Use of Therapeutic Hypothermia after Stroke: Beyond Neuroprotection

by

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

Doctor of Philosophy

University of Alberta

ABSTRACT

Mild therapeutic hypothermia (HYPO), decreasing brain temperature to 32-35°C, is the gold standard neuroprotectant against ischemia. Cooling also ameliorates several cell death mechanisms in other types of brain injury (e.g., traumatic brain injury). As with many other neuroprotectants, HYPO not only reduces neurodegeneration but cooling may affect repair mechanisms. Currently, HYPO is in clinical trials for ischemic and hemorrhagic stroke. Its success highly depends on our understanding of how cooling interacts with stroke pathophysiology and recovery. In turn, this allows us to customize HYPO to different types of brain insults. In this thesis, we carried out a series of experiments to elucidate the impact of HYPO beyond its neuroprotective properties.

First, we conducted a study to determine whether HYPO affected plasticity in the contralesional hemisphere after motor cortex devascularization in rats. Stroke patients get HYPO to the whole brain and this may impact plasticity processes in the intact hemisphere, an important factor in recovery after stroke. We initially hypothesized that contralesional cooling would impair forelimb reaching success. Instead, we found that early contralesional HYPO did not worsen forelimb reaching but reduced the tendency for rats to use their unimpaired limb. Additionally, we tested whether HYPO affected learning of a reaching task in otherwise naïve rats. We found that reaching success was slightly lower in the rats that received cooling, although this was not significant. These findings suggest that even though HYPO is neuroprotective and safe after focal ischemia, it may still have additional effects on functional recovery after stroke.

In the second set of experiments, we assessed the impact of HYPO on an often neglected aspect of intracerebral hemorrhage (ICH) pathophysiology, seizures. Seizures occur in one third

of ICH patients. Cooling confers some benefit after ICH and reduces seizure activity in patients with status epilepticus and in infants with hypoxic-ischemic injury. Therefore, we hypothesized that HYPO would reduce seizure activity after ICH in rats. First, we established that seizures occurred in 66% of collagenase-induced ICH rats, but did not occur in the whole-blood model. Second, using the collagenase model we found that mild localized HYPO reduced the incidence of rats with seizures although this was not significant. Cooling may improve outcome by several mechanisms after ICH (e.g., decreasing edema, inflammation, etc.), however seizure activity may not be one of them.

Altogether, my thesis work shows that HYPO may impact some aspects of stroke pathophysiology (e.g., use of impaired limb) but not others (e.g., seizures after ICH). This has implications to the patient population, as stroke victims are cooled for days or even weeks. Therefore, HYPO may impact plasticity processes that predominate during this period, possibly affecting recovery. The effectiveness of HYPO varies depending on the protocol used (e.g., duration) as well as severity and type of insult (e.g., ischemia vs. ICH). Therefore, further studies should be conducted to elucidate how to maximize HYPO neuroprotective properties while minimizing potential negative side effects (e.g., decreasing plasticity).

PREFACE

This thesis is an original work and received research ethics approval from the University of Alberta Animal Care and Use Committee for Biosciences, Project AUP960.

Part of Chapter 1 of this thesis has been submitted for publication as a book chapter in Therapeutic Hypothermia and Targeted Temperature Management in Acute Neurological Disorders, Editors: Dr. Romer Geocadin and Dr. Neeraj Badjatia, as Klahr AC, Wowk S and Colbourne F, "Neuroprotective Mechanisms of Therapeutic Hypothermia." All authors were involved in the composition of the manuscript, Colbourne F as the supervisory author.

Chapter 2 of this thesis will be published as Klahr AC, Fagan K, Aziz J, John RF, and Colbourne F. Klahr AC was responsible for the concept formation, data collection, data analysis, and manuscript composition. Fagan K, Aziz J, and John RF were involved in the data collection. Colbourne F was the supervisory author, and was involved with concept formation, data analysis, and manuscript composition.

Chapter 3 of this thesis has been published as Klahr AC, Dickson CT, and Colbourne F., "Seizure Activity Occurs in the Collagenase but not the Blood Infusion Model of Striatal Hemorrhagic Stroke in Rats", in the journal Translational Stroke Research (2015), volume 6, pages 29-38. Klahr AC was responsible for the concept formation, data collection, data analysis, and manuscript composition. Dickson CT assisted with the data analysis and manuscript composition. Colbourne F was the supervisory author and was involved in the concept formation, data analysis, and manuscript composition.

Chapter 4 of this thesis has been published as Klahr AC, Dietrich KM, Dickson CT, and Colbourne F, "Prolonged Localized Mild Hypothermia Does Not Affect Seizure Activity after

Intracerebral Hemorrhage in Rats" in the journal Therapeutic Hypothermia and Temperature Management (2016), volume 6, pages 40-47. Klahr AC was involved in the concept formation, data collection, data analysis, and manuscript composition. Dietrich KM was involved in the data collection and manuscript composition. Dickson CT assisted with data analysis and manuscript composition. Colbourne F was the supervisory author and was involved in the concept formation, data analysis, and manuscript composition.

DEDICATION

I dedicate this thesis to mamá, papá, and my amazing brother, Juan.

ACKNOWLEDGEMENTS

I am indebted to Fred Colbourne for his support throughout the last five years. Aside from the broad academic knowledge I acquired during my time in his lab, he taught me how to become a more resilient and independent researcher. Not to mention that when nothing was working for me when I started my Masters, he believed in me when I did not. For this, I am forever grateful.

I would also like to thank my committee members, Dr. Ian Winship and Dr. Karim Fouad. They have always provided great feedback, and helpful words of encouragement. I am also grateful to Dr. Clayton Dickson, who helped me substantially in my seizure work, and has also provided me with much needed academic advice.

Family and friends like Shannon Wowk, Dustin Danyluk, Juan "Siet" Ferrari, and my life partner Matthew Cardinal, have been my rocks during these past years. Thank you for keeping me sane. I would not have been able to go through all the ups and downs that come with graduate school without you.

Last, I would like to acknowledge the additional help I got from undergraduate and other graduate students, our former technician Yonglie Ma, as well as the staff at the Science Animal Support Services, Department of Psychology, and Neuroscience and Mental Health Institute.

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ABBREVIATIONS

BBB	Blood Brain Barrier
BCE	Before Common Era
Bcl-2	B Cell Lymphoma 2
BDNF	Brain-derived Neurotrophic Factor
BP	Blood Pressure
CA1	Cornu Ammonis
CAA	Cerebral Amyloid Angiopathy
CBF	Cerebral Blood Flow
CIMT	Constraint-induced Movement Therapy
CONTRA	Contralateral
EEG	Electroencephalogram
GABA	Gamma-aminobutyric Acid
GluR2	Metabotropic glutamate receptor 2
hrs.	Hours
HYPO	Therapeutic Hypothermia
ICH	Intracerebral Hemorrhage
ICP	Intracranial Pressure
IEGs	Immediate Early Genes
IPSI	Ipsilateral
IQR	Interquartile Range
JNK	c-Jun N-terminal Kinase
LTP	Long-term Potentitation

M	Mean
MCAO	Middle Cerebral Artery Occlusion
ml	Milliliters
min.	Minutes
NMDA	N-methyl-D-aspartic Acid
pMCAO	Permanent Cerebral Artery Occlusion
RMS	Root Mean Square
SD	Standard Deviation
TBI	Traumatic Brain Injury
TIMP	Tissue Inhibitors of Metalloproteinases
tMCAO	Transient Middle Cerebral Artery Occlusion
t-PA	Tissue Plasminogen Activator

CHAPTER 1

General Introduction

1.1. Introduction

Stroke occurs due to a reduction in cerebral blood supply (ischemia) or a leakage from a blood vessel (hemorrhage). A cascade of cell death processes is elicited within minutes, and this is followed by repair mechanisms that develop over time (Lo, 2008; Murphy and Corbett, 2009; Kolb and Teskey, 2012). These structural and functional changes caused by injury and experience are usually referred as neuroplasticity, and they tend to occur both in the affected as well as the intact side of the brain (Murphy and Corbett, 2009; Kolb and Teskey, 2012). When it comes to developing neuroprotective treatments, researchers tend to overlook how therapies used to reduce neurodegeneration impact neuroplasticity.

One of the most promising treatments for ischemic stroke, which has also shown some benefit in intracerebral hemorrhage (ICH), is therapeutic hypothermia (HYPO) (Choi et al., 2012). The history of HYPO as a treatment for brain injury is fascinating. There are reports of individuals that went into cardiac arrest for hours after falling in icy lakes and survived without any signs of brain damage (Schmidt et al., 1995; Varon and Acosta, 2008; Kieboom et al., 2015). For millennia, cooling has been used to reduce edema and inflammation. Hippocrates recommended using ice packs on wounded soldiers (Hippocrates, 460–375 BC). However, it was Napoleon's surgeon Dominique-Jean Larrey (1766–1842) who noted the benefit of HYPO when he realized that wounded soldiers that stayed closer to the fire were likely to die earlier (Remba et al., 2009). In the 1950s there were successful small clinical trials and animal experiments (Williams and Spencer, 1958; Benson et al., 1959; Zimmerman and Spencer, 1959; Wolfe, 1960). Later studies suggested that HYPO was a dangerous therapy, harming the reputation of this treatment for decades (Michenfelder et al., 1976; Michenfelder and Milde, 1977; Steen et al.,

1979; Steen et al., 1980; Colbourne et al., 1997; Choi et al., 2012). In the late 1980s and early 1990s, meticulous animal research showed that HYPO is a powerful neuroprotectant against ischemia (Busto et al., 1987; Busto et al., 1989a; Colbourne and Corbett, 1994; Colbourne et al., 1997; MacLellan et al., 2009; Choi et al., 2012). Cooling has now been tested in other types of brain injury, in some cases with more success (e.g., focal ischemia) than in others (e.g., hemorrhagic stroke) (MacLellan et al., 2009; Choi et al., 2012).

Even though we might have some insight into how cooling confers its neuroprotection (Colbourne et al., 1997; MacLellan et al., 2009; Choi et al., 2012; Yenari and Han, 2012), we do not have an understanding of what impact this treatment may have on other common aspects of stroke pathophysiology (e.g., seizures) or functional recovery after stroke. My thesis describes a series of experiments assessing the effect of HYPO on the contralesional hemisphere after focal ischemia and on seizure activity after ICH in rats. In order to provide some background to the rationale behind these studies, I will first introduce stroke pathophysiology, post-stroke seizures, treatments, and recovery. I will also discuss the importance of temperature on neuroprotection and the use HYPO as a treatment for ischemic and hemorrhagic stroke, as well as seizures.

1.2. Stroke Epidemiology

Stroke is the second leading cause of death in the world, and the third in Canada (Heart and Stroke Foundation, 2015; World Health Organization, 2015). There are approximately 50,000 annual cases in our country, a number that costs over 3 billion dollars to the health system every year (Heart and Stroke Foundation, 2015). The improvement of medical care have increased the incidence of stroke survivors, but also increased the number of people that live with disabilities (World Health Organization, 2015). Several campaigns encourage stroke prevention through

awareness of stroke risk factors (e.g., diabetes). However, these efforts are outweighed by our increasing population, especially the elderly, as well as our sedentary and poor dietary lifestyles, which have increased the incidence of stroke worldwide in the past decades (Feigin et al., 2009; World Health Organization, 2015).

Ischemic strokes occur in 80% of stroke victims and lead to 15% mortality within a month. Ischemic strokes are the result of an embolus, a clot originated elsewhere in the body, or a thrombus, a clot originated at the site of occlusion, occluding a cerebral blood vessel (Adams et al., 2007; Jauch and Stettler, 2015). These clots are originated from accumulated fat and cholesterol in the walls of blood vessels, a condition known as atherosclerosis (Adams et al., 2007; Jauch and Stettler, 2015). Ischemia can also occur in the whole brain (global ischemia) in cases of systemic hypoperfusion, such as during cardiac arrest. There are other causes of ischemia, for instance due to vasoconstriction during migraines (Sacco and Kurth, 2014).

Unlike ischemic strokes, only 15% of stroke victims suffer from ICH. This devastating stroke leads to 40% in-hospital mortality (van Asch et al., 2010). Of those that do survive, 75% will be either disabled or deceased within a year (van Asch et al., 2010). An ICH can occur due to several reasons, such as trauma, tumors, ischemia, arteriovenous malformation, and aneurysms (van Asch et al., 2010; Balami and Buchan, 2012). All strokes have the same risk factors, such as increased age, hypertension, diabetes, obesity, smoking, alcohol consumption, and high cholesterol (van Asch et al., 2010). However, the most common causes for ICH are high blood pressure (BP) and cerebral amyloid angiopathy (CAA) (van Asch et al., 2010; Balami and Buchan, 2012). About 60% of ICH have pre-existing high BP, which can lead to rupture of thin blood vessels such as those in the thalamus, basal ganglia, pons, and cerebellum (Wityk and Caplan, 1992). In normotensive patients one of the highest predictors of ICH is CAA, a condition

in which β-amyloid protein accumulates and weakens blood vessels. Cortical ICHs are more likely to be caused by CAA (Vinters, 1987).

Sub-arachnoid hemorrhage (SAH) is a hemorrhage occurring at the base of the brain leading to a buildup of blood in the subarachnoid space (Becske and Jallo, 2015). This type of stroke occurs in about 5%, and has an extremely high mortality rate (50%). Main causes of a SAH include trauma, tumor, and aneurysms (Becske and Jallo, 2015). Sub-arachnoid hemorrhages have been the least studied, as it is challenging to model due to its high mortality (Silasi and Colbourne, 2009; Becske and Jallo, 2015). Mostly ICH and focal ischemia will be discussed in this thesis as those were the models used in my experiments.

1.3. Pathophysiology

The clinical manifestation of a stroke varies by what part of the brain is affected. For instance, a stroke in the left hemisphere, where language predominates, may lead to aphasia. Stroke should be considered when a sudden neurological deficit or loss of consciousness occurs (Jauch and Stettler, 2015; Liebeskind, 2015). Common acute stroke symptoms involve paresis, hemisensory deficits, vision problems, dysarthria, aphasia, facial droop, ataxia, vertigo, headache and seizures (Jauch and Stettler, 2015; Liebeskind, 2015). Symptoms may occur alone or in combination. Just by symptoms alone, however, it is challenging to discern between focal ischemia and ICH. Therefore, a final diagnosis is made using brain imaging techniques, such as computed tomography (Jauch and Stettler, 2015; Liebeskind, 2015).

1.3.1. Ischemic Stroke

1.3.1.1. Acute Phase

We refer to the first minutes and hours after ischemia onset as the acute phase, in which the main factors contributing to cell death are substantial changes in metabolism, excitotoxicity, and oxidative stress (for a summary of the phases refer to Table 1-1). In focal ischemia, cell death occurs more rapidly (within minutes) in the ischemic core, where cerebral blood flow (CBF) reduction is the most severe (<20% of baseline, below 10ml/100grams/minute) (Morikawa et al., 1992; Kawai et al., 2000; Yanamoto et al., 2001). Necrotic cell death occurs due to lack of oxygen and glucose leading to energy failure as well as loss of normal ionic gradients and membrane potential. Irreversible damage in the core happens by 3 hours after the stroke (Lipton, 1999). Anoxic depolarization leads to activation of Ca^{2+} channels resulting in neurotransmitter release, excitotoxicity, and oxidative stress (Siesjo, 1992a; Dirnagl et al., 1999). Some cells swell and burst due to cytotoxic edema caused by Na^{+} and Cl^{-} entering into the cell (Siesjo, 1992a; Dirnagl et al., 1999). Furthermore, oxidative stress due to impaired increased intracellular Ca^{2+} and mitochondrial depolarization leads to neuronal and glial cell death (Warner et al., 2004). Oxidants (e.g., peroxynitrite, H_2O_2), including free radicals (e.g., $\cdot O_2^-$, OH·, and ·NO), are highly reactive molecules that lead to DNA damage, protein oxidation, and lipid peroxidation directly causing neurodegeneration (Warner et al., 2004).

Surrounding the core, hypoperfusion occurs (50% of baseline, 20ml/100grams/minute) but cells do not experience permanent anoxic depolarization or cell death (Morikawa et al., 1992; Kawai et al., 2000; Yanamoto et al., 2001). This area is called the ischemic penumbra, where energy metabolism is partially preserved (Astrup et al., 1977; Siesjo, 1992a; Dirnagl et al., 1999; Lo, 2008). As neurons in the ischemic core tend to die quickly, many neuroprotective treatments

tend to target penumbral tissue (Siesjo, 1992b; Lo, 2008). However, neurodegeneration is time-sensitive, as cell death may occur within 24 hours in the penumbra if reperfusion is not resolved or if therapies are not applied (Lipton, 1999; (Lo, 2008).

1.3.1.2. Subacute Phase

The subacute phase encompasses the first few days after ischemia in which gene expression and protein synthesis play a major role in inflammatory, survival, neuroplasticity, and cell death pathways. Extracellular accumulation of glutamate can lead to repeated depolarization, oxidative stress, and calcium overload (Siesjo, 1992a). Studies using electron microscopy suggest that necrotic cell death occurs after ischemia even days after the insult (Colbourne et al., 1999). Apoptosis is genetically driven, controlled, and energy dependent cell death. Still, studies show an increase in caspases, pro-inflammatory genes, cytokines, and apoptotic genes (e.g., p53) thought to be involved in delayed apoptotic cell death occurring over days and weeks (Table 1-1) (Siesjo, 1992a; Dirnagl et al., 1999; Lipton, 1999). After focal ischemia, there may also be a raise in intracranial pressure (ICP), thought to be caused by blood brain barrier (BBB) disruption and edema, which also contribute to secondary injury during the subacute phase.

1.3.1.3. Neuroplasticity and the Chronic Phase

Neuroplasticity processes are induced shortly after injury during the acute and subacute phase and continue to be active for weeks (Figure 1-1) (Dirnagl et al., 1999; Bernabeu and Sharp, 2000; Akulinin and Dahlstrom, 2003). Once brain connections are lost, sensory and motor deficits can be restored by creating new connections (Murphy and Corbett, 2009). After stroke, neuroplasticity processes such as synaptogenesis, neurogenesis, angiogenesis, axonal sprouting,

gliogenesis, as well as increased expression of proteins (e.g., brain derived neurotrophic factor or BDNF) and genes, are crucial for recovery (Murphy and Corbett, 2009). For instance, up-regulation of synaptophysin and growth-associated protein 43 (GAP-43), proteins associated with synaptogenesis, begin during the first days after stroke (Schmidt-Kastner et al., 1997; Nishimura et al., 2000). Neurogenesis occurs during the first weeks, primarily in the dentate gyrus of the hippocampus after global ischemia (Liu et al., 1998; Bendel et al., 2005; Salazar-Colocho et al., 2008; Xiong et al., 2011) and in the subventricular and subgranular zones after focal ischemia (Jin et al., 2001).

Most rewiring occurs in the ipsilateral hemisphere after the stroke, especially in areas surrounding the lesion (Cramer, 2008; Murphy and Corbett, 2009; Kolb and Teskey, 2012). However, the contralesional hemisphere undergoes several changes that are thought to also underlie recovery. In patients, some studies suggest that good recovery has been associated with increased activity in the non-affected hemisphere and less lateralization (Butefisch et al., 2005; Cramer, 2008). Conversely, a longitudinal study determined that ipsilesional activation during paretic limb usage was associated with better outcome whereas activation of bilateral networks was associated with poor outcome (Ward et al., 2003). Contralesional plasticity, however, seems to depend on lesion size (Staudt et al., 2002; Gerloff et al., 2006; Hsu and Jones, 2006; Cramer, 2008; Murphy and Corbett, 2009; Kolb and Teskey, 2012). In a study using transcranial magnetic stimulation, when the contralesional hemisphere was stimulated in stroke patients, those with small lesions had almost no response on the affected hand, whereas patients with larger lesions did. Patients with larger lesions, however, seemed to have no response on the paretic hand when the ipsilesional hemisphere was stimulated. This suggests that in those patients the contralesional hemisphere took over the function of the paretic limb. Interestingly,

patients with moderate lesions had a motor response of the paretic hand when each hemisphere was stimulated, implying that the impaired hand was controlled by both motor cortices (Staudt et al., 2002). Given the conflicting data regarding contralesional activation and post-stroke recovery, it has been suggested that lesion location may be a factor influencing whether plasticity in the non-affected hemisphere benefits a subset of patients (Lotze et al., 2006) but not others (Mansur et al., 2005; Fregni et al., 2006).

Contralesional plasticity occurs in the rat as well. For instance, post-ischemic structural changes occur after weeks in the layer V of the contralesional side (Jones and Schallert, 1994). Callosal projections from the contralesional hemisphere sprout into the peri-infarct cortex (Jones et al., 2013). Hyperexcitability has been detected in the contralesional hemisphere, associated with downregulation of GABA and upregulation of NMDA receptors (Buchkremer-Ratzmann et al., 1996; Qu et al., 1998; Witte, 1998). This leads to reduced use of the impaired limb and hyper-reliance of the unimpaired limb for grasping, grooming, support, and balance (Luke et al., 2004). Rodent studies also suggest that contralesional plasticity depends on the size of the stroke. For instance, large lesions induce synaptogenesis and dendritic growth in the contralesional hemisphere (Hsu and Jones, 2006; Gharbawie et al., 2007), but smaller lesions do not (Gerloff et al., 2006; Hsu and Jones, 2006). Several studies have shown that by inhibiting (Biernaskie et al., 2005) or lesioning (Gharbawie et al., 2007) the contralesional hemisphere, forelimb reaching of the impaired hand worsens after focal ischemia in rats. Thus, part of the recovery after focal ischemia is mediated by contralesional plasticity. Other areas of the brain, such as spinal cord and brainstem, also undergo conformational changes (Murphy and Corbett, 2009). Altogether, animal research suggests that diffuse connectivity has an essential role in recovery after stroke (Murphy and Corbett, 2009). Another view is that the contralesional hyperexcitability inhibits

the impaired limb (Schallert et al., 2003). Indeed, some suggest that the neuroplasticity occurring in the intact side of the brain leads to improved use of the non-paretic limb instead of improving the use of the paretic limb (Jones et al., 2013). Therefore, some interventions used in stroke patients attempt to increase activity in the ipsilesional hemisphere by decreasing activity in the intact side of the brain (Hummel and Cohen, 2006).

1.3.2. Hemorrhagic Stroke

As in ischemia, cell death processes predominate within the first hours after ICH (for a summary of the phases refer to Table 1-2). Once neurodegeneration stabilizes, repair processes develop, similar to the ones that occur after ischemia (Aronowski and Hall, 2005; Tang et al., 2007; Nguyen et al., 2008; Keep et al., 2012; Otero et al., 2012). Some mechanisms of injury, however, are very different among ischemic and hemorrhagic stroke. Primary damage is mainly caused by the mechanical trauma of the initial bleed dissecting through the brain parenchyma, pulling apart neuronal connections and damaging cells (e.g., dendrites), and possibly from ischemia surrounding the area where the vessel ruptured (Auer and Sutherland, 2005; Balami and Buchan, 2012; Keep et al., 2012). Secondary injury occurs over time due to mass effect (pathological effect caused by a growing mass that results in displacement of surrounding tissue), toxic blood components (i.e., iron and thrombin), coagulation cascade, inflammation, BBB disruption, edema, and possibly seizures (Wang et al., 2002; Auer and Sutherland, 2005; Balami and Buchan, 2012; Keep et al., 2012). Both primary and secondary injury can be exacerbated due to hematoma expansion, which is defined as an increased in the size of the hematoma by 30–50% or an absolute change in hematoma volume of 12.5–20 ml in patients (Dowlatshahi et al., 2011). Hematoma expansion occurs in 30% of ICH patients within the first 3 hours after the

stroke (Balami and Buchan, 2012). Hematoma enlargement could be due to dysregulation of hemostasis, breakdown of the BBB, tissue distortion caused by increased mass effect, and reduced venous outflow. Those ICH patients with hematoma expansion are at a higher risk of morbidity and mortality due to midline shift and increased ICP. Indeed, increased ICP leads to herniation and neurological deterioration (Balami and Buchan, 2012). There is controversy regarding whether there is ischemia surrounding the peri-hematoma zone, as areas surrounding the bleed tend to show hypoperfusion (Balami and Buchan, 2012). Small ischemic lesions, however, may occur in regions surrounding large hematomas (Gioia et al., 2015b).

Once blood enters the parenchyma, erythrocyte lysis further contributes to secondary damage by releasing iron and thrombin, which are toxic to the brain. This damage occurs over weeks as iron levels remain high even up to a month after ICH in rats (Auriat et al., 2012b) and in patients (Wu et al., 2010). Iron causes damage through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{\cdot}$), producing highly reactive hydroxyl radicals. This leads to a cascade of oxidative stress, in which hydroxyl radicals irreversibly damage cells causing mitochondrial dysfunction which in turn contributes to further free radical production (Wang et al., 2002). Interestingly, animal studies do not show consistent benefit after administering iron chelators, such as deferoxamine and bipyridine. These drugs have been shown to be protective in some studies (Nakamura et al., 2004; Nakamura et al., 2006; Song et al., 2008; Okauchi et al., 2009), but not others (Warkentin et al., 2010; Auriat et al., 2012b; Caliaperumal et al., 2013). The discrepancy between studies could be attributed to differences in the models used (Frantzias et al., 2011).

Thrombin, a protease involved in the coagulation cascade which converts fibrinogen into fibrin creating a seal on the vessel, also has a role in ICH pathophysiology. Thrombin is a fundamental factor contributing to cessation of bleeding (Lee et al., 1997b; Xi et al., 2006).

Injecting high levels of thrombin directly into the brain, however, can be toxic. Thrombin binds to proteases activated receptors (PAR), leading to apoptotic cell death. Bolus injections of thrombin into the brain have been shown to cause neuronal cell death, atrophy, and edema (Lee et al., 1997b; Xue and Del Bigio, 2001; Xue and Del Bigio, 2005; Caliaperumal et al., 2014). There are studies in which a thrombin inhibitor reduced edema, even when administered 24 hours after the ICH (Mutch et al., 2001). Thrombin can also elicit upregulation of iron transporter transferrin receptor and increase iron levels (Xi et al., 2003; Nakamura et al., 2005). Therefore, both thrombin and iron seem to interact, exacerbating secondary injury.

1.4. Acute Stroke Management

Aside from stabilizing the patient (e.g., lowering BP), it is crucial to assess the risks and benefits of the few possible interventions available to stroke victims (Adams et al., 2007; Jauch and Stettler, 2015; Liebeskind, 2015). For ischemic stroke, acute treatments include thrombolytic therapy, with drugs such as tissue plasminogen activator (t-PA) (Adams et al., 2007; Jauch and Stettler, 2015). The window for this therapy, however, is rather narrow as it can be only used within the first 3-4 hours from stroke onset. After that, there is the risk of complications such as hemorrhagic transformation (Adams et al., 2007; Jauch and Stettler, 2015). In some cases, clinicians may administer t-PA outside of this therapeutic window although this is still restricted within 6 hours post-stroke. Another approach is to surgically remove the clot by using stent retriever devices (Adams et al., 2007; Jauch and Stettler, 2015). In patients with large ischemic strokes, such as those with malignant middle cerebral artery infarction, craniotomies may be performed to reduce ICP as well (Adams et al., 2007; Jauch and Stettler, 2015).

In ICH patients, as with ischemic strokes, anticonvulsants may be used in the presence of seizures, and antihypertensive drugs may be used to lower BP (Hemphill et al., 2015; Liebeskind, 2015). Increased ICP can be managed with osmotic diuretics as well (Hemphill et al., 2015; Liebeskind, 2015). Aside from symptomatic approaches to treat these patients, there is no effective therapy for ICH patients. Clinical trials for clotting factors such as recombinant factor VIIa (rFVIIa) have failed (Hemphill et al., 2015; Liebeskind, 2015). Surgery can be performed to stop the bleeding and suction the hematoma. However, this option is only available to some patients (Hemphill et al., 2015; Liebeskind, 2015). Another therapy that has received much attention is to aggressively decrease BP to reduce hematoma growth (Tsivgoulis et al., 2014; Hemphill et al., 2015; Liebeskind, 2015). Currently, there is evidence that this therapy may modestly improve functional outcome although there has been no effect in hematoma reduction or mortality rates (Gioia et al., 2015a). However, this therapy is currently under clinical trials to assess its efficacy and safety.

1.5. Post-Stroke Seizures

The International League Against Epilepsy (ILAE) defines seizures as transient behavioural signs or symptoms due to abnormal excessive and hypersynchronous brain activity (Fisher et al., 2005). Seizures may originate from one network (focal) or can activate bilateral networks (generalized) in the brain (Westbrook, 2000). Secondary generalization occurs when focal seizures spread, making another area of the brain a seizure focus. Focal seizures can be simple (without alteration of consciousness) or complex (with alteration of consciousness). Generalized seizures can be classified as convulsive (e.g., tonic-clonic seizures, or *grand mal*) or nonconvulsive (e.g., absence seizures, or *petit mal*). During generalized seizures, other abnormal

motor movement may occur (myoclonic, tonic, clonic), or total loss of muscle tone (atonic) (Westbrook, 2000).

