 2008). It has also been suggested that ROS play a key role in the microglial activation and proliferation (Wang et al., 2007). Areas of the brain, which are vulnerable to ischemia, including the hippocampal CA-1 region, show activated microglia before evidence of delayed neuronal death (Morioka et al., 1991; Gehrmann et al., 1992). The activation of microglia following ischemia has both neuroprotective and neuropathogenic properties (Wood et al., 1995; Watanabe et al., 2000; Zhang et al., 2005). Activated microglia release a variety of inflammatory mediators and regulators that modulate the activity of
other cells including neurons. Stimulation of microglia via toll-like receptor 9 (TLR9) and subsequent release of nitric oxide and tumor necrosis factor-α (TNF-α) caused neuronal injury in co-cultures with neurons (Iliev et al., 2004). Likewise, *in vitro* models have shown microglial activation to be associated with damage to the blood-brain barrier (BBB) (Yenari et al., 2006). Also, minocycline, an inhibitor of microglial activation, protected the brain following focal (Yrjanheikki et al., 1999) and global ischemia (Yrjanheikki et al., 1998). Furthermore, studies have shown that brain macrophages are involved in propagating inflammation and neurodegeneration rather than circulating macrophages since the depletion of peripheral blood macrophages did not affect injury following cerebral ischemia/reperfusion (Schroeter et al., 1997). These findings strongly suggest that microglia play a detrimental role in ischemic injury. In contrast, other studies have reported the beneficial effects of microglia. For example, pretreatment of cultured neuronal cells with microglial culture medium conveyed an early protection against excitotoxicity (Watanabe et al., 2000).

### 1.7.3 Astrocytes

Astrocytes are the most numerous of the glial cells. They are essential for the function and maintenance of the central nervous system, as they supply energy to neurons, produce precursors to neurotransmitters, form an anti-oxidative defense and secrete neuroprotective and neurogenic factors. Furthermore, their end-feet along with endothelial cells form the BBB and contribute to the maintenance of ionic homeostasis (Petty and Lo, 2002; Nowicka et al., 2008). In response to immunological processes or brain injuries, these cells proliferate and differentiate (astrogliosis), exhibit increased
expression of glial fibrillary acidic protein (GFAP), and form glial scars (Block and Hong, 2005). The glial scars hinder axonal regrowth and reinnervation, but also serve to isolate the damaged tissue from viable tissue (Nowicka et al., 2008). Astrocytic activation also triggers the production of inflammatory mediators and cytotoxic molecules such as ROS, NOS, proteases, etc (Nowicka et al., 2008). High levels of chemokines and their receptors are expressed on astrocytic membranes (Bacon et al., 2000). Taken together, these findings suggest that astrocytic activation represents a potentially damaging mechanism following cerebral ischemia.

1.8 Leukocyte-Endothelial Interactions

Leukocyte-endothelial interactions are a fundamental component of the inflammatory response to ischemia. Endothelial cells become activated during reperfusion following the initial cerebral ischemic event. This is followed by the recruitment of leukocytes to the area of injury (Ishikawa et al., 2003; Gavins et al., 2007). The endothelium is essential for the trafficking of leukocyte by regulating leukocyte chemoattraction, adhesion and transmigration from the peripheral blood (Connolly et al., 1996). Rolling and adhesion of leukocytes on the endothelium is mediated by cellular adhesion molecules (Granger and Kubes, 1994), of which there are three main subgroups: (i) selectins, (ii) immunoglobulin (Ig) superfamily and (iii) integrins (DeGraba et al., 1998; Emsley and Tyrrell, 2002).

1.8.1 Selectins

Selectins are membrane-bound glycoproteins that are necessary for the initial
capture and rolling of leukocytes on the vessel wall during inflammation (McEver et al., 1997). There are three selectins which are characterized by a common sequence and structural features, L- (leukocyte), E- (endothelial), and P- (platelet) selectin. Selectins are generally named for the cell type that expresses them on their surface, however P-selectin is expressed on both endothelial cells and on platelets. Selectins interact with carbohydrate residues [sialyl-Lewis\textsuperscript{X} (sLeX)] on neutrophils and monocytes allowing binding to, and rolling over, the endothelium during the early stage of activation (McEver et al., 1997).

L-selectin can recognize ligands expressed on cytokine-activated endothelial cells as well as those found on other leukocytes (Abbassi et al., 1991; Carlos and Harlan, 1994). L-Selectin is involved in the capture of unstimulated leukocytes from the bloodstream (Bargatze et al., 1994). It is rapidly shed from the surface of leukocytes upon activation, and after chemokine stimulation (Jutila et al., 1989; Kishimoto et al., 1989). Although L-selectins mediate the initial rolling of leukocytes, they do not appear to play a critical role in the development of ischemic injury. Antibody inhibition of L-selectin did not lessen the extent of leukocyte adhesion and transmigration in the areas of damage in a rabbit model of focal cerebral ischemia (Yenari et al., 2001). However, shed L-selectin may be a relevant factor in the recruitment of neutrophils from the bone marrow (van Eeden et al., 1997), and in the regulation of leukocyte rolling velocities (Huppa and Ploegh, 1997).

P- and E-selectin have been more extensively examined than L-selectin and appear to play a critical role in inflammatory damage. Under endogenous conditions, E-selectin is minimally expressed (Simon and Green, 2005). Yet upon activation by cytokines,
thrombin or histamine stimulation, it is rapidly up-regulated (Jones et al., 1993; Lawrence and Springer, 1993). P-selectin is preloaded in the cell and rapidly shuttled to the cell surface in response to ischemia (Simon and Green, 2005). The slow rolling of leukocytes has traditionally been attributed exclusively to E-selectin (Simon et al., 2000; Green et al., 2004). There is evidence in a shear stress cell culture model of leukocyte endothelial interactions that P-selectin mediated slow rolling (DiVietro et al., 2001). The expression of endothelial P- and E-Selectin is increased following cerebral ischemia, and blocking their function has neuroprotective effects in certain stroke models (Huang et al., 2000; Mocco et al., 2002). However, attenuating neutrophil rolling by pre-injury treatment with anti-P-selectin antibody resulted in an increased mortality rate in a gerbil model of global ischemia (Lehmberg et al., 2006).

1.8.2 Immunoglobulin (Ig) Superfamily

The immunoglobulin superfamily class of cell adhesion molecules mediates the adhesion of leukocytes to endothelial cells. Theses molecules are expressed on endothelial cells and interact with the integrin class of adhesion molecules. This interaction is required for the arrest and firm adhesion of leukocytes on the vessel wall (Simon and Green, 2005). In terms of leukocyte-endothelial interactions, the Ig superfamily consists of five molecules: intercellular adhesion molecule-1 and -2 (ICAM-1 or CD54, ICAM-2 or CD102), vascular cell adhesion molecule-1 (VCAM-1 or CD106), platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). After cerebral ischemia, ICAM-1 and -2, VCAM-1 and PECAM-1 have been shown to contribute to the inflammatory
response.

Intercellular adhesion molecule-1 is constitutively expressed at low levels on the cell membrane of endothelial cells. Upon cytokine stimulation, its expression is increased. Under hypoxic conditions the endothelial cells release inflammatory mediators, such as interleukin-1 (IL-1), which signal for the up-regulation of ICAM-1 (Connolly et al., 1996). Transient cerebral ischemia induces expression of ICAM-1 as a homodimer on the membrane of inflamed cerebral endothelial cells, (Staunton et al., 1988) reaching peak levels between 12 and 24 h post-injury (Wang and Feuerstein, 1995). Studies have shown that ICAM-1-deficient mice have smaller infarcts compared to wild-type mice following focal cerebral ischemia (Connolly et al., 1996; Kitagawa et al., 1998). These findings help emphasize the critical role of leukocyte adhesion in furthering inflammatory injury following cerebral ischemia.

Vascular cell adhesion molecule-1 is up-regulated following stimulation by cytokines [i.e., IL-1 and tumor necrosis factor-α (TNFα)] (Yonekawa and Harlan, 2005). However, VCAM-1’s role in inflammatory injury is not completely understood. Inhibition of VCAM-1 expression was neuroprotective in a model of transient global cerebral ischemia (Zhang and Wei, 2003), while inhibition of VCAM-1 was not neuroprotective in a focal cerebral ischemia model (Justicia et al., 2006).

Lastly, increased plasma and cerebral spinal fluid (CSF) concentrations of soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) were measureable in patients shortly following cerebral ischemic events and these concentrations correlated with the severity of injury (Simundic et al., 2004; Ehrensperger et al., 2005).
1.8.3 Integrins

The integrins are a family of adhesion molecules that respond to a variety of inflammatory mediators, including cytokines, chemokines and chemoattractants (Smith, 1993). The binding of integrins to endothelial cell adhesion molecules mediates leukocyte adhesion to the endothelium, from the slow rolling of leukocytes, to their firm arrest (Hynes, 1992; Taylor et al., 1996). Integrins are transmembrane surface proteins that consist of a common β-subunit dimerized with a variable α-subunit (Albelda, 1991). The β2-integrin subunit, CD18, can dimerize with the αL (CD11a) and αM (CD11b) subunits as well as other CD11 α-subunits. The CD11a/CD18 integrin is referred to as leukocyte function associated antigen-1 (LFA-1), and CD11b/CD18 is referred as macrophage-1 antigen (Mac-1). Both LFA-1 and Mac-1 are expressed on neutrophils, monocytes and lymphocytes. Their primary ligand is ICAM-1, but these molecules can bind to a wide variety of molecules including myeloperoxidase, elastase, heparin, complement fragment C3bi, fibronectin, and albumin (Ross and Lambris, 1982; Diamond and Springer, 1994; Diamond et al., 1995; Cai and Wright, 1996; Johansson et al., 1997). Leukocyte factor antigen-1 bonds are associated with slow rolling, while Mac-1 are believed to maintain stable adhesive bonds (Hentzen et al., 2000). Hence, there is an overlap in function between the selectins and integrins during the capture of leukocytes in the microvasculature to their firm arrest.

Beta-2-integrins are constitutively expressed on leukocytes in an unactivated, low avidity state. Through selectin-mediated rolling or by stimulation with chemotactic agents, integrins’ affinity and avidity for ICAM-1 is induced within seconds with a conformation change to the active state (Simon and Green, 2005). Various inflammatory
mediators are known to induce a high avidity and affinity state of β2-integrins for their ligand. These include chemokines, interleukin-8 (IL-8) (Kuijpers et al., 1992), monocyte chemoattractant protein-1 (MCP-1) (Kumar et al., 1997), and macrophage inflammatory protein-1 (MIP-1) (Tessier et al., 1997) and the growth factor cytokine, granulocyte/macrophage colony-stimulating factor (GM-CSF) (Huber et al., 1991).

1.9 Inflammatory Mediators

1.9.1 Cytokines

Cytokines are inflammatory mediators produced by leukocytes, macrophages, endothelial cells and the resident cells within the CNS, including glial cells and neurons, in response to a diverse range of injuries (Liu et al., 1994; Sairanen et al., 2001). They are known to regulate cell activation, proliferation, and differentiation (Sriram et al., 2007; Zhao and Schwartz, 1998). Generally, cytokines and their receptors are nearly undetectable under normal conditions. However following cerebral ischemia, pro- and anti-inflammatory cytokines are quickly and highly up-regulated in the brain (Huang et al., 2006; Wang et al., 2007). Cytokines known to be involved with ischemia-induced inflammation are interleukin-1 (IL-1), tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6) and interleukin-10 (IL-10) (Allan and Rothwell, 2001) as well as the growth factor cytokines, granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Schneeloch et al., 2004; Matchett et al., 2007). It is important to note that cytokines cannot unequivocally be categorized as either pro- or anti-inflammatory, and may exert neurotoxic as well as neuroprotective effects (Vitkovic et al., 2001). Hence, their ultimate effect on the brain depends on the physiological and
biochemical context (Vitkovic et al., 2001).

Interleukin-1 is a major mediator of the inflammatory response following ischemia, with potentially neurotoxic effects. There are two isoforms, IL-1α and IL-1β. It is regulated by an endogenous inhibitor, IL-1 receptor antagonist (IL-1ra) (Arend et al., 1991). There are two types of IL-1 receptors that are expressed by both neuronal and glial cells throughout the CNS (Touzani et al., 1999). IL-1 and IL-1 receptor type I play an important role in neuronal death following cerebral ischemia (Basu et al., 2005). Interleukin-1 binding to IL-1 receptor Type I leads to a pro-inflammatory response, while IL-1 binding to IL-1 receptor Type II does not propagate a signal. Because IL-1 receptor Type II competes for IL-1 with IL-1 receptor Type I, the over-expression of this receptor can reduce IL-1 pro-inflammatory signaling (Kuno and Matsushima, 1994; Basu et al., 2005). Of the two isoforms, IL-1β has been implicated in the inflammatory response to cerebral ischemia (Davies et al., 1999). Interleukin-1β expression is biphasic following both global and focal cerebral ischemia, with significant peaks in the brain within hours of injury and at approximately 24 hours post-ischemia (Haqqani et al., 2005). Exogenous IL-1β has been shown to exacerbate ischemic injury (Yamasaki et al., 1995), whereas IL-1 inhibition decreases infarct size (Yang et al., 1997; Mulcahy et al., 2003) in rodent models of focal ischemia. The exact mechanism of IL-1β mediated cellular injury is unknown; however, it has been shown to induce neurotoxic molecules, such as phospholipase A2, cyclooxygenase, nitric oxide and presumably other unidentified mediators (Rothwell and Luheshi, 2000).

Another major player in the inflammatory response is tumor necrosis factor-α (TNFα), which is expressed in both neurons (Liu et al., 1994) and glial cells (Uno et al.,
Its expression pattern is similar to that of IL-1β following ischemia, in that it is biphasic with an increase in the brain within the first few hours of reperfusion (Liu et al., 1994) and with a second peak at between 24 and 36 hours post-injury (Murakami et al., 2005). In contrast to IL-1 there are conflicting findings on how TNFα affects brain cell survival following ischemia. The inhibition of TNFα was found to reduce ischemic brain injury (Yang et al., 1998). However, TNFα-deficient mice exhibit larger infarcts, possibly due to a lack of TNFα-mediated tolerance to ischemic damage (Ginis et al., 2002). Still other studies have shown that TNFα does not have any effect, either positive or negative, on the outcome following ischemia (Murakami et al., 2005). These differing outcomes with regards to the effects of TNFα inhibition in different models of ischemia may be attributable to differential TNFα receptor expression (Hallenbeck et al., 2002).

Interleukin-6 is generally considered to be a pro-inflammatory cytokine. It is detected 4 hours after ischemic onset, with peak concentrations after 24 hours and remains detectable for up to 14 days (Amantea et al., 2009; Nilupul et al., 2006; Kim, 1996). Secreted by activated microglia, astrocytes, neurons, and invading cells of the immune system, its role in cerebral ischemia-induced inflammation is controversial. Similar to TNFα, IL-6 has been known to be both harmful (Smith et al., 2004), and beneficial (Herrmann et al., 2003) following ischemic insults. It is known to attract T-lymphocytes to the brain and generally contribute to an exacerbation of the inflammatory response. In support of this hypothesis, transgenic mice over-expressing IL-6 are more vulnerable to neurodegeneration (Campbell et al., 1993). However, IL-6 can also up-regulate IL-1ra, and IL-6-deficient mice do not show improved outcome after ischemia (Huang et al., 2006). Furthermore, it has been shown to inhibit neuronal damage caused
by NMDA infusion (Toulmond et al., 1992) and following focal cerebral ischemia (Loddick et al., 1998). In clinical studies, serum levels of IL-6 were the strongest single predictor of in-hospital mortality in patients that had suffered an acute ischemic stroke (Rallidis et al., 2006).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine released by activated microglia and astrocytes in response to cerebral ischemia. Generally considered to be neuroprotective, IL-10 suppresses cytokine release as well as the expression and activity of the cytokine receptors (Strle et al., 2001), leading to a reduction in inflammation. Specifically, it acts by inhibiting the function of IL-1 and TNFα. Exogenous IL-10 injection following global cerebral ischemia increased the survival of hippocampal CA-1 neurons in rodents (Dietrich et al., 1999).