Even though seizures might be a presenting sign of stroke, they can also occur after days, weeks, months or years as well (Bladin et al., 2000; De Reuck, 2009; Balami and Buchan, 2012; Guekht and Bornstein, 2012; Hemphill et al., 2015). Indeed, 30% of seizures occurring in patients over 60 years of age are attributed to stroke (Camilo and Goldstein, 2004). Early onset seizures, within the first week after stroke, arise from structural (e.g., lost inhibitory circuitry) and biochemical (e.g., excessive neurotransmitter release) dysfunction whereas later ones are thought to be attributed to glial and meningocerebral scars (contiguous brain and meninges gliosis) (Camilo and Goldstein, 2004; Balami and Buchan, 2012). Clinical studies are highly variable in their report of seizure activity. This is due to small patient samples, differences in the definition of seizures, and lack of continuous EEG monitoring. This latter one is especially concerning considering that in the follow-ups patients are asked to report whether they had seizures and over 60% may not be aware that they did (Guekht and Bornstein, 2012). Taking this information into account, incidence of seizures after ischemic stroke has been reported to be anywhere from 2% to 33% within the first two weeks. In ICH studies, clinical seizures occur between 4% and 30% of ICH patients (Balami and Buchan, 2012; Hemphill et al., 2015). However, subclinical seizures, those only detected with continuous monitoring with an electroencephalogram (EEG), have a much greater incidence, occurring anywhere between 16% and 31% after ICH (Balami and Buchan, 2012). Non-convulsive seizures are rare after focal ischemia, occurring in 5% of patients (Camilo and Goldstein, 2004).

In those patients who suffer from epileptiform activity, the incidence is greatest within the first 24 hours (~70%) and 72 hours after the stroke (~90%), whereas later occurrence is less

likely (~8%) (Balami and Buchan, 2012; Hemphill et al., 2015). Still, epileptic activity is twice as more likely to occur after hemorrhagic stroke (Bladin et al., 2000). This could be attributed to the impact of toxic blood components such as iron and thrombin in the brain. Indeed, these two compounds cause seizures when experimentally infused in the rodent brain (Willmore et al., 1978; Lee et al., 1997a). Risk factors for seizures involve stroke size, poor functional outcome, lobar/cortical location, and younger age for ischemic stroke (Serafini et al., 2015). Predictors of post-ICH seizure activity involve lobar location, hematoma volume, hydrocephalous, intracranial midline shift, and severe neurological deficits (De Reuck, 2009; Garrett et al., 2009; Haapaniemi et al., 2014).

Epilepsy is a condition characterized by two or more unprovoked seizures occurring more than 24 hours apart (1993). Post-stroke epilepsy develops in 2% of patients. Early seizures, occurring within the first week after the stroke, increase the likelihood to develop late seizures (Balami and Buchan, 2012; Haapaniemi et al., 2014; Serafini et al., 2015). Also, those individuals with early seizures are more likely to develop drug-resistant epilepsy (de Greef et al., 2015). Prophylactic administration of antiepileptic drugs would be an intuitive approach to avoid early occurring seizures. However, anti-convulsants such as phenytoin have been associated with fever and poor outcome after ICH (Naidech et al., 2009). Animal studies have also suggested that drugs that depress the nervous system (e.g., GABA agonists), which can also act as anticonvulsants, impair recovery in animal models of stroke (Schallert et al., 1986; Feeney and Sutton, 1987; Hernandez and Schallert, 1990; Hernandez, 1997; Goldstein, 2003). Even though there are not a lot of studies on this topic, the latest guidelines from the American Heart Association/ American Stroke Association advises against prophylactic use of these drugs in ICH patients (Hemphill et al., 2015).

Seizures after stroke can be detrimental to patient outcome in several ways. It is important to note that most seizures, and their potential side effects, occur during the acute stroke period when the risk of complications (e.g., hematoma expansion in ICH) is the greatest. First, systemic effects due to epileptic activity involve release of catecholamines in the blood stream leading to increased heart rate, arrhythmias, fever, pulmonary edema, acidosis (Walton, 1993). Second, seizure activity could potentially affect hemostasis in ICH. For instance, tonic and tonic-clonic seizures (convulsions), types of clinical seizures, are known to increase CBF and metabolism during seizure activity (Bode, 1992; Takano et al., 2011). Hyperperfusion occurs over several minutes prior to and during focal seizures (Federico et al., 2005b; Federico et al., 2005a). Also, increased BP and ICP have also been reported during seizure activity (Perlman and Volpe, 1983; Dunn, 2002). Third, seizure activity could lead to a reduction in CBF, which is a concern in already hypoperfused/ischemic areas of the brain. Even though the epileptic focus (where the seizure originates) tends to be hyperpefused, surrounding areas display low CBF (Federico et al., 2005a). Also, increased ICP caused by seizure activity can lead to hypoperfusion as well (Balami and Buchan, 2012; Hemphill et al., 2015). Fourth, it has been shown that seizures increase excitotoxicity experimentally, which in turn augments metabolic demand and oxidative stress (Willmore and Ueda, 2009; Shin et al., 2011). Fifth, seizures can lead to increased mortality due to raised ICP and/or sudden unexpected death caused by autonomic dysfunction (Walton, 1993; Balami and Buchan, 2012; Hemphill et al., 2015). Last, seizures may also be interfering with recovery by altering plasticity after stroke. In experimental models of epilepsy, it has been found that cortical maps are enlarged (Teskey et al., 2008). However, it has not been yet studied how this may affect rehabilitation in animal models of stroke.

Intuitively, seizures have the potential to worsen outcome after stroke. Yet, researchers have not been able to find a clear link between epileptic activity and patient prognosis (Balami and Buchan, 2012; Hemphill et al., 2015). Several studies have failed to find a relationship between seizures and outcome (Kilpatrick et al., 1990; Reith et al., 1997; Labovitz et al., 2001; Camilo and Goldstein, 2004; Alberti et al., 2008). Still, in a study it was found that a two-fold increase in 30 day mortality have been associated with early seizure incidence even when other factors were accounted for (e.g., hemorrhage) (Szaflarski et al., 2008). Even in that study, seizures were not an independent predictor of poor functional outcome (Szaflarski et al., 2008). A Canadian study also associated higher seizure activity with mortality, and found longer hospitalization time and higher morbidity among those patients with seizures (Burneo et al., 2010). The lack of consensus among these reports could be attributed to how difficult it is to quantify seizure activity in patients, as previously mentioned. Studies should therefore use continuous EEG monitoring to be able to appropriately relate epileptic activity to patient prognosis. As this can be cumbersome to be done in patients, animal research may be a more practical way to study the relationship between seizure activity and outcome after stroke.

1.6. Functional Recovery after Stroke

Behavioural improvements occur within the first months after the stroke, and tend to plateau over time (Jorgensen et al., 1995; Jorgensen et al., 1999; Murphy and Corbett, 2009). Patients with more severe strokes tend to recover slower than those with smaller strokes. In many instances, however, this improvement reflects compensatory behaviours rather than true neurological recovery. In terms of motor function, this means that the kinematics of the movement pre-stroke are not the same as before the injury, but rather the individual uses

different motions to perform a certain task (Murphy and Corbett, 2009). Neurological impairment tends to always be worse than functional outcome (Jorgensen et al., 1999).

Rehabilitation therapy aims to reduce impairment (e.g., paresis) and also disability (i.e., inability to perform activities). Even though there is a relationship between impairment and disability, rehabilitation tends to improve disability more than reduce neurological impairment (Roth et al., 1998). Motor rehabilitation therapy uses neuroplasticity principles to enhance functional recovery, such as repetitive and intensive training (Kleim and Jones, 2008). Most clinical and animal studies suggest that rehabilitation improves functional outcome after stroke, and in some pre-clinical research that this treatment may be neuroprotective (Jorgensen et al., 2000; Caliaperumal and Colbourne, 2014). The extent of recovery provided by rehabilitation depends on several factors, such as lesion type, size, location, age, and co-morbidities (Jorgensen et al., 1999). Given that plasticity processes are more prevalent during the first week after the stroke, it is recommended for rehabilitation therapy to start as early as possible (Nudo and Milliken, 1996; Paolucci et al., 2000; Biernaskie et al., 2004).

Some therapies prevent patients from engaging in compensatory behaviours that may be maladaptive. Learned non-use is an example of such behaviour, as this occurs when the patient stops using their impaired limb and relies on the use of their non-affected limb instead. Learned non-use was first noted in monkeys that tended to solely use their unimpaired limb after the other forelimb was deafferented (Taub, 1980). The monkeys learned that they could perform tasks more successfully if they used their non-paretic limb. A form of rehabilitation called constraint-induced movement (CIMT) therapy was then developed to treat learned non-use. During CIMT monkeys were forced/encouraged to use their affected arm, as their unimpaired limb was either restrained and/or discouraged from use (Taub et al., 1999). Currently, this treatment has been

used in patients that also suffer from learned non-use (Taub and Morris, 2001). Generally, early CIMT is suggested as well, as the brain tends to be more plastic within the sub-acute stage. However, it is not clear whether intervening too early may be detrimental as it may exacerbate damage (e.g., via hyperthermia) (Kozlowski et al., 1996; Humm et al., 1998; DeBow et al., 2004). This is also the case in patients, as early high intensity CIMT (within 10 days of the stroke) resulted in less motor improvement (Dromerick et al., 2000). The intensity of the treatment may also have an impact. For instance, a clinical trial showed that applying CIMT (from 2 weeks to 3 months after the stroke) in patients was not more or less beneficial than intervening years after the stroke (Boake et al., 2007; Wolf et al., 2010).

1.7. Animal Models of Stroke

1.7.1. Ischemic Stroke

The use of animal models allows us to further understand stroke pathophysiology to develop adequate treatments in a more controlled setting (Carmichael, 2005; Kleim et al., 2007). Focal ischemia has been modeled in several species, but I will just focus on the rat as that is the model used in this thesis. Most models involve the occlusion of the middle cerebral artery (MCA), as MCA territory strokes are common in humans (Carmichael, 2005; Kleim et al., 2007). Proximal middle cerebral artery occlusion models usually mimic malignant middle cerebral artery infarctions, which are very severe and large strokes with high mortality rates and not as common as smaller strokes (Carmichael, 2005). Transient or permanent middle cerebral artery occlusion (tMCAO or pMCAO) can be achieved by insertion of an intraluminal suture through the internal common carotid, which in turn blocks flow to the MCA (Longa et al., 1989). This method is widely used, although it is technically challenging as hemorrhages may occur if the MCA is

punctured. Also, either tMCAO or pMCAO may be performed by surgically clamping or cauterizing the proximal or distal MCA. This technique causes consistent lesions, but requires a craniotomy. Embolic strokes can be mimicked by injecting clots or artificial spheres into the MCA (Kaneko et al., 1985; Futrell et al., 1989). Even though this simulates what occurs in stroke patients better, the variability in the size of the lesions makes it a less consistent model to work with. Focal ischemia can also be induced by injecting endothelin-1, a vasoconstrictor, in different brain structures (e.g., striatum, cortex-(Agnati et al., 1991)). Small and consistent lesions can be achieved by injecting photothrombotic dyes into the cortex as well (Futrell et al., 1988). Last, cortical focal ischemia can be modeled via devascularization of the cortex, either by cauterization or pial stripping (Gonzalez and Kolb, 2003). Pial stripping, however, may also lead to hemorrhage. Along with photothrombotic strokes, devascularization has also been criticized as they both cause some extent of mechanical damage and edema (Carmichael, 2005; Kleim et al., 2007). Still, these models are able to cause consistent unilateral lesions.

1.7.2. Hemorrhagic Stroke

Compared to focal ischemia, there are substantially fewer animal models of ICH. One of the most widely used methods involves stereotaxically infusing bacterial collagenase, which degrades the basal lamina, the extracellular layer that holds vascular endothelial cells together (Rosenberg et al., 1990). Another commonly used ICH model involves injecting autologous whole blood into the brain (Bullock et al., 1984; Manaenko et al., 2011; MacLellan et al., 2012). Both of these models are often used to create striatal ICH, as this stroke location tends to occur most commonly in patients. The collagenase model better mimics hematoma expansion (MacLellan et al., 2008). In turn, this model also tends to create bigger lesions, with more

inflammation and edema, increased ICP, and worse behavioural deficits compared to the whole blood model (MacLellan et al., 2008; Hiploylee and Colbourne, 2014). Although not commonly used, spontaneously hypertensive rats will also suffer of spontaneous ICHs (Okamoto et al., 1973). This model, however, is highly variable as rats may have more than one hemorrhage, and location may vary, making it difficult to compare among individuals in experiments. Another model involves stereotaxically inserting a "microballoon", which once inflated it mimics mass effect produced by hematoma expansion (Sinar et al., 1987). Other more simplistic methods, such as bolus iron or thrombin injections, have also been used to study the impact of toxic blood factors released during ICH (Willmore et al., 1978; Lee et al., 1997b; Nakamura et al., 2006; Caliaperumal et al., 2012; Caliaperumal et al., 2014).

1.8. Temperature and Neuroprotective Treatments

Neuroprotective therapies aim to prevent salvageable neurons from dying by targeting one or more neurodegenerative or protective pathways. Common targets include excitotoxicity, inflammation, oxidative stress, etc. Despite the plethora of mechanisms and drugs, clinical success has been abysmal. For instance, one review identified over 1000 neuroprotectants that were tested in animal models of focal ischemia, of which none had shown clinical efficacy (O'Collins et al., 2006). In an effort to improve upon translational success, numerous key issues have been identified regarding the quality of animal neuroprotection studies, such as the lack of both blinding and randomizing (O'Collins et al., 2006). Physiological confounds were also widely acknowledged as an important and common design weakness (O'Collins et al., 2006). Accurate and complete reporting of experimental details (e.g., exclusions and mortality) and data

is widely noted as a key way to improve translational success (e.g., ARRIVE guidelines) (Kilkenny et al., 2011).

Besides the fact that temperature can be a major confound in neuroprotective studies, as it is a key factor in drug kinetics, intra- and post-insult temperature has an important role in determining the extent of brain injury. Indeed, it has been long recognized that HYPO lessens whereas hyperthermia worsens ischemic and traumatic brain injury (TBI) (Colbourne et al., 1997; Polderman, 2008; MacLellan et al., 2009). In hindsight, the early clinical cases and experiments along with animal work should have been enough to convince everyone of the need for proper temperature measurement and control in neuroprotection studies (Colbourne et al., 1997; Polderman, 2008; MacLellan et al., 2009).

A major discovery during the 1990s was the finding that the N-methyl-D-aspartic acid (NMDA) receptor antagonist, MK-801, which was widely thought to be a leading candidate for stroke neuroprotection, worked largely through drug induced HYPO (Buchan and Pulsinelli, 1990; Corbett et al., 1990; Colbourne et al., 1997). Next, a number of studies showed that post-ischemic temperature critically impacts brain injury (Colbourne et al., 1997; Polderman, 2008; MacLellan et al., 2009). Thus, it came as no surprise that drugs given after ischemia could also affect outcome via temperature confounds (e.g., NBQX). Furthermore, numerous studies have documented spontaneous temperature changes in many models (e.g., focal ischemia), which may vary by species, age, sterility, and other factors (Colbourne et al., 1997; Polderman, 2008; MacLellan et al., 2009). These changes in temperature are an important additional complication warranting consideration.

Along with these studies were those that illuminated the harmful effects of hyperthermia (Colbourne et al., 1997; Polderman, 2008; MacLellan et al., 2009). Fever is a common

complication after brain injury and an independent predictor of poor outcome in patients (Polderman, 2008; Badjatia, 2009). One of the first studies suggesting that hyperthermia was associated with poor patient prognosis in ischemic stroke was published in the 1970s (Hindfelt, 1976). Hyperthermia after brain injury can be attributed to several factors such as hypermetabolism, excitotoxicity, inflammation, infection, ischemic depolarizations, and thermopooling problems caused by edema and vascular blockage (Badjatia, 2009). In contrast to HYPO, hyperthermia worsens outcome by increasing the metabolic rate, excitotoxicity, and oxidative stress, among others (Polderman, 2008; Badjatia, 2009). Hyperthermia has also been found to block neuroprotective agents, such as MK-801 and thrombolytic drugs after ischemia (Colbourne et al., 1997; Polderman, 2008; Badjatia, 2009).

1.9. Therapeutic Hypothermia

Of over one thousand treatments developed in animal models, HYPO is the only one that has been repeatedly successful in clinical trials (O'Collins et al., 2006). In 2002, HYPO was shown to be an effective treatment for cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002). These reports show that mild HYPO for 12-24 hours reduced morbidity and mortality. Cooling was also found to improve outcome in neonates with hypoxic-ischemic injury (Shankaran et al., 2005). Phase II clinical trials have confirmed the safety of this treatment for patients with focal ischemia. Clinical trials to test the effectiveness of HYPO after focal ischemia are currently underway (Hemmen et al., 2012); (Lyden et al., 2014; van der Worp et al., 2014). Moreover, converging evidence from animal studies as well as the positive correlation between fever and poor outcome after ischemic stroke strongly suggests that HYPO, and perhaps just fever prevention, will be a promising treatment for focal ischemia (see

reviews (van der Worp et al., 2007; Polderman, 2008; MacLellan et al., 2009). However, clinical studies have shown conflicting results. For instance, there have been reports suggesting that prophylactic administration of the antipyretic agent paracetamol is associated with worsened outcome after focal ischemia in patients (Frank et al., 2013). Another study showed that paracetamol improved functional outcome when given to stroke patients with temperatures between 37-39 °C (den Hertog et al., 2009). Currently, there is a clinical trial testing whether paracetamol improves outcome in stroke patients with a temperature higher than 37 °C (de Ridder et al., 2015).

Experimental studies on HYPO began in the late 1800s and early 1900s, when it was noted by researchers Stefani, Deganello, and Trendelenburg that cooling reduces brain activity and therefore metabolism (Rothman, 2009). Cooling has been studied as a treatment for brain injury since the 1940's. Problems were encountered in many of the earlier animal and human studies with some of these complications causing death (Colbourne et al., 1997; Varon and Acosta, 2008). The treatment was then largely abandoned due to failed clinical trials and perhaps the belief at the time that other therapies, such as steroids and barbiturates, could be more effective while being more easily given (Shapiro, 1985; Norris and Hachinski, 1986). During the late 1980's interest in HYPO was revived when researchers noted that even very mild cooling potently reduced cell death in animal models (Busto et al., 1987). Currently, HYPO is one of the most extensively studied treatments for ischemia, with over one thousand published studies and over a hundred reviews on the topic (Figure 1-2).

Certainly HYPO is a potent neuroprotectant whose efficacy varies with depth, delay and duration of treatment (MacLellan et al., 2009). Notably, studies in the late 1980's and early 1990's showed that very mild drops in temperature during ischemia conveyed remarkable

neuroprotection. For instance, Busto and colleagues (Busto et al., 1987) showed that intra-ischemic HYPO at 34°C prevented 50% of cell death in the cornu ammonis 1 (CA1) region of the hippocampus, a structure highly susceptible to global ischemia (Kirino, 1982; Pulsinelli et al., 1982). As previously mentioned, researchers noticed that several neuroprotective drugs were mainly effective through drug induced HYPO (Buchan, 1990; Corbett et al., 1990). Owing to the limited clinical application of intra-ischemic cooling, investigators also studied delayed HYPO with seemingly contradictory reports of permanent and transient protection (Dietrich et al., 1993; Colbourne and Corbett, 1994). Colbourne and Corbett (Colbourne and Corbett, 1994) noticed that longer durations (e.g., 24 hours) of mild cooling (32°C) resulted in enhanced and long-lasting CA1 sector neuroprotection after global ischemia, unlike other studies showing that prolonged moderate cooling (29°C) was harmful (Michenfelder and Milde, 1977) or brief mild HYPO was ineffective (Dietrich et al., 1993). Similar parameters have been successfully applied in animal models of focal ischemia although there is still controversy regarding what is the best depth and duration of HYPO (Kollmar et al., 2007; van der Worp et al., 2007; MacLellan et al., 2009). A thorough review and meta-analysis revealed that cooling can reduce infarction by up to 44% after focal ischemia (van der Worp et al., 2007). Therefore, decreasing body temperature for brief to prolonged periods can reduce cell death in animal models of both focal and global ischemia, even when HYPO is started after several hours into or after the ischemic event (Figure 1-3 and Figure 1-4).

A few words of caution are in order before discussing HYPO's mechanisms of action. First, many factors pertaining to the insult will clearly influence not only HYPO efficacy, but potentially the underlying mechanisms (see Table 1-3). So far HYPO has been tested in many ischemia models with varying insult severity, in many species, in males and females, in animals

across the age range and including some with co-morbidities such as hypertension (O'Collins et al., 2006). As expected, these data show that the protection afforded by HYPO varies with such factors (e.g., young animals are better protected than old animals). Despite the fact that each model has limitations in reflecting and predicting clinical situations, they provide considerable insight into the underpinnings of HYPO, but one should be cognizant of the limitations and need for converging evidence.

Second, intervention delay is critically important. For instance, whereas some mechanisms of action overlap between intra and delayed HYPO, differences may arise from targeting early versus more downstream mediators of injury. Although the underlying pathophysiology is somewhat similar between focal and global ischemia, important differences exist, such as in the maturation rate of cell death. One of the most studied and vulnerable area of the brain after global ischemia is the CA1 region of the hippocampus, which undergoes cell death within 2-4 days. Conversely, neurodegeneration starts quickly in the core, the area with the least blood flow during focal ischemia, and slower secondary injury occurs mostly in the ensuing hours and first day in the penumbra, which surrounds the core and is partially fed by collateral arteries (Astrup et al., 1977). However, studies have shown much later cell death here too, depending upon the severity of ischemia and treatments administered (Valtysson et al., 1994; Du et al., 1996). Therefore, the timing at which different endpoints are measured (e.g., cell death, edema, etc.) and the location of injury (e.g., CA1 vs. cortex) need to be considered when assessing the effectiveness and mechanisms of action of HYPO. Indeed, compared to intra-ischemic HYPO, there are still comparatively few studies on the neuroprotective mechanisms of delayed HYPO.

Third, one must consider the substantial influence of treatment parameters, namely the depth and duration of cooling and re-warming rate, on HYPO mechanisms of action (Kollmar et al., 2007). Lower temperatures may confer better neuroprotection considering that most factors involved in stroke pathophysiology are temperature dependent. However, the side effects of applying a more aggressive treatment may counteract the benefits of deeper HYPO (MacLellan et al., 2009). In some cases, milder temperatures (34ºC) have been shown to be more protective than colder temperatures (Kollmar et al., 2007). Therefore, the optimal depth of cooling is still controversial. Longer treatments and quicker interventions often seem the most effective. Although one meta-analysis suggested that shorter durations of HYPO were more effective than prolonged cooling (van der Worp et al., 2007), several studies directly evaluating this hypothesis report that a longer treatment is more effective (Carroll and Beek, 1992; Colbourne and Corbett, 1994; Clark et al., 2009). Regardless, cooling parameters should be customized to the type and severity of the ischemic insult, among other factors. For instance, longer durations may be needed when cooling onset does not begin until hours after the stroke or cardiac arrest, an unfortunate clinical inevitability (MacLellan et al., 2009; van der Worp et al., 2010). Furthermore, slow re-warming is fundamental to achieve the most neuroprotection (Berger et al., 2007).

Last, the method of cooling may also affect its neuroprotective properties. Animal research uses both focal and whole-body HYPO; methods that have been used in clinical studies as well (Gluckman et al., 2005; MacLellan et al., 2009). Systemic cooling, as commonly done in rodent experiments, induces shivering that drives up the brain's metabolic activity, an important mechanisms of action discussed later (Colbourne et al., 1997; Ueda et al., 2004). Shivering is normally prevented in humans with anesthetics and also by drugs such as meperidine that are

used to decrease discomfort in conscious patients (Guluma et al., 2006; Polderman and Herold, 2009; Choi et al., 2011). The interaction of these drugs on the effectiveness and mechanisms of neuroprotection afforded by HYPO has not been sufficiently studied. There is only one study suggesting that meperidine does not alter neuroprotection provided by cooling in a rat model of focal ischemia (Sena et al., 2012). Further studies should determine if other pharmaceuticals used in patients interact with cooling.

1.9.1. Effects of Therapeutic Hypothermia during the Acute Phase of Ischemia

Decreases in CBF and BP due to cooling have been reported in normal (non-ischemic) animals under anesthesia (Rosomoff and Holaday, 1954; Michenfelder and Milde, 1991, 1992). Fortunately, HYPO does not potentiate the decrease in CBF during ischemia (Busto et al., 1987) but reduces metabolism more so than decreasing CBF ('luxury perfusion'). The effects of post-ischemic HYPO on CBF are not clear. Studies have reported increases, decreases, and no changes in CBF after cooling was completed, perhaps of methodological or timing differences (Baldwin et al., 1991; Lo and Steinberg, 1992; Huang et al., 1998). Some researchers have found a dual effect of HYPO by ameliorating the immediate hyperperfusion and delayed hypoperfusion that occurs after ischemia, possibly reducing reperfusion injury (e.g., oxidative stress) and providing normal perfusion to enhance repair processes (Karibe et al., 1994; Huang et al., 1998). Re-warming could also potentially affect CBF (Suehiro and Povlishock, 2001; Ueda et al., 2004). For instance, healthy human subjects underwent an increase in peripheral blood flow after re-warming (Savard et al., 1985). Furthermore, in canine model of cardiac arrest, complications such as cardiovascular collapse, tissue hypoxia, and acidosis occurred during rewarming as well

(Steen et al., 1980). Therefore, it is important to assess the effects of re-warming on CBF and BP after ischemia.

Increased BP, a sympathetic response observed during cooling and shivering, has been noticed during prolonged HYPO in awake animals (MacLellan et al., 2004). It is possible that the elevated BP could enhance CBF via collateral perfusion, a strategy which is being evaluated for its neuroprotective potential (Clark et al., 2008; Armitage et al., 2010; Shuaib et al., 2011). Collateral reperfusion contributes to further angiogenesis and neuroprotective factors, such as vascular endothelial growth factor and basic fibroblast growth factor (Kawamata et al., 1997; Hayashi et al., 1998; Lin et al., 2002). Conversely, in some cases HYPO decreases heart rate and BP as we observed in old (1 year) and young (3 months) hypertensive rats without an ischemic lesion (MacLellan, Wiltshire, and Colbourne, unpublished data). Such effects could negate some or all of the therapeutic value of HYPO in animal studies (i.e., underestimating efficacy) (Michenfelder and Theye, 1968; Michenfelder and Milde, 1992).

Animals that hibernate, which also undergo a decrease in temperature and metabolism, are more resilient to ischemic episodes (Drew et al., 2007). In fact, one of the longstanding hypotheses put forth to explain HYPO neuroprotection is its ability to reduce metabolism. Specifically, drops in temperature to 32 °C lower cerebral metabolic rate for glucose by 15-30%, cerebral metabolic rate for oxygen by 30-40% in normal rats and depolarization time by up to a minute in ischemic rats (Rosomoff and Holaday, 1954; Hagerdal et al., 1975; Michenfelder and Milde, 1991; Nakashima et al., 1995; Okubo et al., 2001; Erecinska et al., 2003). Likewise, there are many studies indicating that intra-ischemic HYPO delays ATP, lactate, and pyruvate expenditure but does not prevent it (Nilsson et al., 1975; Welsh et al., 1990; Sutton et al., 1991; Ibayashi et al., 2000; Kimura et al., 2002; Erecinska et al., 2003). Nonetheless, HYPO improves

recovery of high-energy phosphate metabolites and reverses acidosis produced by lactate accumulation during reperfusion (Chopp et al., 1989; Sutton et al., 1991; Kimura et al., 2002; Erecinska et al., 2003). Mild delayed HYPO applied for 12 hours after ischemic onset also ameliorates energy delivery failure for the first days after the arrest in neonatal pigs (Thoresen et al., 1995). Yet there is not much research on the effects of delayed HYPO on metabolism after global or focal ischemia in adults.

Intra-ischemic HYPO can partially mitigate Ca^{2+} influx thereby ameliorating cell death (Kristian et al., 1992; Moyer et al., 1992). Delayed and prolonged HYPO also presumably attenuates delayed excitotoxicity that occurs through the ischemia-induced down-regulation of the metabotropic glutamate receptor 2 (GluR2) subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) channels, which normally prevents Ca^{2+} and Zn^{2+} influx, in the CA1 region after global ischemia (Colbourne et al., 2003) (for a review on GluR2 and cell death see (Bennett et al., 1996). However, others have reported that post-ischemic cooling did not mitigate the decrease in glutamate receptor subunits, but this may be due to the use of brief cooling (Friedman et al., 2001). As discussed, short bouts of post-ischemic HYPO are substantially less effective at reducing cell death (MacLellan et al., 2009).

Many neurotransmitters are released after ischemia due to spreading depression and the inability to regulate their release. Glutamate, dopamine, serotonin, norepinephrine, glycine, gamma-aminobutyric acid (GABA), and aspartate are significantly increased in the extracellular space after ischemia (Globus et al., 1989; Baker et al., 1991; Globus et al., 1991; Takagi et al., 1993). The quantity of neurotransmitter released is proportional to the duration of ischemia (Baker et al., 1991). Cooling decreases the propagation of spreading depolarization (Takaoka et al., 1996), thereby ameliorating total neurotransmitter release and the possibility of further

excitotoxic injury. In a study by Okuda and others (Okuda et al., 1986) deep HYPO (21°C) decreased levels of norepinephrine and serotonin but not dopamine after global ischemia. However, Globus and colleagues (Globus et al., 1991) found that dopamine, which has been attributed to worsening cell death in global ischemia, was attenuated by 60% at 33°C. Glycine and GABA release also increase after ischemia and are attenuated by HYPO (Globus et al., 1991; Takagi et al., 1993). Overall, decreases in neurotransmitter release are associated with less excitotoxicity and less cell death.