The growth factor cytokines, G-CSF and GM-CSF, play an important role during inflammation. In addition to stimulating leukocyte trafficking into tissues, these factors promote leukocyte differentiation in the bone marrow. However, G-CSF pre-treatment has been shown to be neuroprotective after global cerebral ischemia (Matchett et al., 2007). The expression of G-CSF in the brain activates anti-apoptotic pathways, promotes neurogenesis and decreases infarct volume following experimental stroke (Gibson et al., 2005; Schneider et al., 2005). Both growth factor cytokines share similar expression patterns as well as neuroprotective properties (Schabitz et al., 2008).

### 1.9.2 Chemokines

Chemokines are regulatory proteins that are involved in cell communication and recruitment during inflammation. They are structurally and functionally related to
cytokines, and are known for their chemotactic activity with leukocytes (Yamagami et al., 1999; Bacon et al, 2000; Chen et al., 2003). These members of the G-protein coupled receptor superfamily are categorized by the position of their cystine residues (Bajetto et al., 2001). Constitutively, chemokines and their receptors are expressed in very low concentrations (Bacon and Harrison, 2000). They are up-regulated following ischemia and signal leukocytes to traffic on the inflamed cerebral endothelium (Chen et al., 2003). Chemokines that have been associated with ischemia/reperfusion injury are mouse chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), and interleukin-8 (IL-8), to which keratinocyte-derived chemokine (KC) is the mouse homologue.

Inhibition of chemokines during ischemic injury is associated with improved outcomes (Garau et al., 2005), while an over-expression of chemokines exacerbates injury through increased recruitment of inflammatory cells (Chen et al., 2003). A pharmacological inhibitor of IL-8, Repartaxin, is neuroprotective in a rodent model of transient brain ischemia and its beneficial effects have been attributed to the inhibition of neutrophil recruitment and decreased secondary injury (Garau et al., 2005). Monocyte chemoattractant protein-1 and MIP-1α’s deleterious actions following cerebral ischemic insult have also been attributed to their role in neutrophil recruitment (Zhang and Chopp, 1998). Following global cerebral ischemia, there is an increase in the expression of MCP-1 in the astrocytes of the hippocampus, a selectively vulnerable area of the brain that exhibits delayed neuronal cell death (Sakurai-Yamashita et al., 2006).
1.9.3 Other Molecular Mediators Associated with Neuroinflammation

Other mediators that may play important roles in furthering secondary injury following cerebral ischemia/reperfusion, and that we investigated in my thesis, include matrix metalloproteinase-9 (MMP-9) and plasminogen activator inhibitor-1 (PAI-1).

Matrix metalloproteinase (MMP) is a family of proteins that breakdown extracellular components and are involved in matrix remodeling. Matrix metalloproteinase-2 and MMP-9 have been implicated in cerebral ischemia, specifically with their roles in inflammation, BBB dysfunction and cellular damage (Meli et al., 2003). Matrix metalloproteinase-9 is considered the chief MMP associated with neuroinflammation. It is not detectable in brain tissue under normal conditions, but elevated levels were found in brain tissue and in serum from patients with acute ischemic stroke and in animal models of stroke at 12 h after permanent middle cerebral artery occlusion (MCAO) (Park et al., 2009). Early (within 24 h) MMP-9 inhibition reduced infarct size at day 14, but the benefit was lost when the treatment was delayed until 72 hours, and stroke pathology was exacerbated when administration was delayed until day 7 (Zhao, 2006). Taken together, these findings suggest that MMP inhibition could have a beneficial effect on the outcome of ischemic stroke, but the effect will depend on the treatment schedule in relation to the stage of the injury.

Plasminogen activator inhibitor-1 acts with tissue plasminogen activator (t-Pa) to maintain a balance between the formation and breakdown of fibrin during blood clotting. It is associated with neurodegeneration in models of ischemia/reperfusion injury but not in permanent ischemia (Nagai et al., 2003). Excess PAI-1 is pro-thrombotic and can exacerbate injury following transient cerebral ischemia (Jansson et al., 1991; Held et al.,
Over-expression of PAI-1 in a mouse model of transient focal cerebral ischemia resulted in larger infarct volumes (Nagai et al., 2003).

1.10 Mechanisms of Cell Death after Global Cerebral Ischemia

The process of programmed cell death, apoptosis, acts as a defense mechanism to remove damaged, unwanted or potentially harmful cells. This active form of cell death is present in all higher eukaryotic cells and is a part of normal cell homeostasis. At the molecular level, apoptosis is orchestrated by cysteine proteases called caspases (MacManus and Buchan, 2000). It requires active protein synthesis as well as intact mitochondria (Newmeyer et al., 1994; Kroemer et al., 1995; Zamzami et al., 1996; Martin et al., 1998). Apoptosis can occur via the intrinsic and extrinsic pathways, the former of which is triggered by mitochondrial stress caused by factors such as DNA damage and heat shock (Adrain et al., 2002), and the latter of which is triggered by the activation of transmembrane death receptors such as the binding of Fas ligand receptor (Cispo et al., 1998).

Apoptosis can be contrasted with necrosis, which is a passive process that occurs after massive exogenous insult causing depletion of ATP. Other key distinctions can be made between apoptosis and necrosis. First, apoptosis is a highly controlled and organized cellular dismantling while necrotic death is characterized by a rapid deregulation and dysfunction of normal cellular physiology (Martin et al., 1998). Second, necrosis is characterized by cellular swelling, membrane disruption, random DNA breakage, and inflammation. The release of cellular contents into the interstitium is a potent inflammatory stimulus and leads to extensive damage to surrounding cells (Wyllie
et al., 1980; Martin et al., 1998). In contrast, apoptosis involves nuclear and cytoplasmic condensation, DNA fragmentation, blebbing of the plasma membrane into membrane bound vesicles, and ultimately phagocytosis of these vesicles without induction of an inflammatory response (Hale et al., 1996).

While apoptosis plays a critical role in healthy tissues, it can also lead to further damage after stroke and other brain injuries (Linnik et al., 1993; Steller, 1995). Intracellular Ca$^{2+}$ concentrations (Coyle and Puttfarcken, 1993), excess glutamate (Coyle and Puttfarcken, 1993; Thompson et al., 1995), and free radical release (Kiyota et al., 1993) have all been shown to initiate apoptosis and are associated with the progression of ischemia/reperfusion injury.

After transient global cerebral ischemia, cell death takes place in two major phases: a fast rapid phase of necrotic cell death, and a second delayed phase that lasts from hours to days and produces cell death with more characteristics of apoptosis (Balduini et al., 2004). Indeed after global ischemia, apoptosis is reported to be responsible for a significant proportion of the final cell loss in the brain (Russell et al., 2006; Zhu et al., 2007). It occurs in selectively vulnerable areas of the brain including the hippocampus, the basal ganglia and the watershed areas of the cerebral cortex following transient global cerebral ischemia in the rat (MacManus et al., 1993). Cell death peaks between 3 and 4 days post-ischemia (Kihara et al., 1994; Nitatori et al., 1995). In experimental cerebral ischemia, the severity of damage depends on the severity and duration of the reduction of CBF (Pulsinelli et al., 1982). Moreover, the intrinsic vulnerability of specific cell types and systems in the brain may be more important in determining the final pattern of damage and functional disabilities. For the purposes of
measuring cell death in this thesis, we used FluoroJade B staining which is non-specific and likely measures both apoptotic and necrotic cell death. Of the apoptotic cells, FluoroJade B also does not differentiate cells that die via the intrinsic or extrinsic apoptotic pathways.

1.11 Therapeutic Interventions

1.11.1 Acute Interventions

Global cerebral ischemia/reperfusion leads to cell death through multiple molecular pathways. A therapeutic neuroprotective approach that acts via several different important molecular mechanisms would be ideal. A number of therapeutic strategies have been shown to inhibit or reduce injury via the previously discussed mechanisms. For example, genes that are anti-apoptotic (i.e., bcl-20) have been over-expressed in transgenic mice and have provided moderate resistance to cerebral ischemic damage in both focal and global ischemic models (Martinou et al., 1994). The over-expression of free radical scavenging enzymes has reduced cerebral ischemic injury (Nogawa et al., 1997; Sheng et al., 2000; Yamamoto et al., 1997). Use of some NMDA receptor antagonists (MK-801 and remacemide) have only shown some efficacy in reducing ischemic injury, whereas the application of peptides that disrupt the interaction between the NMDAR with the postsynaptic density protein PSD-95 has been reported to block downstream neurotoxic signaling without impeding essential neuronal excitation (Aarts et al., 2002). The administration of Na$^+$ and Ca$^{2+}$ channel blockers (i.e., tetrodotoxin, SB206284) following both focal and global cerebral ischemia has been shown to be effective (Small et al., 1999). Despite the large number of therapeutic interventions that
decrease ischemic brain damage in experimental models, they have not translated well into the clinical setting (Grotta, 1997; The American Nimodipine Study Group, 1992).

1.11.2 Anti-inflammatory Therapies

Manipulation of the expression and release of inflammatory mediators in the brain is being investigated as a therapeutic approach to reduce secondary ischemic brain damage. In principle, anti-inflammatory therapy should be considered as an adjuvant therapy to thrombolytics. Several therapeutic strategies that directly or indirectly target post-ischemic brain inflammation have been developed and tested in animal and human studies. The two most important approaches have been: i) reducing the overall immune response with non-selective immunosuppressants, and ii) targeting a single inflammatory mediator.

Non-selective immunomodulation consists of using drugs such as glucocorticoids, cyclosporine A, or non-steroidal anti-inflammatory drugs (NSAID). Animal studies have shown that cyclosporine A and NSAIDs ameliorate brain damage after global and focal ischemia (Li et al., 2000; Zhang et al., 2000; del Zoppo et al., 1997; Jean et al., 1998).

Targeting immune cells and inflammatory mediators (i.e. leukocyte and endothelial cell adhesion molecules and cytokines) has had variable success. Blocking IL-1β, Il-1ra over-expression, and IL-1β converting enzyme (ICE) have been reported to be neuroprotective following experimental cerebral ischemia (Yamasaki et al., 1994; Yang et al., 1999; Scheele et al., 1998). A neutralizing anti-IL8 antibody and broad-spectrum inhibitor of chemokines (NR58-3.14.3) reduced brain edema and infarct size in experimental MCAO models (Matsumoto et al., 1997; Beech et al., 2001). Systemic
injections of the TNFα inhibitor CNI-1493, as well as the administration of anti-TNFα
antibody, have reduced brain infarct volumes after MCAO (Barone et al., 1997). Although anti-TNFα strategies have not been tested in clinical trials of stroke, TNFα
neutralization has been documented in trials for heart failure, and rheumatoid arthritis
(Camussi and Lupia, 1998). Neutrophil depletion and blocking or deficiency of adhesion
receptors on either the neutrophil (i.e., CD18, Mac-1) or the endothelial cells (ICAM-1)
has also shown benefit in animal models of cerebral ischemia (Chopp et al., 1994; Chopp et al., 1996; Kogure et al., 1996; Soriano et al., 1996; Zhang et al., 1994). The initial
clinical experience with leukocyte adhesion molecule inhibitors has demonstrated safety,
and in some cases efficacy, as an adjuvant therapy for stroke (The Abciximab in Ischemic
Stroke Investigators, 2000). However, in contrast to the protective effect of anti-adhesion
molecules in animal models, recent failure of a murine anti-ICAM antibody in human
stroke illustrates the difficulties in extrapolating therapeutic effects observed in the
laboratory to clinical benefits in patients (DeGraba et al., 1998). An additional difficulty
in these approaches is associated with the redundancy of inflammatory mediators. For
example, blocking a single mechanism of inflammatory injury is likely offset by the
detrimental effects of an alternate pathway, and is unlikely to be effective in the clinical
setting.

1.12 Importance of Hypothermia Therapy

1.12.1 Historical Perspective

Temperature modulation has now been proven to have significant benefits
following brain injury including positive histological and behavioral outcomes (Corbett
and Thornhill, 2000; Colbourne et al., 1997; Ginsberg et al., 1993). Unlike studies using pharmaceutical intervention, the observed histological protection is long lasting (i.e., 60 days to 1 year) if not permanent. Consequently, hypothermia has been examined as a potential therapy for numerous insults including cerebral ischemic injury (Corbett et al., 2000) and traumatic brain injury (Clark et al., 1996).

Hypothermia has been defined as mild (34-36 °C), moderate (28-32 °C), and deep (15-25 °C) (Safar et al., 1996), though considerable divergence from this classification occurs so prudence must be utilized in reviewing the literature. The use of hypothermia to prevent injury during cardiac surgery and GCI dates back more than 60 years (Bigelow et al., 1950). Subsequently hypothermia has been used to treat cerebral ischemia following asphyxia and cardiac arrest (Westin et al., 1959). Despite intensive investigations into the use of hypothermia, the temperatures used early on were too hypothermic (4-32 °C) and led to too many adverse effects such as infection (Biggart and Bohn, 1990), and cardiac arrhythmias (Mouritzen and Andersen, 1966).

In the late 1980s, interest in hypothermia therapy increased again with reports that lowering brain temperature to a milder degree (32-34°C) provided considerable neuroprotection following GCI in the laboratory setting (Busto et al., 1987, Buchan and Pulsinelli, 1990; Coimbra and Cavalheiro, 1990). It is now an established neuroprotectant in the laboratory, showing remarkable and consistent effects across multiple laboratories and models of brain injury (Yenari et al., 2010). Furthermore, the risks of adverse effects (i.e., bleeding, arrhythmia, sepsis) appear to be minimal following mild hypothermia.

Currently, hypothermia therapy is the recommended treatment by the Advanced Life Support Task Force of the International Liaison Committee on Resuscitation
following ventricular fibrillation-induced cardiac arrest in adults (Nolan et al., 2003). There is limited data of its application in the pediatric population, as reflected in the American Heart Association guidelines, which recommended that hypothermia therapy be considered in children who remain comatose after resuscitation (ILCOR, 2006). This recommendation was based on the extrapolation of data from adult resuscitation trials to the pediatric population.

Hypothermia has been shown to be beneficial in patients during cardiac surgery, neurosurgical procedures and following traumatic brain injury, and neonatal resuscitation (Wagner et al., 1999). Two case control studies reported that 12 (Bernard et al., 1998) and 24 (Holzer et al., 1997) hours of mild hypothermia following cardiac arrest improved neurological outcome in adult patients when compared to historical controls. In addition, survival and functional outcomes were also ameliorated in two randomized controlled trials of hypothermia, of 12-24 hours duration, initiated within 4 hours following ventricular tachydysrhythmia-induced cardiac arrest in adults (Bernard et al., 2002; The Hypothermia after Cardiac Arrest Study Group, 2002). In the pediatric population, hypothermia therapy appears to be safe and improves neurological outcome following birth asphyxia (Gunn et al., 1998; Shankaran et al., 2005) but the beneficial effects have not been proven in older children (Doherty et al., 2009).