Evoked potentials may be restored with cooling after focal ischemia. Four hours of HYPO at 33 °C was able to partially restore evoked potentials (18% of baseline) whereas cooling to 30 °C had a greater effect (43% of baseline) (Lo and Steinberg, 1992). This is indicative of normalized ionic homeostasis, which in turn will ameliorate the excitotoxic cascade (Busto et al., 1989b). Even though excitotoxicity occurs early after ischemia, secondary cascades are elicited for hours to days making HYPO's potential for neuroprotection plausible even when applied after 12 hours of ischemia onset (Coimbra and Wieloch, 1994; Colbourne et al., 1999).

Cooling decreases immediate post-ischemic hyperperfusion as well as glutamate and intracellular Ca^{2+} toxicity, thereby reducing injury and thus the inflammatory response (Karibe et al., 1994; Huang et al., 1998). By affecting these processes HYPO indirectly affects $\cdot O_2^-$ production. In addition, research shows a beneficial effect of HYPO on reperfusion injury in TBI (Clifton et al., 2011) and myocardial infarction (Gotberg et al., 2010), which may be attributable to a reduction in $\cdot O_2^-$ production. Intra-ischemic HYPO attenuates the rise in $\cdot O_2^-$ during global ischemia (Koda et al., 2010). Both intra-ischemic HYPO and cooling initiated immediately upon reperfusion mitigates the reperfusion-induced $\cdot O_2^-$ spike and brings levels back to baseline within an hour (Koda et al., 2010). In an animal model of focal ischemia, the reduction of $\cdot O_2^-$

production via intra-ischemic HYPO was found only in the penumbra and not in the ischemic core (Maier and Chan, 2002), which fits with the pattern of protection. Cooling applied either during or after ischemia also reduces the accumulation of $OH\cdot$ and H_2O_2 after reperfusion whereas hyperthermia has the opposite effect (Globus et al., 1995; Kil et al., 1996; Lei et al., 1997; Horiguchi et al., 2003; Van Hemelrijck et al., 2005).

1.9.2. Effects of Therapeutic Hypothermia during the Sub-acute Phase of Ischemia

Immediate early genes (IEGs) are important transcription factors for inducing protein expression associated with the inflammatory responses, tissue damage, and regeneration after ischemia (for more information see review (Akins et al., 1996)). Some early genes, such as *c-jun*, *jun-B*, *c-fos* and *fos- b*, are augmented after 1-2 hours in the dentate gyrus post-global ischemia (Kamme et al., 1995). This early phase of IEGs has been described as a stress response to the ischemic insult and this may be one reason why the dentate gyrus is more resistant to cell death than the CA1 zone (Kamme et al., 1995). A second peak occurs around 36 hours in the CA1-CA3 regions after global ischemia. Both intra- and post-intra-ischemic HYPO accelerates the second phase of early gene expression in the CA1, which has been associated with neuroprotection by restoring protein synthesis (Kamme et al., 1995; Kamme and Wieloch, 1996; Kumar et al., 1996). Intra-ischemic HYPO also augments one of the early genes belonging to the *jun* family, *jun-D*, in the dentate gyrus while preventing delayed expression in the CA1-CA3 areas of the hippocampus. Early *jun-D* expression is involved in the protective stress response whereas the late induction may be a transcriptional factor for delayed cell death pathways (Kamme and Wieloch, 1996). A similar biphasic temporal profile of early gene expression has been reported in focal ischemia. Immediate early genes are expressed after hours of ischemic onset with a second peak found several days later (Johansson et al., 2000; Akaji et al., 2003).

Intra-ischemic HYPO accelerates IEGs as seen after focal ischemia (Akaji et al., 2003) although the effects of delayed cooling on IEGs expression have not been tested.

Protein synthesis is disrupted for several days after global ischemia in both hippocampus and cortex (Bergstedt et al., 1993; Widmann et al., 1993; Berger et al., 1998). In healthy animals HYPO transiently slows protein synthesis (Sayegh et al., 1992). Intra-ischemic HYPO does not protect against the first depression in protein synthesis but is able to rescue it after 6 hours from end of ischemia (Bergstedt et al., 1993; Widmann et al., 1993; Wu et al., 1995; Berger et al., 1998). However, the efficacy of HYPO in improving protein synthesis, which is related to CA1 protection, largely depends on the age of the animal as younger animals benefit more from the treatment (Berger et al., 1998).

Therapeutic hypothermia reduces inflammation as well. Cooling leads to a reduction in both the total number of microglia as well as reactive microglia (Inamasu et al., 2000b; Deng et al., 2003; Han et al., 2003; Fukui et al., 2006; Florian et al., 2008; Webster et al., 2009; Ceulemans et al., 2011; Drabek et al., 2012; Fries et al., 2012). Cooling also reduces edema (Karibe et al., 1994; Preston and Webster, 2004; Xiao et al., 2004; Kallmunzer et al., 2012). For instance, cooling initiated prior to global ischemia normalizes brain water content (Xiao et al., 2004). Furthermore, cooling moderates ICP and appears to promote survival in stroke (Schwab et al., 1998) and TBI patients (Sinclair and Andrews, 2010) although rebounds in ICP levels need to be monitored during rewarming, with slower re-warming (over 24 hours) likely linked to survival (Schwab et al., 1998).

Both intra- and post-ischemic cooling can lessen the amount of BBB disruption (Karibe et al., 1994; Wagner et al., 2003; Hamann et al., 2004; Preston and Webster, 2004; Lee et al., 2005; Nagel et al., 2008; Baumann et al., 2009) possibly by preserving the shape of endothelial

cells and preventing pericyte disassociation (Duz et al., 2007). In addition, HYPO ameliorates the decrease of basement membrane proteins, such as agrin and secreted protein acidic and rich in cysteine (SPARC), after global ischemia (Baumann et al., 2009). Nevertheless, HYPO delayed for 2 hours after reperfusion had no effect on BBB disruption (Preston and Webster, 2004). Furthermore, in a clinical study stroke patients given HYPO had greater levels of intact laminin than patients given tPA (Horstmann et al., 2003). Cooling attenuates the increase in matrix metalloproteinases (MMPs) in experimental (Wagner et al., 2003; Hamann et al., 2004; Lee et al., 2005; Nagel et al., 2008; Kallmunzer et al., 2012) and clinical stroke (Horstmann et al., 2003) thereby diminishing BBB disruption. Cooling effects on tissue inhibitor metalloproteinases (TIMP) are unclear with some studies reporting that HYPO increases TIMP levels (Horstmann et al., 2003; Lee et al., 2005) and others showing no effect (Kallmunzer et al., 2012).

Cooling also affects cell death pathways. Mild intra-ischemic and post-ischemic HYPO decreases DNA fragmentation, release of cytochrome C, apoptosis-inducing factor, B cell lymphoma 2 (Bcl-2) associated protein X (BAX), c-Jun N-terminal kinase (JNK), and caspase expression after ischemia (Maier et al., 1998; Ferrand-Drake et al., 1999; Inamasu et al., 2000a; Phanithi et al., 2000; Prakasa Babu et al., 2000; Zhang et al., 2001; Eberspacher et al., 2003; Zhao et al., 2005; Zhao et al., 2007; Hu et al., 2008). It should be also noted that intra-ischemic HYPO affects early and late processes in cell death. Many early processes are directly affected whereas later ones, such as inflammation, are attenuated because there is simply less cell death (a correlation no causation). Similar concerns must be raised with use of delayed HYPO as well; that is, we cannot assume that every effect of HYPO is necessary for histological protection.

1.9.3. Effects of Therapeutic Hypothermia on Plasticity and the Chronic Phase

There are many factors that have a biphasic role in neurodegeneration and neuroplasticity (Lo, 2008). For instance, increases in NO are involved in oxidative stress but NO also sustains collateral blood flow and contributes to angiogenesis after ischemia (Iadecola and Zhang, 1994; Lo et al., 1996). After ischemia, Zn^{2+} accumulates in neurons causing cell death, but at later stages Zn^{2+} modulates neuroplasticity (Galasso and Dyck, 2007). The JNK pathway has also been associated with neuroprotection during the chronic stages and its inhibition leads to worsened neurological and functional outcome (Murata et al., 2012). There is also a temporal and spatial overlap between these processes. For instance, within the penumbra the area closest to the core is heavily exposed to neurodegenerative factors coming from dead tissue. However, the healthy tissue surrounding the penumbra is also causing repair processes. Both secondary injury and repair processes are highly active during the subacute phase (Lo, 2008), which is when neuroprotective treatments are usually applied in patients. Accordingly, treatments aimed at reducing neurodegeneration may also target common factors involved in neuronal repair.

Several studies have shown that HYPO is relatively safe. In rats without a stroke, an extreme duration of localized HYPO (21 days at 32 °C) did not affect subsequent behaviour, nor did it cause any neuronal atrophy or brain damage (Auriat et al., 2012a). Long durations of delayed HYPO enhanced neurogenesis in the dentate gyrus after global ischemia, although we were not able to find any neurogenesis in the CA1 contradicting previous reports (Silasi and Colbourne, 2011). Short HYPO durations also induced neuronal proliferation in neonatal rats with hypoxic-ischemic injury (Xiong et al., 2011). These new neurons were less likely to co-label with caspases and were highly co-expressed with Bcl-2. Also, when a Bcl-2 inhibitor was applied, caspases increased in the HYPO group suggesting that this up-regulation in

neuroplasticity is accompanied by enhanced expression of proteins involved in cell survival (Xiong et al., 2011). Neurotrophins, such as BDNF, aid the formation of new cells (Scharfman and Hen, 2007). Long durations of HYPO increased neurotrophin gene expression after ischemic stroke and cardiac arrest (Vosler et al., 2005; Ohta et al., 2007). Although there is an increase in BDNF during the first 24 hours after asphyxia cardiac arrest, HYPO seems to increase the transcription of a specific BDNF exon as well (D'Cruz et al., 2002; Vosler et al., 2005). In turn, this increase in BDNF expression could potentially account for the neuroprotection in the CA1 (Vosler et al., 2005). However, most of these changes have been assessed earlier during ischemia. Fortunately, even very prolonged HYPO did not affect BDNF expression or other neuroplastic factors such as Zn^{2+} after global ischemia (Silasi et al., 2012). Intra- and post-ischemic HYPO are also able to restore evoked potentials and maintain normal electrophysiology in the CA1 after weeks in global ischemia models (Nurse and Corbett, 1994; Dong et al., 2001) suggesting that the rescued neurons work normally. Conversely, one study found that post-ischemic HYPO decreased long-term potentiation (LTP) in gerbils (Miyamoto et al., 2000).

Pre-clinical research suggests that longer durations of HYPO should be applied to enhance neuroprotection in patients, which could potentially interfere with repair mechanisms (Colbourne et al., 1997; Lo, 2008; MacLellan et al., 2009; van der Worp et al., 2010). In this regard, HYPO does not seem to interfere with neuroplasticity in global ischemia models. However, this needs to be further studied in several animal models by varying type of insult and using clinically relevant HYPO protocols (e.g., several days of HYPO). Furthermore, HYPO is usually applied systemically in the clinic (Polderman and Herold, 2009). This means that the whole brain gets cooled, even the contralesional hemisphere. It has not been yet investigated whether HYPO has an impact on plasticity occurring in the contralesional hemisphere.

1.9.4. Therapeutic Hypothermia and ICH

One of the earliest reports in which mild HYPO (30-32°C) was applied in ICH patients comes from a small study in 1956 (Howell et al., 1956). Even though only 2 out of 8 patients survived, the authors hinted that cooling might have reduced ICP and edema in 7 patients. Indeed, in a study in which ICH patients were cooled for 10 days, HYPO reduced edema and improved outcome but did not have an effect on hematoma size (Staykov et al., 2013). Other small clinical studies have also been carried out (Feng et al., 2002; Abdullah and Husin, 2011; Su et al., 2015). Currently, randomized controlled trials are testing cooling in ICH patients (Kollmar et al., 2012).

Many cell death mechanisms overlap between ischemia and ICH, such as excitotoxicity, inflammation, oxidative stress, increased edema (Balami and Buchan, 2012). Cooling ameliorates some of those mechanisms of injury in the same fashion as in ischemia. For instance, several studies show that prolonged HYPO reduces edema, inflammation, BBB disruption in several animal models (Kawai et al., 2001; Kawanishi, 2003; MacLellan et al., 2004; Dai et al., 2006; MacLellan et al., 2006; Fingas et al., 2007; Kawanishi et al., 2008). Some of these experiments, however, fail to show a consistent reduction in lesion size and improvement in functional outcome (MacLellan et al., 2004; MacLellan et al., 2006; Fingas et al., 2007). This could be due to the toxicity of blood components in ICH compared to ischemia. Our studies have shown that cooling does not mitigate thrombin or iron induced injury (Wowk et al., 2014, In press). Also, in some instances HYPO cannot be applied early as in ischemia because cooling can aggravate bleeding either by increasing BP or due to coagulopathy (MacLellan et al., 2004; John et al., 2015). Therefore, many mechanisms of injury can act for longer prior to cooling administration and in turn causing more damage. Furthermore, fever does not seem to affect

outcome after ICH as much as it does after focal ischemia (MacLellan and Colbourne, 2005; Penner et al., 2011).

1.9.5. Effect of Therapeutic Hypothermia on Seizures

There is a relationship between temperature and seizure activity which has been known since the times of Hippocrates (Rothman, 2009). Hyperthermia increases whereas HYPO decreases seizure susceptibility. This is mainly thought to occur due to cooling's ability to reduce neuronal activity, metabolism, and pre- as well as post-synaptic neurotransmitter release (Rosomoff and Holaday, 1954; Moseley et al., 1972; Yang et al., 2005). Indeed, in vitro and in vivo epilepsy experiments have demonstrated that lower temperatures reduce epileptiform activity (Hill et al., 2000; Yang et al., 2002; Burton et al., 2005; Yang et al., 2005; Motamedi et al., 2006). Also, in humans it has been shown that local cooling with saline can stop paroxysmal discharges during intraoperative surgical mapping (Sartorius and Berger, 1998; Karkar et al., 2002).

Mild HYPO successfully reduced seizure activity after different types of brain injury both in animal and clinical studies. For instance, 4 hours of HYPO reduced seizure susceptibility after TBI in rats (Atkins et al., 2010). Mild HYPO has been shown to reduce seizures after hypoxic-ischemic injury in infants (Harbert et al., 2011). This could be attributed to the protective effect of HYPO rather than cooling directly reducing seizures. In humans with status epilepticus, there are several small clinical trials suggesting that seizures are ameliorated by cooling. For instance, a study showed that mild cooling (31–36° C) reduced seizure activity in 5 out of 6 patients with status epilepticus (Vastola et al., 1969). In another study, anesthetized patients that suffered from chronic motor seizures had their body temperature lowered to 29° C with cold air, and then iced

saline was applied to the cortical surface to lower temperature to 24° C. After 1 year, 11 out of 15 patients showed a reduction in seizure incidence, and 4 were seizure free (Sourek and Travnicek, 1970). This has spiked an interest in cooling locally in patients and animal models with cortical seizures, as focal HYPO may have less systemic complications and allows for the use of lower temperatures (Yang et al., 2002; Bakken et al., 2003; Burton et al., 2005; Imoto et al., 2006; Tanaka et al., 2008; Polderman and Herold, 2009).

1.10. General Hypothesis

This thesis aims to further our understanding regarding how HYPO may affect aspects of stroke pathophysiology and recovery that go beyond neuroprotection. Given that cooling reduces neuronal activity (Rosomoff and Holaday, 1954; Moseley et al., 1972; Yang et al., 2005), it is likely that it may have an impact on both plasticity and seizure activity. Several studies suggest that cooling does not negatively impact plasticity after ischemia (Nurse and Corbett, 1994; Dong et al., 2001; D'Cruz et al., 2002; Vosler et al., 2005; Ohta et al., 2007; Silasi and Colbourne, 2011; Xiong et al., 2011; Silasi et al., 2012). However, cooling reduced LTP even a week after global ischemia (Miyamoto et al., 2000). Still, it is not known how cooling may affect contralesional plasticity after unilateral strokes. This is especially important considering that stroke patients get both hemispheres cooled during systemic HYPO, the most common method to induce cooling in the clinic (Polderman and Herold, 2009). Thus, in the first set of experiments described in this thesis we assessed the impact of cooling the contralesional hemisphere on functional outcome after a motor cortex devascularization. We also assessed the impact of HYPO on learning of a forelimb reaching task in otherwise naïve rats. We hypothesized that cooling would impair behaviour both after focal ischemia and also in healthy rats.

Moreover, seizures occurring after ICH have never been studied in animal models. In the second set of experiments, we assessed seizure activity in the most widely used animal models of ICH, the collagenase and whole-blood model. Our main objective was to find a model that we could use to study the effect of HYPO on seizure activity after ICH. Cooling ameliorates edema, inflammation, and oxidative stress after ICH (MacLellan et al., 2009). Cooling also reduces seizure activity in animal and human studies (Rothman, 2009; Zeiler et al., 2015). Therefore, we hypothesized that 1) seizures would occur after ICH in both models, and that 2) cooling would decrease seizure activity after ICH.

1.11. References

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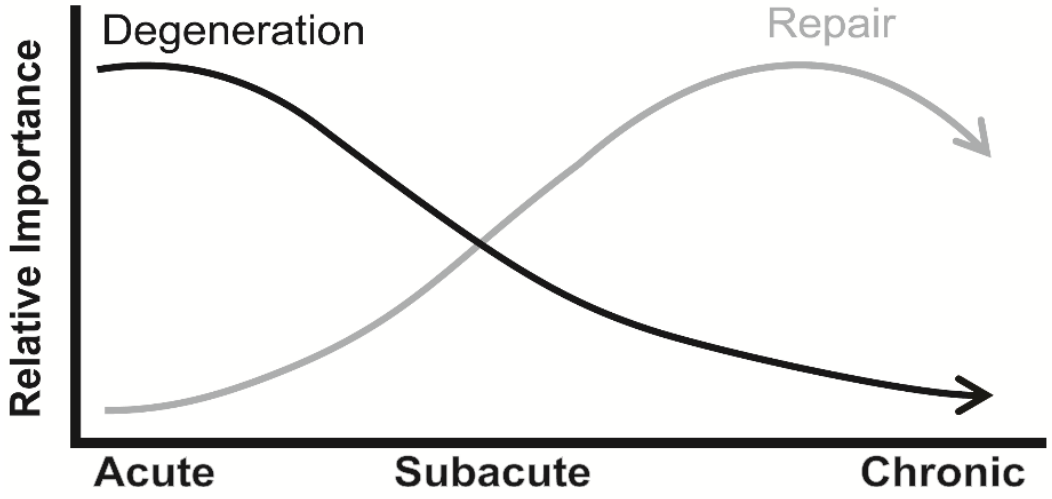

Figure 1-1. Temporal and spatial overlap of neurodegenerative and neuroplastic processes after ischemia. Neurodegenerative processes predominate during the first hours and days after ischemia. The rate of cell death slows after several days but still persists for weeks and months in some cases (e.g. mild insults, and use of neuroprotectants). Processes of neuronal repair temporally coincide with neurodegeneration. However, neuroplasticity predominates after weeks. Cell injury and repair processes also overlap spatially after ischemia. For instance, cell death and repair mechanisms are occurring simultaneously in penumbral tissue. Therapies targeting neurodegeneration should take into account that cell repair and death processes share some of the same factors. For instance, glutamate toxicity is one of the main causes of cell death. Nevertheless, decreasing glutamate and targeting receptors for extensive periods may interfere with glutamate related neuroplasticity (e.g., NMDA-mediated long-term potentiation; for a review see (Lo, 2008)**.**

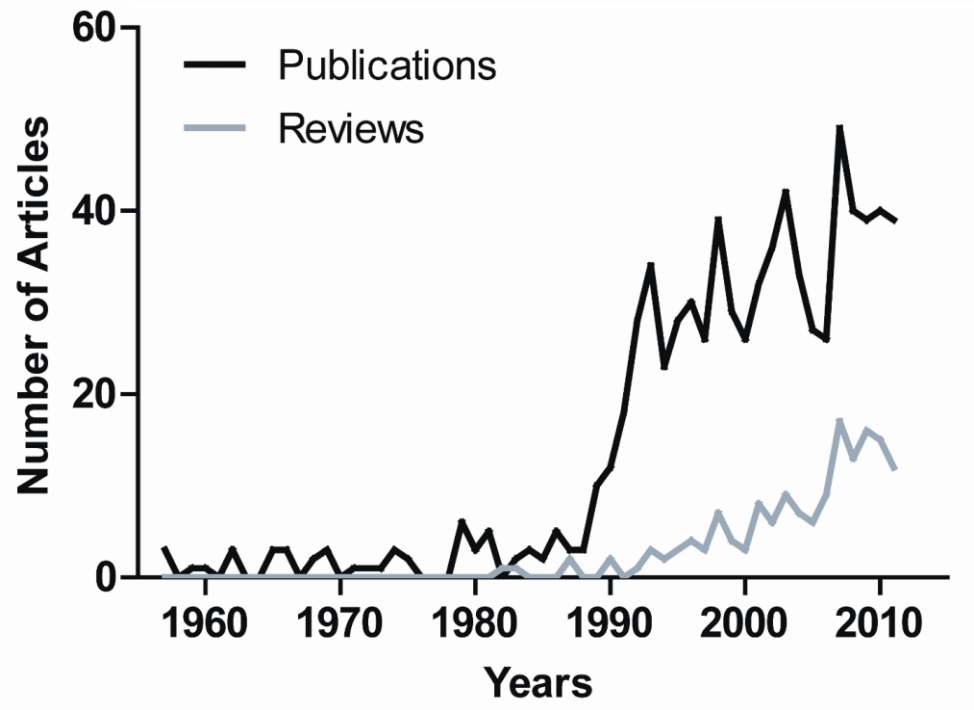

Figure 1-2. Articles on TH and neuroprotection. We conducted a Pub Med search of all articles containing the words "Hypothermia and cerebral ischemia" and "Hypothermia and ischemic stroke" related to neuroprotective mechanisms. We found 785 publications and 155 reviews from 1957 to 2011. This is a rough estimate as we missed articles published before 1957 and we did not use alternative search terms that might have detected other papers.

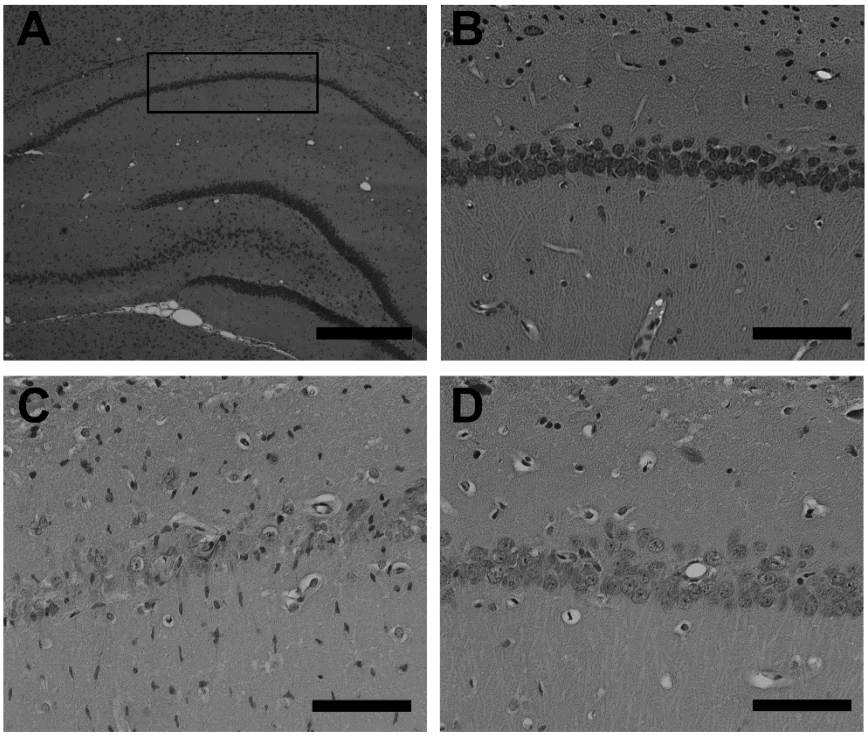

Figure 1-3. Long durations of post-ischemic HYPO (24 hours at 33 ºC followed by 24 hours of 35 ºC starting one hour from ischemic onset) provide neuroprotection as assessed at a long survival time (42 days post global ischemia) . A) Photomicrograph of a hippocampus stained with H&E of a sham-operated animal. Photomicrographs B, C, and D were taken from the CA1 zone illustrated in the square (bar = 200 μm). B) Sham hippocampus is depicted with intact CA1 cells. C) Normothermic ischemia results in the death of most CA1 neurons. D) Long durations of post-ischemic TH are able to rescue most CA1 neurons. Slides were taken from previous published experiments (Silasi and Colbourne, 2011).

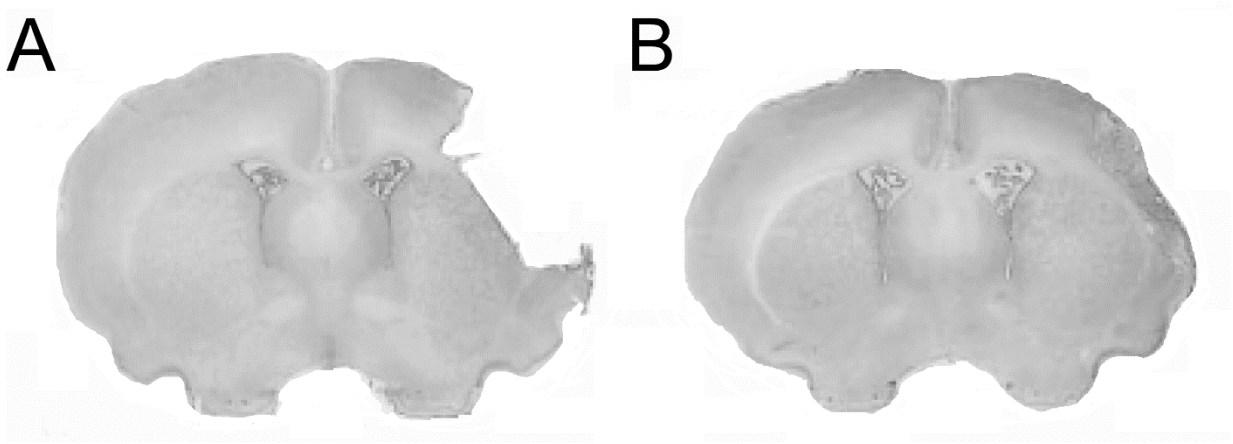

Figure 1-4. Long durations of delayed HYPO (48 hours at 33 ºC starting one hour from ischemic onset) provide neuroprotection as illustrated here at 32 days post focal ischemia. A) Coronal section stained with cresyl violet of permanent MCAO (electrocautery of MCA). Average lesion volume was approximately 150 mm^3. B) Long durations of TH are able to partially rescue cortical tissue. Average lesion volume was reduced to approximately 80 mm^3. Slides were taken from previous published experiments (Clark et al., 2009).

Table 1-1. Phases of ischemia. Several factors contribute to cell death and repair during the acute phase of ischemia. In this table, we list some of the main players of the acute, subacute and chronic phases of ischemia.

Acute phase (minutes-hours)	Subacute phase (several hours to days)	Chronic phase (weeks to months)
Decline in cerebral blood flow	Decrease in protein synthesis	Resolution of diaschisis
ATP loss	Blood brain barrier disruption	Neuroplasticity
Ionic homeostasis imbalance	Inflammation	Angiogenesis
Spreading depression	Diaschisis	Debris removal
Neurotransmitter release	Exictotoxicity	Cell death pathways
Increase in intracellular Ca^{2+}	Spreading depression	Increases in spine density
Excitotoxicity	Activation of cell death and survival pathways	Neurogenesis
Acidosis	Stress response	
Oxidative stress	Cell death pathways	
Cytotoxic edema	Vasogenic edema and ↑ ICP	
Stress response	Neuronal atrophy	
Cell death pathways	Zinc neuroplasticity	
Zinc accumulation	Increases in growth factors (e.g. BDNF)	
	Seizures	

Table 1-2. Phases of ICH**.** In this table, we list some of the main players of the acute, subacute and chronic phases of cell death after ICH. Many of these mechanisms of injury overlap with ischemia.

Acute phase (minutes-hours)	Subacute phase (several hours to days)	Chronic phase (weeks to months)
Mechanical damage	Iron toxicity	Iron toxicity
Mass effect	Thrombin toxicity	Resolution of diaschisis
Decline in cerebral blood flow	Blood brain barrier disruption	Neuroplasticity
ATP loss	Inflammation	Angiogenesis
Ionic homeostasis imbalance	Diaschisis	Debris removal
Spreading depression	Exictotoxicity	Cell death pathways
Neurotransmitter release	Spreading depression	Increases in spine density
Increase in intracellular Ca^{2+}	Activation of cell death and survival pathways	Neurogenesis
Excitotoxicity	Stress response	
Acidosis	Cell death pathways	
Oxidative stress	Vasogenic edema and ↑ ICP	
Cytotoxic edema	Neuronal atrophy	
	Seizures	
Stress response		
Cell death pathways		

Table 1-3. Principles of hypothermic neuroprotection. Many factors influence the mechanisms of neuroprotection provided by TH, including the insult, animal and treatment parameters. All these factors influence efficacy, but relatively few consider their impact in mechanistic studies.

Parameters	Principle	Description
Insult	Type of ischemia	Focal vs. global ischemia vs. hypoxic-ischemic injury.
	Insult model	Method of producing the insult (e.g., craniotomy vs. intraluminal MCAO models).
	Duration	Duration of ischemia determines extent and rate of injury (e.g., 5 vs. 10 minute global ischemia) with several studies showing less benefit against more severe ischemia.
Animal	Species	Species, strain and supplier effects have been shown to influence ischemic damage.
	Age	With age there is reduced capacity for plasticity.
	Sex	Hormonal changes between sexes will have an impact on the insult severity and repair.
	Co-morbidities	Co-morbidities (e.g., hypertension) will impact many factors (e.g., CBF).
	Depth	Depth of HYPO (30ºC vs. 35 ºC) will impact HYPO neuroprotection effectiveness and potentially mechanisms of action.
	Duration	Longer durations (e.g., 48 hours) have been associated

	with better neuroprotection. Although they could impede repair processes (i.e., diminishing returns).
Delay	Shorter delays are associated with more HYPO neuroprotection by targeting earlier cell death.
Method of cooling	The method of induction (e.g., anesthetics vs. water misters) may positively or negatively interact with injury and neuroprotective processes.
Monitoring of temperature	Different sites predict brain temperature to varying levels of accuracy.
Re-warming	Fast re-warming increases metabolism and CBF leading diminishing HYPO neuroprotection.
Co-treatment	Interactions of HYPO and other drugs (tPA) will affect cooling effectiveness and mechanisms of neuroprotection.

CHAPTER 2

Contralesional Localized Therapeutic Hypothermia Reduces Use of the Unimpaired Limb in a Skilled Reaching Task after Motor Cortex Devascularization in the Rat

2.1. Introduction

Therapeutic hypothermia (HYPO), reducing brain temperature to ~ 33°C, is the gold standard neuroprotectant against ischemia (Choi et al., 2012). Currently, HYPO is provided as a treatment for cardiac arrest patients and infants with hypoxic-ischemic injury (The Hypothermia After Cardiac Arrest Study Group, 2002; Gluckman et al., 2005). Cooling is also being tested in clinical trials for ischemic and hemorrhagic stroke (Kollmar et al., 2012; Lyden et al., 2014; van der Worp et al., 2014). Neuroprotectants, such as HYPO, act by reducing acute and chronic brain damage. Even though cell death processes predominate during the first hours after injury, many neuroplasticity processes initiate within minutes and last for weeks (Lo, 2008). Also, these processes not only occur simultaneously, but also in the same location (e.g., penumbra). Moreover, these mechanisms have overlapping factors. For instance, HYPO reduces neuronal activity and therefore reduces metabolism. Normal neuronal activity is essential for plasticity (Moser et al., 1993; Lo, 2008). Given that HYPO is applied for days after injury, cooling may not only be reducing neurodegeneration but also affecting neuroplasticity after stroke.

There are conflicting reports regarding the impact of HYPO on repair processes after stroke. For instance, HYPO enhanced neuronal proliferation after global ischemia and hypoxic-ischemic injury in the rat (Silasi and Colbourne, 2011a; Xiong et al., 2011). Also cooling increased brain-derived neurotrophic factor (BDNF) gene expression after ischemic stroke, cardiac arrest, and asphyxia (Vosler et al., 2005; Ohta et al., 2007) (D'Cruz et al., 2002; Vosler et al., 2005). In a previous study, however, we found that four days of HYPO did not affect BDNF expression after global ischemia (Silasi et al., 2012). Both intra- and post- ischemic HYPO restored evoked potentials in the CA1 after weeks in global ischemia (Nurse and Corbett, 1994; Dong et al., 2001). Conversely, another study found that in gerbils post-ischemic HYPO

decreased long-term potentiation (LTP) within a week after the insult (Miyamoto et al., 2000). These discrepancies in the findings could have been due to factors such as differences in the type of insult, insult severity, species, age, etc. Still, it is not clear whether HYPO negatively impacts plasticity after ischemic stroke.

Systemic HYPO, where the whole body gets cooled, is the most commonly used method in the clinic (Polderman and Herold, 2009). Localized forms of cooling have been used, although with more success in infants. Therefore, in most patients not only the affected hemisphere gets HYPO, but also the contralesional side. Most neuronal reorganization occurs in the areas surrounding the lesion and related networks (Cramer, 2008; Murphy and Corbett, 2009; Kolb and Teskey, 2012). However, several human and animal studies show that the contralesional hemisphere undergoes substantial neuroplasticity, especially after a very large injury or major destruction to a network (Jones et al., 1996; Chu and Jones, 2000; Gonzalez et al., 2004; Gharbawie et al., 2007). In patients, both increased activity in the non-affected hemisphere and less lateralization have been associated with improved recovery after stroke (Cramer, 2008). In the rat model of focal ischemia dendritic arborization and synaptogenesis occurs in the layer V of the contralesional hemisphere for weeks after the insult (Jones and Schallert, 1994). Clinical and animal studies also suggest that there is hyperexcitability occurring after stroke in the contralesional hemisphere, which inhibits the use of the affected limb (Floel et al., 2004; Murase et al., 2004; Ward and Cohen, 2004; Cramer et al., 2006; Dancause et al., 2015). Thus, therapies make use of non-invasive methods such as transcranial magnetic stimulation and transcranial direct current stimulation to reduce contralesional hemisphere activity in order to improve activation of the ipsilesional side (Hummel and Cohen, 2006). Therefore, there is the possibility

that neuroprotectants, HYPO included, could impact plasticity and/or excitability in the region surrounding the lesion as well as the repair processes occurring in the contralesional side.

It is challenging to disentangle the effect of HYPO on behavioural outcome after stroke. Given that this treatment provides neuroprotection on the ipsilesional side, it is nearly impossible to discern whether cooling may have a negative effect on plasticity. Therefore, our objective in this study was to determine whether selectively cooling the contralesional hemisphere affected reaching success after motor cortex devascularization, a model of focal ischemia (Gonzalez and Kolb, 2003; Gharbawie et al., 2007). Rats tend to use their unimpaired paw after stroke (Whishaw et al., 2008). This change also occurs in patients, and it is a phenomenon termed "learned non-use" (Taub et al., 1999; Taub et al., 2006). Learned non-use develops during the initial post-lesional phase when the individual is unable to properly use the affected limb either due to cortical shock caused by brain injury and/or diaschisis (loss of function in a portion of the brain connected to a distant damaged brain area). Therefore, the subject learns that using the impaired limb leads to punishment (e.g., dropping objects) and they learn via negative reinforcement to use the good limb instead. This in turn leads to decreased use of the impaired arm and depression of ipsilesional activity, making it less likely that the impairment will resolve. Therapies have been developed to treat learned non-use. For instance, constrained-induced movement therapy (CIMT) is a treatment that encourages the use of the paretic limb by restricting or discouraging the use of the non-paretic limb (Taub et al., 1999). Therefore, we also assessed whether rats tended to rely on their unimpaired paw after the stroke. In another experiment, we determined whether HYPO affected learning of a reaching task in otherwise naïve rats. We locally cooled the cortex ipsilateral or contralateral to the preferred paw. We also

had an additional group in which rats were maintained normothermic. This was done to explore the impact of cooling on neuroplasticity driven by learning rather than injury.

2.2. Methods

Subjects

All procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. One hundred and sixteen male Sprague-Dawley rats (300-350 g, ~ 3 months old) were obtained from the Biosciences breeding colony at the University of Alberta. Food (Purina rodent chow) and water were provided ad lib, unless food deprived for behavioral testing. Rats were housed in a temperature and humidity-controlled room (lights on from 7 a.m.–7 p.m.). All surgeries were performed aseptically.

In experiment 1 (N=3), we measured temperature from the cooled motor cortex (Ipsi), the contralateral cortex (Contra), and rectum (Body) of anesthetized naïve rats to test the selectivity of the focal cooling device (Clark and Colbourne, 2007; Silasi and Colbourne, 2011b). Rats were anesthetized with isoflurane (4% induction, 1.5–2.5% maintenance in 60 % N2O, balance O2) and a cooling strip was implanted (see below). Two holes were drilled in the skull (AP=+1.5, ML=+2.5 and ML=-2.5) and a 20G 1 cm cannula was attached to each hole with dental cement. The skin was pulled and stapled in order to cover the skull. Two thermocouple probes (HYP1-30-1/2-T-G-60-SMP-M, Omega, Stamford, CT, USA) were lowered 3 mm below the skull surface in order to measure temperature from both the ipsilateral (side of cooling) and contralateral cortices. Temperature was measured for 15 minutes prior to cooling, which lasted

30 minutes, until 15 minutes after cooling. Cold water was pumped at a rate of 110 ml/hour, a similar rate to those used in other experiments.

In experiment 2 (N=80), we tested the impact of cooling the contralesional side on behavioural outcome after a motor cortex devascularization. Rats were trained 5 days/week for 4 weeks in the single pellet reaching task (see below), and were also trained on a ladder task. The single pellet reaching task assesses reaching success whereas the ladder task evaluates foot placement while the rat crosses a series of parallel bars spaced 1-3 cm apart (Metz and Whishaw, 2002; Clark et al., 2008). After baseline behavioural assessment, rats underwent a motor cortex devascularization contralateral to their preferred limb. Briefly, rats were anesthetized and four holes were drilled using a fine dental burr in the skull overlaying the target area AP=-1mm, ML=+1mm; AP=-1mm, ML=+4mm; AP= 4mm, ML =+1mm; AP=4.0 mm, ML=4.0 mm (Gharbawie et al., 2007). The area enclosed by the burr holes was trephinated, the dura mater was removed, and the tissue was devascularized with a scalpel blade and cotton swab. The area was then covered with a sterilized piece of dental cement. Then the rats were implanted with a focal cooling device (see below). After surgery, rats were randomly assigned to normothermia (NORMO), cooling 1-49 (HYPO-1-49hr), 1-97 (HYPO-1-97hr), or 48-96 (HYPO-48-96) hours after the stroke. After cooling, animals were brought to normothermia over 6 hours. At 4 days after the injury, all animals were anesthetized and untethered. Rats were then tested on single pellet from days 7-10 and days 27-30, and on the ladder task on days 7-8 and 29-30 (Metz and Whishaw, 2002; Schallert, 2006). For the ladder task, the success rate on four ladder crosses was counted. Success rate was expressed as the number of successful foot placements/total number of steps×100.

At day 30, all rats were injected with sodium pentobarbital (100 mg/kg, i.p.) and then transcardially perfused with 0.9 % saline followed by 10 % neutral buffered formalin. Brains were extracted, cryostat sectioned at 40 μm, and stained with cresyl violet. Coronal sections taken every 200 μm were then analyzed with Image J (Klahr et al., 2015; Klahr et al., 2016). The volume of each hemisphere was calculated as follows: (average area of complete coronal section of the hemisphere−area of damage− ventricle) × interval between sections × number of sections. This method accounts for injury, atrophy and ventricular dilation (Klahr et al., 2015; Klahr et al., 2016).

In experiment 3 (N=33), we tested the impact of cooling the contra- or ipsilateral side to the preferred paw on learning of a skilled reaching task. First, all rats were implanted with the focal cooling device. The side was randomly assigned. The day after focal cooling implantation, rats were trained for single pellet reaching for two days to establish paw preference. Then, rats were randomly assigned to contralateral HYPO (CONTRA-HYPO), ipsilateral HYPO (IPSI-HYPO), or normothermia (NORMO). Rats were tested on the single pellet task while being cooled or tethered for 5 days. Then, all rats were untethered and tested for another 5 days. At 10 days after randomization, rats were injected with intraperitoneally with sodium pentobarbital and transcardially perfused with 0.9 % saline.

Single-pellet reaching task

Starting 3 days prior to training, rats were food deprived to 90% of free feeding weight and weighed daily to maintain weight at this level. Food restriction took into account the natural gain in body weight during the training period. Rats were placed in a clear plexi-glass box (length: 60 cm, width: 14 cm and height: 35 cm for Experiment 2 and 20 cm for Experiment 3)

and trained to reach through a 1 cm wide opening to retrieve a food pellet placed on the ledge in front of the opening (Greenough et al., 1985; MacLellan et al., 2006). Pellets were placed in both wells and reaching was followed by immediately replacing retrieved or displaced pellets.

In Experiment 2, once rats displayed a paw preference and were reliably reaching, the pellet was placed in the well contralateral to their preferred paw to prevent simultaneous use of the non-preferred paw. Each rat received one daily test session consisting of 20 trials. The last four sessions were used in calculating average baseline performance (MacLellan et al., 2006). In Experiment 3, there was no baseline, just training. In both experiments, a reach was considered a "success" if in one attempt the rat grasped the pellet, brought it inside the box using its paw, and placed the pellet into its mouth. A "failed" reach is one in which an animal advanced the paw through the slot but missed the pellet or knocked it off the ledge. Reaching success (for each type of reach) was defined as (number of successful retrievals/20)×100. During baseline, if rats reached <20% of the trials, they were excluded. Also, only rats that attempted to reach at all time points (baseline, day 7, and day 27) were included. In experiment 3, if the rats did not decide on a paw preference during those two days they were excluded.

Some rats tend to use their unimpaired limb instead of their affected paw after stroke. Therefore, in Experiment 2 we recorded whether rats changed their paw preference in the single pellet task. We then computed a bias ratio, which is calculated by (total reaches with the unimpaired paw – total reaches with impaired paw)/total number of reaches. A bias ratio of 1 depicts that the rat is using its originally preferred paw, whereas a ratio of -1 depicts that the rat solely reaches with its unimpaired paw after the stroke. Rats were considered to have meaningfully altered paw preference if their bias ratio was below 0.75.

Focal Cooling Device Implantation

A cooling strip was surgically implanted and secured with screws and dental cement (Clark and Colbourne, 2007; Silasi and Colbourne, 2011b). At the end of surgery, this device was connected via silastic tubing to an overhead swivel and a cold-water source. For those rats that received HYPO, the flow of chilled water averaged ~110 ml/hour over the 48-hour treatment, as previously done (Silasi and Colbourne, 2011b). This protocol causes brain temperature to drop to ~ 33°C in the hippocampus while body temperature remains normothermic in non-anesthetized rats (Silasi and Colbourne, 2011b). Control rats were treated similarly except they did not have water perfused through the device and thus they remained normothermic.

Statistical Analysis

Data are presented as mean ± SD for normally distributed data, or median ± IQR for non-parametric and not normally distributed data. All data was analysed using SPSS (v.17.0, SPSS Inc., Chicago, IL). ANOVA or Fisher's Exact test were performed depending on the nature of the data.

2.3. Results

In Experiment 1, we measured temperature during HYPO in otherwise naïve rats. As expected, ipsilateral cortical temperature was lowered to ~29°C during cooling (baseline temperature ~35.5°C), whereas the contralateral side dropped to ~33°C (baseline temperature ~35°C, Figure 2-1).

In Experiment 2, there were initially 20 rats in each group. Thirteen rats were excluded due to technical complications with the cooling system (rats remaining HYPO-1-49hr N=16, HYPO-1-97hr N=16, HYPO-48-96hr N=18, NORMO N=17). There was no significant difference in lesion volume among groups (p= 0.371, Figure 2-2). In the single pellet task, eight rats were excluded due to failed criteria (rats remaining, HYPO-1-49hr N=16, HYPO-1-97hr N=14, HYPO-48-96hr N=14, NORMO N=15). Motor cortex devascularization impaired reaching success as well as walking on the ladder after the stroke in all conditions ($p\leq$ 0.0074). We did not find a treatment effect in the single pellet task (p= 0.523, Figure 2-3). However, we found that in the ladder task the affected and unaffected forelimb had a treatment effect ($p\leq$ 0.0481), and the unaffected limb also had an interaction effect (p= 0.0001, Figure 2-4). For the impaired paw, the HYPO-48-96hr group performed worse on days 29-30 compared to NORMO and HYPO-1-49hr ($p\leq$ 0.038). Moreover, for the unimpaired paw, the HYPO-48-96hr group performed worse than all groups on days 7-8 ($p\leq$ 0.001). In the HYPO-1-48hr condition there were no rats that switched paw preference at any of the time points (Figure 2-5). This difference was statistically significant when HYPO-1-48hr was compared to NORMO and HYPO-48-96hr groups at days 7-10 (p <0.0177) but ceased being significant at days 27-30 (p>0.0996).

In Experiment 3, six rats were excluded due to complications with the cooling system. One additional rat had to be euthanized early due to health issues seemingly unrelated to the experiment (rats remaining HYPO CONTRA N=8, HYPO IPSI N=8, NORMO= 10). As expected, rats reaching success improved in the last 5 days in all groups (p<0.0001). However, there were no interactions among group, day of training, and/or period (tethered versus untethered; p>0.592). Once untethered both HYPO groups performed slightly worse than NORMO (<10% difference), although this was not significant (p>0.197, Figure 2-6).

2.4. Discussion

In this study, we found that early cooling for 48 hours lessened reliance on the unimpaired limb after stroke. Some rats still shifted limb preference with longer cooling (HYPO-1-96hr) although not as many as in the HYPO-48-96hr and NORMO groups. This suggests that early contralesional HYPO acted similarly to CIMT, as cooling the contralesional side of the brain discouraged the use of the unimpaired paw. Contralesional HYPO did not reduce lesion volume or affect reaching success in a single pellet task. Cooling may not be worsening ipsilesional plasticity or disturbing ipsilateral projections from the intact hemisphere to the affected limb. Rats displayed impaired walking on both forelimbs in the HYPO-48-96hr group. However, this behavioral effect was small (<10%) and we do not think it is biologically important. Additionally, we tested whether learning skilled reaching in the single pellet task was affected by HYPO in rats without a stroke. We found that reaching success was not significantly worsened by cooling. Therefore, cooling did not impair learning or remembering of the reaching task in otherwise naïve animals.

Several animal and human studies have shown that contralesional plasticity aids recovery after stroke (Schallert et al., 1997; Schaechter, 2004; Biernaskie et al., 2005; Hsu and Jones, 2006; Gharbawie et al., 2007; Murphy and Corbett, 2009). Substantial conformational changes occur in the contralesional hemisphere, ranging from synaptogenesis, motor remapping, dendritic arborization, etc. These repair processes tend to correlate with improved behavioral performance (Schaechter, 2004; Hsu and Jones, 2006; Kolb and Teskey, 2012). For instance, rats that partially recovered after focal ischemia display enhanced forelimb impairments when the contralesional motor cortex is either inhibited (Biernaskie et al., 2005) or lesioned (Gharbawie et al., 2007). Conversely, some clinical studies suggest that the contralesional hemisphere may create an

imbalance or inhibit use of the affected limb (Floel et al., 2004; Murase et al., 2004; Ward and Cohen, 2004; Cramer et al., 2006; Dancause et al., 2015). The imbalance between hemispheres is thought to be mediated by hyperexcitability in the contralesional cortex, characterized by both a reduction in GABA and an increase in NMDA receptors (Meyer et al., 1985; Sakatani et al., 1990; Kozlowski et al., 1994; Witte and Stoll, 1997; Qu et al., 1998; Mohajerani et al., 2011). Therefore, some studies actually attempt to reduce activity in the contralesional hemisphere (Hummel and Cohen, 2006). A study performed in rats showed that applying GABA-A agonist in the contralesional hemisphere for up to 14 days led to improvement in the use of the impaired limb in a reaching task and reduced bias in a spontaneous limb use task (Mansoori et al., 2014). As HYPO reduces neuronal metabolism and activity, it was possible that it may have had an improvement in reaching. However, it may also have been that HYPO decreased anatomical changes occurring in the contralesional hemisphere, therefore affecting recovery. Previous research showed that the motor cortex devascularization model used in our experiments elicited contralesional plasticity that aided recovery of the affected side (Gharbawie et al., 2007). We did not find that contralesional HYPO affected performance of the impaired paw, just the bias in shifting paw preference. In the future, it would be interesting to assess how our cooling protocol affected reaching trajectory or how it may have affected mechanisms of plasticity.

Individuals with stroke tend to rely on their unimpaired limb to compensate for their behavioral impairment, a phenomenon termed learned non-use (Schallert et al., 1997; Taub et al., 1999). The first study on learned non-use was carried out in monkeys with a deafferented limb. The monkeys tended to solely rely on the use of its non-impaired limb after deafferentation. Even though the monkeys were able to use their affected limb, they did not have sensory feedback and could not use it properly. Therefore, they learned to use their unimpaired limb to

compensate for the loss of function in the other arm (Taub, 1980). In order to treat learned non-use, rehabilitation therapies such as CIMT discourage the use of the unimpaired limb and encourage use of the affected limb (Taub et al., 1999). Use of the unimpaired limb not only leads to compensatory behaviors but may also worsen performance of the impaired paw (Allred and Jones, 2008). Therefore, HYPO applied to the contralesional hemisphere may be beneficial to stroke patients if this leads to decreased learned non-use. The effect of HYPO on this type of lesion may vary depending on lesion size as most studies find that the intact hemisphere tends to be mostly involved in patients with large lesions (Schaechter, 2004; Hsu and Jones, 2006; Murphy and Corbett, 2009; Kolb and Teskey, 2012; Dancause et al., 2015). In instances where there is not much neuronal tissue left in the affected hemisphere for true recovery to occur, compensatory behaviors may be the only option available to reduce disability in these patients. Indeed, rehabilitation therapies may lead to a reduction in disability but not impairment (Roth et al., 1998). In this regard, CIMT tends to encourage reduction in impairment (Taub and Morris, 2001).

Extensive research shows that cooling is one of the best neuroprotective treatments for ischemia (O'Collins et al., 2006). Pre-clinical research shows that HYPO reduces lesion volume and robustly improves outcome after focal ischemia (Miyazawa et al., 2003; Clark et al., 2008; Clark et al., 2009; Choi et al., 2012). The potential benefit of HYPO acting as CIMT could only occur when cooling is provided only to the contralesional hemisphere, which is not the case in most patients. Still, given that most plasticity processes occur on the lesioned hemisphere (Murphy and Corbett, 2009), there is the possibility that these reorganizational processes could be hindered by HYPO. Impaired neuroplasticity, however, may be masked by the decrease in neuronal injury as this could be a stronger determinant of functional outcome. Most cell death

mechanisms predominate within the first hours and days after the stroke (Lo, 2008; Murphy and Corbett, 2009). Thus, prolonged HYPO may have a detrimental effect on recovery. This is important to determine as stroke patients tend to be cooled for days or even weeks (Polderman and Herold, 2009; Staykov et al., 2013). If patients are undergoing HYPO, it is unlikely that they will be involved in any rehabilitation therapy. This delay may influence patient prognosis as well. Furthermore, the use of sedatives such as meperidine, are used to decrease discomfort during cooling (Polderman and Herold, 2009). The impact of such drugs on post-stroke recovery has not been properly assessed.

There are only a few studies assessing how HYPO may affect post-stroke plasticity. Cooling enhances cell proliferation and angiogenesis in an animal model of TBI (Kuo et al., 2010). However, we did not find that cooling impacted neurogenesis, synaptophysin, or brain derived neurotrophic factor levels after global ischemia in the rat (Silasi et al., 2012). Another study using younger animals and deeper cooling (30°C) found that cell proliferation was decreased in the hippocampus after post-ischemic cooling (Kanagawa et al., 2006). Furthermore, cooling decreased NMDA receptor NMDAR1 expression after global ischemia in the gerbil. Even though reducing NMDAR1 conferred neuroprotection by ameliorating excitotoxicity, it also led to a depressed LTP even a week after the ischemic insult (Miyamoto et al., 2000). These studies, however, were mainly carried out in global ischemia models in which damage occurs in both sides.

Cooling for 21 days does not have any impact on physiological variables (e.g., blood pressure), behavioural outcome, or cortical neuronal morphology in otherwise naïve animals (Auriat et al., 2012). However, in that study there was no factor that was driving plasticity (e.g., injury, learning, etc.). Interestingly, a previous study assessing spatial learning in healthy rats

showed that the rats cooled during training in the Morris water maze performed as well as NORMO rats despite HYPO lowering evoked potentials in the hippocampus (Moser and Andersen, 1994). In this case, cooling could have acted as a motivator, as the discomfort of being cold could have improved the rats' ability to perform the task, or it could be mimicking our results. However, when their hippocampal temperature went below 30°C, rats were not able to perform the task as well (Moser and Andersen, 1994). It is possible that deeper cooling temperatures, which are not currently used in the clinic, could have impaired learning.

In our study, we initially hypothesized that cooling the contralateral side to the preferred paw of naïve rats would impair reaching or make rats switch limb preference. Instead we found that both HYPO conditions, although not a large or significant effect, similarly reduced reaching success. These could be explained by different factors. First, cooling could be acting as a stressor. This could be further assessed in a future study by measuring cortisol levels and correlating these to outcome. Second, our cooling device may not be selective enough to just cool one side, as shown in the anesthetized animals. This is challenging to address, as it would be cumbersome to have a device that would cool one side and keep the other normothermic. This would imply having to measure brain temperature, which in itself can lead to injury. Third, there is the possibility that cooling the contralesional hemisphere hinders balance of the unimpaired paw therefore also affecting reaching. This could be further studied by measuring spontaneous limb use, for instance by testing the rats in a cylinder task. Last, it may be a combination of all of these factors. Overall, our results suggest that HYPO is a safe treatment, as it did not have a detrimental effect on learning. In order to determine whether learning-driven plasticity was affected by cooling, we will also measure dendritic arborization in the motor cortex with Golgi Cox stain. The data is currently being analyzed.

In conclusion, contralesional HYPO does not hinder reaching success of the impaired limb but reduces shift in limb preference. This might be helpful when combined with rehabilitation therapy (MacLellan et al., 2005). Also, cooling does not impair learning of a forelimb reaching task in otherwise naïve rats. Indeed, HYPO may be beneficial for recovery by reducing learned non-use. However, bilateral cooling applied for extended periods may have a detrimental effect (e.g., diminishing returns), as it may not act only by reducing cell death but may begin to negatively interact with plasticity processes. Further animal research must assess when HYPO ceases to be neuroprotective and starts hindering outcome.

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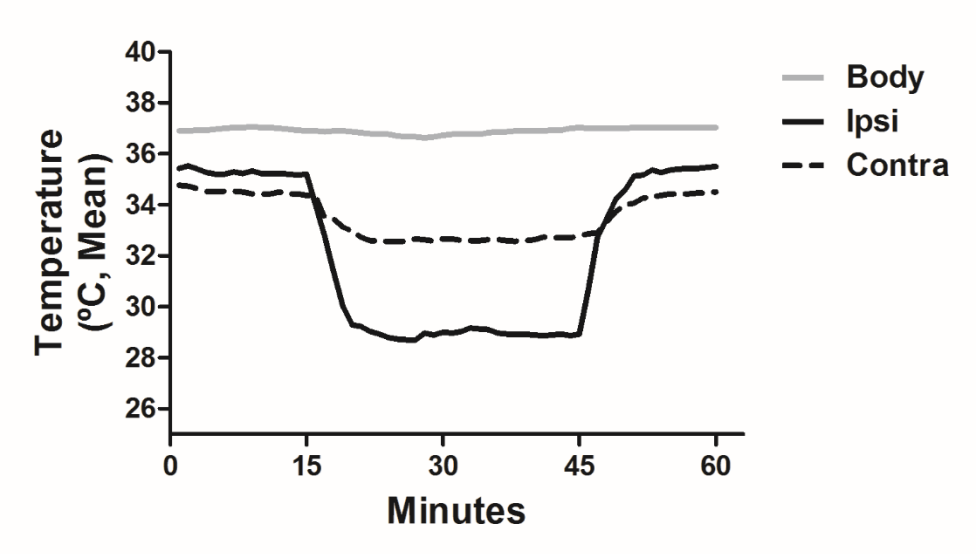


Figure 2-1. Temperature measurements from rectum (Body), ipsilateral (Ipsi), and contralateral (Contra) cortex prior, during, and after localized cooling in anesthetized rats (Experiment 1).

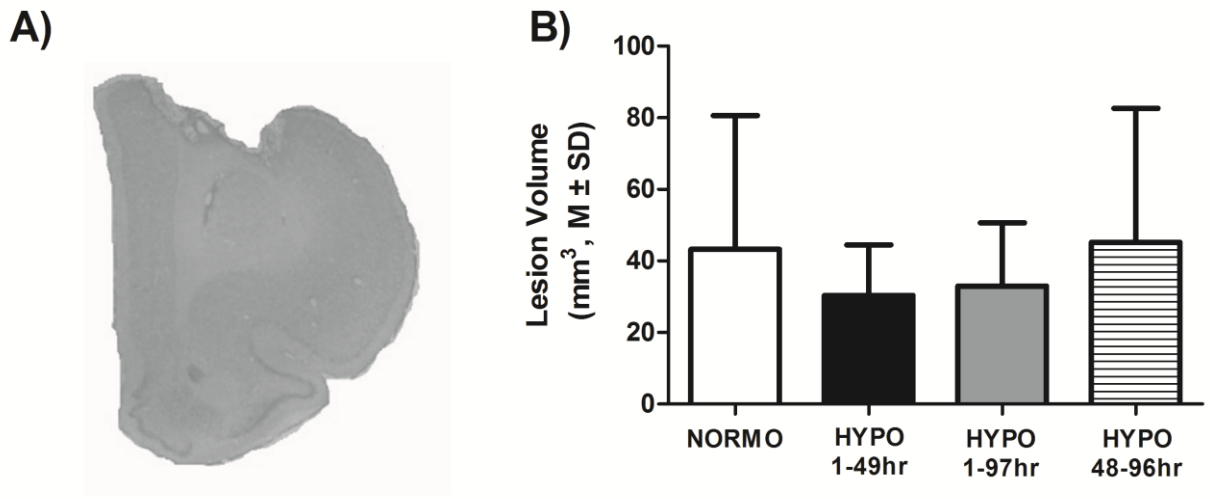


Figure 2-2. A) Profile of injury after motor cortex devascularization. B) Lesion volume was not affected by contralesional HYPO (Experiment 2).