1.12.2 Intra-ischemic and Post-ischemic Hypothermia

Following the findings by Buchan and Pusinelli in 1990, there has been a surge of animal research investigating the benefits of hypothermia following cerebral ischemia. In vitro models have provided added support showing that hypothermia protects murine...
astrocytes and neuronal cells in culture against simulated ischemia (Shuaib et al., 1993). Many animal studies have shown that moderate intra-ischemic hypothermia reduces cerebral ischemia (Buchan and Pulsinelli, 1990; Busto et al., 1987, Coimbra and Wieloch, 1992), and provides prolonged behavioral and histological protection (Corbett and Thornhill, 2000). After GCI, intra-ischemic hypothermia provided virtually complete hippocampal CA1 pyramidal cell preservation at 2 months compared to normothermic ischemic animals (Green et al., 1992). Similar neuroprotection has been reported in other rat models of GCI (Minamisawa et al., 1990; Dietrich et al., 1993), and in FCI models (Chen et al., 1992; Barone et al., 1997; Connolly et al., 1996), spanning from young to aged animals (Corbett et al., 1997). However, the utility of cooling during cerebral ischemia is a restricted therapeutic tool because many patients who suffer ischemic insults do not reach treatment centers until hours after the ischemic insult. A clinically more relevant question is whether post-ischemic hypothermia is beneficial.

Numerous studies in models of GCI demonstrate considerable benefits with post-ischemic hypothermia (Busto et al., 1987; Safar et al., 1996; Coimbra et al., 1990). A large portion of this work has been done in gerbils, but similar results have been reported in the mouse (Yang et al., 1997), rat (Colbourne et al., 1999; Coimbra and Wieloch, 1992), pig (Thoresen et al., 1995), and dog (Safar et al., 1996). Moderate post-ischemic hypothermia protects against neuronal loss in the hippocampus (CA1) and improves neurobehavioral outcome following GCI in the gerbil (Colbourne et al., 2000; Corbett et al., 1997). Nonetheless, controversy arises regarding how long moderate hypothermia must be maintained to provide prolonged neuroprotection. Evidence suggests that short duration (<3 h) post-ischemic hypothermia does not provide protection (Welsh et al.,
brief duration (3-4 h) simply delays cerebral ischemic damage (Dietrich et al., 1993), and that 12-24 h hypothermia is required to provide long-lasting (1 year) protection (Colbourne et al., 1997). Moreover, moderate hypothermia is most beneficial when applied immediately following the cerebral ischemic insult and for longer durations (i.e., 48 h). Longer durations of hypothermia therapy (48 h) are effective even when the onset of hypothermia is delayed 6 h after severe forebrain ischemia (Colbourne et al., 1999).

The investigation of post-ischemic hypothermia in focal cerebral ischemia models has been less extensive. A number of animal models have been used including mouse (Chen et al., 1992; Connolly et al., 1996; Yang et al., 1997), and rat (Kawai et al., 2000; Maier et al., 1998; Huh et al., 2000; Zhang et al., 1993). Brief duration (1-3 h) moderate post-ischemic hypothermia has been shown to provide short-term neuroprotection (Zhang et al., 1993; Maier et al., 1998). Although extended survival times have not been investigated thoroughly, one report does suggest that 24 h post-ischemic hypothermia following MCAO in a rat model does provide prolonged (1 month) neuroprotection (Corbett et al., 2000).

1.13 Potential Mechanisms of Hypothermic Neuroprotection

The precise mechanism(s) by which mild hypothermia protects brain cells remains to be elucidated, but it is likely that hypothermia acts upon multiple pathways to ultimately prevent cell death (Liu and Yenari, 2007; Lyden et al., 2006). The best-known feature of hypothermia is the associated reduction in cerebral metabolism of approximately 5% for every degree of temperature reduction (Laptook et al., 1995).
Cooling during hypoxia-ischemia is partially protective via the delay of the onset of anoxic cell depolarization. However, the neuroprotective effects of hypothermia cannot be simplified to reduced metabolism, because mild cooling disproportionately improves outcome even when the absolute duration of depolarization is controlled (Bart et al., 1998). Hypothermia’s effect also cannot be solely attributed to a reduction in glutamate release, as hypothermia still decreases infarct volume when initiated in the subacute phase of the ischemic cascade, at which time the initial increase in glutamate has already passed (Van Hemelrijck et al., 2003; Sakoh et al., 2003).

After reperfusion, cerebral blood flow generally recovers transiently, followed by secondary hypoperfusion. Hypoperfusion is often seen during the latent phase; and its duration is highly correlated to the severity of the insult (Karlsson et al., 1994). Hypothermia initiated at either 1.5 hours or 5.5 hours after ischemia has been shown to prevent the later development of hypoperfusion during the secondary phase (Karlsson et al., 1994). It has no effect on the cerebral blood flow in the penumbra during ischemia, but can block hyperperfusion after the ischemic insult (Liu et al., 2007).

Mitochondrial dysfunction is a hallmark of secondary brain injury (Bennet et al., 2006). Maintaining mitochondrial function is crucial in promoting cell survival after hypoxia-ischemia. Post-ischemic hypothermia has been reported to preserve mitochondrial respiratory activity after 2 hours of reperfusion in the adult gerbil (Canevari et al., 1998). Hypothermia has also been shown to suppress ROS-mediated mitochondrial damage and rescue mitochondrial membrane potential in cultured myocytes (Huang et al., 2009).

There is growing evidence that hypothermia may have a key role in attenuating the
evolution of post-ischemic apoptosis, which can be triggered by such mechanisms as glutamate receptor excitotoxicity and resultant intracellular calcium accumulation, inflammation, and oxidative stress. The regulation of apoptosis involves alterations in the ratio of various intracellular factors such as Bcl-2, which inhibits apoptosis, Bax, which promotes it, and the activation of caspases. *In vitro* studies of hypothermia after severe hypoxia in developing rat neurons have shown that hypothermia exerts neuroprotective effects by depressing cell activity and minimizing hypoxia-associated protein synthesis.

Hypothermia has a pan-inhibiting effect (Zhao et al., 2007; Lyden et al., 2006). It has been reported to decrease BBB permeability (Dietrich et al., 1990; Smith and Hall, 1996; Jaing et al., 1992), reduce metabolic rate and O$_2$ consumption (Thoresen et al., 1995), attenuate post-ischemic hypoperfusion (Karibe et al., 1994), decrease glutamate release and enhance re-uptake (Zhao et al., 1997; Busto et al., 1989), decrease the generation of free radicals and lipid peroxidation (Lei et al., 1994; Thoresen and Wyatt, 1997), reduce apoptosis (Leonov et al., 1990; Edwards et al., 1995), and anti-inflammatory effects (Maier et al., 1998; Toyoda et al., 1996; Ishikawa et al., 1999; Whalen et al., 1997). It is likely that a combination of several mechanisms accounts for the reduced neuronal injury and improved functional outcome produced by hypothermia (Leonov et al., 1990; Clark et al., 1996; Colbourne and Corbett, 1995).

### 1.14 Hypothermia and Inflammation

Hypothermia exerts both stimulating and inhibiting effects on the neuroinflammatory response (Ceulemans et al., 2010). For example, hypothermia inhibits the proliferation of superoxide and NO production in cultured microglia (Si et al., 1997).
Moderate hypothermia (29°C) has been reported to reduce ovine leukocyte migration toward chemotaxic stimuli *in vitro* (Akriotis and Biggar, 1985). Several studies have subsequently demonstrated that intra-ischemic (2-3 h) moderate hypothermia reduces leukocyte infiltration into the brain following transient FCI (Toyoda et al., 1996; Maier et al., 1998; Inamasu et al., 2001). Post-traumatic hypothermia provided similar protection (Whalen et al., 1997), and also reduced IL-1β production (Goss et al., 1995). Another study has revealed that 16 h of post-ischemic hypothermia only delayed neutrophil accumulation from 2 to 3 days following cerebral ischemia, implying that post-ischemic hypothermia may only extend the window of opportunity for further intervention (Inamasu et al., 2000). This widespread reduction in inflammatory signaling may offer significant mitochondrial protection. For example, cytokine-mediated iNOS expression increases NO levels, which then compete with molecular oxygen at its binding site on cytochrome oxidase (Brown et al., 1997), and therefore hinder oxidative metabolism and reduce ATP levels. Also, TNFα has a similar effect by inhibiting complex-1 of the electron transport chain. Lastly, TNFα and interferon-γ-mediated iNOS expression is closely linked to mitochondrial DNA damage and apoptosis in cultured oligodendrocytes (Druzhyna et al., 2005). These findings support the hypothesis that reducing inflammation in the ischemic brain is a potential mechanism of hypothermic neuroprotection.

Although there is a large amount of data showing that hypothermia is neuroprotective, its mechanisms of action are not fully understood, and require further investigation. An elucidation of the effects of post-ischemic hypothermia on the cerebral and systemic inflammatory responses will strengthen our understanding of the therapeutic
effects of hypothermia, and potentially provide insight into future combination therapeutics.

1.15 Animal Models of Transient Global Cerebral Ischemia

One of the earliest models of global ischemia was described in 1964 by Lowry and colleagues. Here, the animal was decapitated and ischemia was stopped by the rapid freezing of the brain (Lowry et al., 1964). Other models of global ischemia include the use of neck tourniquets (Siemkowicz and Hansen, 1978) or neck cuffs (Kabat et al., 1942). These earlier irreversible models have several drawbacks. They did not allow reperfusion or long-term recovery or assessment. Cardiac arrest models such as those achieved through ventricular fibrillation have also been used to produce GCI (Michael et al., 1984). However while a whole-body ischemia model would more closely mimic the conditions of cardiac arrest, such a model would make it difficult to delineate the effects of the cerebral ischemia alone.

Rodent Bilateral Common Carotid Artery Occlusion Model

The two-vessel occlusion (2VO) model was developed in the rat by Bo Siesjo (Eklof and Siesjo, 1972). To achieve a consistent global ischemic injury (severe incomplete ischemia) in this model, hypotension was induced to prevent forebrain perfusion via the vertebral arteries and the posterior communicating arteries. The selectively vulnerable areas in this model include the hippocampus, particularly the CA1 region, thalamus and cortex (Smith et al., 1984). Another rodent model of reversible GCI that allowed for long-term recovery was the four-vessel occlusion (4-VO) model in rats developed by Pulsinelli and Brierley (Pulsinelli and Brierley, 1979). In this model the
vertebral arteries were permanently occluded and then the common carotid arteries are transiently occluded for a specific duration of ischemia. Cerebral blood flow is reduced to less than 3% of the baseline volume.

Our model - C57Bl/6 Mice and the Bilateral Common Carotid Artery Occlusion Model

We elected to use a modified version of the bilateral common carotid artery (two vessel occlusion, 2VO) model. In this ischemic injury model, damage to the hippocampus relies on the absence of blood flow to the forebrain via the posterior communicating arteries (Murakami et al., 1998). Because C57Bl/6 mice have hypoplastic or atretic posterior communicating arteries they were deemed ideal, and chosen for our study.

Following 2VO, forebrain ischemia occurs with maintenance of cerebral blood flow to the brainstem and therefore no apnea or need for mechanical ventilation. Unlike in the rat 2VO model, the mean arterial blood pressure is not reduced in the murine GCI model. The model is much simpler to perform compared to the 2VO model in the rat. There is reliable and reproducible GCI with a low mortality rate (approximately 10% in our hands). The pattern of hippocampal, cortical and thalamic selective damage is reasonably consistent in this model. The C57Bl/6 strain of mice were shown to be the most susceptible to this 2 vessel occlusion when compared to ICR, BALB/c, C3H, CBA, ddY and DBA/2 strains, based on neurological tests and histological evaluation (Yang et al., 1997).

1.16 Hippocampal-based Learning and Memory

The hippocampus, specifically the CA-1 region (field 1 of Ammon’s horn), is one of the most vulnerable regions of the brain following GCI. Ischemic periods as brief as 2
37

minutes have been shown to significantly damage this region (Smith et al., 1984). The hippocampus plays a critical role in the spatial memory and learning of rodents (Cammalleri et al., 1996; Burgess et al., 2002). Spatial memory is required by animals to learn the location of a reward, such as food, and is an essential feature of higher cognitive processing (Silva et al., 1998). This memory system stores reference knowledge about the environment, making it possible to distinguish one location from another (Morris et al., 1982). Lesions in the rat and mouse hippocampus are associated with an inability to learn or recall spatial memory tasks.

Neurological impairment such as decreased intellectual capacity, apraxia, spatial disorientation and memory deficits have been shown in patients who have suffered from GCI (Godefroy et al., 1994; Bokura and Robinson, 1997). The hippocampus and related structures are involved in spatial memory in humans as well as other higher cognitive functions, such as episodic memory (Aguirre et al., 1996; Burgess et al., 2002). This emphasizes the importance of analysis of hippocampal function following GCI.

### 1.17 Assessing Spatial Memory after GCI using the Morris Water Maze

The Morris Water Maze (MWM) was first described by Richard G. Morris in 1981 as an alternative to the radial maze, and remains one of the most widely used paradigms to test spatial learning and memory (D’Hooge and De Deyne, 2001). The test allows for the accurate and reproducible study of reference memory, spatial working memory and learning (D’Hooge and De Deyne, 2001; Dudchenko, 2004). It consists of a round pool filled with opaque water, in which the animal learns to locate the platform hidden below the water from four different starting points. Over a number of training
trials, the animals learn to locate the platform based on distal cues. This learning is measured by the decreasing time latency to locate the platform. The strength of the memory is tested afterwards by a probe trial, in which the platform is removed, and the animal’s search pattern for the former region of the platform is measured (Morris et al., 1981). Various parameters can be measured with this maze, including latency to find the platform, swim path, swim velocity, time spent in each quadrant of the pool, number of platform crossings, and percent trials with failures (the animal was unable to find platform before the time limit elapsed). According to Maei et al. (2009), the mean proximity to a former location of the platform during a probe test is the most sensitive measure to assess in water maze performance. The animals’ performance can also be influenced by the number of trials per day, number of days of training, duration of each trial, and the inter-trial interval (Zhou et al., 2009). Mice can use three types of strategies to locate the platform: a praxic strategy, whereby the animal learns a sequence of movements needed to reach the platform, a taxic strategy, whereby the animal uses cues or visual proximal guides to reach the platform, or a spatial strategy, whereby the animal reaches the target using information about the spatial location of the platform according to the spatial configuration of distal cues (Brandeis et al., 1989).

From the many established behavioral protocols to assess spatial memory in mice, we have chosen to use the MWM because of its relative simplicity, diverse applicability and high sensitivity in the assessment of damage to the hippocampus (Bannerman et al., 1999; Morris et al., 1982, Sutherland et al., 1982). Indeed, rats with a damaged hippocampus are unable to learn the platform location (Morris et al., 1982; Sutherland et al., 1982). This finding was also reported in mice with hippocampal lesions (Cho et al.,
The MWM has been show to be sensitive to GCI in a number of rodent studies (Brandeis et. al, 1989). There is a direct relationship between the hippocampus CA1 damage and impairment of spatial learning and memory, when most (> 80%) of the CA1 region of the hippocampus is damaged (Nelson et al., 1997). Severe CA-1 damage is a feature of our mouse model, which strongly supports our use of the Morris water maze.