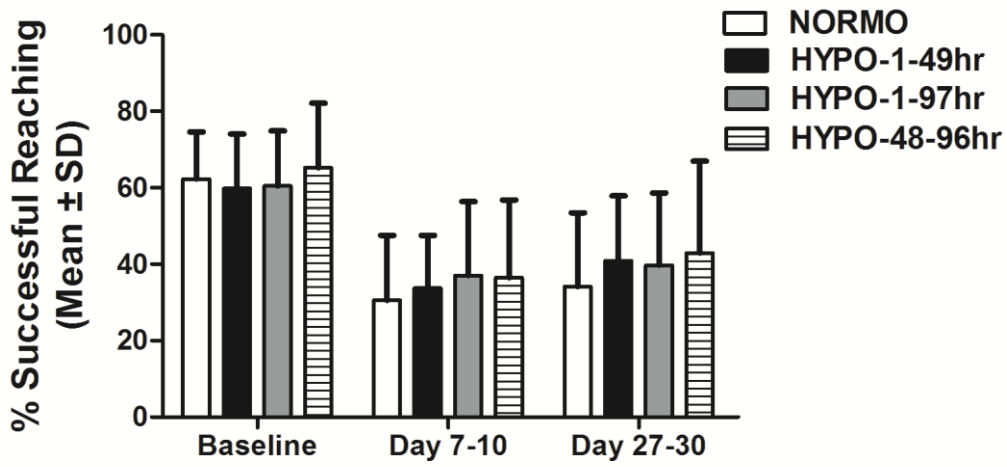


Figure 2-3. Successful reaching in the single pellet task was not different among groups. Overall performance was worse due to the motor cortex devascularization at both time points after the stroke but there were no differences among groups (Experiment 2).

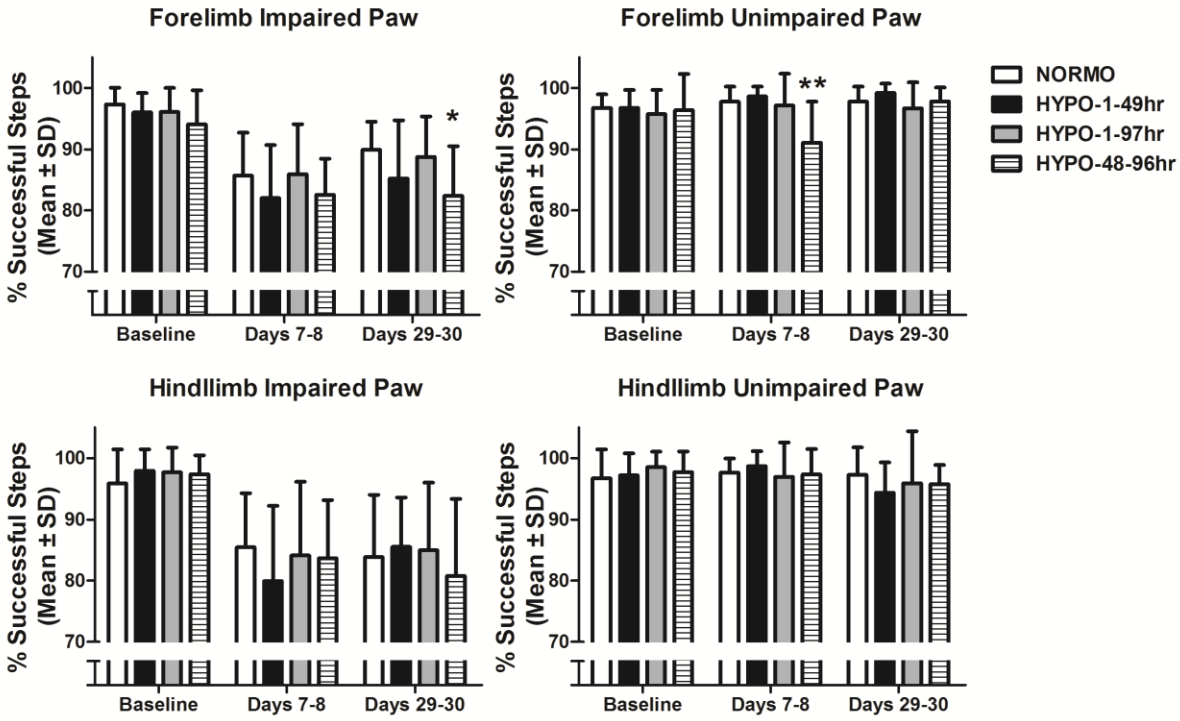
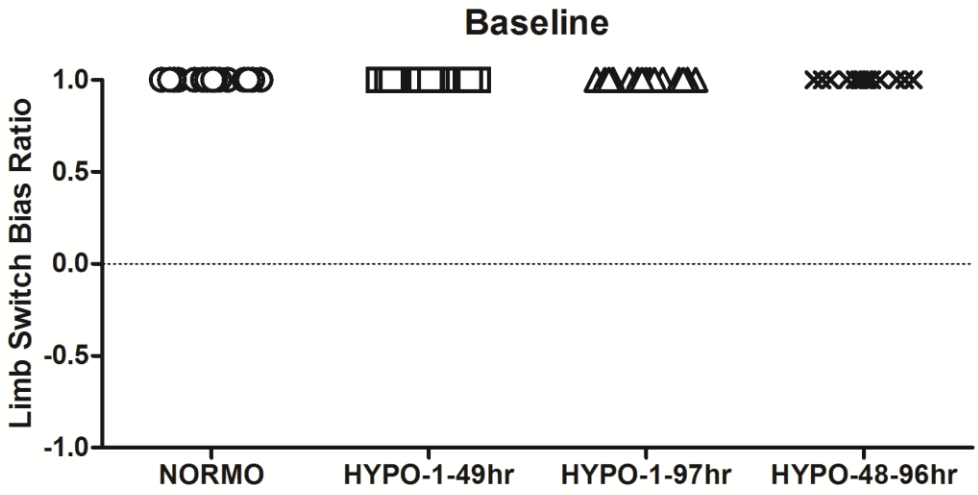
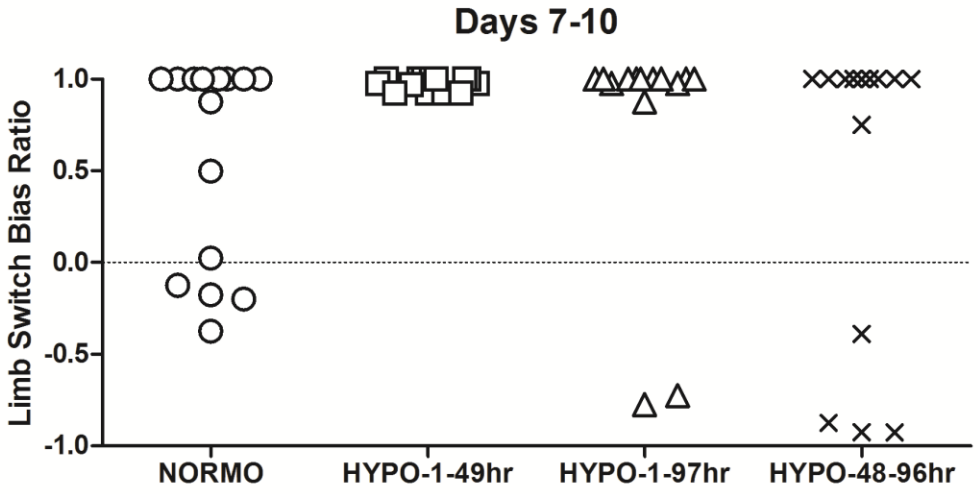


Figure 2-4. Motor cortex devascularization reduced the percentage of successful steps on the ladder task in the impaired limbs. There were treatment effects for both forelimbs, in which HYPO-48-96hr had lower successful steps (*p<0.05 compared to HYPO-1-49hr and NORMO on days 29-30, and **p<0.001 compared to all groups on days 7-8; Experiment 2).


Baseline
Limb Switch Bias Ratio
1.0
0.5
0.0
-0.5
-1.0
NORMO
HYPO-1-49hr
HYPO-1-97hr
HYPO-48-96hr


Days 7-10
Limb Switch Bias Ratio
1.0
0.5
0.0
-0.5
-1.0
NORMO
HYPO-1-49hr
HYPO-1-97hr
HYPO-48-96hr

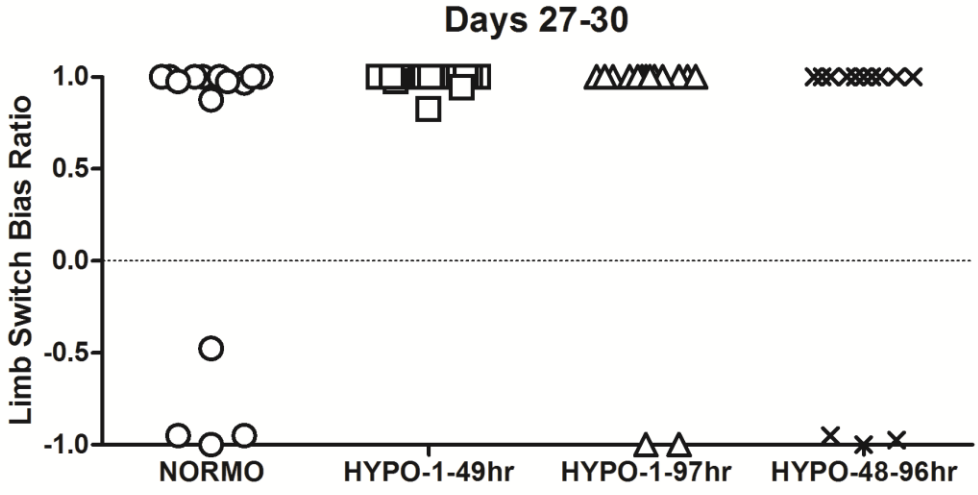

Days 27-30
Limb Switch Bias Ratio
1.0
0.5
0.0
-0.5
-1.0
NORMO
HYPO-1-49hr
HYPO-1-97hr
HYPO-48-96hr

Figure 2-5. Bias ratio for limb preference. Ratio of 1 depicts use of the affected paw only, whereas ratio of -1 depicts use of the unaffected limb. At baseline, all rats used their preferred paw (score of 1), which was later impaired by the motor cortex devascularization. NORMO and HYPO-48-96hr rats switched limb preference significantly more than HYPO-1-49hr at days 7-10 (Experiment 2).

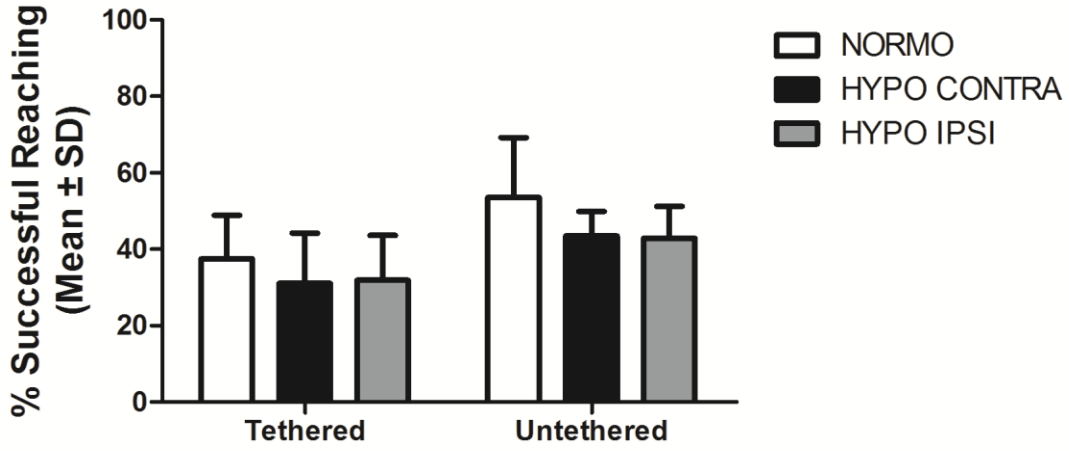


Figure 2-6. Performance on the single pellet task was not affected in those rats that were trained while being cooled. The "Tethered" period displays the averages of the first five days of training, while the "Untethered" displays the last five days of training.

CHAPTER 3

Seizure Activity Occurs in the Collagenase but not the Blood Infusion Model of Striatal Hemorrhagic Stroke in Rats

3.1. Introduction

Intracerebral hemorrhage (ICH) occurs in ~15% of stroke patients, leading to ~50% mortality and significant disability in survivors (Broderick et al., 1989; Balami and Buchan, 2011). So far, there are no specific neuroprotective therapies for ICH although survivors benefit from rehabilitation. Thus, it is important to fully understand those factors that affect outcome after ICH in order to improve medical management and further limit death and disability. For instance, seizures are a common occurrence after ICH, or even a presenting sign of an ICH. About 4-20% of ICH patients will suffer from clinical seizures (e.g., convulsions) whereas 30% of ICH victims will have subclinical seizures observable on an electroencephalogram (EEG; (Bladin et al., 2000; Passero et al., 2002; Balami and Buchan, 2011)). Current data suggests that the risk of seizures occurring within the first month is 8% (Passero et al., 2002), and the risk of a seizure occurring after the first month and within the first year is 3% (Bladin et al., 2000). Still, this could be an underestimate caused by the lack of continuous EEG monitoring in patients.

Intuitively, seizures are expected to worsen outcome after an ICH. Seizures can exacerbate excitotoxicity and oxidative stress (Willmore and Ueda, 2009; Shin et al., 2011), augment metabolic rate (Meldrum and Nilsson, 1976), and cause re-bleeding or increased bleeding due to elevated BP and blood flow during seizures (Meldrum and Nilsson, 1976; Goodman et al., 1990; Passero et al., 2002). Intracranial pressure (ICP) also rises due to seizures (Vespa et al., 2007), which can cause complications after ICH (e.g., herniation) and increase mortality (Balami and Buchan, 2011). Seizures may also cause aberrant brain plasticity (e.g., larger cortical maps), and impair recovery (Teskey et al., 2008). Lastly, even though the number of ICH patients that develop epilepsy is relatively low (2-5%), the incidence of one seizure increases the chance of developing epilepsy (Bladin et al., 2000; Passero et al., 2002; Balami and

Buchan, 2011).

All of this illustrates why seizures could be harmful. Clinical studies on this topic, however, have not consistently found that seizures are detrimental (Bladin et al., 2000; Passero et al., 2002; Alberti et al., 2008; Balami and Buchan, 2011), although some support this notion (Arboix et al., 1997; Szaflarski et al., 2008; Messe et al., 2009). This variability among clinical studies could be attributed to several factors, such as inclusion criteria, methods for measuring EEG, lack of continuous EEG monitoring, use of anti-epileptic drugs (AEDs), among others. Current guidelines suggest that ICH patients with a depressed mental state should have continuous EEG monitoring, as most seizure activity occurring after ICH is subclinical (Bladin et al., 2000; Passero et al., 2002; Morgenstern et al., 2010; Balami and Buchan, 2011). Any seizure activity ought to be treated intravenously with an AED, and if seizure activity persists, AED treatment may continue orally. Prophylactic administration of AEDs, however, has been discouraged after evidence from studies indicating a worsening of outcome caused by administration of phenytoin before any signs of seizure activity not only after ICH (Naidech et al., 2009; Morgenstern et al., 2010) but also traumatic brain injury (TBI (Temkin et al., 1990).

The incidence and consequences of seizures after an ICH have not been well studied in animal models. Thus far, swine studies have shown that excitability increases in certain areas of the brain after ICH (Mun-Bryce et al., 2006). Others have shown that in rodents, the most widely used ICH model, intracerebral infusions of blood components such as thrombin (Lee et al., 1997) and iron (Willmore et al., 1978) cause seizures. To our knowledge, there have been no formal evaluations of seizure activity in the common rodent models of ICH, which involve injecting autologous blood (Bullock et al., 1984) or collagenase (Rosenberg et al., 1990) into the brain. Unlike the whole blood injection, bacterial collagenase, an enzyme that breaks down the basal

lamina, causes bleeding over hours mimicking what frequently occurs in ICH patients (Rosenberg et al., 1990; MacLellan et al., 2008). Often, investigators target the striatum as it is a common site of ICH in humans and because it can contain a large hematoma that results in persistent, easily quantified, behavioural impairments (MacLellan et al., 2006). In this study, we induced a moderate-sized striatal ICH in rats by injecting collagenase or whole blood, and we monitored rats with an implanted EEG telemetry probe for a week after the stroke. By using telemetry, we were able to continuously record EEG in freely moving untethered animals, which is the least stressful method for these animals. The objective of this study was to determine the incidence and characteristics of electrographic epileptiform activity (seizures) that occur in these animal models of ICH.

3.2. Methods

Subjects

All procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. Twenty male Sprague-Dawley rats (250-400 g, ~ 3 months old) obtained from the Biosciences breeding colony at the University of Alberta were assigned to either the collagenase, whole blood or saline group. As a positive control, a rat received an injection of $FeCl_2$. Food (Purina rodent chow) and water were provided ad lib and rats were house individually in a temperature and humidity controlled room (lights on from 7 am–7 pm).

EEG Probe Implantation

Surgical procedures were performed aseptically. Rats were anesthetized with isoflurane (4% induction, 1.5–2.5% maintenance in 60% N_2O, balance O_2) and body temperature was maintained at 37 °C during anesthesia with a heated water blanket and a rectal temperature probe. An EEG telemetry probe (F40EET, Data Sciences International, St. Paul, MN) was inserted either in the peritoneal cavity or the neck (dorsal S.C. placement), and the leads were channeled under the skin and attached to screws stereotaxically placed ipsilateral (AP: -1.5, ML: 4) and contralateral (AP: -1.5, ML: -4) to the injection site (see Figure 3-1). The leads were secured to the screws (0-80x3-32; Plastics One, Roanoke, VA) with dental cement. These telemetry probes measure EEG (sampled at 500 Hz, low-pass filtered at 100 Hz) and also temperature and movement activity. The latter is detected by changes in signal strength as the probe moves across the receiver (Data Sciences International), providing a relative measure of activity (Colbourne et al., 1998).

Iron injection

As a positive control to assess the ability of the EEG probe to detect seizures, we injected iron in the rat striatum (N=1). Immediately following attachment of the electrical leads, a hole was drilled (AP: 0.5, ML: 3.5) and a Hamilton 26 gauge needle was inserted 6.5 mm into the striatum to infuse 38.0 μg of $FeCl_2$ in 30 μL of saline (Nakamura et al., 2006; Caliaperumal et al., 2012). The injection was completed over 10 minutes and the needle was removed after an additional 10 minutes. Clips were used to close the wound. The rat was euthanized 1 week later.

Collagenase, Blood, and Saline injections

For the saline (N=3) and five of the collagenase rats (N=10) a baseline recording period of one week was undertaken before the injection. No baseline recording was done for the whole-blood rats (N=6). Following baseline recordings, or directly after the securing of electrical leads, a hole was drilled (AP: 0.5, ML: 3.5) and a Hamilton 26 gauge needle was inserted 6.5 mm into the striatum. Either saline, 100 µL of autologous whole blood (from tail artery), or 0.14 U of bacterial collagenase in 0.7 µL of sterile saline was infused over 5 or 10 (blood model) minutes and the needle was removed after an additional 5 or 10 minutes (MacLellan et al., 2008). Clips were used to close the wound. Saline control and whole-blood rats were euthanized after 7 days whereas the collagenase rats were euthanized between 11 and 66 days. This difference in survival times was due to differences in probe placement and technical difficulties that ensued. For instance, probe placements in the neck region caused irritation due to collection of fluid (seroma).

Euthanasia and lesion volume assessment

Rats were injected with sodium pentobarbital (100 mg/kg, i.p.) and then perfused with 0.9% saline followed by 10% neutral buffered formalin. Brains were extracted, cryostat-sectioned at 40 µm and stained with Cresyl Violet. Coronal sections taken every 200 µm were then analyzed with Image J (Schneider et al., 2012), as routinely done on digitized images extending anterior, through and posterior to the lesion. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere − area of damage − ventricle) × interval between sections × number of sections (MacLellan et al., 2008;

Caliaperumal et al., 2012). This method takes into account both areas of injury as well as atrophy and ventricular dilation.

EEG Analysis

Baseline and post-infusion EEG traces were visualized with Dataquest A.R.T 2.3 system (Data Sciences International) and the incidence, duration of seizures, as well as the time to seizure onset from injection were recorded. In three rats, an extended period of 30 days was further analyzed in this way. We compared five-minute epochs of non-epileptiform activity from the day prior to the injection with recordings taken at least three days post-stroke from the rats that had a one week baseline recording (collagenase N=4; and saline N=3) in order to detect any changes in otherwise normal-looking EEG. We did not find a significant difference between the day prior to collagenase infusion and day 3 after stroke. Therefore, we considered traces from the third day post-collagenase infusion to be an additional substitute baseline measure for all collagenase rats. Baseline and putative epileptiform traces were exported and analyzed using custom code written in MATLAB (R2012a, Mathworks, Natick, MA). We computed for each signal the root mean square (RMS), the power spectral density using Welch's averaged modified periodogram method (6 s window, 2 s overlap), as well as dual channel coherence for ipsilateral and contralateral recordings (3 s window; 1 s overlap). Field signals and spectra were plotted for comparison with baseline measures using Origin 9.1 (Microcal Software, Northampton, MA). We compared all putative epileptiform measures, including RMS and 95% confidence intervals of amplitude fluctuations to those taken during baseline/control conditions in order to confirm that the events were abnormal and therefore they were classified as seizures. For ictal traces longer than 25 seconds, power spectra were compared to those baseline traces equal in duration

to determine total increases in power, frequencies significantly affected by the seizures, as well as bilateral coherence. A randomized coherence distribution based on a series of sequential time-shifted (and also time-reversed) coherence computations from these actual traces was computed to calculate the coherence significance level. The maximum 95% confidence limit for this randomized distribution (throughout the frequency range) across multiple datasets having durations ranging from 27 to 87 seconds ranged from 0.22 to 0.061, respectively. In order to determine cross-hemispheric coupling changes during epileptiform activity we subtracted the coherence values of normal activity from those of epileptic traces and considered that any increases equal to or larger than the confidence limit for that trace was significant. For shorter duration aberrant activity (i.e. interictal spikes) we performed a detection analysis based on a threshold amplitude beyond the amplitude distribution of the normal traces (3.5 standard deviations from the mean (Nazer and Dickson, 2009) and computed the average waveform and number of events occurring per unit time.

Temperature and Activity

We computed temperature averages (F40EET probe) of 5-minute periods taken from the hour after the seizure as the difference from the hour prior (e.g., one hour before seizure average – 5 minute temperature average) and statistically analyzed it for comparison. If a seizure occurred in the hour before another seizure, then it was excluded. In the same manner, one hour activity measures before and following seizure were averaged and compared to assess the impact of seizures on activity (as measured by signal strength changes as the probe moved across the receiver).

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by repeated measures analysis of variance (ANOVA) and student's t-tests (SPSS v.17.0, SPSS Inc., Chicago, IL). The Fisher's exact test was used to compare seizure incidence between models.

3.3. Results

Baseline EEG in the Collagenase Group

Baseline recordings allowed us to relate different behaviours to the EEG traces (Figure 3-2), which were helpful for detecting abnormal activity. When we compared the RMS of 5-minute traces of the day prior to stroke/sham surgery and days 1, 2, and 3 after the injection of either saline or collagenase (including only those ICH rats with seizures), we found a group (saline vs. collagenase) effect for the ipsilateral channel ($p = 0.050$, Figure 3-3) depicting an increase in RMS in non-epileptic EEG traces after the injection in the collagenase group. This means that the average amplitude fluctuation of traces was higher for the collagenase group even during non-epileptic EEG. Moreover, there was a time effect for the contralateral side ($p = 0.028$), the RMS on the first day after injection was larger than the third day ($p = 0.040$) in both groups although there was no treatment effect.

Seizures after Collagenase and Iron Injection

One collagenase rat had to be excluded due to equipment failure that did not allow for adequate EEG recordings. Six out of the nine remaining rats (66%) had seizures within the first two days after the stroke with the earliest occurring after ~10 hours and the latest occurring after ~36 hours. Seizures ranged in duration from ~5 to 90 seconds, and their averages ranged from 14

to 54 seconds (Table 3-1, Figure 3-4B-D). Iron also caused seizures within hours of application (Figure 3-4A), which displayed a similar pattern of tonic-clonic ictal events followed by notable EEG suppression and occasional after discharges. Seizures or other electrographic abnormalities were not observed in any of the 6 rats infused with 100 µL of autologous blood ($p = 0.028$ for comparing number of rats with seizures between models). Not surprisingly, both the number ($p = 0.036$) and duration of seizures ($p = 0.036$) were significantly greater in the collagenase model.

In two collagenase rats, we detected extended periods of interictal epileptiform discharges, ranging from ~1.5 to 14 hours (Figure 3-5). Over the span of 30 minutes in which seizures occurred in one animal, we detected abnormal interictal events in-between obvious ictal activity. Interictal activity did not last more than 2 minutes; therefore we were not able to analyze them using our event detection analysis due to their short duration. For the three rats in which 30 days of EEG were screened, two had seizures within 36 hours, but none of them had any noticeable EEG abnormalities after that period. Therefore, none of our collagenase rats appeared to have developed epilepsy *per se*. Most of the seizures were bilateral, which were likely generalized from an initiation zone located in the affected hemisphere and propagated across contralateral hemisphere. There was only one case in which the seizure activity occurred only ipsilaterally (Table 3-1).

Severity of Seizures in the Collagenase Group

We calculated RMS and quantified power increases of seizure traces and compared them to non-epileptic activity taken from day 3 post-collagenase surgery as an indicator of increases in amplitude at different frequencies (power) and fluctuations in the amplitude (RMS) of the ictal events. The smallest RMS fold increase for the ipsilateral side was 1.58 times larger than non-

epileptiform activity, and the largest was 4.69 times larger during ictal activity. Similarly, for the contralateral side, the smallest epileptiform RMS was 1.45 times larger, and the largest was 4.96 larger. This indicates that during seizure activity there was about a 50-500% increase in amplitude fluctuations in these traces. In general, increases in power were seen at all frequencies up to 38 Hz, with a decrease in frequencies across the 0.5-5.8 Hz bandwidth detected in a single rat (Table 3-1). Also, in most cases the ipsilateral channel had an equal or greater increase in power than the contralateral, although this was not the case for two rats. This could support the notion of an ipsilateral focus that propagates, and/or generalizes, to the contralateral hemisphere.

For coherence calculations, we concentrated on increases in coherence at the frequencies in which we noted a change in power. Coherence values range from 0 to 1; values of 0 indicating that signal-specific frequencies between the two channels are completely unrelated, whereas values of 1 indicate that they are completely related. In most instances, we found significant increases in cross-hemispheric coherence during epileptiform activity as compared to normal traces taken 3 days after the collagenase injection (average increase across frequencies 0.28 ± 0.14). Two cases showed exceptions to this rule, one in which no increase was observed and another in which coherence values were significantly decreased despite prominent bilateral seizure activity in both cases. In one rat we also detected that coherence was decreased for frequencies lower than 6 Hz but that for higher frequencies coherence was increased. Indeed, increases in power for higher frequencies (12 - 40 Hz) during seizure events were all associated with significantly increased coherence (see example in Figure 3-6). This might indicate that the more severe seizures were, the more likely that both hemispheres were engaged in epileptic activity.

Lesion volume

The infusion of collagenase or blood caused significant damage and inflammation, as depicted in Fig 7A ($p = 0.22$ for lesion volume). In the collagenase model, we found no significant relationships between lesion volume and seizure incidence ($r = 0.18$, $p = 0.65$; see Figure 3-7B), time of onset after stroke ($r = 0.30$, $p = 0.56$), total ($r = 0.37$, $p = 0.31$) and average time spent in seizure activity ($r = 0.18$, $p = 0.63$), RMS ($r = 0.18$, $p = 0.65$), and power increase ($r = 0.29$, $p = 0.63$). Thus, we did not find that lesion volume predicted seizure characteristics in this model. Owing to the lack of seizure activity we did not perform this analysis with the whole blood model data.

Temperature and activity data

A repeated-measures ANOVA did not depict a difference among the twelve 5-minute average intervals (i.e., 1 hour) following the seizure (p=0.382), indicating that there were no changes in temperature after the seizure. Even though there was no pattern in the temperature change among rats, one rat had a seizure leading to about 90 minutes of hypothermia (Figure 3-8). Moreover, a paired t-test on all of the rats' movement activity indicated no difference between activity before and after the seizure ($p = 0.7968$).