1.18 Contextual Fear Conditioning

Examining the learning and memory of mice involves the use of multiple behavior tests that are designed to assess specific parts of their memory. Another simple and robust test that provides a reliable assessment of learning and memory in mice is the fear-conditioning test. Contextual fear conditioning involves a classical conditioning paradigm, whereby an association is formed between a distinctive place (conditioned stimulus [CS]) and an aversive event (unconditioned stimulus [US]) (Fanselow et al., 2000). It is possible to demonstrate that an animal has formed a specific CS-US association by showing that re-exposure to the conditioned context, but not dissimilar contexts, evokes conditioned fear behavior (Fanselow et al., 1980).

Specific to our study, the mouse will be placed in a visual context-controlled conditioning chamber, and then a series of foot shocks will be administered. The rationale underlying this experiment is that the mouse will then associate the contextual features with the pain received from the foot shock. Accordingly, re-introducing the mouse to the context after the training should elicit a freezing response even though no shock is applied.

Hence, when testing contextual memory the mouse will be placed in the cage where
it was trained with all the contextual cues preserved. It is hypothesized that the mouse will freeze when it recognizes the context. Freezing is defined as the absence of all movements except those related to breathing. Memory can then be assessed and expressed as the percentage of time that the mice spent freezing, which is commonly used as an index of fear in mice. Contextual memory is thought to be largely dependent on the hippocampus (Lynch, 2004). Therefore, we elected to use the contextual fear-conditioning test as another probe of how hypothermia therapy affects hippocampal function after GCI.

1.19 Rationale

Very little is known about neuroinflammation. Specifically, our knowledge about the timing, the role of cytokines, chemokines, and the role of specific inflammatory cells following global cerebral ischemia is not fully elucidated. As well, the anti-inflammatory mechanisms of hypothermia therapy following global cerebral ischemia are poorly understood. A clearer understanding of anti-inflammatory mechanisms of hypothermia therapy might aid in the design of novel anti-inflammatory therapies, which could extend the therapeutic window, or help us develop new therapies that could be combined with hypothermia therapy to improve outcome following global cerebral ischemia. Furthermore, an elucidation of the effects of hypothermia on neurodegeneration post-ischemia may be even more important for novel therapies. Figure 1 outlines what is known and not known about the effects of hypothermia therapy on inflammatory mechanisms leading to cell death following global cerebral ischemia.
Chapter 2

Hypothesis and Objectives

2.1 Hypothesis

Bilateral common carotid artery occlusion will induce an inflammatory response associated with secondary brain injury. Mild hypothermia therapy will be anti-inflammatory as well as neuroprotective following transient global cerebral ischemia. We hypothesize that hypothermia therapy induced attenuation of release of pro-inflammatory cytokines and chemokines into the brain and peripheral blood and activation of immune cells in the brain, in the acute phase of injury, will be associated with improvements in long-term neurobehavioral outcomes following global cerebral ischemia.

2.2 Objectives

To test this hypothesis following 10 minutes of bilateral common carotid artery occlusion and reperfusion we will:

i) Determine the systemic inflammatory response at 24 hours following global cerebral ischemia, by measuring inflammatory mediators in the peripheral blood, and determine the effect of hypothermia therapy on this inflammatory response.

ii) Determine whether hypothermia therapy reduces cytokine and chemokine expression in the hippocampus at 72 hours following cerebral ischemia and inflammatory cell activation in the hippocampus, dentate gyrus and cerebral cortex at 72 hours post-ischemia.
iii) Determine neuronal cell death in the brain regions that are selectively vulnerable to ischemia at 72 hours post-ischemia and the effect of hypothermia therapy on the cell death.

iv) Determine the effect of hypothermia therapy on spatial memory following global cerebral ischemia using a series of hippocampal-dependent spatial learning and memory tasks.

v) Determine the effect of hypothermia therapy on fear learning and memory following global cerebral ischemia using a contextual fear paradigm.
Chapter 3

Material and Methods

3.1 Animals

Male C57Bl/6 mice (Charles River, Montreal, QC), weighing between 19-22g before experimentation, were housed in cages in groups of five. The light/dark cycle was 12:12 with lights on at 06:00, and all treatments were performed between 08:00 and 11:00. The animals were randomized into either global ischemia or sham procedure with normothermia or hypothermia therapy post-surgery. All animal protocols used were approved by Laboratory Animal Service at the Hospital for Sick Children, Toronto, Canada.

3.2 Murine Global Cerebral Ischemia Model

Male C57Bl/6 mice were anesthetized with isoflurane (2.5 % for induction, 1.5-2 % for maintenance) mixed with medical air and oxygen (700:300 ml/min) via face-mask and randomly subjected to either bilateral common carotid artery occlusion (two vessel occlusion, 2VO) as previously described (Kelly et al., 2001; Murakami et al., 1998, Olsson et al., 2003) or sham-operated surgery. Depth of anesthesia was measured by hindlimb pinch every 15 min and rectal temperature was continuously recorded during ischemia and reperfusion, and kept at 37.0±0.5°C throughout the experimental protocol until recovery from anesthesia, using a heated water mat and an overhead-heating lamp until the mouse had fully recovered. Common carotid arteries were isolated with 5/0 non-absorbable sutures. Atraumatic aneurysm clips were placed to occlude cephalad blood
flow. After 10 min, the clips were removed, and reperfusion was observed. Wound sites were then closed with interrupted 4/0 non-absorbable sutures. For the sham-operated animals, the carotid arteries were isolated and exposed for 10-mins, but the clips were not applied.

3.3 Brain Inflammation Model

A model of pure brain inflammation without brain cell injury was studied as a positive control (Deng et al., 2003). Seven (7) mice were given 5 mg/kg lipopolysaccharide (LPS, E Coli endotoxin, E Coli serotype 055:B5; Sigma, St. Louis, MO) into the peritoneal cavity (IP) as it produces a robust inflammatory response without causing brain cell death (Deng et al., 2003). This model has been shown to lead to peak inflammatory responses 8-24 hours post-LPS injection, which resolves without histologic and biochemical evidence of brain injury as far out as 72 hours (Deng et al., 2003; Han et al., 2002). Male C57Bl/6 mice were anesthetized with isoflurane by face mask, then injected with LPS and maintained at 37 °C for 4 hours. Brains were harvested 24 hours later.

3.4 Postoperative Recovery and Hypothermia Therapy in Global Cerebral Ischemia

Ischemic and sham-operated mice were randomly rendered hypothermic (HT, 32±0.5 °C) or normothermic (NT, 37 ± 0.5 °C) immediately after wound closure. The animal’s rectal temperature was maintained at the appropriate range for 4 hours using overhead heating lamps and heated water mats. The 4 hour duration was chosen for the
hypothermia therapy as it has been shown to exert significant neuroprotection in C57Bl/6 mice (Sutcliffe et al., 2001), whilst limiting the animal’s time under anaesthesia. We have previously shown that rectal temperature correlates closely with brain temperature in this model (Hutchison et al., unpublished data). After the 4 hours, hypothermic mice were rewarmed by removing the animals from hypothermic conditions, and placing them on a heating blanket. The process of rearming to a rectal temperature of 37 °C took approximately 20 min. Animals were allowed to recover from anesthesia, then they were individually housed in a temperature-controlled environment with a 12-hour light/dark cycle and access to standard laboratory chow and water ad libitum. The mice were then given buprenorphine 0.05 mg/kg (Schering Plough, Pointe-Claire PQ) every 6 to 8 hours for 24 hours beginning just prior to emergence from anesthesia. Following surgery, animals were closely monitored while being allowed to survive for 24 hours to 28 days, and then euthanized. The mortality in the long-term studies (survival to 4 weeks) was approximately 5 percent.

3.5 Assessment of Systemic and Cerebral Inflammatory Mediators Post-GCI

Quantification of systemic inflammatory mediators

Serum Sample Preparation

Blood samples from animals from the four experimental groups (NT-sham, NT-2VO, HT-sham, HT-2VO) were collected via decapitation at 24 hours post-surgery. Blood samples were allowed to clot for 30 minutes on ice and then were centrifuged at room temperature for 10 minutes at 20,000 rpm. The supernatant was collected and stored
at \(-80^\circ\text{C}\) (n=4-5 mice/group). Please refer to Fig 2 for timeline of all molecular experiments.

*Soluble Adhesion Molecule/MMP-9/PAI-1 Microbead Array*

Serum samples taken at 24 hours post-surgery were analyzed using Millipore Multiplex technology. This time point was chosen because it coincides with the peak expression timepoints (12-24 hours) for selectins, ICAMs, VCAMs, MMPs, and PAI after cerebral ischemia (please refer to Introduction Section 1.8 for further details). Soluble E-selectin (sE-selectin), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble inter-cellular adhesion molecule-1 (sICAM-1), matrix metalloproteinase-9 (MMP-9), and plasminogen activator inhibitor-1 (PAI-1) were analyzed using a Multiplex Map Kit Mouse CVD1 Panel (MPXMCVD1-77AK-05, Millipore, Billerica, MA). Median fluorescent intensity was read using a Luminex 100SI apparatus and software (Luminex Corp. Austin, TX, USA). The kits include samples for generation of a 5-parameter logimetric standard curve and quality control samples. The total protein concentration was determined using a standard Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON).

*Quantification of cerebral inflammatory mediators*

*Brain Homogenate Sample Preparation*

Animals from the four experimental groups (NT-sham, NT-2VO, HT-sham, HT-2VO) were sacrificed 72 hours post-surgery (n=4-5 mice/group). The cortex, hippocampus, and cerebellum were dissected from the brain and manually homogenized on ice using glass tissue mortars and pestles in an ice cold solution of 0.32M sucrose in 50mM Tris-HCl, at pH 7.4, containing complete mini protease inhibitor cocktail (Roche
Applied Sciences, Laval, QC). The brain homogenate was centrifuged at 20,000 rpm for 20 minutes at 4°C and the supernatant was collected and stored at -80°C.

**Cytokine/Chemokine Microbead Array**

Brain homogenate samples were analyzed using Millipore Multiplex techniques. Interleukin 1-Beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), keratinocyte chemoattractant (KC), tumor necrosis factor-alpha (TNFα), and monocyte chemotactic protein-1 (MCP-1) were analyzed using a Multiplex Map Kit Mouse Cytokine/Chemokine Panel (MPXMCYTO-70K-08, Millipore, Billerica, MA) at 72 hours post-surgery. This timepoint was chosen because it reflects the peak inflammatory response and expression of inflammatory cytokines/chemokines post-ischemia (please refer to Introduction section 1.9). Median fluorescence intensity was read using a Luminex 100SI apparatus and software (Luminex Corp. Austin, TX, USA). The kits include samples for generation of a 5-parameter logimetric standard curve and quality control samples. The total protein concentration was determined using a standard Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON).

### 3.6 Immunohistochemistry

**Tissue Preparation**

Following a survival time of 72 hours, mice from the four experimental groups (NT-sham, NT-2VO, HT-sham, HT-2VO) were deeply anaesthetized with ketamine/xylazine and sacrificed by transcardiac perfusion with normal saline via the left ventricle for 10 mins at the pressure of 160 mmHg (n=7/group). Mice were then perfused
with 20 mL of 10% formalin. The brains were extracted by careful removal of the cranium, and then stored in 10% formalin, dehydrated in ethanol, and embedded in paraffin. The paraffin-embedded brains were cut in serial coronal (5 μm) sections, which were mounted on silane-coated slides. For immunohistochemistry, sections were deparaffinized with two 10-mins washes with xylene, rehydrated to distilled water through decreasing concentrations of ethanol, and subjected to antigen retrieval.

*Citrate Buffer Antigen Retrieval*

Because formalin forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection, we elected to use a citrate buffer antigen retrieval protocol to break the protein cross-links and unmask the antigens and epitopes in our formalin-fixed and paraffin embedded tissue sections. This protocol was used to enhance antibody binding to antigens to increase staining. Antigen retrieval was achieved by incubating the deparaffined sections in a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 mins in a water bath maintained at 100 °C.

*Immunohistochemistry*

Immediately after antigen retrieval, slides were allowed to cool to room temperature. Next, slides were incubated for 15 mins at room temperature with a peroxidase blocking solution (10% H₂O₂ in methanol). Non-specific staining of immunoglobulin was blocked with 5% Bovine Serum Albumin (85041C, Sigma Aldrich, Oakville, ON) for 1 hour at room temperature. Afterwards, sections were incubated at 4 °C overnight with rabbit anti- ionized calcium binding adaptor molecule 1 (anti-Iba1) antibody (2ug/mL, Wako Chemicals USA, Richmond, VA), which is specific to
microglia and macrophage. Negative controls were generated by replacement of the primary antibodies with PBS. Visualization was achieved with a standard immunoperoxidase reaction using donkey anti-rabbit secondary antibody (1:100, Santa Cruz, Santa Cruz, CA) and diaminobenzidine and H$_2$O$_2$ (Sigma Fast DAB, Sigma Aldrich, Oakville, ON) to yield an insoluble brown deposit. Sections were counterstained with hematoxylin to identify nuclei, and then rinsed for 10 mins with distilled water. Next, the sections were rehydrated with increasing concentrations of ethanol, then immersed in two 5-mins changes of xylene, and mounted with VectaMount mounting medium (H-5000, Vector Laboratories Inc., Burlingame, CA).

**Image acquisition and analysis**

Sections were imaged using a Nikon TE2000 inverted microscope with a Bright Field filter at a magnification of 20x and 40x. Hippocampal regions CA-1, CA-2, CA-3, DG, and the barrel field cortex were examined in 2 adjacent sections at -1.53 mm to -1.96 mm from the bregma. Two visual fields were taken per region per section. All images were acquired and analyzed using Volocity software v. 5.5 (Improvision, Image Processing & Vision Company Limited, Coventry, England). The cells were counted by two researchers, who were blinded to the experimental group of the mice.

### 3.7 Histological Analysis

**Short-term (72 hours post-surgery): FluoroJade B Histochemistry**

At 72 hours after sham surgery or ischemia, brains were harvested (n=7 mice/group). After induction of ketamine/xylazine, the mice were transcardially perfused with normal saline via the left ventricle for 10 mins at the pressure of 160 mmHg. Mice were then perfused with 20 mL of 10% formalin. The animals were decapitated, and the
brain extracted by careful removal of the cranium. The brain was then placed in 10% formalin, dehydrated in ethanol, and embedded in paraffin. Coronal slices of 5 µm were mounted and stained with FluoroJade B (Chemicon International Temecula, CA) to determine the extent of neurodegeneration in the mouse hippocampus. FluoroJade B is a polyanionic fluorescein derivative that binds to degenerating neurons. We opted to use this method for measuring cell death because of its simplicity of staining procedures and visual interpretation. However, the mechanism by which FluoroJade B works is unknown. Of note, it has a non-specific binding profile, which does not differentiate apoptotic from necrotic cells.

Sections were deparaffinized through immersion in xylene for 20 min, and then in 100% ethanol for 3 min. This was followed by 1 min in 70% ethanol, 1 min in 30% ethanol, and 3 min in distilled water. Sections were then incubated in 0.06% potassium permanganate (KMnO$_4$) for 20 min to decrease background staining and protect the tissue against photobleaching and fading, and then placed in distilled water for 3 min. The sections were subsequently transferred to the FluoroJade B solution (0.001% FluoroJade/0.1%acetic acid) and were gently agitated for 30 min. After this period, the sections were rinsed with three 1-min changes of distilled water, dried, cleared with two 5-min washes in xylene, and mounted with Vectamount mounting medium for fluorescent observation. Sections were observed under an epifluorescent microscope with blue (450-490 nm) excitation light and a barrier filter allowing passage of all wavelengths longer than 515 nm, thus resulting in a yellow-green emission color. Hippocampal regions CA-1, CA-2, CA-3, DG, and barrel field cortex were examined in 6 sections, at 6 specific locations, ranging from -1.53 mm to -2.80 mm from the bregma. The cells were
counted by a researcher, who was blinded to the experimental group of the mice, using Volocity software v. 5.5 (Improvision, Image Processing & Vision Company Limited, Coventry, England).

3.8 Morris Water Maze and Spatial Memory Analysis

Training 1

All behavioral testing was done in a circular water maze (diameter 130 cm, depth 50 cm) in a dimly lit room (please refer to Fig 3. for timeline of all behavioural experiments). The water maze tank was filled with water to a depth of 40 cm. Non-toxic white paint was added to make the water opaque and the escape platform (diameter 10 cm) was submerged 0.5 cm below the water level to ensure that there were no visual cues in the tank. Surrounding the tank were white curtains with distinctive visual cues painted on them. All training and probe tests were video recorded and data were analyzed using an automated tracking system (Noldus Ethnovision XT v.5, Attleboro, MA). The mice were handled twice a day, with each session lasting 2 minutes, for one week prior to water maze training. For each trial, the mouse was placed facing the wall of the pool at one of the four start locations, which were determined pseudo-randomly. The trial was completed when the mouse found the platform and remained there for at least 3 seconds, or the 60-second time limit elapsed. If the mouse was unable to find the platform, the water maze operator guided the mouse to the platform. The training day consisted of 6 trials (presented in 2 blocks of 3 trials; inter-block interval was 3 hours; inter-trial interval was approximately 15 seconds). The mice received 5 days of training prior to ischemia or sham surgery. During training the time required to reach the platform was recorded. The
mice were then randomized to 3 experimental groups: 1) sham surgery (NT-sham; n=14); 2) 10 minutes of global cerebral ischemia with 4 hours of hypothermia therapy (HT-2VO; n=16) or 3) ischemia with 4 hours of normothermia (NT-2VO; n=16).

Testing 1

On the morning of the 7th day following surgery, spatial memory was assessed in a 60 s probe test in which the platform had been removed from the pool. The mice were placed in the pool at the same location (in the opposite quadrant as the previous trained platform) and allowed to search for the platform. During the probe test, a number of parameters were recorded, including the time spent in the each zone, swim speed, the latency to each zone, and the frequency in entering each zone. The target or trained zone was an area of the water maze with a 20 cm radius, centered on the former location of the platform and represented 11% of the total surface area of the tank. The time spent in zones of equal size and location in the other 3 quadrants of the pool was also recorded.

Training 2

To assess if the animals could relearn to find the platform, training procedures were recommenced from day 7 to 11 following surgery. The water maze training again consisted of 6 trials (presented in 2 blocks of 3 trials; inter-block interval was 3 hours; inter-trial interval was approximately 15 seconds). Training sessions were performed as described above.

Testing 2

Twenty-one (21) day post-surgery, spatial memory was assessed in another 60-second probe test, which was carried out as described above. All recordings in the Morris
water maze were done by a researcher who was blinded to the experimental group of the mice.

### 3.9 Contextual Fear Conditioning

**Training**

A standard fear-conditioning paradigm was used. The conditioning chamber (Mouse Test Cage; 17.5 cm × 17.5 cm × 30 cm; Coulbourn Instruments, Whitehall, PA) had two sides made of transparent acrylic, and the other sides with controlled contextual cues made of black and white ceramic tiles arranged in a checkered pattern. The floor of the chamber (Shock Floor for Mouse Test Cage; H10-11M-TC-SF; Coulbourn Instruments, Whitehall, PA) consisted of eight (8) parallel stainless-steel grid bars, each measuring 0.3 mm in diameter and spaced 0.8 mm apart. The grid was connected to a device (Precision Animal Shocker, H13-15, Coulbourn Instruments, Whitehall, PA) that delivered the foot shocks. The conditioning chamber was housed in a larger isolation cubicle (H10-24T, Coulbourn Instruments, Whitehall, PA) made of monolithic rigid foam walls (57.5 cm x 50 cm x 60 cm). Training consisted of placing the mice individually in the chamber and allowing a 2 min acclimatization period. After this period, the mice received three shocks (0.5 mA, 2 s) with an inter-shock interval of 30s. The mice remained in the chamber for an additional minute and were returned to their home cages. Each training session was videotaped to calculate freezing. Percentage freezing was calculated before the first and after the last shock to measure the response to the adverse stimuli in the NT-sham, NT-2VO, and HT-2VO mice.
Testing

Contextual fear conditioning during the test session was evaluated 7 days after training by placing the mice in the training environment for 4 min in the absence of the foot shock. Each test was videotaped to calculate freezing. Memory was assessed and expressed as the percentage of time that the mice spent freezing, which is commonly used as an index of fear in mice. Freezing was defined as the absence of all movements except those related to breathing. It was scored according to an instantaneous time-sampling procedure in which each animal was observed every 2 s in a 240 s testing period in the case of contextual test (120 measures).

Data acquisition and analysis

Data acquisition was done using FreezeFrame 2.0 software (Coulbourn Instruments, Whitehall, PA). Mouse behavior was recorded using the TruScanLinc laser grid. Parameters recorded included: number of movements, movement time, rest time, distance travelled, and movement speed. An adjusted value of rest time was used as an indicator of freezing. To calculate freezing time the rest time of the mice during the testing phase was used. Rest time prior to the foot shock was calculated as normal, baseline resting time. The increase in rest time post shock was judged to be freezing. For a particular group of mice, values for freezing during the contextual test were then normalized using the average freezing time found post shock during the training phase. The data was analyzed using ANOVA test and Bonferroni post-hoc analysis in SPSS (v.19.0.1, IBM Corporation, Chicago, IL). In addition, manual recordings of freezing were also performed to confirm the results from the digitally collected data.
3.10 Statistical Analysis

One-way analyses of variance (ANOVA) were done with post-hoc Student-Newman-Keuls method for pairwise comparisons of subgroups when data followed a normal distribution. A Mann-Whitney U test or Kruskal-Wallis test with post-hoc Mann-Whitney U test method for pairwise comparison of subgroups with Bonferroni correction was done when non-parametric distribution of data was observed. Normality was determined by the Kolmogorov-Smirnov test. Results were considered to be statistically significant if p<0.05. All statistics were performed using SPSS (v.19.0.1, IBM Corporation, Chicago, IL). All data are expressed as mean ± standard error of the mean.
Chapter 4

Results

4.1 Maintenance of Animals’ Rectal Temperature

Ischemia/sham mice were maintained at 37 ± 0.5 °C during 10-minutes of bilateral common carotid artery occlusion or sham operation (Fig 4A), then at normothermia (37 ± 0.5 °C) or hypothermia (32 ± 0.5 °C) for 4 hours post-surgery (Fig 4B). Positive control mice that received lipopolysaccaride (LPS) injections were also maintained at 37 ± 0.5 °C for 10 minutes after injection (Fig 4C), then at normothermia for 4 hours post-injection (Fig 4D). Temperature recordings were made every 1 minute over the 10-minute occlusion/injection time interval, then every 15 minutes over the 4 hours post-operation interval. The data shown are means ± SEM of temperature recordings taken from 5 animals per group at the indicated time-points.

4.2 Hypothermia Did Not Affect the Systemic Concentrations of Soluble Adhesion Molecules 24 hours Following GCI

No significant differences between the 3 experimental groups were found in the peripheral blood concentrations of soluble E-selectin (sE-selectin; Fig 5A), soluble vascular adhesion molecule-1 (sVCAM-1; Fig 5B), and soluble intercellular adhesion molecule-1 (sICAM-1; Fig 5C). An increase in the peripheral blood concentration of matrix metalloproteinase-9 (MMP-9) was found 24 hours post-ischemia in the NT-2VO mice when compared to those in the NT-sham group. However levels of MMP-9 in the
HT-2VO mice were not significantly different from those found in the NT-sham and NT-2VO mice (Fig 5D). Decreased systemic concentrations of plasminogen activator inhibitor (PAI-1) were found in the HT-2VO group when compared to NT-sham. Meanwhile levels of PAI-1 in the NT-2VO group were not significantly different from those measured in the NT-sham and the HT-2VO group (n=4-5 mice per group).

4.3 Hypothermia Blocks Inflammatory Mediators IL-1β and KC in the Hippocampus 72 hours Following Cerebral Ischemia

At 72 hours post-ischemia, concentrations of pro-inflammatory cytokines IL-1β (Fig 6A) and KC (Fig 6C) in the NT-2VO mice were significantly elevated compared to NT-sham tissue. This inflammatory response was attenuated by hypothermia therapy (p<0.05). Mean concentrations of IL-6 (Fig 6B) were found to be mildly elevated post-ischemia in the NT-2VO group when compared to NT-sham, while those of IL-10 (Fig 6D), GM-CSF (Fig 6E), and G-CSF (Fig 6F) were slightly decreased. While such trends were found, no statistically significant differences were found between the 3 experimental groups of each of these analytes. There were also no significant changes in the hippocampal concentrations of chemokines MCP-1 (Fig 6G) and TNFα (Fig 6H) between the 3 experimental groups (n=4-5 mice per group).

4.4 Mild Hypothermia Reduces the Inflammatory Response 72 hours Post-ischemia

Positively-stained cells were quantified by two researchers, who were both blinded to the experimental groups of the mice. The inter-observer Cohen’s kappa coefficient was
0.96. At 72 hours post-ischemia, there were significant increases in Iba-1 staining in the hippocampus CA-1, CA-2, CA-3 and dentate gyrus (DG) regions and in the cerebral cortex of the normothermic-ischemic (NT-2VO) brains compared to normothermic-sham (NT-sham) brains (p<0.05). Hypothermic-ischemic (HT-2VO) brains showed significantly decreased numbers of activated microglia or macrophages in all brain regions (Figs 7-9). Activation of microglia or macrophages was not detected in the normothermic-sham (NT-sham) brains, and at very low levels in the hypothermic-sham (HT-sham) brains, and there was no significant difference between the number of Iba-1 stained cells in the brains of NT-sham and HT-sham mice in all brain regions except for the hippocampus CA-2 and cerebral cortex (p<0.05). Brains from mice injected with LPS showed similar levels of Iba-1 staining in the cerebral cortex when compared to normothermic-ischemic brains, but very low levels of positive staining were detected in the hippocampus of these animals.

4.5 Hypothermia Improved Survival of Brain Cells 72 hours after Global Cerebral Ischemia

Degenerating neurons were examined in brain sections stained with FluoroJade B in the areas previously described as being vulnerable in this model, specifically the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) and cerebral cortex (Fig 10). There were significant increases in cell death in the CA-1, CA-2 and DG but not in the CA-3 or cerebral cortex in the brains of normothermic-ischemic (NT-2VO) mice, when compared to normothermic-sham (NT-sham) mice (p<0.05) (Fig 11). Hypothermic-ischemic mice showed significantly attenuated levels of cell death in the CA-1 and CA-2 but not in the
DG when compared to normothermic-ischemic mice (p<0.05). Quantification of degenerating neurons showed a 39.3% reduction in cell death in the HT-2VO group compared to the NT-2VO brains in the CA-1 region, and a 47.2% decrease in the CA-2 region. A strong trend in hypothermic protection against cell death could also be found in the DG however these differences between the two groups were not statistically significant (p=0.17) (Fig 11). HT-2VO mice showed significantly higher levels of neurodegeneration in the cerebral cortex when compared to NT-sham mice (p<0.05). These levels were higher than, but not statistically different from those found in NT-2VO mice (p>0.05).

4.6 Hypothermia Therapy Preserved Spatial Memory after Global Cerebral Ischemia

To assess spatial memory, mice were first trained to locate a submerged platform in a pool (Fig 12B) with 2 blocks of 3 trials per day for five consecutive days (Fig 12A; please also see Fig 3 for timeline of Morris water maze experiments). Performance in the task improved over the course of the training days, as evidenced by decreases in escape latency in all 3 experimental groups (Fig 12C, p<0.05). No difference in performance was found between the 3 experimental groups by the last day of training (Day -3). Memory was subsequently tested in a probe test 7 days post-surgery. The platform was removed from the pool and the mice were allowed to search for it for 60 seconds. The HT-2VO and NT-2VO mice spent significantly less time in the trained zone (TZ) than the NT-sham mice (p<0.05; Fig 12D). However, the HT-2VO mice also spent significantly more time in the TZ than the NT-2VO mice (p<0.05; Fig 12D). The HT-2VO mice also spent
similar amounts of time searching around the perimeter of the pool when compared to NT-sham mice (p>0.05; Fig 12E). Both of these groups spent significantly less time by the pool wall than the NT-2VO group (p<0.05; Fig 12E). The swim speeds were similar between all experimental groups during the probe test (Fig 12F).

To assess if the animals could re-learn to find the platform after the surgery, mice were trained again using the same training protocol for 5 days from day 7 to 11 post-surgery (Fig 13A). As this training period progressed, mice from all 3 experimental groups showed improvements in their ability to locate the platform (Fig 13B). By the last day of training (Day 11), there was no significant difference in performance between the 3 experimental groups (Fig 13B). Twenty-one (21) days following ischemia/sham surgery, spatial memory was re-assessed with another 60-second probe test. Hypothermic-ischemic (HT-2VO) mice spent similar amounts of time in the TZ, where the platform was previously located, as normothermic-sham (NT-sham) mice (p>0.05; Fig 13C). Both of these groups spent significantly more time in the TZ than the normothermic-ischemic (NT-2VO) group (p<0.05). During this probe test, the NT-2VO group spent significantly more time searching around the periphery of the pool when compared to the NT-sham group (p<0.05; Fig 13D). The HT-2VO mice on average spent more time by the pool wall than NT-sham mice, but less than the NT-2VO mice. However, these differences between these means did not reach statistical significance (p>0.05; Fig 13D). The swim speeds were again similar between all 3 experimental groups (Fig 13E) during this probe test.
4.7 Normothermic-ischemic Mice (NT-2VO) Exhibit Lowered Fear Response 21 days Post-ischemia

Twenty-one (21) days after the ischemia/sham surgeries, mice were trained in a classical contextual fear-training paradigm (Fig 14A). They were introduced to a chamber where they received 3 mild foot shocks. During this training session, percent freezing before the first shock and after the last shock were measured to determine the animal’s response to the adverse stimuli (Fig 14B). Before the first shock, NT-2VO mice exhibited less freezing behavior than mice from the other 2 experimental groups. However, this difference was not statistically significant. After the last shock, NT-2VO mice continued to freeze less than both the NT-sham and HT-2VO mice (p<0.05 in both comparisons) (Fig 14B). The HT-2VO mice exhibited similar freezing responses as NT-sham mice (p>0.05; Fig 14B).