3.4. Discussion

We expected seizures to occur in the blood infusion model, but this was not observed. Our results did confirm our hypothesis that seizures commonly occur after striatal ICH in the collagenase model. Sixty-six percent of rats in the collagenase group suffered seizures during the first 36 hours following their stroke. Seizures commonly occur after brain injury and stroke, both

in patients (Annegers et al., 1998; Camilo and Goldstein, 2004; Balami and Buchan, 2011) and in other animal models of brain injury (Hartings et al., 2003; Kadam et al., 2010; Bolkvadze and Pitkanen, 2012). Thus, it is not surprising that collagenase rats would also display abnormal electrical activity as we demonstrated in this study, including both full-blown ictal and abnormal interictal activity, which mostly occurred bilaterally. Increased cross-hemispheric coherence coinciding with increased power suggests that the activity in both hemispheres during seizure activity was coupled, and that more severe seizures recruited both hemispheres. Although there were some exceptions to this, coherence remained significantly increased, especially at the higher frequencies. We also demonstrated that even normal-looking electrical activity has an increased RMS for the first three days after collagenase-induced ICH, which could indicate that there were abnormalities in non-epileptic EEG activity during this limited time frame. Although robust epileptiform activity was a consistent phenomenon in our collagenase group, we did not find lesion volume to be a predictor for any of the seizure characteristics. Likewise, the lack of seizures in the blood model, which had a comparable lesion, argues against lesion or hematoma volume as key predictors of seizure activity.

The incidence of electrographic seizures in our collagenase group (66%) is more than double that documented in ICH patients (Balami and Buchan, 2011). The difference in incidence might be attributed to the greater range in patient characteristics (e.g., ICH locations, severity) in clinical studies, along with other factors such as species differences. Interestingly, other pre-clinical studies of brain insults, namely hypoxic-ischemic injury (Kadam et al., 2010), focal ischemia (Hartings et al., 2003), and traumatic brain injury (Bolkvadze and Pitkanen, 2012), all report a much higher percentage of animals developing seizures and epilepsy than what is

reported in the clinic. However, continuous monitoring of EEG for many weeks or months is rare in clinical studies, making it difficult to compare animal and clinical data.

There are key differences between the collagenase and whole blood models that may explain the discrepancy in seizure incidence between these models. For instance, the whole-blood model of ICH provides a somewhat different profile of injury (see Figure 3-7A) with less secondary injury, inflammation, blood brain barrier damage and smaller ICP spikes (MacLellan et al., 2008; MacLellan et al., 2012; Hiploylee and Colbourne, 2014), but these may vary by species (Barratt et al., 2014). As with inflammation (Vezzani et al., 2010), it is possible that the timing, extent and localization of thrombin production vary between models and this might account for differences in seizure activity. Note that intra-cerebral infusions of thrombin induces seizure activity (Lee et al., 1997). Iron infusions also cause epileptogenic activity (Willmore et al., 1978), as we presently confirmed. However, given the timing of iron release, which in our collagenase model occurs between 24 and 72 hours (Auriat et al., 2012), it is unlikely that iron causes seizures as they began between 10 and 22 hours, and stopped by 36 hours. As well, the hematoma volume is expected to be larger in rats infused with 100-μL of blood than those given collagenase (MacLellan et al., 2008). Thus, if iron were the primary cause of seizures, there should have been more seizures in the whole blood model.

Early seizures are predictors of future epilepsy in stroke patients (Szaflarski et al., 2008); although a study by Bladin and colleagues (2000) showed that all of the patients that had late onset seizures, which were those at 2 or more weeks after the stroke, developed epilepsy. We did not find recurrent seizures after the first 36 hours of the stroke, even though we screened EEG for up to a month after collagenase infusion. While this suggests that this model does not lead to epilepsy, a much larger sample size is needed especially given the small percentage expected to

develop that condition. There is also the possibility that seizures may develop later than a month after ICH in rats, as occurs in other animal models such as TBI (Bolkvadze and Pitkanen, 2012).

There are some limitations to this study. First, we did not video record any of the seizure events, so the type of behavioural manifestations with these electrographic seizures remains unknown. Although, we occasionally noticed behavioural signs of focal seizures, such as clonic paw movements. Second, the relatively limited number of animals in this study cannot exclude the possibility that occasional seizure activity occurs in the whole-blood model. Third, with larger sample sizes a modest relationship between lesion volume and seizure characteristics may have been detected. Indeed, others have reported a relationship between epileptiform activity and infarct size after focal ischemia (Williams et al., 2004). In a clinical study, however, small lesion size was a better predictor of seizure incidence (Passero et al., 2002). Fourth, while we recommend use of telemetry probes, tethered systems have the advantage of allowing monitoring from more locations, which would be advantageous in future studies (e.g., to identify seizure focus). The use of telemetry probes also had some additional disadvantages (e.g., greater cost) including technical problems we encountered with use of lead extenders and of course the inevitable loss of battery power. Last, while EEG eventually returned to normal, it is likely that seizure thresholds were altered as found in traumatic brain injured (TBI) rats given a pro-convulsant challenge (Bolkvadze and Pitkanen, 2012).

Further studies should be carried out to advance our knowledge of seizures occurring after ICH. Seizure incidence should be studied after changing the location of the lesion, as lobar/cortical location has been associated with more seizure activity in patients (Bladin et al., 2000; Passero et al., 2002). Even though the striatal model of ICH is a common one, other structures have also been targeted, such as cortex (Mun-Bryce et al., 2006) and hippocampus

(Song et al., 2008), and we are presently evaluating these models. It is possible that whole blood injections in different locations may elicit seizure activity. Also, patients with an ICH also have increased ICP after the insult (Morgenstern et al., 2010), which is also common after a collagenase-induced ICH in rats (Hiploylee and Colbourne, 2014). This sustained rise in ICP could be associated either to the mass effect arising from the hematoma and edema or to seizure activity, which could especially be related to ICP spiking (Vespa et al., 2007). This could be elucidated by simultaneous EEG and ICP monitoring. Furthermore, future research should focus on the relationship between seizures, cell death, and recovery. Some clinical studies have related seizures with worsened outcome and mortality (Arboix et al., 1997; Szaflarski et al., 2008; Messe et al., 2009), although others failed to do so (Bladin et al., 2000; Passero et al., 2002; Alberti et al., 2008; Balami and Buchan, 2011). In animal models, we can experimentally increase seizure activity with convulsant drugs or diminish it with AEDs and test its impact on several markers of cell death (e.g., neurodegeneration) and functional outcome.

In conclusion, seizures occur in the majority of rats subjected to a collagenase-induced striatal ICH but did not occur after infusion of whole-blood - models widely used to study the pathophysiology of ICH and to assess neuroprotectants and rehabilitation therapies (MacLellan et al., 2012). As ICH patients also suffer from seizures early after the stroke, the rat collagenase model has good face validity to model seizures occurring after ICH although further work is needed to determine whether the underlying cause is the same as in patients. This is a key factor for translational purposes, as others have raised concerns regarding differences between animal and human ICH pathophysiology (NINDS ICH Workshop, 2005; James et al., 2008; Adeoye et al., 2010; Kirkman et al., 2011; Krafft et al., 2012; MacLellan et al., 2012). Researchers should also consider that seizures could potentially impact their studies. For instance, seizure activity

may exacerbate the damage caused by the stroke, altering the effectiveness of neuroprotective therapies. Also, treatments may indirectly reduce cell death by ameliorating seizure activity. We recommend the use of the whole-blood model when seizures may be a confounding factor. As this is the first study to find that seizures occur after collagenase-induced ICH in rats, we encourage further research to understand the relationship between seizures, cell death, and recovery after ICH. This way, we will be able to enhance therapies currently provided to ICH patients.

3.5. References

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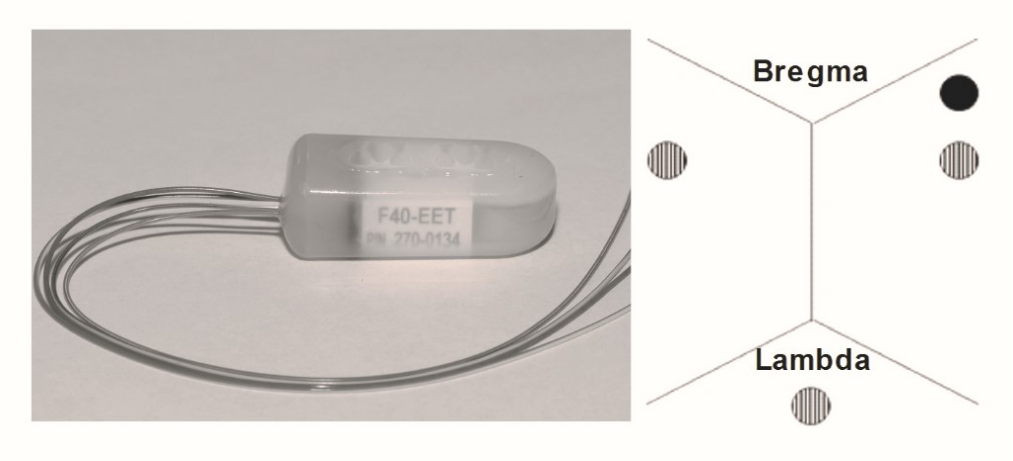

Figure 3-1. Telemetry probe inserted either in the peritoneum or under the skin of the neck (left). Leads were attached to screws (striped circle) on the skull and cemented. One channel recorded from the ipsilateral hemisphere next to the site of injection (full circle) and the other channel recorded from the contralateral side. Negative leads were connected to a screw posterior to Lambda. No further grounding was required.

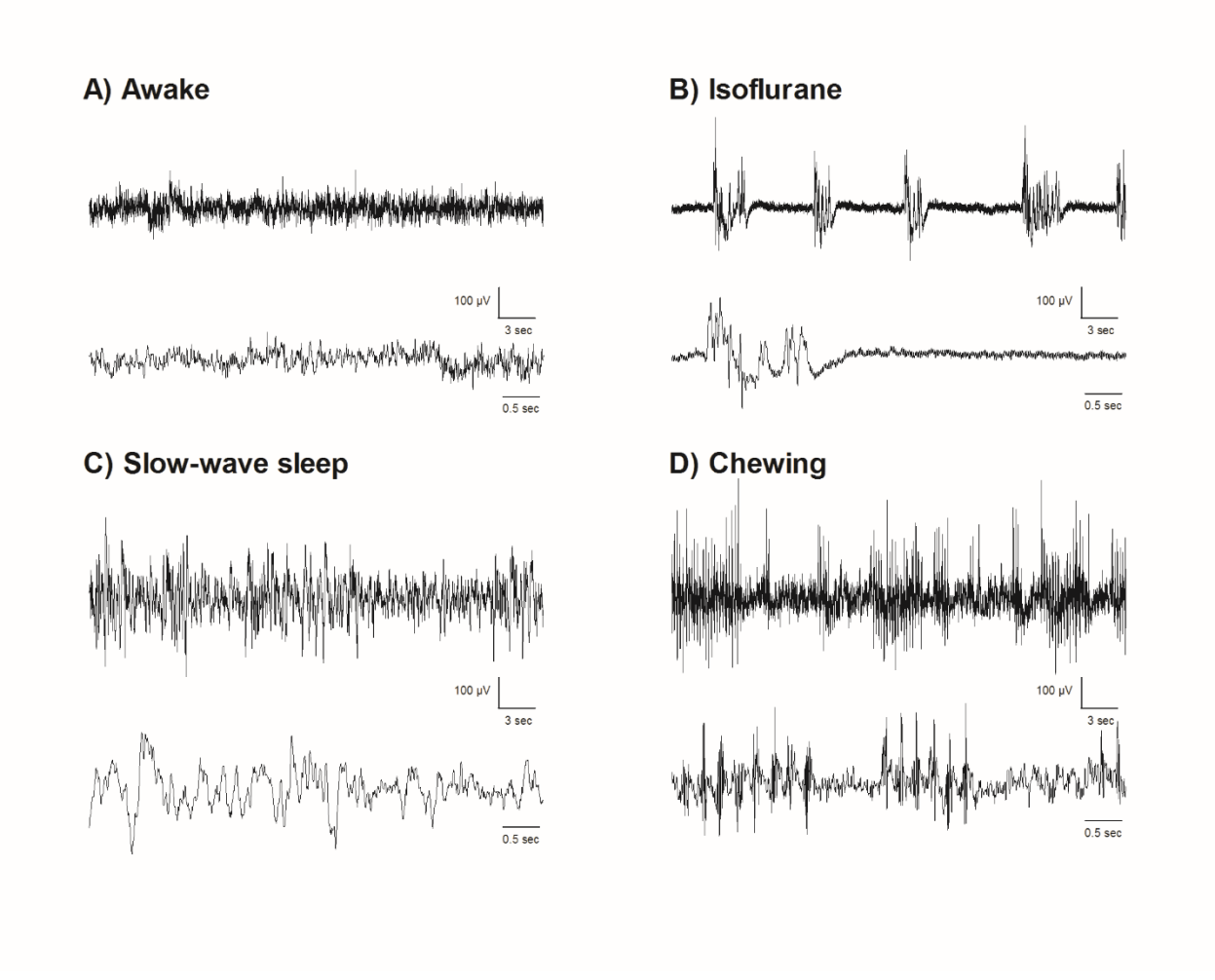

Figure 3-2. EEG traces during normal behaviour. These are examples of EEG activity during A) awake and alert periods, which display higher frequency and lower amplitude than C) slow-wave sleep. During anesthetics, such as B) isoflurane, it is common for EEG to display burst-suppression in rats. We were also able to detect artifacts such as during D) chewing in the EEG traces. In these instances, the experimenter observed all these behaviours.

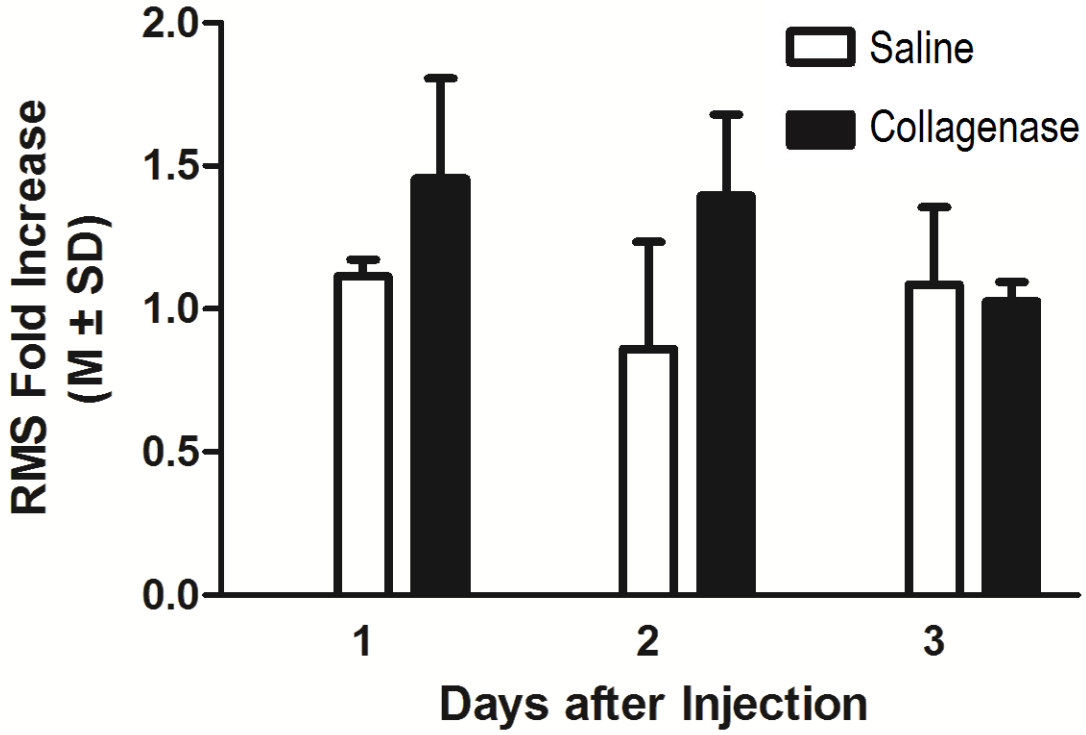

Figure 3-3. RMS increased in normal EEG after collagenase-induced ICH in the ipsilateral side. An overall RMS-fold increase from the day prior to stroke for a period of 3 days was detected in the collagenase rats that had seizures (N=4) compared to saline injection in the ipsilateral side ($p= 0.05$). This indicates that there were more fluctuations in the EEG traces relating to normal activity after collagenase than after saline (sham surgery) infusion. This was not the case for the contralateral side, for which there was a day effect for both groups but no impact of the treatment.

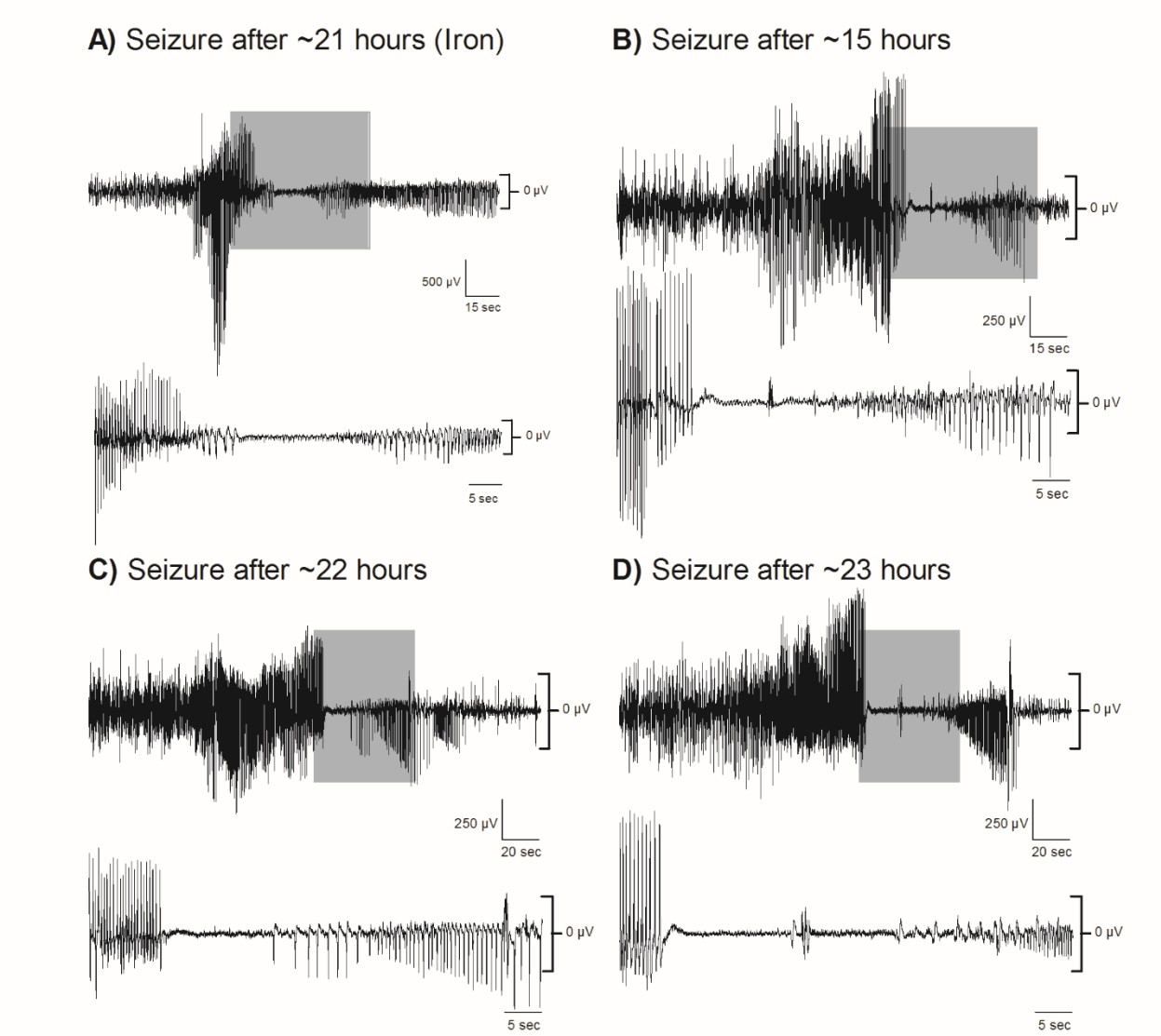

Figure 3-4. Seizures occurring after iron injection and collagenase-induced ICH. Examples of long-lasting seizures after A) iron injection as well as B)C)D) in three different rats given a striatal collagenase-induced ICH. Onset of seizures would be delayed by 10 h or more, and they would range anywhere from 5 to 90 s. Note the characteristic extended periods of suppression (highlighted in gray and expanded on the bottom panels) after high frequency and amplitude bursting. The confidence intervals (95 %) of normal activity 3 days post-stroke are indicated on the right of the traces.

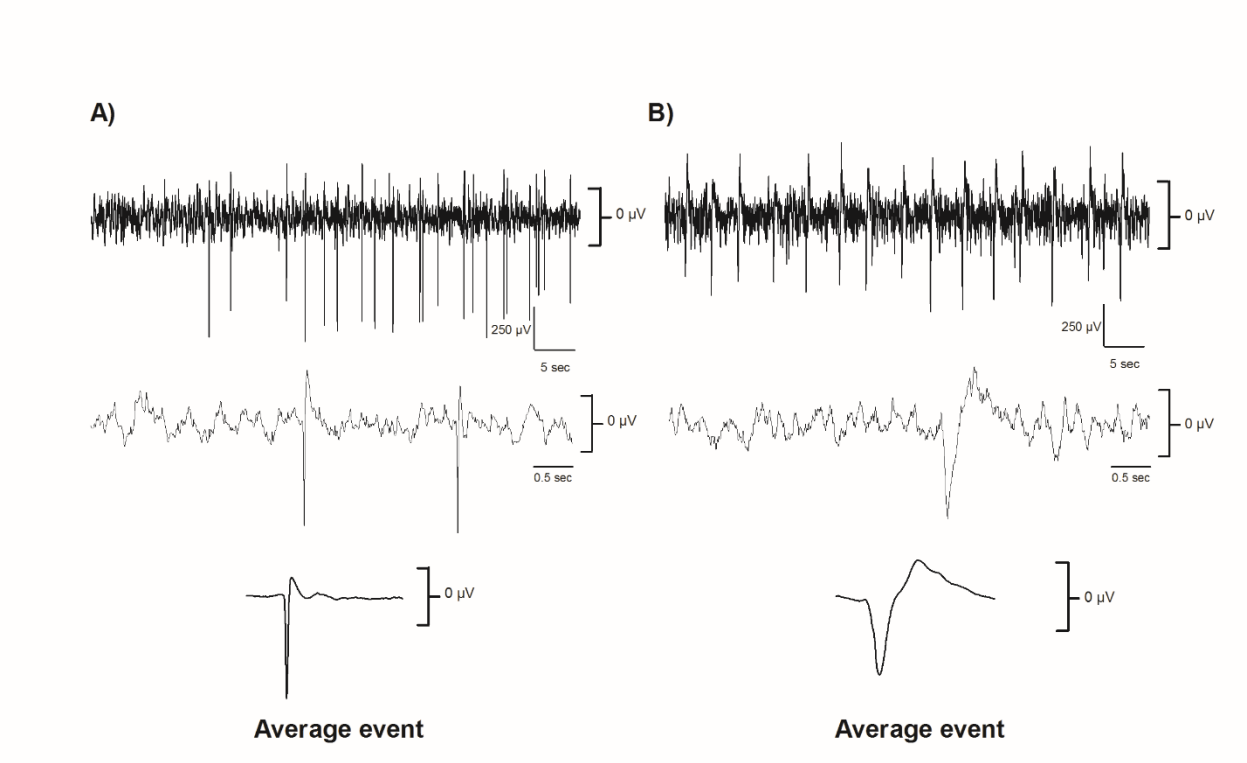

Figure 3-5. Interictal epileptiform activity in two collagenase rats. In our study, these rats (A, B) suffered extended periods of aberrant interictal activity. Note the characteristic downward spiking. For rat A, spikes occurred about 20 times per minute, and in rat B, they occurred 10 times per minute. The interictal events elicited over 1 h were averaged, as depicted at the bottom of the figure. The confidence intervals (95 %) of normal activity 3 days post-stroke are indicated on the right of the traces.

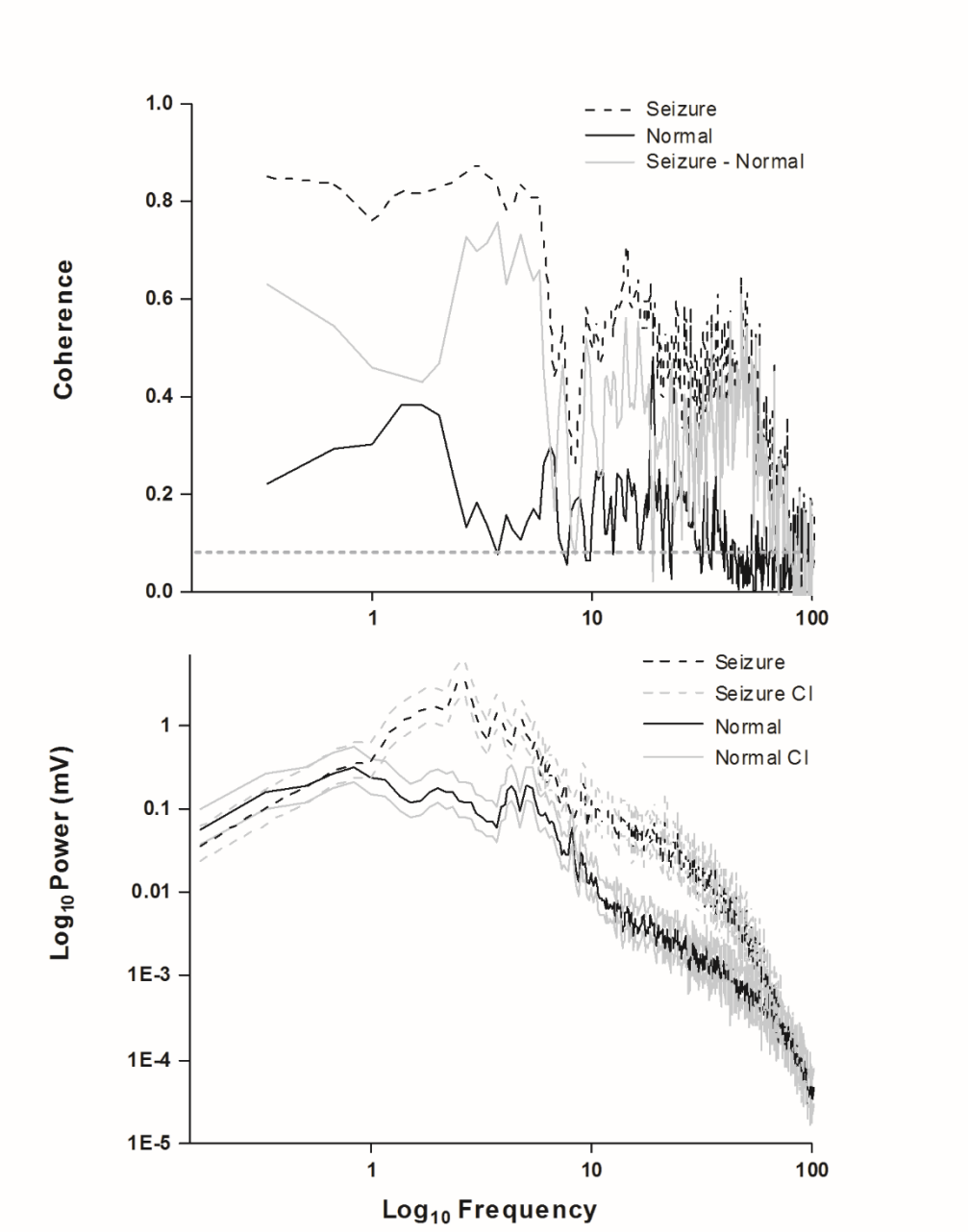

Figure 3-6. Seizures displayed higher power and increased coherence than normal EEG. This is an example of the coherence (top panel) and power spectrum (bottom panel) for the epileptiform event in Fig. 4d (dashed line) compared to normal EEG activity 3 days after collagenase-induced ICH (solid line). Any increase in coherence above the confidence interval limit (dotted line) of the difference between seizure and normal activity (gray solid line) was significant. The gray

lines in the power spectrum represent the 95 % confidence interval (CI) and the black lines the mean values for the spectrum. For those increased frequencies, there were also increases in coherence.