After a 7-day delay, contextual fear memory was assessed for 4 mins by re-introducing the mice to the training chamber with the contextual cues preserved. The HT-2VO mice exhibited similar freezing response patterns as the NT-sham mice, with more freezing behavior than those shown by NT-2VO mice. However, no statistically significant difference was found between the groups when percent freezing responses measured for each minute over the entire testing period (Fig 14C), and over the entire 4 mins testing period were analyzed (Fig 14D).
Chapter 5

Discussion

In both laboratory and clinical studies, mild hypothermia has been shown by several labs to improve neurological outcome from the effects of GCI brain injury at both the histological and behavioral levels (Colbourne and Corbett, 1994, 1995; Colbourne et al. 1999; Dietrich et al. 1993; Lyden et al. 2006). Several mechanisms by which mild hypothermia exerts its protective effects have been characterized, including salutary effects of blood flow reduction and excitotoxin accumulation, preservation of brain metabolites, and inhibition of molecular pathways such as apoptosis, and inflammation.

5.1 Hypothermia Protects Against Cell Death

In our study, significantly reduced levels of degenerating cells were observed in the CA-1 and CA-2 regions of the hippocampus, while the reduction that was observed in the CA-3, dentate gyrus, and cortex did not reach the significance level. Pyramidal neurons of the CA-1 region are among the cells most vulnerable to loss of blood supply, and death frequently occurs days after the initial ischemic insult (Schmidt-Kastner and Freund, 1991). Our histological result and its corresponding time-point are consistent with the delayed neuronal death observed in previous studies (Kelly et al., 2001).

The use of FluoroJade B is controversial as the specific protein or lipid component that it labels is not known. FluoroJade B is a fluorescent anionic xanthene dye derived from fluorescein. There is speculation that, like fluorescein, it binds to basic proteins, which occur at high concentrations in the nuclei and cytoplasm of dead cells
Correlative studies with traditional neuronal degeneration stains such as hematoxylin and eosin (H&E) and de Olmos’ cupric staining have demonstrated that Fluoro-Jade B consistently reproduced insult-specific staining patterns of neurodegeneration that were identical to H&E and de Olmos’ cupric staining after injection of kainic acid, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and multivalent metals (Schmued et al., 1997). However, FluoroJade B has also been found to occasionally bind to astrocytes (Colombo et al., 2002). It is believed that such labeling represent astrocytic activation and not death as is the case for neurons (Ye et al., 2001; Anderson, 2003).

5.2 Hypothermia Attenuates Microglial/Macrophage Activation

The role of inflammation in GCI models has been less studied compared to FCI. We found robust increases in microglial/macrophage immunoreactivity in the normothermic-ischemic (NT-2VO) mouse brain. Meanwhile, microglial/macrophage staining was reduced to levels found in sham mice in the hypothermic-ischemic (HT-2VO) mouse brain. This finding is consistent with other studies that report that hypothermia decreased microglial activation and generation of a variety of cytotoxic immune mediators. Although these findings are not especially surprising, they are important to document since the study of genetic mouse models is becoming increasingly popular, while most reports of GCI are in the rat.

We found similar increases in microglial staining in the cerebral cortex of mice given LPS. These latter observations are an important control, since the extent of inflammation in ischemia models is likely directly correlated to the amount of cell death.
Because hypothermia decreases overall cell death, the amount of inflammation should also be proportionately decreased, and any anti-inflammatory effects may be secondary to an upstream mechanism. However in the LPS model, a robust inflammatory response occurs, but in the absence of cell death (Deng et al., 2003).

Much of the work attempting to elucidate the mechanisms of hypothermic protection in GCI models has focused on pathways largely involving neurons. Other cell types, especially microglia, are likely to participate in determining the ultimate fate of the brain following GCI, but this has not yet been studied extensively. Prior studies have shown that activated microglia and circulating macrophages increase neuronal injury following hypoxia treatment when co-cultured with neurons (Flavin et al., 1997; Flavin and Ho, 1999; Flavin et al., 2000; Giulian et al., 1993; Giulian and Vaca, 1993; Lehnardt et al., 2003), and inhibiting microglia seems to lead to reduced cell death in GCI (Yrjanheikki et al., 1998). Furthermore, mild hypothermia was found to inhibit the transformation of microglia to a phagocytic phenotype \textit{in vitro}, and reduced overall microglial cell death (Webster et al., 2009). This observation may suggest a potentially added benefit of hypothermia, in that by preserving microglial viability, microglia may be available to perform restorative functions such as phagocytosing necrotic debris and elaborating pro-survival factors.

Interestingly we did find an increase in microglial/macrophage activation in the CA-3 of the hippocampus and cerebral cortex, but there was no increase in neurodegeneration in those same regions with the FluoroJade B histochemical stains. These findings suggest that microglial/macrophage activation and neurodegeneration are not directly related. While it is generally regarded that activated microglial cells in
ischemic injury are neurotoxic, the results of several studies revealed, that in certain conditions, microglia may exert neuroprotection (Kitamura et al., 2004; Kitamura et al., 2005). Microglia may be involved in regeneration and remodeling of the brain via production of neurotrophic molecules such as brain-derived neurotrophic factor (BDNF) (Batchelor et al., 1999; Miwa et al., 1997; Nakajima and Kohsaka, 2004), and insulin growth factor-1 (IGF-1) (O’Donnell et al., 2002; Lalancette-Hebert et al., 2007). Moreover, Neumann et al. (2006) using organotypic hippocampal brain slices exposed to oxygen/glucose deprivation (OGD), an in vitro model of stroke, showed that activated microglial cells can protect neurons by direct physical contacts (Neumann et al., 2006) and/or by the phagocytosis of activated and neurotoxic polymorphonucelar neutrophils (PMNs) (Neumann et al., 2008). Interestingly, Lai and Todd (2008) reported that severity of neuronal injury may indeed influence whether microglia would exert neurotoxic or neuroprotective effects. Nonetheless, the fact that activated microglia can produce and secrete growth factors raises the possibility that inflammatory cells may have a role in tissue remodeling after cerebral ischemia and ultimately affect brain plasticity and functional recovery after ischemia.

We also found that hypothermic-sham (HT-2VO) mice had increased macrophage/microglial activation compared to normothermic-sham (NT-2VO) animals. This finding may be reflective of a systemic stress response induced by hypothermia. Indeed, the clinical use of hypothermia leads to some adverse effects including multi-organ dysfunction in children after cardiac arrest (Doherty et al., 2009).
5.3 The Systemic Inflammatory Response in the Peripheral Blood

Soluble ICAM-1 and soluble VCAM-1 have been shown to be increased in the serum following an inflammatory insult, as well as acting as a marker for stroke severity in humans (Blann et al., 1999). We however observed the opposite finding with decreasing trends in serum concentrations of sICAM-1, and sVCAM-1 following global ischemia. The reduction below sham control concentrations is consistent with finding presented by Clark and colleagues (Clark et al., 1993), who observed a reduction in circulating ICAM-1, even though neutrophil adhesion was significantly increased. Furthermore in our study, hypothermia did not significantly affect the levels of sVCAM-1 and sICAM-1 compared to sham levels 24 hours post-ischemia. Hypothermia was also ineffective in increasing the levels of PAI-1, sE-selectin and MMP-9.

One major caveat that must be considered when interpreting this data is that some of the measured levels of inflammatory mediators in the peripheral were below the multiplex immunoassay’s sensitivity threshold. According to the manufacturer, the standard curve range for this panel is 3.2-10,000 pg/mL. The protein concentrations in many of our sE-selectin, sVCAM-1, sICAM-1, MMP-9 and PAI-1 samples fell below this range. When below-range readouts were encountered, we eliminated these samples from our study and statistical calculations altogether. This issue with the sensitivity threshold could account for the large within-group variability, and consequently our negative results. The multiplex immunoassays were ultimately not sensitive enough to detect the protein levels of our experiment. Repeated experiments using more sensitive protein detection methods, such as enzyme-linked immunosorbent assay (ELISA) whose
range is 1.50 pg/mL-50 pg/mL, are needed before firm conclusions can be made regarding hypothermia’s effect on the systemic inflammatory response post-ischemia.

5.4 The Cerebral Inflammatory Response

We found that mild hypothermia significantly reduced the generation of cytotoxic immune mediators. This is consistent with numerous reports that largely suggest that ischemia-induced inflammation is detrimental (Tang and Yenari, 2010), and also directly supports our hypothesis that hypothermia attenuates the cerebral inflammatory response following global ischemia.

At 72 h post-ischemia, we found that hypothermia decreased the elaboration of pro-inflammatory cytokines IL-1β and KC. Similar trends were found with IL-6, MCP-1 and TNF-α, however these differences were not significant. Hypothermia also showed a general trend to simultaneously increase the anti-inflammatory cytokine IL-10, and growth factor cytokines G-CSF and GM-CSF in the hippocampus, although these differences were again not found to be statistically significant. Taken together, these results show an inflammatory response in our murine ischemic model, which was mildly lowered by the application of 4 hours of hypothermia therapy immediately after reperfusion.

We encountered the same sensitivity-threshold issue with these immunoassay panels as those described in section 5.3. Again, some of the levels of hippocampus cytokines and chemokines were below the multiplex immunoassay’s sensitivity threshold. According to the manufacturer, the standard curve range for this panel is 3.2-10,000 pg/mL. The protein concentrations in many of our IL-6, IL-10, GM-CSF, G-CSF,
MCP-1 and TNFα samples fell below this range, while IL-1β and KC levels were well within range. When below-range readouts were encountered, we eliminated these samples from our study and statistical calculations altogether. This issue with the sensitivity threshold could account for the large within-group variability, and ultimately our negative results.

5.5 Spatial Memory Assessment

Previous studies using the Morris water maze following global cerebral ischemia have found consistent deficits in spatial learning and memory in rats (Jaspers et al., 1990; Nunn et al., 1994). Despite the fact that rats and mice differ in collateral circulation and anatomy of the circle of Willis in the brain, both species have shown impaired memory after ischemia (Jaspers et al., 1990). In general, the spatial memory deficit is directly related to the hippocampal damage post-ischemia, specifically with CA-1 injury (Nelson et al., 1997; Block, 1999). Our FluoroJade B staining results demonstrated that BCCAO robustly increased the number of degenerating neurons in the hippocampus CA-1 and CA-2 regions. These results were consistent with previous reports about neuronal damage in the hippocampus induced by ischemia. It was shown that after cerebral ischemia, a significant loss (p<0.01) of hippocampal hilus neurons took place (Johansen et al., 1987). Other studies also found that following global cerebral ischemia, an early development of neurodegeneration occurred in specific brain regions, including the hippocampal hilus and CA1 areas (Cizkova et al., 2000). The hilus belongs to one of the layers of dentate gyrus, which is believed to be involved in learning and memory. Hence, the loss of hilus neurons in elderly people has been linked to the decline in relational memory (West,
1993). Thus our present data, together with findings by other investigators, suggest that BCCAO-induced spatial memory impairment may be due to the hippocampal neuronal injury or neurodegeneration caused by the artery occlusion.

The probe test that we used has been shown to be sensitive to global ischemia induced spatial memory deficits (Wright et al., 1996; Block and Schwarz, 1997; Nelson et al., 1997). During the first probe test (on day 7), the normothermic-ischemic (NT-2VO) mice exhibited decreased spatial memory of the original platform, as evidenced by their spending less time in the target zone (TZ). On the other hand, hypothermic-ischemic (HT-2VO) mice performed at levels similar to that of sham-operated mice (NT-sham).

Thigmotaxis, the tendency for the animal to follow the walls around the perimeter of the tank, is one index to reflect that mouse is not problem-solving. Excessive thigmotaxis indicates that the animal is not remembering the location of the platform, because one of the first things that the animals must learn is that there is no escape located around the perimeter of the tank. As the mice begin to learn the task, most animals swim away from the pool wall and then, by weaving searching patterns, eventually find the platform. Thigmotaxis does not reflect an effective searching behavior for the submerged platform, and is routinely observed in untrained animals. A return to this behavior, consistent with our findings, has been shown to be associated with hippocampal injury (Block and Schwarz, 1998), and may also indicate that the animal does not have adequate awareness of its surroundings.

Consistent swim speeds have been reported in multiple studies with global cerebral ischemia (Nelson et al., 1997; Block and Schwarz, 1998). Likewise in our study, an analysis of the swim speeds during the probe test revealed that they were similar
across experimental groups. Therefore, we attribute the memory deficit found in the NT-2VO mice to the hippocampal injury caused by the ischemia rather than motor performance impairment. Taken together, our findings suggest that 4 hours of mild hypothermia applied immediately after reperfusion seems to preserve spatial memory in mice 7 days post-ischemia.

The BCCAO model of ischemia has been shown to induce hippocampal injury along with a spatial memory deficit, both of which were reduced with mild hypothermia. During the second training series between day 7 and day 11 post-ischemia, working memory was evaluated. Although a greater escape latency time was observed among the normothermic-ischemic (NT-2VO) mice, during the final trials on day 11 all mice were able to find the platform equally well, thereby demonstrating an intact working memory system. This finding has also been observed in other rodent models of global ischemia (Block and Schwarz, 1998) and suggests that the areas of the hippocampus damaged by global ischemia plays less of a role in working memory compared to its role in spatial memory referencing. Interestingly, there were also some improvements in performance in the NT-2VO and the HT-2VO mice at the day 21 probe test compared to their day 7 probe test, especially in time spent in TZ. This presents the possibility that global ischemia induced spatial memory impairment is only transient. However, studies have shown that the behavioral deficits evaluated using a Morris water-maze can be detected up to 20 weeks post injury (Nelson et al., 1997). Therefore an alternative possible explanation for the improved memory retention and recall test results could be due to neurogenesis in the hippocampus. Beginning 21 days post-injury and continuing for 90 days, there was evidence of newly formed neurons in rat studies of global cerebral
ischemia. This neurogenesis was associated with improved performance in the Morris water maze (Bueters et al., 2008). One caveat of this theory is that this neurogenesis and cognitive improvement was only temporary with all newly derived neurons and improved spatial disappearing by approximately 150 day post injury (Bueters et al., 2008). It would be interesting to investigate whether neurogenesis occurred in our model, but carrying out immunohistological experiments in which we double stain brain sections with NeuN (for post-mitotic neurons) and BrdU (newly formed cells) at 21 days post-ischemia.

5.6 Contextual Fear

On the training day, relatively high freezing responses were observed in the NT-sham (µ=18.4%) and HT-2VO (µ=19.9%) mice even prior to the first shock. This behavior can be explained by the fact that these animals were trained in the contextual fear conditioning chamber 1 hour after the second MWM probe test. It is possible that the abrupt change in protocol and environment, especially after an intense 21 day MWM experimental protocol, would cause high stress in these animals with a functional hippocampus. Hence, ideally two separate cohorts of experimental animals would be used for the Morris water maze and fear experiments, or a longer delay between the two sets of experiments would be observed.

Comparisons between the pre and post-shock freezing responses reveal that animals from all three experimental groups did not associate the context with the adverse stimuli. One possible explanation for the NT-2VO’s lack of fear response may be associated with changes in their pain perception. However, this deficit may also be attributable to locomotor hyperactivity, an established effect of hippocampal lesions
According to this view, animals with hippocampal lesions prior to training fail to exhibit normal freezing because hyperactivity caused by the lesion disrupts freezing and behavioral inhibition directly (Good and Honey, 1997; Blanchard et al., 1977; Douglas, 1967). It is important to note that this is a performance account, because in this view hippocampal lesions disrupt the freezing response rather than fear memory.

As such, caution must be taken when interpreting the fear testing data. While the NT-2VO mice showed a slightly lowered freezing response compared to the HT-2VO and NT-sham mice, it was not statistically different from those groups. Furthermore, even though a strong trend exists, we cannot definitively rule out the possibility that NT-2VO mice’s hyperactivity during the training session disrupted their freezing response, rather than their fear memory.