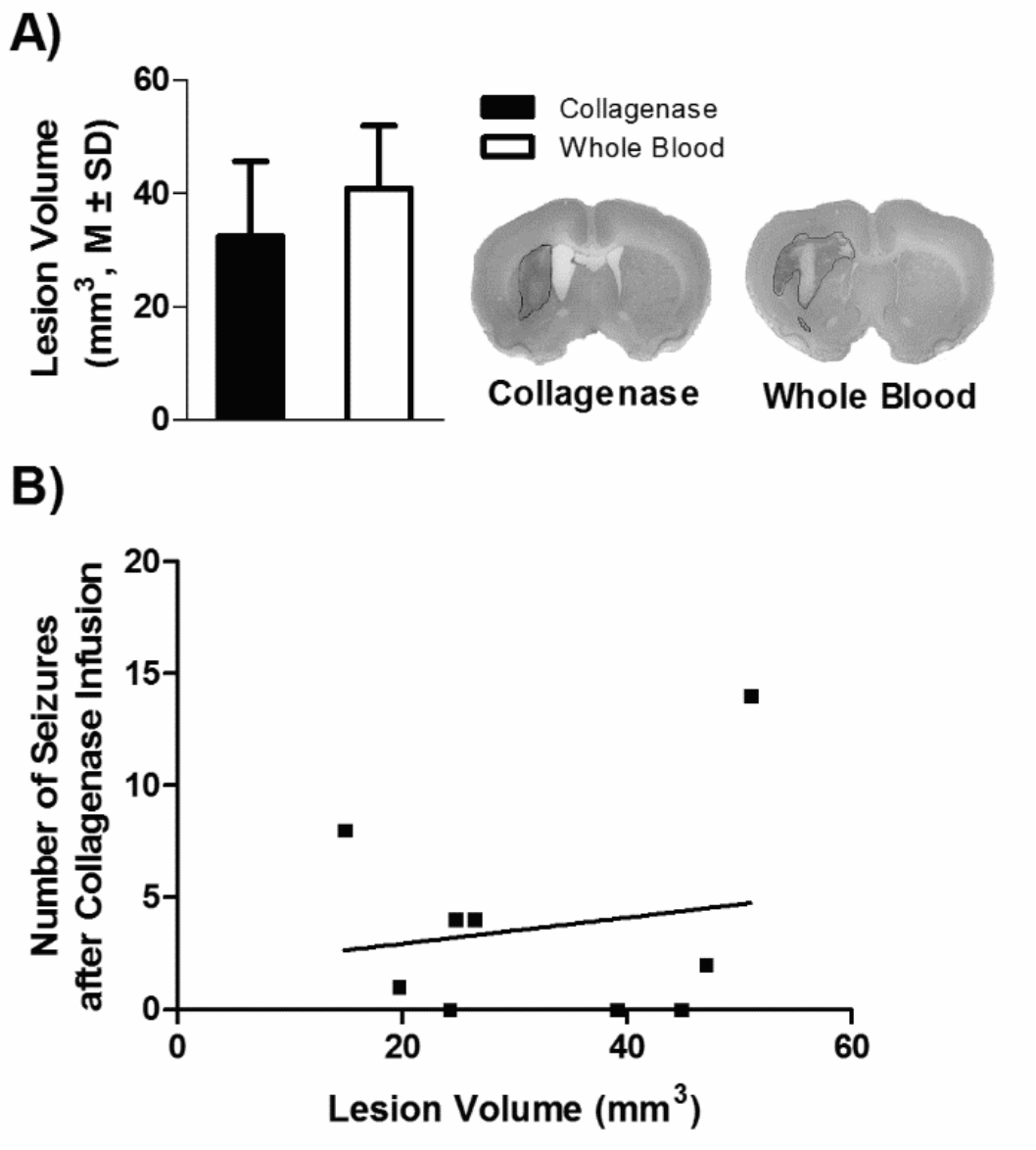

Figure 3-7. Lesion volume had no relationship with seizure incidence. The ICH typically damaged a substantial portion of the striatum with some damage to corpus callosum. No injury was found in sham-operated rats, other than a needle track. A) Total lesion volume (M±SD in mm^3) for the collagenase and whole blood models was not significantly different. Photomicrographs illustrate each model's profile of injury at the level of maximal damage (cresyl violet stain). B) There was no significant correlation between lesion volume (black squares) and the incidence of seizures in the collagenase model (r=0.16, p=0.67).

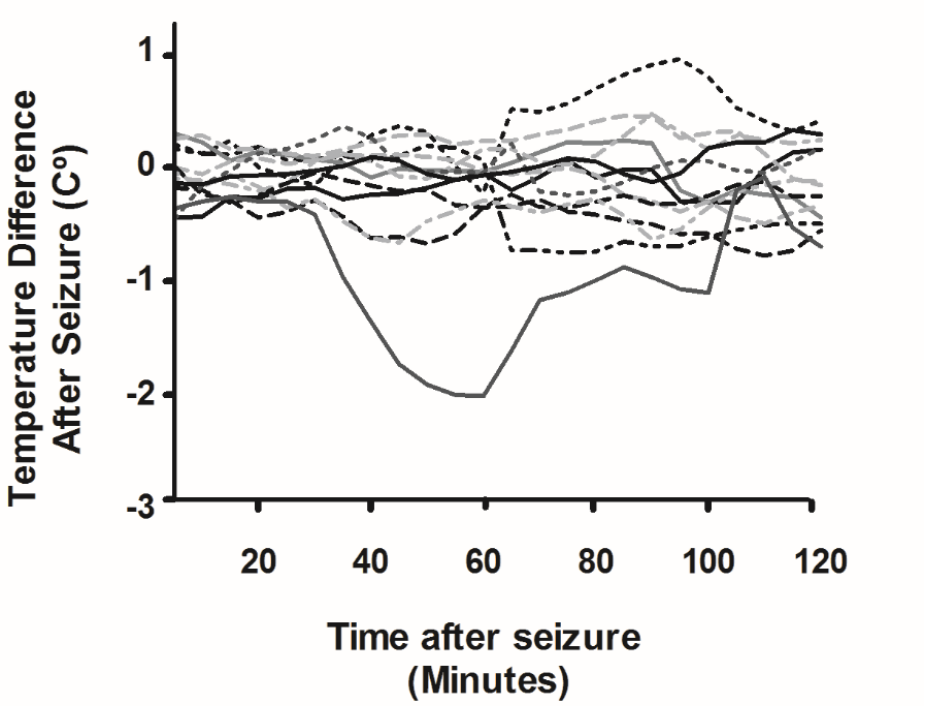

Figure 3-8. Temperature changes (post-seizure minus pre-seizure values) for 2 h post-seizure activity. Values are expressed as difference between baseline average and temperature (e.g., values below 0 indicate that hypothermia occurred after the seizure). Time 0 would be the time at which rats suffered of a seizure. There were no consistent changes in temperature among the rats.

ID	Incidence	Laterality	Total duration (Seconds)	Time of onset after ICH	RMS Ratio	Frequencies affected (Hz)	Power increase (mV^2)	Coherence change
1	1	Ipsilateral	14	11 hrs., 52 min.	2.44	N/A	N/A	N/A
2	2	Bilateral	102 (51 ± 19.80)	18 hrs., 50 min.	1.9 ± 0.33	↑ 0 - 4.6	34.11 ± 14.66	-0.53 ± 0.11
3	3	Bilateral	163 (54.33 ± 35.57)	22 hrs., 7 min.	1.92 ± 0.29	↓ 0.5 - 0.94, ↑1.32 - 1.5, ↑ 1.78 - 5.8, ↑12.38 - 38	16.40 ± 5.23	-0.19 ±0.10*, 0.14 ± 0.16
4	4	Bilateral	108 (27 ± 4.9)	16 hrs., 25 min.	2.65 ± 0.23	↑ 1.07 – 6.2	16.01 ± 3.99	0.34 ± 0.022
5	8	Bilateral	173 (21.62 ± 17.76)	11 hrs., 37 min.	4.13 ± 0.43	↑ 0 - 39.9	71.03 ± 14.57	0.42 ± 0.19
6	14	Bilateral	616 (44 ± 46.24)	9 hrs., 57 min.	2.53 ± 0.53	↑ 0 - 47	33.77 ± 16.07	0.24 ± 0.025

*Occurring in frequencies below 5.8 Hz

Table 3-1. Characteristics of seizures after collagenase-induced ICH. EEG activity occurring in the first week after collagenase injection were visualized and analyzed. These are the characteristics for the seizures that occurred within the first 36 h after the stroke; no seizures were detected afterwards. For each rat, the number of seizures, laterality, total duration, and time of onset were documented. We also reported other factors to determine the variability of the traces as depicted by the RMS ratio (RMS seizure/RMS non-epileptic activity) and changes in voltage according to their frequency, as depicted by the power increased and frequencies affected. Also, for those frequencies in which the power was affected, coherence was assessed as an indicator of how coupled the activity was in between both channels. Here, coherence was computed as an increase from baseline coherence. Traces shorter than 25 s were not analyzed for power and coherence. Data expressed as M±SD.

CHAPTER 4

Prolonged Localized Mild Hypothermia Does Not Affect Seizure Activity after Intracerebral Hemorrhage in Rats

4.1. Introduction

Intracerebral hemorrhage (ICH) causes 40% in-hospital mortality and leaves many survivors significantly impaired (van Asch et al., 2010; Balami and Buchan, 2012). Primary injury, the mechanical force of blood dissecting through brain, has proven challenging to prevent. Therefore, experimental treatments focus on decreasing secondary injury, such as that caused by oxidative stress, inflammation and edema. Epileptiform events complicate and potentially contribute to secondary injury after ICH (Balami and Buchan, 2012; Hemphill et al., 2015). Clinical studies estimate that up to 31% of patients experience seizures during the first three days after the ICH (Balami and Buchan, 2012; Hemphill et al., 2015). Most occur during the acute period when the risk of hematoma expansion and mortality is the highest. Thus, prophylactic administration of anticonvulsants seems logical. However, these drugs have the potential to worsen outcome after ICH (e.g., phenytoin causes fever) or otherwise appear to have no benefit (Naidech et al., 2009; Hemphill et al., 2015). Thus, recent guidelines advise against prophylactic anticonvulsants usage after ICH (Hemphill et al., 2015). Several animal studies have also shown that administering different drugs that depress the nervous system, such as anticonvulsants (e.g., diazepam), are associated with poor outcome after brain injury (Schallert et al., 1986; Feeney and Sutton, 1987; Hernandez and Schallert, 1990; Hernandez, 1997; Goldstein, 2003).

Therapeutic hypothermia (HYPO) is used to improve outlook after cardiac arrest in adults and in infants with hypoxic ischemic injury (Polderman, 2008; MacLellan et al., 2009). Based upon extensive animal data and promising clinical work, HYPO is also being evaluated for other conditions, notably ischemic and hemorrhagic stroke (Kollmar et al., 2010; Kollmar et al., 2012; Staykov et al., 2013; Lyden et al., 2014; Rincon et al., 2014; van der Worp et al., 2014). While

numerous studies show that HYPO improves outlook after ischemia and trauma (Crossley et al., 2014; MacLellan et al., 2009), cooling is not consistently or robustly beneficial against ICH, at least in our lab (MacLellan et al., 2006a; Fingas et al., 2007; MacLellan et al., 2009), perhaps in part because of occasional complications (e.g., worsened bleeding – (MacLellan et al., 2004; John et al., 2015). Nonetheless, small clinical trials suggest that HYPO decreases edema and improves outcome after ICH (Kollmar et al., 2010; Staykov et al., 2013) and larger trials are planned (Kollmar et al., 2012; Rincon et al., 2014). Interestingly, small studies report that HYPO reduces seizures after status epilepticus (Bennett et al., 2014; Zeiler et al., 2015), and in infants with hypoxic ischemic injury (Harbert et al., 2011; Boylan et al., 2015). Accordingly, we tested the hypothesis that HYPO will diminish electrographic seizure activity after collagenase-induced ICH in rats. We also assessed whether HYPO affects lesion size, behavioural outcome, and bleeding after ICH.

4.2. Methods

Subjects

Seventy-one male Sprague-Dawley rats (350-450 grams, 10-12 weeks old) were obtained from the Biosciences breeding colony at the University of Alberta. Food (Purina rodent chow) and water were provided ad lib and rats were housed individually in a temperature- and humidity-controlled room (lights on from 7 a.m.–7 p.m.).

In Experiment 1 (N=3), we measured temperature from the cooled striatum, the contralateral striatum, and rectum of anesthetized naïve rats to test the selectivity of the focal cooling device (Clark et al., 2007). Rats were anesthetized with isoflurane (4% induction, 1.5–2.5% maintenance in 60 % N_2O, balance O_2) and a cooling coil was implanted (see below). Two

holes were drilled in the skull (AP 0.5, ML 3.5 and ML -3.5) and a 20G 1 cm cannula was attached to each hole with dental cement. The skin was pulled and stapled in order to cover the skull. Two thermocouple probes (HYP1-30-1/2-T-G-60-SMP-M, Omega, Stamford, CT, USA) were lowered 6 mm below the skull surface in order to measure temperature from both the ipsilateral (side of cooling) and contralateral striatum. Temperature was measured for 15 minutes prior to cooling, which lasted 30 minutes, until 15 minutes after cooling. Cold water was pumped at a rate of 110 ml/hour, a similar rate to those used in other experiments.

In Experiment 2 (N=15), rats were given an ICH, had a cooling coil implanted, and were randomly assigned to normothermia (NORMO, N=7) or 18 hours of HYPO with a 6 hour delay (N=8). All surgical procedures in this and the following experiments were performed aseptically. This delay was chosen in order to avoid worsening bleeding (MacLellan et al., 2004; John et al., 2015) while still treating prior to the onset of seizures, which we previously observed as early as 10 hours after ICH (Klahr et al., 2015). At 24 hours post-ICH, all rats were anesthetized, decapitated, and the brain was extracted to measure ipsilateral (side of the stroke) and contralateral blood volume with a hemoglobin assay, as previously done (John et al., 2015). Animals were anesthetized with isoflurane and decapitated. The brain was removed and dissected into left (contralateral) and right (ipsilateral) hemispheres. Each sample was weighed and transferred to a tissue homogenizer (7 mL; Pestle ‘‘B,’’ Kimble Chase). Distilled water was added in a 1:4 tissue to water ratio (weight:volume) before homogenization. Tissue homogenates were incubated on ice for 7 minutes as this caused additional osmotic lysing of red blood cells. Duplicate homogenate samples were transferred to 1.5-mL Eppendorf tubes and centrifuged for 35 minutes at 15,800 g. Subsequently, 100 µL aliquots of hemoglobin-containing supernatant were mixed with 600 µL of diluted Drabkin’s reagent (Sigma) in 1.5-mL cuvettes and allowed to

react for 15 minutes. Absorbance was measured at 540 nm (spectrophotometer model 4001/4; Thermo Fisher Scientific), and compared with a previously generated standard curve to determine total blood volume in each brain region.

In Experiment 3, six naïve rats were implanted with the cooling coil and electroencephalographic (EEG) probe (ipsilateral channel only). They all received 48-hour HYPO and six hours rewarming five days after the surgery. Rats were euthanized 7 days after the cooling protocol started and histological assessment as well as EEG analysis was performed.

In Experiment 4 (N=47), we tested whether HYPO reduced seizures occurring after ICH. Rats received a unilateral ICH, a cooling device implanted ipsilateral to the side of infusion, and an EEG probe implantation (one surgery). They were randomly assigned to NORMO (N=23) or six hour delay, 48 hour HYPO (N=24) with six hour rewarming. Behavioral deficits were assessed with a composite neurological deficit score ranging from 0 (not impaired) to 14 (severe impairment), which included circling, hind limb retraction, bilateral forepaw grasp, contralateral forelimb flexion, and beam walking (MacLellan et al., 2006b; Fingas et al., 2007). Rats were assessed one day before ICH (Baseline), and 7 and 14 days after ICH. All rats were euthanized at 14 days post-ICH, at which time histological assessment and EEG analysis was performed.

Focal Cooling Device Implantation

A cooling coil was surgically implanted between the right temporalis muscle and the skull, and secured with dental cement (Clark and Colbourne, 2007; Fingas et al., 2007). At the end of surgery, this device was connected via silastic tubing to an overhead swivel and a cold-water source. For those rats that received HYPO, the flow of chilled water averaged ~108 ml/hour over the 48-hour treatment, as previously done (Clark and Colbourne, 2007; Fingas et

al., 2007). This protocol causes brain temperature to drop to ~ 33°C while body temperature remains normothermic in non-anesthetized rats (Clark and Colbourne, 2007; Fingas et al., 2007). Control rats were treated similarly except they did not have water perfused through the device and thus they remained normothermic (NORMO).

Collagenase-induced ICH

Rats were anesthetized with isoflurane and body temperature was maintained at 37°C with a blanket and a rectal temperature probe. Local anesthetic, Marcaine (Sigma), was applied under the scalp prior to surgery. A hole was drilled (AP 0.5, ML +3.5) and a Hamilton 26-gauge needle was inserted 6.5 mm into the striatum. A total of 0.14 U of collagenase in 0.7 μL of sterile saline was infused over 5 minutes and the needle was removed after an additional 5 minutes (Klahr et al., 2015).

EEG Probe Implantation

Rats were anesthetized and an EEG telemetry probe (F40EET, Data Sciences International, St. Paul, MN) was implanted to continuously measured brain electrical activity (sampled at 500 Hz, low-pass filtered at 100 Hz) until euthanasia. The probe was inserted either in the peritoneal cavity or the neck (dorsal S.C. placement) while the leads were channelled under the skin and attached to screws stereotaxically placed ipsilateral (AP −1.5, ML +4) and contralateral (AP −1.5, ML −4) to the collagenase injection site (Klahr et al., 2015). The leads were secured to the screws (B000FMUH4M, Small Parts, Miami Lakes, FL, USA) with dental cement (Klahr et al., 2015).

Histology

All rats were injected with sodium pentobarbital (100 mg/kg, i.p.) and then transcardially perfused with 0.9 % saline followed by 10 % neutral buffered formalin. Brains were extracted, cryostat sectioned at 40 μm, and stained with cresyl violet. Coronal sections taken every 200 μm were then analyzed with Image J. The volume of each hemisphere was calculated as follows: (average area of complete coronal section of the hemisphere−area of damage− ventricle) × interval between sections × number of sections. This method accounts for injury, atrophy and ventricular dilation (Klahr et al., 2015).

EEG Analysis

All EEG traces were visualized with Dataquest A.R.T. 2.3 system (Data Sciences International). For Experiment 3, three five-minute traces were averaged to compare baseline (1 day before cooling), HYPO day 1, HYPO day 2, first (STEP 1) and second (STEP 2) half of rewarming. A customized MATLAB code (R2012a, Mathworks, Natick, MA) computed the root mean square (RMS), a measure of the fluctuation in the EEG signal, for each epoch of interest. For Experiment 4, we used 4-min epochs of non-epileptiform slow-wave activity from the first 24 hours post-ICH. Control and putative epileptiform traces were exported and analyzed using custom code written in MATLAB for detection of seizures. The code identified epileptiform peaks that were above 4 standard deviations (SD) from the mean of the control traces, and peak clusters occurring within one second apart (3 spikes per second) were considered seizures (Smith, 2005). Each seizure had a minimum of 10 peaks. Seizures detected by the MATLAB code were also visually verified for further accuracy.

Statistical Analysis

Data are presented as mean ± SD for normally distributed data, or median ± IQR for non-parametric and not normally distributed data. All data was analysed using SPSS (v.17.0, SPSS Inc., Chicago, IL). ANOVA or non-parametric tests (e.g., Mann-Whitney U test) were performed depending on the nature of the data.

4.3. Results

Five HYPO rats were excluded altogether from Experiment 2, 3 and 4 due to technical problems with the cooling system. One NORMO rat from Experiment 4 unexpectedly died during surgery before any invasive procedures were performed. In Experiment 1, we measured temperature during HYPO in naïve rats. As expected, ipsilateral striatal temperature was lowered to 30.9 ± 1.3 °C during cooling, whereas the contralateral side was only slightly affected (34.8 ± 0.4 °C) and core temperature remained comparable to baseline levels (Figure 4-1). In Experiment 2, cooling delayed 6 hours did not increase hematoma volume at 24 hours after ICH (~40 µL p= 0.276; Figure 4-2). In Experiment 3, naïve rats undergoing local brain cooling did not show any alterations in their EEG amplitude during HYPO or the re-warming steps (p= 0.944; Figure 4-3). As expected, there was no injury in these rats (data not shown).

In Experiment 4, out of the 22 rats included in each group (after exclusions), 13 and 10 had seizures in the NORMO and HYPO conditions, respectively (Figure 4-4A and 4-4B). This difference was not significant, neither was there a difference between number of seizures per group ($p\geq$ 0.079 Figure 4-4C).

Seizures were detected at an onset of between 2 and 27 hours after collagenase injection, and were detected as late as 4 days after the ICH. There were no group differences for times of

seizure onset or cessation ($p \geq 0.253$; Figure 4-4D). Note that 2 HYPO rats had seizures before cooling began. Still cooling did not seem to block further seizure episodes. The total duration was not different between groups ($p = 0.068$; Figure 4-4E), although the average duration per rat was higher in the ipsilateral side of the HYPO group ($p = 0.0133$; Figure 4-4F). The total number and average number of peaks per seizure were not different between groups ($p \geq 0.088$; Figure 4-4G and 4-4H).

Cooling did not affect lesion volume ($p = 0.695$; Figure 4-5A and 4-5B) that largely damaged the striatum with some additional damage (e.g., corpus callosum). We performed different curve fittings (linear, logarithmic, and inverse) to identify the best relationship between lesion volume, and number, duration, and peaks of seizures (Figure 4-5C, 4-5D, 4-5E, respectively). We also tested whether there was a relationship with NDS scores on day 7 and 14 and seizure characteristics (Figure 4-5F). We found that lesion volume was negatively related to the number, duration, and number of peaks, although this was not significant ($p = 0.060$-0.104). There was no relationship between contralateral seizure characteristics and lesion volume ($p > 0.168$). Behavioral outcome at day 7 or 14 did not predict ipsilateral or contralateral seizure characteristics ($p > 0.483$). Last, there was no significant correlation between lesion volume and NDS scores, although there was a trend suggesting more deficits at 14 days in those with larger lesions ($p = 0.065$).

All rats were impaired on day 7 ($p < 0.001$) and even though their behavioral outcome improved on Day 14, it was did not go back to Baseline levels ($p < 0.001$). Groups were comparable in performance prior to ICH (Baseline $p = 0.674$). Although the HYPO group had lower post-ICH NDS scores, this was not significant (Day 7 $p = 0.099$; Day 14 $p = 0.085$; Figure 4-6).

4.4. Discussion

In this study we found that mild HYPO did not affect post-ICH electrographic seizure activity (incidence, duration, etc.), which occurred to a similar extent as we previously reported for this model (Klahr et al., 2015). As well, HYPO did not affect lesion volume or behavioral impairments, which is similar to some previous studies (Fingas et al., 2007). Furthermore, there was no clear relationship among seizure measures and behavioral deficits, although there was a trend to a negative relationship between lesion volume and seizure characteristics. The lack of benefit was not due to bleeding complications with our HYPO protocol (John et al., 2015), which we excluded, and we ensured that our cooling method indeed caused brain HYPO. Finally, the mild level of cooling had no discernible impact in naïve animals on EEG activity, which coincides with other studies in which similar depth of cooling was used (Jia et al., 2008).

This study replicates our previous findings for untreated collagenase-induced ICH with regard to electrographic seizure characteristics and incidence (Klahr et al., 2015). While we did not systematically assess whether rats convulsed during seizure activity, we did notice only focal neurological effects as well as behavioral arrest, with the latter being a possible indication of absence seizures. Generalized convulsions were not observed. This time we used a much larger sample, so we found somewhat more heterogeneity in seizure characteristics (e.g., earlier occurrence) than our previous study found. The lack of histological and behavioral protection with HYPO also replicates some of our earlier work (Fingas et al., 2007), but there are considerable differences among studies with regard to whether HYPO is efficacious after ICH (MacLellan et al., 2006a; Fingas et al., 2007; Kawanishi et al., 2008; Sun et al., 2015). The reasons for such are only partly known (e.g., intermittent bleeding complications – John et al., 2015). It should be noted that there were some trends for benefit with HYPO (e.g., number of

rats that had seizures, and NDS data), but also those rats that suffered of seizures seemed to have longer average durations. Yet it is possible that modest treatment effects could have been missed despite using large group sizes (N=22/group in Experiment 4), especially compared to what is commonly used in the ICH literature (MacLellan et al., 2012). For instance, HYPO may reduce late onset seizures, if they occur, by reducing seizure susceptibility (Atkins et al., 2010). Further work is needed to assess this with long survival times, as has been done for traumatic brain injury (Atkins et al., 2010). Moreover, systemic HYPO that cools the entire brain may be more effective against seizures. Still local cooling methods have been investigated for stroke (MacLellan et al., 2009) and focal epilepsy (MacLellan et al., 2009; Rothman, 2009; Bennett et al., 2014), and it makes sense that focal cooling would target the focus of seizure activity along with some of the complications of ICH (e.g., inflammation and edema). Regardless, much deeper cooling (e.g., 20°C - (Niquet et al., 2015)) would certainly have a greater impact; however, those HYPO protocols are not as clinically relevant given the risk of severe complications to ICH patients (MacLellan et al., 2009).

The lack of obvious benefit against post-ICH seizures contrasts with findings in ischemia and hypoxia models (Atkins et al., 2010; Harbert et al., 2011; Boylan et al., 2015). Perhaps this is because cooling provides better protection in those models. Alternatively, the type of seizure and mechanisms of seizure initiation may differ among these insults with those following ICH being less amenable to HYPO. For instance, HYPO may fail to mitigate seizures in ICH because cooling does not effectively target iron and thrombin toxicity (Wowk et al., 2014); Wowk et al., submitted), factors involved in triggering seizures (Willmore et al., 1978; Lee et al., 1997). One might also argue that if our HYPO protocol had been neuroprotective, it would have ameliorated seizure activity. The lack of a clear relationship between seizure activity and lesion size,

however, argues against that. It might also be argued that the known and presumptive benefits of this particular HYPO protocol (e.g., on reducing edema) do not influence seizure activity. Yet a review of clinical studies using mild TH for a median of 48 hours showed that cooling stopped epileptic activity in 65% of status epilepticus patients (Zeiler et al., 2015). Therefore, HYPO may have different actions for different mechanisms of epileptic activity.

Seizures and their potential side effects following ICH tend to occur during the acute period when the risk of hematoma expansion, a main predictor of morbidity and mortality, is the highest. Seizures after ICH can be detrimental to patient outcome in several ways either through systemic effects (e.g., arrhythmias) or more directly by exacerbating brain injury through increasing edema and bleeding (Perlman and Volpe, 1983; Dunn, 2002; Takano et al., 2011). Also, early seizures can lead to post- stroke epilepsy (Balami and Buchan, 2012). Despite all the negative effects seizures might have, clinical studies have not elucidated the link between initial lesion size, seizure characteristics and outcome (Balami and Buchan, 2012). In two rat studies, including this one, we could not find a clear relationship between these factors either (Klahr et al., 2015). We recognize that it would have been a good positive control had lesion volume clearly predicted behavioral outcome in this study. However, earlier work shows that such correlations are often modest at best, especially in small data sets with a relatively restricted range in injury (MacLellan et al., 2006b). Still, it is likely that the seizures were not severe enough to cause further histological damage or behavioural deficits, and this may be the case in most patients too. Regardless, it seems prudent to assess alternative ICH models and endpoints to fully explore the effects of seizures, and whether anti-convulsants or neuroprotectants can impact outcome in some cases.

In conclusion, mild localized brain cooling did not reduce seizures after ICH in the collagenase model of ICH in rat. This lack of benefit cannot be easily explained by treatment parameters and bleeding complications. However, before firm conclusions are drawn, further study is needed to understand the relationship between seizures and outcome after ICH (e.g., testing other protocols), and it remains possible that HYPO may attenuate seizure activity or injury in certain ICH cases. If so, reducing some of the complications associated with ICH, such as seizures, could potentially lessen morbidity and mortality.

4.5. References

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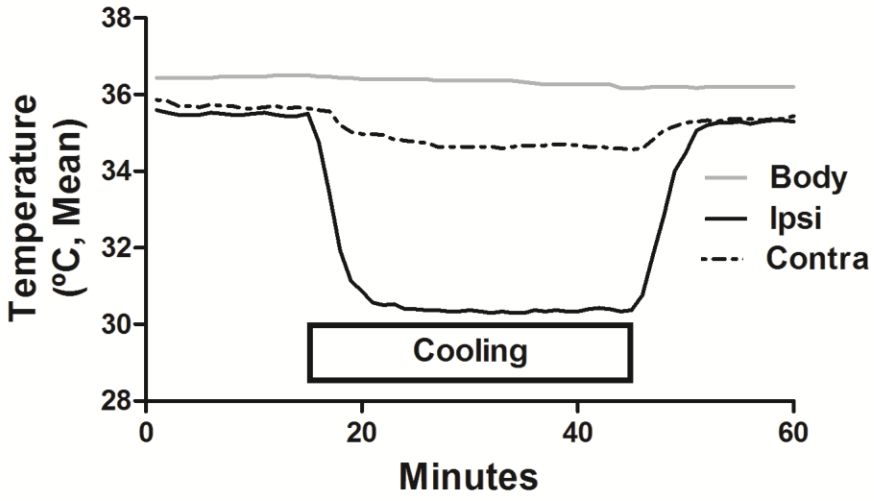

Figure 4-1. Temperature measurements from rectum (Body), ipsilateral (Ipsi), and contralateral (Contra) striatum prior, during, and after localized cooling in anesthetized rats (Experiment 1).