Freezing behavior in mice has been used a quantifiable measure of fear memory, which in turn has been associated with the amygdala. In this project, we did not study the amygdala, and how hypothermia may affect its fate after global cerebral ischemia. It is possible that the differential response to fear training may be due to molecular/biochemical changes within the amygdala.

5.7 Relevance to Clinical Trials

In clinical studies mild hypothermia therapy has shown benefits in adults who suffered cardiac arrest and in neonatal hypoxic-ischemic brain injury. A review of the literature has shown that in order to achieve the maximum benefits of hypothermia, brain
cooling needs to begin soon after the insult, maintained for relatively long periods of time, and in the case of ischemic stroke, should be applied in tandem with the re-establishment of cerebral perfusion. Translating this to the clinical arena can be challenging, especially rapid cooling and the re-establishment of perfusion. The use of a second neuroprotectant could potentially lead to the enhancement of overall protection, the prolonging the temporal therapeutic window for hypothermia, and/or to supplement protection in areas where hypothermic treatment is only transient or weak. Combination therapies resulting in recanalization following ischemic stroke would improve the likelihood of a good outcome, given that the experimental literature suggests that they provide more consistent neuroprotection against ischemia with reperfusion than ischemia without. And because recombinant tissue plasminogen activator (rt-PA) is the only FDA approved treatment for acute ischemic stroke, and its mechanism of action includes the recanalization of occluded vessels, it is a clear candidate for combination therapy with hypothermia. However, caution must be exercised because the effects of thrombolytics are temperature-dependent, and the risk of hemorrhage is high. Nonetheless, experimental data seem to favor such a combination approach. Therefore in order to apply hypothermia therapy to a broader range of patients, combination strategies should be further investigated.

5.8 Limitations

_Bilateral Common Carotid Artery Occlusion Model_

The experimental model we opted to use, the murine bilateral common carotid artery occlusion model, is widely used and extensively studied. However, it has some
drawbacks. First, its abrupt onset of ischemia and subsequent complete restoration of blood flow do not reflect a realistic reperfusion paradigm. Furthermore, the model itself is highly variable in ischemic outcome when used in mice. This variability can be attributed to the application of a universal occlusion time in strains of mice with different genetic backgrounds. Indeed, there is an emerging body of evidence that makes it apparent that the sensitivities to ischemic insult between different mouse strains are a problematic issue (Sheldon et al., 1998; Wellons et al., 2000). To address this problem, it is imperative that the ischemic duration be optimized for each mouse strain. To complicate the issue further, variability stemming from individual differences in collateral flow through the circle of Willis, even within one strain, has been widely reported (Beckmann, 2000, Fujii et al., 1997, Kitagawa et al., 1998, Sheng et al., 1999 and Wellons et al., 2000). Hence, it would be ideal to verify the patency of the posterior communicating arteries of an individual mouse before conducting surgery to optimize this model. This is a step that we did not take. Also since we only used male mice, we may not have accounted for sexual dimorphic differences in the response to global cerebral insults.

Mild hypothermia therapy applied immediately after the ischemic insult has been reported to provide significant neuroprotection. In our study, we elected to manipulate the animals’ body temperatures immediately after reperfusion. This treatment schedule, while commonly used in experimental studies, is unlikely to be achievable in a clinical setting. Patients suffering from cardiac arrest and drowning incidents may not reach an institution where hypothermia therapy is instituted for hours. Further studies in which the onset of
hypothermia therapy is delayed and varied following ischemia would be needed to establish the therapeutic window of hypothermia therapy.

**Neurodegeneration after global cerebral ischemia**

The extent of neuronal degeneration in response to ischemia/reperfusion (I/R) injury has been shown to be influenced by age, sex, and background. Age is an important factor in determining an animal’s vulnerability to I/R injury. It has been attributed to the link that exists between mitochondria and cerebral damage following ischemia as well as the phenomenon of ischemic tolerance. In fact, it is now well recognized that mitochondria are the principal targets, and effectors, of the progression of the neurodegenerative process that occurs in the brain with aging as well as after I/R injury (Bertoni-Freddari et al., 2006; Friberg et al., 2002; Lesnefsky et al., 2001). Females have been observed to show a lower susceptibility to post-ischemic and post-traumatic brain injury (Roof and Hall, 2000). Backgrounds can also be different quantitatively in their sensitivity to ischemia/reperfusion including BCCAO (Kelly et al., 2001 and Majid et al., 2000). We have shown here that hypothermia therapy provides a robust resistance to neurodegeneration in the hippocampus in 6-8 week old (adolescent) male C57Bl6 mice after 10 minutes of BCCAO. This finding is consistent with existing evidence that reports that mild hypothermia reduces ischemic damage in the CA-1 hippocampus region (Webster, 2009).

While our studies showed that hypothermia provided clear neuroprotective benefits at 72 hours, we have not thoroughly investigated whether these benefits were sustained in the long-term. Prior studies have shown only transient protective effects of hypothermia in the global cerebral ischemia model when cooling begins during
reperfusion and is maintained for 3 hours (Dietrich et al., 1993; Shuaib et al., 1995). However, long-term protection has been documented in several studies. Following global cerebral ischemia, hypothermic protection was sustained out to 6 months provided cooling was maintained for 24 hours (Colbourne and Corbett, 1995), but the effect was not as robust as at 1 month post-ischemia. In a study of tMCAO, intraischemic hypothermia for 2 hours resulted in protection that was sustained up to 2 months (Maier et al., 2001). Therefore, long-term protection can be attained following relatively short durations of cooling provided that hypothermia begins immediately after ischemic onset, otherwise cooling can be delayed but the duration of cooling must be prolonged.

**Morris Water Maze**

While the MWM has been widely used to assess learning and memory in various contexts, it does have some limitations regarding the translatability of its findings to humans. Indeed neurobehavioral deficits are less translatable than other toxic end-points, such as pathological change. Behaviors tend to be highly species-specific, as they have been adapted to the survival needs of the species. And while spatial learning is a good behavioral end-point in rodents, it is not as well developed or used as a measure of cognitive function in humans. Hence, the direct extrapolation of the MWM task is ill advised. However, the functional mechanisms of the brain and their involvement in mediating behaviors are shared among most mammalian species. Therefore, changes in murine behavior are likely to reflect differences found in human, albeit they may be manifested in a different pattern of behavior.
**Contextual Fear Conditioning**

The hyperactivity account discussed in section 5.6 raises an important issue. Any physiological manipulation (i.e. lesion, drug, mutation) of the hippocampus has the possibility of disrupting performance of the measured response (activity versus freezing), rather than the learning and/or memory that mediate these behaviors. It is possible that the hippocampal damage caused by ischemia caused the ischemic animals to become hyperactive. This change ultimately interfered with our study of the animal’s fear learning and memory. Hence, it is critical to include controls for such performance effects. One possible experiment to test whether the ischemic animals are indeed hyperactive would be the open-field test. This test is used to analyze the animal’s exploratory behavior after placing it into an empty bright square arena surrounded by walls. The animal’s hyperactivity can be quantified with the distance traveled, the number of entries into a given area, resting/slow/fast movements, speed, etc.

**5.9 Conclusions**

We have shown that transient global ischemia leads to increased neurodegeneration in the hippocampus, and mild systemic and cerebral inflammatory responses characterized by increases in cytokines IL-1β and KC in the hippocampus and MMP-9 in the peripheral blood. Also global cerebral ischemia increased microglia/macrophage activation in the hippocampus 72 hours post-ischemia. We have demonstrated that mild hypothermia therapy applied for 4 hours immediately after reperfusion attenuated these inflammatory responses. Mild hypothermia preserved the cellular integrity of the hippocampus, and lowered the inflammatory response of this
structure. The preservation of the hippocampus was further examined by a series of behavioral experiments in which memory was assessed. At 7 days post-ischemia, hypothermic-ischemic mice demonstrated improved spatial memory compared to normothermic-ischemic mice in the water maze task. Their superior performance in comparison to the normothermic-ischemic mice was again observed on day 21. Training in a contextual fear paradigm revealed that hypothermia preserved contextual fear response 21 days post-ischemia. Taken together, these results indicate that hypothermia therapy can attenuate the global ischemic inflammatory response, which may provide long-term benefits in memory performance.
Chapter 6

Future Directions

While our studies showed that hypothermia provided neuroprotective benefits at 72 hours, we have not thoroughly investigated whether these benefits were sustained in the long-term. Prior studies have shown only transient protective effects of hypothermia using the global cerebral ischemia model with cooling beginning during reperfusion and maintaining the cooling for 3 hours (Dietrich et al., 1993; Shuaib et al., 1995). However, long-term protection has been documented in several other studies. Following global cerebral ischemia, hypothermic protection was sustained for up to 6 months provided cooling was maintained for 24 hours (Colbourne and Corbett, 1995), but the effect was not as robust as at 1 month. In a study of tMCAO, intra-ischemic hypothermia for 2 hours resulted in protection that was sustained up to 2 months (Maier et al., 2001). Therefore, long-term protection can be attained for relatively short durations of cooling provided that hypothermia begins immediately after ischemic onset. If the onset of cooling is delayed the duration of cooling must be prolonged. To determine whether hypothermia’s neuroprotection is sustained, we will further assess the long-term histological outcome at 28 days post-ischemia by doing hematoxylin and eosin staining.

Histopathological examination of the mice used for the behavioral tests at 28 days could also be helpful in demonstrating the extent of hippocampal damage in these animals. This experiment would allow us to directly attribute the differences in learning and memory found in the Morris water maze and fear conditioning experiments to
hippocampal damage. Furthermore, we also hope to do immunohistological labeling for neurogenesis in the hippocampus at this late time-point to determine whether neurogenesis occurred in our model, and whether hypothermia therapy has an effect on this neurogenesis (please see section 5.5 for discussion on neurogenesis and spatial learning).

In the future, we hope to repeat our studies of the systemic and cerebral inflammatory response post-ischemia using a more sensitive protein detection method. We also hope to expand these studies. Specifically, we have banked serum and tissue taken at 24 hours, 72 hours, 1 week, and 3 weeks post-surgery, and plan to run multiplex immunoassay panels at each of these timepoints for both the peripheral blood and cerebral tissue, namely the hippocampus, cortex, striatum, midbrain, and cerebellum. These additional experiments will provide more detailed temporal and spatial expression profiles post-ischemia. Studying the molecular responses at 1 week and 3 week will help elucidate the mechanisms underlying the spatial and fear learning and memory performance of the animals in the 3 experimental groups.

Although hypothermia has been shown to effectively exert neuroprotection in experimental models, its performance in the clinical setting has been less than ideal. This may be due to the fact that neuroprotection can be age, sex, and background dependent. These variables are highly variable across in vivo studies, and have been a major confounder when interpreting findings. Therefore to determine whether the neuroprotective effects of hypothermia therapy can be generalized across age, sex, and background, it would be necessary to repeat these experiments in both young and aged, male and females, and in multiple genetic backgrounds.
Our microglial (Iba-1) stains showed a clear inflammatory response in the normothermic-ischemic group, and an apparent decrease in microglial staining in the hypothermic-ischemic group. However, additional IHC experiments would provide a more thorough understanding of the inflammatory profile in the hippocampus. In particular, we plan to study the extent of neutrophil infiltration into the hippocampus, the presence of activated astrocytes, the elaboration of inflammatory mediators (IL-1β, TNFα, IL-6, etc.), and the role of NF-κB, a major transcription factor involved in the inflammatory response.

To study the therapeutic window of hypothermia therapy in mice, we plan to repeat our experiments in normothermic-ischemic and hypothermic-ischemic mice with delayed temperature regulation. These findings may be clinically relevant in that they more closely mimic real-world scenarios in which there is a delay before patients reach treatment centers.

We completed the water maze experiments to study both the effects of pre-training and post-training ischemia on spatial memory. During the second training sessions from days 7 to 11, we observed that the animals’ performance did not reach the levels previously seen in training trials from the first set of training (days -7 to -3). This difference in learning can be attributable to the fact that the second task is more complex, requiring the mice to both recognize that the former platform is no longer in the original location and that it is now in another place. Taking this increase in task complexity into account, we plan to study the spatial memory in mice that have suffered an ischemic insult prior to any training.
We have mentioned the issue of hyperactivity in our normothermic-ischemic mice during the contextual fear training. To address this issue, we plan to first determine whether these mice’s behavior is due to changes in locomotor activity or to changes in responses to fear. If in fact the cause is the former, we hope to continue studying how hypothermia affects fear memory by using other measures of fear and stress, such as fecal amount, urine quantity, and borrowing, grooming and stereotypic behaviors.
References


Chen Y, Hallenbeck JM, Ruetzler C, Bol D, Thomas K, Berman NE, Vogel SN (2003) Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates...


Thoreson M, Penrice J, Lorek A, Cady EB, Wylezinska M, Kirkbride V, Cooper CE,


List of Figures and Tables

Figure 1. Ischemia/Reperfusion cascade. Global cerebral ischemia occurs when there is a reduction of blood flow to the brain. It is representative of cardiac arrest as opposed to focal ischemia, which is the experimental correlate to stroke. The return of blood flow, reperfusion, is necessary for the ultimate survival of brain tissue, but is also associated with secondary brain injury. Together, they initiate a complex cascade of pathological events that ultimately lead to ischemic brain damage. The focus of this thesis is neuro-inflammation, which is believed to develop as a consequence of microglia and astrocyte activation, and the mobilization and infiltration of peripheral inflammatory cells into the brain. The development of this inflammation is coordinated by activation, expression, and secretion of numerous pro-inflammatory genes/mediators from the brain parenchyma and vascular cells, including cytokines/chemokines, and adhesion molecules. These changes then disrupt the blood-brain barrier, increase neuronal vulnerability and feedback to further stimulate gliosis. Ultimately, cell death occurs. Therefore, the therapeutic targeting of the neuroinflammatory pathways in post-ischemia is an important area of research. This figure shows a simplified flow-chart that outlines the elements of the ischemic/reperfusion cascade that are most relevant to this thesis. In reality, the exact mechanisms of neuroinflammation are not known, and the relationship between inflammatory mediators and cell death are also not understood. The anti-inflammatory mechanisms of hypothermia therapy are also not understood. There is a strong need for this research because cardiac arrest, particularly in the pediatric population, is associated low survival rates and severe neurological deficits.
Global Cerebral Ischemia/Reperfusion

- Neuro-inflammation
  - Microglial activation
  - Astrocytic activation
  - Mobilization/infiltration of leukocytes
  - Release of inflammatory mediators: NO, superoxide, interleukins, MMP, TNF, MCP-1, adhesion molecules
  - BBB breakdown
  - Neuronal vulnerability

- Stimulate gliosis

Neuronal death
**Figure 2. Timeline for molecular and behavioral experiments.** For molecular experiments, ischemia/sham surgeries with normothermia/hypothermia therapy occurred at day 0. Twenty-four (24) hours later, mice were sacrificed and the systemic inflammatory response in the peripheral blood was measured. At 72 hours post-ischemia, the hippocampal inflammatory response was quantified, as well as the extent of neurodegeneration and microglial/macrophage activation in the hippocampus and barrel field cortex. All molecular experiments are noted in bold text. For details on the timelines of the behavioural experiments, please refer to figure 3.
Figure 3. Timeline for Morris water maze experiments. The mice were trained, for 5 days in 2 3-trial blocks per day, to locate a submerged platform in a circular pool using spatial cues. The animals were then randomized to the 3 experimental groups, NT-sham, NT-2VO, and HT-2VO, after which they underwent sham surgery with 4h of normothermia, ischemia with 4h of normothermia, or ischemia with 4h of hypothermia therapy, respectively. Seven (7) days later, the first 60s probe test was performed. One hour after the first probe test, platform training was resumed for 5 days as previously described. A ten (10) day period elapsed before the second 60s memory probe test was performed.
Figure 4. Rectal temperature recordings of experimental animals during 10-mins of surgery and 4 hours post-surgery. Ischemia/sham mice were maintained at 37 ± 0.5 °C during 10-minutes of bilateral common carotid artery occlusion or sham operation (A), then at normothermia (37 ± 0.5 °C) or hypothermia (32 ± 0.5 °C) for 4 h post-surgery (B). Positive control mice that received lipopolysaccharide (LPS) injections were also maintained at 37 ± 0.5 °C for 10 minutes after injection (C), then at normothermia (37 ± 0.5 °C) for 4 h post-injection (D). Temperature recordings were made every 1 minute over the 10-minute occlusion/injection time interval, then every 15 minutes over the 4 h post-operation interval. The data shown are means ± SEM of temperature recordings taken from 5 animals per group at the indicated time-points.
Figure 4

A

B

C

D

Mean temperature (°C)

Time (min)

Mean temperature (°C)

Time (min)
Figure 5. Hypothermia did not significantly affect the systemic concentrations of inflammatory mediators 24 hours following GCI. (A-C) No differences were found in the concentrations of soluble E-selectin (sE-selectin; A), soluble vascular adhesion molecule-1 (sVCAM-1; B), soluble intercellular adhesion molecule-1 (sICAM-1; C) between the 3 experimental groups. (D) An increase in the peripheral blood concentration of matrix metalloproteinase-9 (MMP-9) was found 24 hours post-ischemia in the NT-2VO mice when compared to those in NT-sham group. However levels of MMP-9 in the HT-2VO mice were not significantly different to those found in both the NT-sham and NT-2VO mice. (E) Decreased systemic concentrations of plasminogen activator inhibitor-1 (PAI-1) were found in the HT-2VO group when compared to NT-sham. Meanwhile levels of PAI-1 in the NT-2VO mice were not significantly different from those measured in the NT-sham and the HT-2VO group. (n=4-5 mice/group) Data shown as mean ± s.e.m. *p<0.05 compared to NT-sham, **p<0.05 compared to NT-2VO.
Figure 5

(A) **sE-selectin**

(B) **sVCAM-1**

(C) **sICAM-1**

(D) **MMP-9**

(E) **PAI-1**
Figure 6. Hypothermia attenuated the concentrations of pro-inflammatory mediators IL-1β and KC in the hippocampus 72 hours following cerebral ischemia. Concentrations of the pro-inflammatory cytokines (A-C), anti-inflammatory cytokine (D), growth factor cytokines (E-F) and chemokines (G-H) were measured in the hippocampus using multiplex immunoassays 72 hours after global cerebral ischemia. At this timepoint, levels of IL-1β (A) and KC (C) of the NT-2VO mice showed significant increases compared to NT-sham tissue. This inflammatory response was attenuated by hypothermia therapy (p<0.05). Mean concentrations of IL-6 (B) were found to be mildly elevated post-ischemia in the NT-2VO group when compared to NT-sham, while those of IL-10 (D), GM-CSF (E), and G-CSF (F) were slightly decreased. While such trends were found, no statistically significant differences were reported between the 3 experimental groups for each of these analytes. There were also no significant changes in the hippocampal concentrations of chemokines MCP-1 (G) and TNFα (H) between the 3 experimental groups. (n=4-5 mice/group) Data shown as mean ± s.e.m. *p<0.05 compared to sham; ** p<0.05 compared to NT-2VO.
Figure 6

A. IL-1β

B. IL-6

C. KC

D. IL-10

E. GM-CSF

F. G-CSF

G. MCP-1

H. TNF-α
Figure 7. Hypothermia reduced microglia and macrophage activation 72 hours after global cerebral ischemia (GCI). Representative images from the Iba-1 histochemical stains of the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) regions and cerebral cortex from normothermic-sham (NT-sham), hypothermic-sham (HT-sham), normothermic-ischemic (NT-2VO), and hypothermic-ischemic (HT-2VO) mice. Mice that received intraperitoneal injections of lipopolysaccharide (LPS) to induce bacterial meningitis were used as positive controls. Increased Iba-1 staining was found in the NT-2VO brains, but was nearly undetectable among NT-sham, HT-sham and HT-2VO animals. Scale bars = 50 μm. 20x, 5μm.
Figure 7

<table>
<thead>
<tr>
<th></th>
<th>CA-1</th>
<th>CA-2</th>
<th>CA-3</th>
<th>DG</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td><img src="image1" alt="LPS CA-1" /></td>
<td><img src="image2" alt="LPS CA-2" /></td>
<td><img src="image3" alt="LPS CA-3" /></td>
<td><img src="image4" alt="LPS DG" /></td>
<td><img src="image5" alt="LPS Cortex" /></td>
</tr>
<tr>
<td>NT-sham</td>
<td><img src="image6" alt="NT-sham CA-1" /></td>
<td><img src="image7" alt="NT-sham CA-2" /></td>
<td><img src="image8" alt="NT-sham CA-3" /></td>
<td><img src="image9" alt="NT-sham DG" /></td>
<td><img src="image10" alt="NT-sham Cortex" /></td>
</tr>
<tr>
<td>HT-sham</td>
<td><img src="image11" alt="HT-sham CA-1" /></td>
<td><img src="image12" alt="HT-sham CA-2" /></td>
<td><img src="image13" alt="HT-sham CA-3" /></td>
<td><img src="image14" alt="HT-sham DG" /></td>
<td><img src="image15" alt="HT-sham Cortex" /></td>
</tr>
<tr>
<td>NT-2VO</td>
<td><img src="image16" alt="NT-2VO CA-1" /></td>
<td><img src="image17" alt="NT-2VO CA-2" /></td>
<td><img src="image18" alt="NT-2VO CA-3" /></td>
<td><img src="image19" alt="NT-2VO DG" /></td>
<td><img src="image20" alt="NT-2VO Cortex" /></td>
</tr>
<tr>
<td>HT-2VO</td>
<td><img src="image21" alt="HT-2VO CA-1" /></td>
<td><img src="image22" alt="HT-2VO CA-2" /></td>
<td><img src="image23" alt="HT-2VO CA-3" /></td>
<td><img src="image24" alt="HT-2VO DG" /></td>
<td><img src="image25" alt="HT-2VO Cortex" /></td>
</tr>
</tbody>
</table>
Figure 8. Mild hypothermia reduced microglia/macrophage staining 72 hours after global cerebral ischemia. Representative images from the Iba-1 histochemical stains of the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) regions and cerebral cortex of a normothermic-ischemic (NT-2VO) mouse (A-E, respectively), and a hypothermic-ischemic (HT-2VO) mouse (F-J) shown at 40x magnification to show cell morphology. Scale bars =50 µm. 40x, 5µm.
Figure 8
Figure 9. Hypothermia decreased microglia and macrophage staining in C57Bl/6 mice 72 hours after global cerebral ischemia. Positively-stained cells from the Iba-1 stained sections from normothermic-sham (NT-sham), hypothermic-sham (HT-sham), normothermic-ischemic (NT-2VO), hypothermic-ischemic (HT-2VO), and lipopolysaccharide-injected (LPS) mice were quantified in the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) regions and in the cerebral cortex. Among the HT-2VO mice, cell counts were significantly lower than normothermic (NT-2VO) in all regions. Among the LPS mice, cell counts were not significantly different from those found in NT-sham mice in all regions, except for the cortex. Iba-1 staining was nearly undetectable among NT-sham animals, and detected at low levels in the HT-sham mice. Scores were no different between HT-sham and NT-sham groups in all regions, except the CA-2 and cerebral cortex. Data shown as mean ± s.e.m. n=7 per group. * p<0.05 vs. NT-sham, ** p<0.05 NT-2VO.
Figure 9

![Bar charts showing cell count in different regions (CA-1, CA-2, CA-3, DG, Cortex) across different conditions (NT-1h, NT-2VO, HT-1h, HT-2VO, LPS).](image-url)
Figure 10. Hypothermic-ischemic (HT-2VO) mice showed fewer FluoroJade B-positive cells 72 hours post-ischemia. Representative images of the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) regions, and the cerebral cortex stained with FluoroJade B to detect degenerating neurons in normothermic-sham (NT-sham), hypothermic-sham (HT-sham), normothermic-ischemic (NT-2VO), and hypothermic-ischemic (HT-2VO) mice. FluoroJade B-positive cells were nearly undetectable in both sham groups, and were less detectable in the HT-2VO mice in comparison to NT-2VO mice. Scale bar: 62 µm.
Figure 10

<table>
<thead>
<tr>
<th></th>
<th>CA-1</th>
<th>CA-2</th>
<th>CA-3</th>
<th>DG</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT-sham</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>HT-sham</td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>NT-2VO</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>HT-2VO</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 11. Mild hypothermia reduced neurodegeneration in the hippocampus CA-1 and CA-2 regions 72 hours following global cerebral ischemia. Cell counts of FluoroJade B positive cells in the hippocampus CA-1, CA-2, CA-3, dentate gyrus (DG) regions, and the cerebral cortex of normothermic-sham (NT-sham), hypothermic-sham (HT-sham), normothermic-ischemic (NT-2VO), and hypothermic-ischemic (HT-2VO) mice. HT-2VO mice showed significantly decreased numbers of degenerating neurons following ischemia in the CA-1 and CA-2 regions of the hippocampus compared to NT-2VO mice (p<0.05). No difference in cell counts were found between the NT-2VO and HT-2VO groups in the CA-3, DG, and cerebral cortex. Data shown as mean ± s.e.m, n=7 per group. *p<0.05 compared to NT-sham group, and **p<0.05 compared to NT-2VO.
Figure 11

Cell Count

**CA-1**

- NT-sh
- HT-sh
- NT-2VO
- HT-2VO

**CA-2**

- NT-sh
- HT-sh
- NT-2VO
- HT-2VO

**CA-3**

- NT-sh
- HT-sh
- NT-2VO
- HT-2VO

**DG**

- NT-sh
- HT-sh
- NT-2VO
- HT-2VO

**Cortex**

- NT-sh
- HT-sh
- NT-2VO
- HT-2VO
**Figure 12. Hypothermia improves spatial memory 7 days following global cerebral ischemia.** (A) Prior to ischemia or sham operation the mice were trained, for 5 days with 2 sessions per day, to locate a submerged platform in a circular pool using spatial cues. 

(B) Schematic map of the zones of the water maze, including the target zone (TZ) where the submerged platform was located, adjacent left (AdjL), opposite (Opp), and adjacent right (AdjR) zones. (C) Time required to reach the platform for each group of animals was recorded. Mice showed improved performance, as exhibited by steady decreases in escape latency, over the 5-day training period. No difference in performance was found between the 3 experimental groups by the last day of training. (D) Three days after the end training, mice were randomized to one of the normothermic-sham (NT-sham, n=14), normothermic-ischemic (NT-2VO, n=16), or hypothermia-ischemic (HT-2VO, n=16) groups. At 7 days following surgery, a 60s trial memory retention probe test was given, in which the platform was removed from the pool and the mouse was allowed to search for it. HT-2VO mice spent significantly more time in the TZ compared to NT-2VO mice (p<0.05), but still significantly less time than NT-sham mice (p<0.05). (E) The percent of time spent in an area within 10 cm of the tank wall was recorded. HT-2VO mice spent similar amounts of time in this zone as NT-sham mice, and significantly less time there than NT-sham (p<0.05). (F) The average swim velocities of the animals were similar across experimental groups during the probe test. All data displayed as mean ± s.e.m. *p<0.05 compared to NT-sham, and **p<0.05 compared to NT-2VO.
Figure 12

A

Training 1
Days -7 to -3
NT-sham
NT-2VO
HT-2VO

7 d
Probe 1
Day 7

Training 2
Days 7 to 11

10 d
Probe 2
Day 21

Ischemia/Sham surgeries
Day 0

B

Opp
AdjR
AdjL
TZ

C

D

Escape latency (sec)

Day

-7
-6
-5
-4
-3

Time spent in zone (sec)

NT-sham
NT-2VO
HT-2VO

E

Time spent by pool wall (sec)

NT-sh
NT-2VO
HT-2VO

F

Swim speed (cm/sec)

NT-sham
NT-2VO
HT-2VO
Figure 13. Hypothermia improves spatial memory 21 days following global cerebral ischemia. (A) One hour following the first probe test, platform training was resumed for 5 days as previously described. (B) As the training progressed, mice from all 3 experimental groups showed improvements in their ability to locate the platform with general decreasing trends in escape latency. By day 11, there was no significant difference in performance between the 3 experimental groups. (C) Another single 60s trial memory retention probe test was administered 7 days following this training period. HT-2VO mice spent similar amounts of time in the TZ as NT-sham mice, which was significantly more time than NT-2VO mice (p<0.05). (D) The time spent in an area within 10 cm of the tank wall was recorded. NT-2VO animals spent significantly more time in this zone than NT-sham mice (p<0.05). (E) The average swim speed of the animals was similar across experimental groups during the probe test. All data displayed as mean ± s.e.m. *p<0.05 compared to NT-sham; **p<0.05 compared to NT-2VO.
Figure 13

A

Training 1
Days -7 to -3

NT-sham

NT-2VO

HT-2VO

7 d

Probe 1
Day 7

Training 2
Days 7 to 11

Ischemia/Sham surgeries
Day 0

10 d

Probe 2
Day 21

B

C

D

E

Escape latency (sec)

Time spent in zone (sec)

Time spent by pool wall (sec)

Swim speed (cm/sec)

Day

NT-sham

NT-2VO

HT-2VO

TZ

AdjL

Opp

AdjR

NT-sham

NT-2VO

HT-2VO

NT-sham

NT-2VO

HT-2VO

NT-sham

NT-2VO

HT-2VO
Figure 14. Hypothermia preserves contextual fear response 21 days post-ischemia.

(A) Twenty-one (21) days after the ischemia/sham surgeries, animals underwent a classical contextual fear training paradigm, whereby they were acclimatized to a chamber for 120 s before receiving 3 mild foot shocks (0.5 mA) at 210, 240, and 270 s, and then allowed to remain in the chamber for an additional 30s. (B) During this training session, percent freezing before the first shock and after the last shock were measured to determine the animal’s response to the adverse stimuli. HT-2VO mice exhibited freezing responses similar to that of NT-sham mice. Animals from the NT-2VO group showed less freezing than both the NT-sham and HT-2VO mice (p<0.05). (C) After a 7 d delay, contextual fear memory was assessed for 4 mins by re-introducing the mice to the training chamber with the contextual cues preserved. HT-2VO mice exhibited similar freezing response patterns to NT-sham mice. However, the freezing responses in these two groups were not significantly different than NT-2VO mice when percent freezing responses were measured for each minute over the entire testing period (C), and when (D) total percent freezing over the entire 4 mins testing period was analyzed. All data displayed as mean ± s.e.m. *p<0.05 compared to NT-sham, **p<0.05 compared to NT-2VO.
Figure 14

A

B

C

D

Figure 14

A

B

C

D