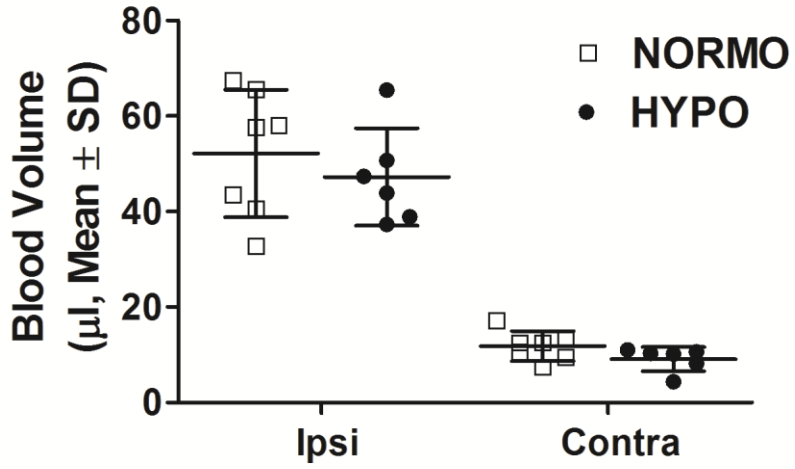

Figure 4-2. Cooling for 18 hours starting 6 hours after collagenase injection (HYPO) did not exacerbate bleeding after ICH at 24 hours (Experiment 2). Values of blood volume displayed from the side of the ICH (Ipsi) and the control hemisphere illustrating normal blood levels (Contra).

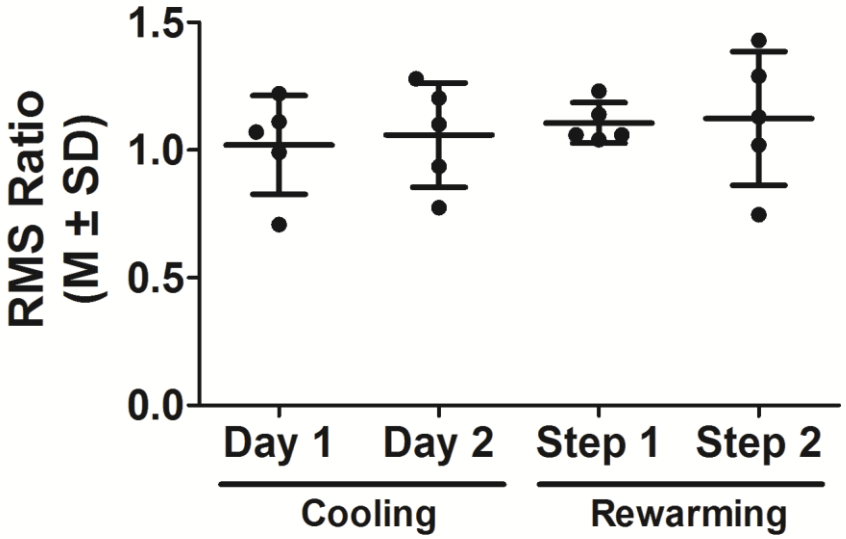

Figure 4-3. Our cooling protocol did not cause any alterations in the EEG activity of naïve rats, depicted as the ratio of RMS values taken at baseline versus the time points indicated (Experiment 3).

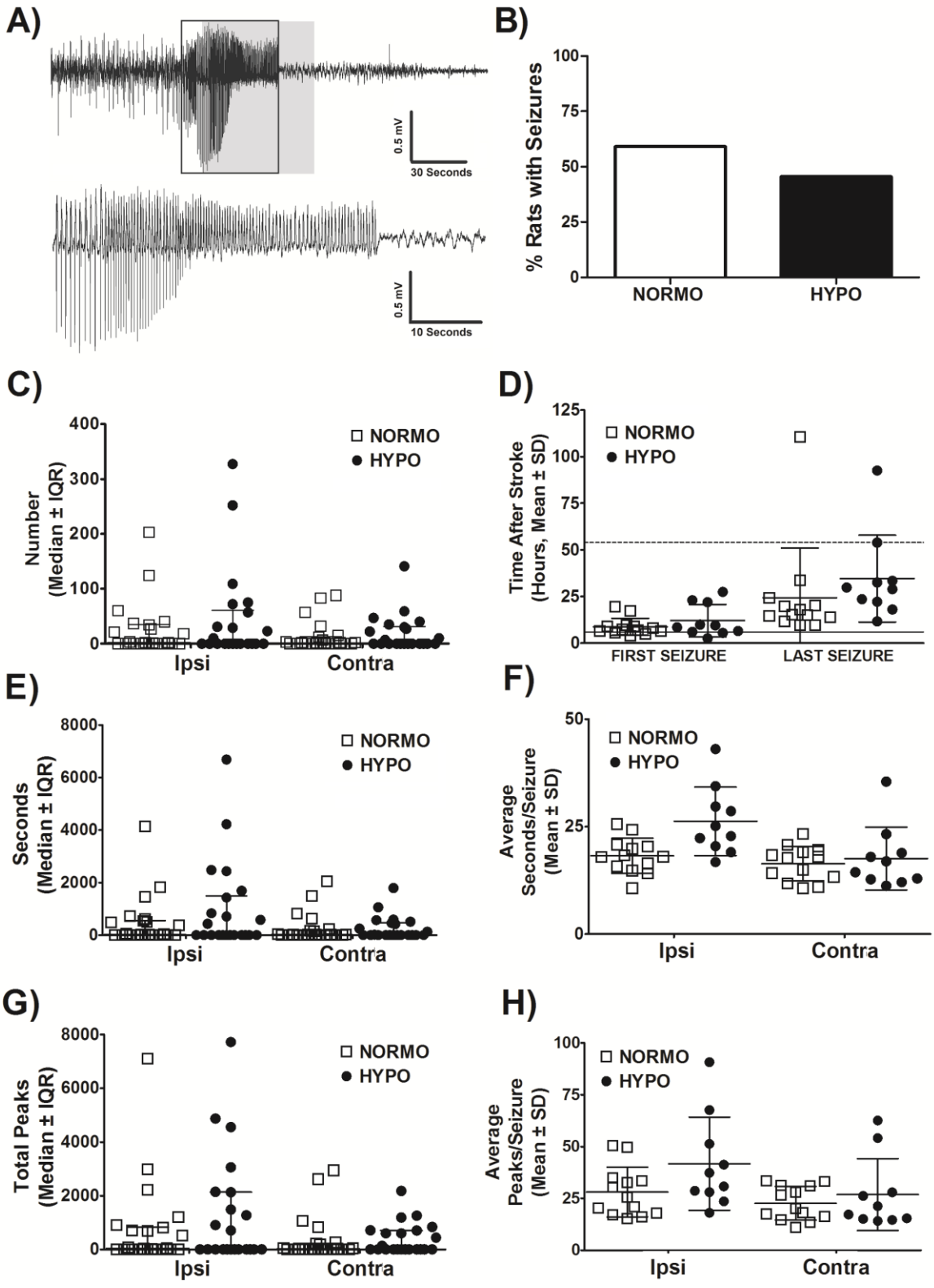
A)
0.5 mV
30 Seconds
0.5 mV
10 Seconds
B)
% Rats with Seizures
100
75
50
25
0
NORMO
HYPO
C)
Number (Median ± IQR)
400
300
200
100
0
NORMO
HYPO
Ipsi
Contra
D)
Time After Stroke (Hours, Mean ± SD)
125
100
75
50
25
0
NORMO
HYPO
FIRST SEIZURE
LAST SEIZURE
E)
Seconds (Median ± IQR)
8000
6000
4000
2000
0
NORMO
HYPO
Ipsi
Contra
F)
Average Seconds/Seizure (Mean ± SD)
50
25
0
NORMO
HYPO
Ipsi
Contra
G)
Total Peaks (Median ± IQR)
8000
6000
4000
2000
0
NORMO
HYPO
Ipsi
Contra
H)
Average Peaks/Seizure (Mean ± SD)
100
75
50
25
0
NORMO
HYPO
Ipsi
Contra

Figure 4-4. A) Example of seizure activity in a NORMO rat (Experiment 4). Seizure is highlighted within the black-lined rectangle. The bottom panel shows a close-up of the grey area from the trace above. B) Percentage of rats that had seizures in each group. The difference was not significant. C) Number occurring on the side of the stroke (Ipsi) and the opposite side (Contra). D) Time of onset of seizures and the last seizure seen after stroke total duration. Dash line at 6 hours depicts the time when cooling started. E) Total and F) average duration of seizures. Average duration was significantly larger in the ipsilateral side of the HYPO group. G) Total and H) average peaks of seizures.

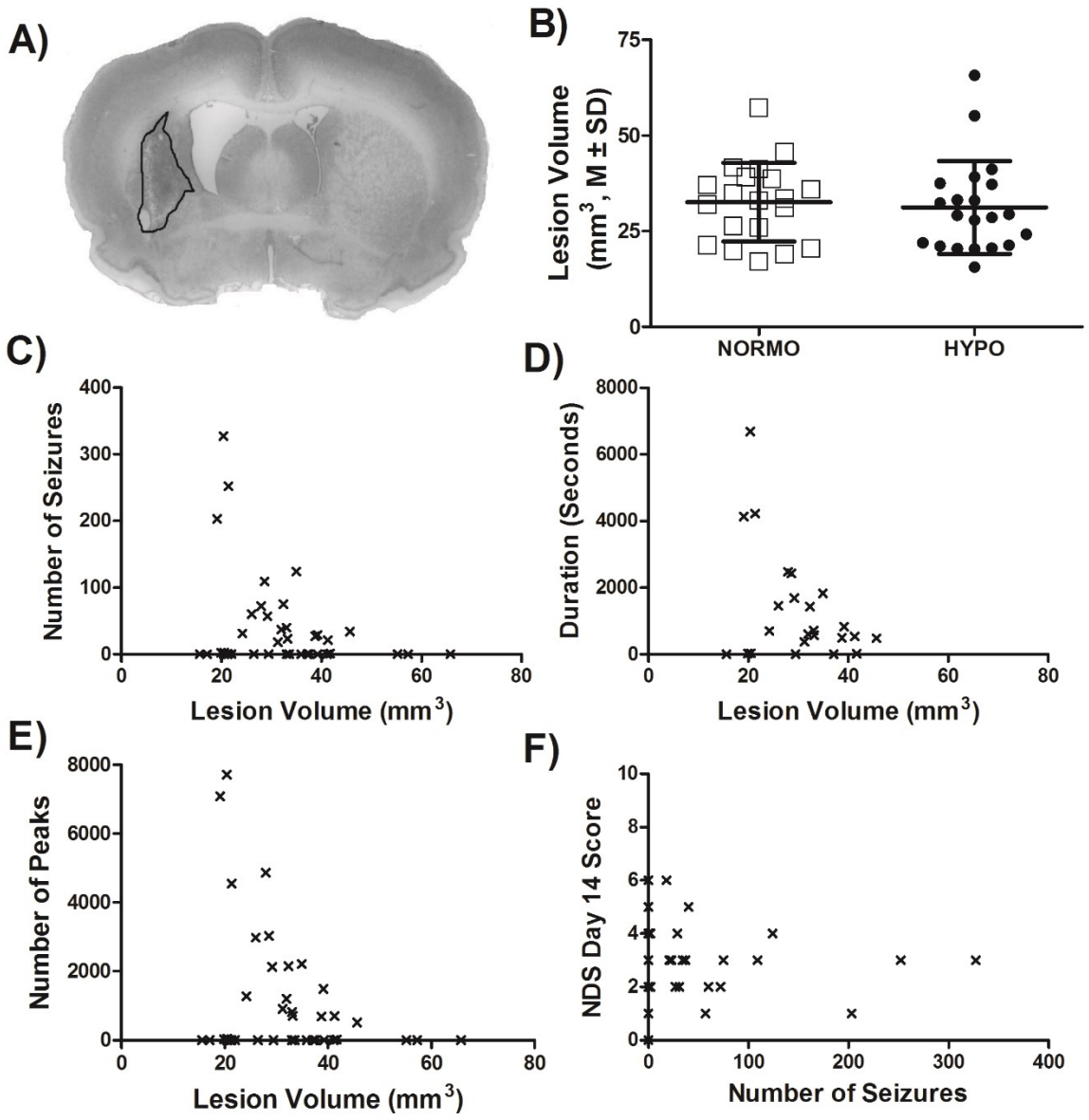

Figure 4-5. A) Example of profile of injury after collagenase injection from a HYPO rat (Experiment 4). Inside the box, arrows highlight the hematoma border zone. Scale bar depicts 20 μm. B) Lesion volume was not different between NORMO and HYPO. Lesion volume was not affected by cooling. Ipsilateral C) number, D) duration, and E) peaks of seizures plotted against lesion volume. Although not significant, lower lesion volume was associated with higher seizure severity. Seizures did not predict behavioral outcome. F) NDS scores on day 14 plotted against ipsilateral number of seizures.

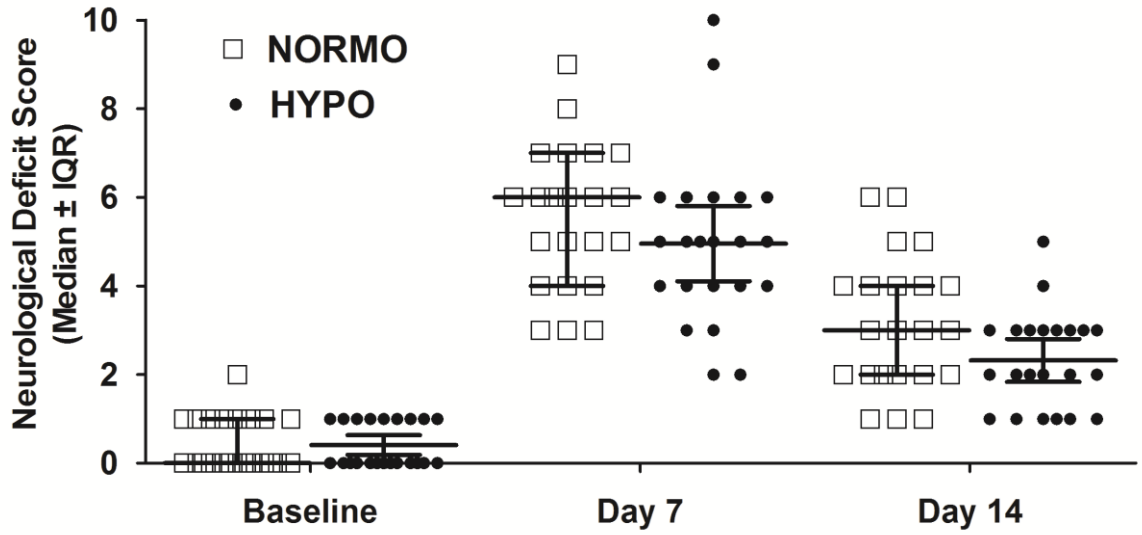

Figure 4-6. Baseline, day 7, and day 14 post-ICH NDS scores show that HYPO did not significantly lessen functional impairments, although there was a trend for benefit (Experiment 4).

CHAPTER 5

General Discussion

5.1. Summary of Findings

The main objective of this thesis was to investigate the impact of post-stroke hypothermia (HYPO) beyond its neuroprotective properties. Researchers tend to focus on mechanisms of action and efficacy of neuroprotective treatments, including HYPO. Side effects of cooling, such as the potential impact of cooling on motor recovery independently of neuroprotection, have been largely ignored. Our results suggest that early contralesional cooling applied for 48 hours reduced the likelihood to shift paw preference to the unimpaired limb. This suggests that contralesional HYPO may be acting as constraint-induced movement therapy (CIMT). This therapy aims to reduce impairment in the affected limb by restraining the use of the unimpaired limb. We also carried out another study in which we trained rats in the single pellet task while undergoing HYPO. Cooling did not impair reaching during or after tethering. This suggests that HYPO is a relatively safe treatment, and may have some beneficial effects after stroke, such as reducing the use of the non-paretic limb.

Another factor that has been understudied are seizures after ICH. Most importantly, it is not well understood what the impact is (if any) of neuroprotective therapies, such as HYPO, on seizure activity after stroke. This topic has been ignored probably due to how challenging it is to appropriately measure seizures in patients and relate epileptiform activity to outcome. In animal studies, there have been no reports of whether seizures occur after ICH. Therefore, we carried out a study to determine the incidence and characteristics of seizures in two of the most commonly used animal models of ICH, collagenase and whole-blood (Chapter 3) .We found that seizures occurred after collagenase injection in 66% of the rats, but did not detect any epileptiform activity in the whole blood model. We then designed an experiment using the collagenase model to test whether HYPO reduced post-stroke seizure activity (Klahr et al.,

2015). Cooling is known to decrease seizure activity in patients with status epilepticus, as well as in other forms of brain injury. However, seizures were not significantly decreased by cooling in the collagenase model of ICH (Chapter 4) (Klahr et al., 2016). In the following sections, I will discuss future directions of my thesis work.

5.2. Future Directions of Therapeutic Hypothermia and Plasticity Studies

There are several future studies that can address issues raised by our research. First, HYPO might be inhibiting electrical activity in the contralesional hemisphere, therefore reducing use of the unimpaired limb. Electrophysiological recordings can be used to assess whether cooling decreases evoked activity in the cortex. Second, HYPO might have affected mechanisms of plasticity. In the contralesional cooling study our main endpoint was behavioural outcome. In order to further explain our findings, follow up studies can determine how contralesional HYPO impacted representative mechanisms of plasticity. Repair processes predominate within the first weeks after stroke (Lo, 2008; Murphy and Corbett, 2009; Kolb and Teskey, 2012). Given that the rats were euthanized 30 days after the motor cortex devascularization, we had limited options for measuring plasticity. Intracortical microstimulation could be used to determine whether contralesional HYPO affected motor cortical maps post-stroke (Nudo and Milliken, 1996). Also, cortical dendritic changes can be determined with 2-photon imaging (Winship and Murphy, 2009) or with Golgi-Cox Stain (Greenough et al., 1985). Third, cooling did not impair learning in otherwise naïve rats. However, it might have affected spontaneous limb, which could be assessed with the cylinder task (Schallert et al., 2000).

Future studies should also determine the impact of cooling on post-stroke ipsilesional plasticity. Neuroplasticity develops over hours after the injury and overlaps with

neurodegenerative processes in the ipsilesional hemisphere (Lo, 2008). Repair processes predominate for weeks after cell death mechanisms stabilize, and correlate with recovery after stroke (Warraich and Kleim, 2011; Kolb and Teskey, 2012). Neurodegeneration and plasticity can also differ depending on the lesion location and size. Originally, we designed a study in which we created an ischemic insult by injecting endothelin-1 (ET-1) into the striatum (Agnati et al., 1991; Kleim et al., 2007). We aimed to find if there was a point at which HYPO stopped being neuroprotective and started being detrimental to plasticity and recovery by cooling for different durations (e.g., 2 versus 4 days) and in different locations (cortical versus striatal). We found that small ischemic lesions were protected by both systemic and focal HYPO (Figure A-1). Unfortunately, due to the small size of the lesion we were not able to relate neuroprotection to an improvement in behaviour (Figure A-2, Appendix). When we tried to create a moderate size lesion, the mortality rate went up to 50% and we considered it unethical to continue with the study. We think that the high mortality rate was due to heterogeneity in the ET-1 concentration of each batch provided by the company. Other models of focal ischemia, such as permanent and transient middle cerebral artery occlusions, can be too large and the animals may be too impaired to perform in behavioural tasks (Carmichael, 2005; Kleim et al., 2007). Other ischemia models may be small and cause little behavioural impairment, or lack a penumbra (e.g., motor cortex devascularization) (Carmichael, 2005; Kleim et al., 2007). A good alternative would have been to use the photothrombotic model (Futrell et al., 1988). We still consider that the impact of HYPO on ipsilesional neuroplasticity deserves further investigation considering our findings in the contralesional hemisphere. A model of brain injury that may allow for determining whether HYPO affects ipsilesional plasticity without having the confound of neuroprotection would be the suction model, in which the cortex is aspirated with a syringe (Mathew et al., 1994). Finally,

by varying duration and depth of HYPO, it is possible to also assess to what extent this treatment can impact post-stroke plasticity.

Most studies, even those that use focal cooling devices and drug-induced HYPO, tend to cool the whole brain (Polderman and Herold, 2009). Our studies show that cooling the contralesional hemisphere of the rat can reduce the tendency to use the unimpaired limb on a reaching task. In the case of moderate size strokes in which learned non-use should be prevented to reduce impairment, the use of HYPO may be beneficial (Taub and Morris, 2001). Still, reaching success in the impaired limb remained similar to NORMO, which suggests that contralesional cooling does not negatively impact reaching. As shown in previous experiments, NORMO (and possibly rats with delayed HYPO in this study) underwent contralesional plasticity that aided recovery (Biernaskie et al., 2005; Gharbawie et al., 2007). However, HYPO may be reducing early hyperexcitability of the contralesional hemisphere. This excessive activity in the intact side of the brain may be inhibiting use of the affected limb (Floel et al., 2004; Murase et al., 2004; Ward and Cohen, 2004; Cramer et al., 2006; Dancause et al., 2015). Therefore, the rats that received earlier HYPO may have suppressed activity in the contralesional hemisphere, either through the effect of cooling or by not using the unimpaired limb as much. The lack of use of the unimpaired limb, which encouraged use of the impaired limb, likely induced plasticity in the ipsilesional hemisphere. These theories, however, can only be investigated with further experiments that use markers for mechanisms of neuroplasticity. Alternatively, patients with large infarctions may rely on contralesional plasticity to recover (Hsu and Jones, 2006). As the findings in our study may also apply to a larger lesion, this should be further examined in a larger stroke model.

5.3. Future Directions of Post-ICH Seizure Studies

Both of our studies showed that seizures occurred within the first days post-ICH. Future experiments should assess whether seizure activity occurred after 30 days of the insult. Indeed, there are trauma studies suggesting that seizures can re-occur even a year after the initial insult (Kharatishvili et al., 2006). This is worth investigating considering that it may be possible that HYPO could have decreased the incidence of late post-stroke epilepsy. Also, it could be that HYPO reduced later seizure susceptibility, which could be determined with a pro-convulsant challenge experiment as previously demonstrated in an animal model of traumatic brain injury (Atkins et al., 2010). Alternatively, a different HYPO protocol with deeper cooling may be more effective at decreasing seizure activity post-ICH. There are a few clinical cases in which deep cooling (24 ºC) was used to stop paroxysmal activity in humans (Sourek and Travnicek, 1970). Localized forms of cooling are being developed to use lower HYPO temperatures for cortical epilepsy (Rothman, 2009). However, mild systemic HYPO has been more commonly used in the clinic, and has shown success in patients with status epilepticus (Vastola et al., 1969; Corry et al., 2008; Zeiler et al., 2015).

A limitation of our study in which we assessed HYPO as a treatment for post-ICH seizures is that we did not use a battery of test to assess functional outcome. Cooling can improve behavioural outcome after ICH in rats (MacLellan et al., 2009), but we did not detect that effect possibly because the neurological deficit score used was not sensitive enough. However, we did not find that behaviour correlated with seizure incidence. Fortunately, now that we found a model to study post-stroke seizures (Klahr et al., 2015), further studies can be carried out to determine whether by increasing (e.g., pro-convulsants) or decreasing (e.g., anti-convulsants) the number of seizures after ICH, we can impact outcome after ICH. Given the

inconsistency in clinical studies regarding outcome and seizure activity (Balami and Buchan, 2012), there is the possibility that seizures may not be detrimental after stroke. It is also possible that seizures in our study were not frequent or severe enough to impact outcome. Still, we found that seizures occurred within the same time frame as what is seen in patients (Balami and Buchan, 2012).

Further experiments can also be carried out to investigate how seizure incidence may vary depending on the nature of the stroke, such as by varying size and location. For instance, many studies show that patients with cortical ICH have a higher risk for seizure activity (Balami and Buchan, 2012). Cortical and hippocampal ICH have been modeled in animals, but these studies did not assess seizure activity (Mun-Bryce et al., 2006; Song et al., 2008). Also, we found a trend of smaller lesion leading to higher seizure incidence. This has been reported in the past studies (Balami and Buchan, 2012) and it could be due to the possibility that with larger hematomas there is less circuitry left for epileptic activity to start and spread. Increased intracranial pressure (ICP) in large ICHs can lead to herniation and suppressed neuronal activity (Balami and Buchan, 2012). This is unlikely considering that we modeled a moderate sized hematomata, that would not have such an increase in ICP (Hiploylee and Colbourne, 2014). We were surprised that seizure activity was not decreased after HYPO in the ICH model, considering the positive results found after ischemia (Harbert et al., 2011; Boylan et al., 2015). However, HYPO has shown to be a far better neuroprotectant after ischemia than after ICH (MacLellan et al., 2009). Damage caused by thrombin and iron outweighs some benefits that HYPO may confer on the overlapping neurodegenerative factors between ischemia and ICH (e.g., excitotoxicity). Furthermore, iron and thrombin are both epileptogenic factors, making HYPO less likely to work after ICH compared to ischemia (Willmore et al., 1978; Lee et al., 1997). Regardless, it is

important to test different neuroprotectants, HYPO included, with varying stroke types, sizes and locations as their effectiveness to reduce seizure activity may depend on these factors.

5.4. Conclusions

Prior to the clinical use of HYPO for ischemic and hemorrhage stroke, which is already underway, it seems prudent to fully examine potential side effects and alternative benefits of cooling. In the current set of experiments I focused on the impact of using HYPO on the contralesional hemisphere after focal ischemia, and on seizure activity after ICH. Cooling has the ability to reduce neuronal activity and neurotransmitter release, aspects that can affect both plasticity and seizure activity. We found that cooling can affect behaviour when applied to the contralesional hemisphere. Even though HYPO reduces edema, ICP, and inflammation after ICH, our protocol did not reduce seizure activity in rats. In conclusion, our experiments show that HYPO does not only ameliorate cell death after ischemia, but it can also have an impact on other aspects of stroke pathophysiology (e.g., use of impaired limb). This may be different depending on the type of injury, as HYPO seems less effective after ICH in reducing lesion size and seizures. Fortunately, cooling does not seem to have severe side effects, and it may even be beneficial if it reduces learned non-use. Further investigation will elucidate how this treatment can be customized to each brain insult to maximize its effectiveness.

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APPENDIX

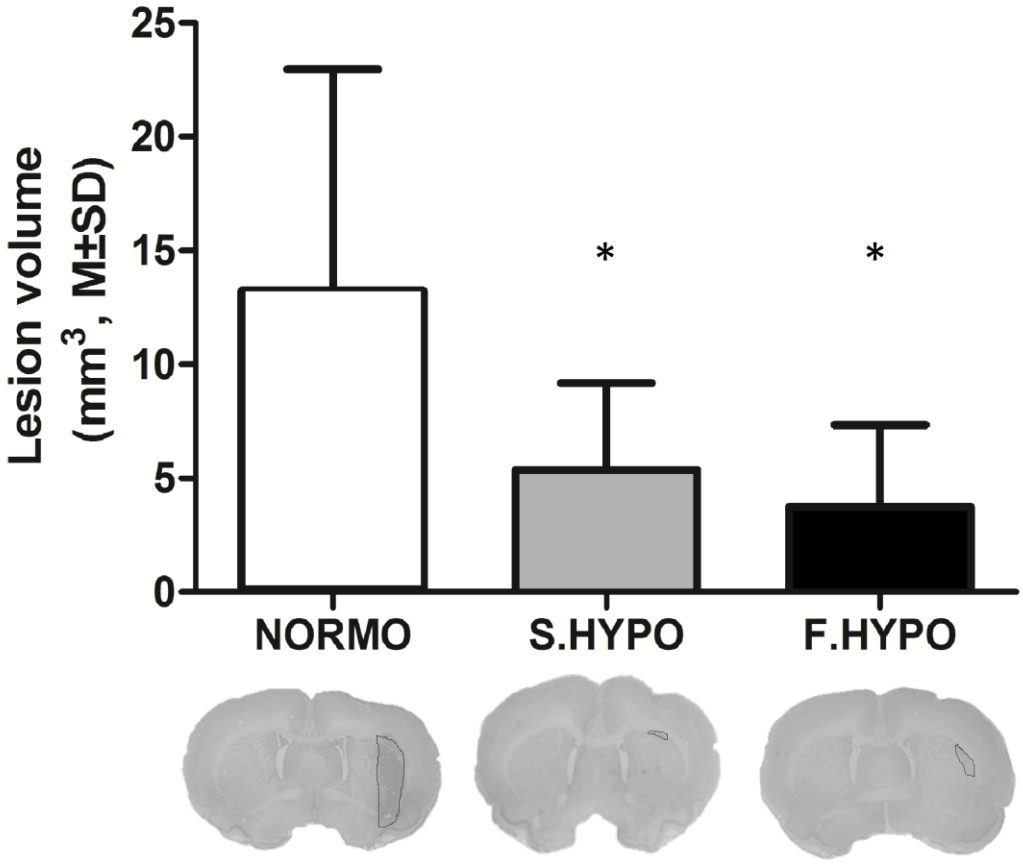

Figure A-1. Rats were stereotaxically injected with 400 pmol ET-1 in the striatum (AP=+0.5, ML=+3.5, DV=-6.5; Agnati et al., 1991) and randomly assigned to NORMO (N=9), systemic HYPO (S.HYPO, N=7) and focal HYPO (f.HYPO N=7) starting 1 hour after injection. Duration of HYPO in both conditions was 48 hours plus 6 hours of rewarming and followed previously used protocols (DeBow and Colbourne, 2003; Clark and Colbourne, 2007). Rats were euthanized 15 days post-stroke and tissue was stained with cresyl violet. A one-way ANOVA depicted a main effect (p=0.0165). Both systemic S.HYPO and F.HYPO significantly decreased lesion volume (*p<0.032 post hoc comparisons versus NORMO).

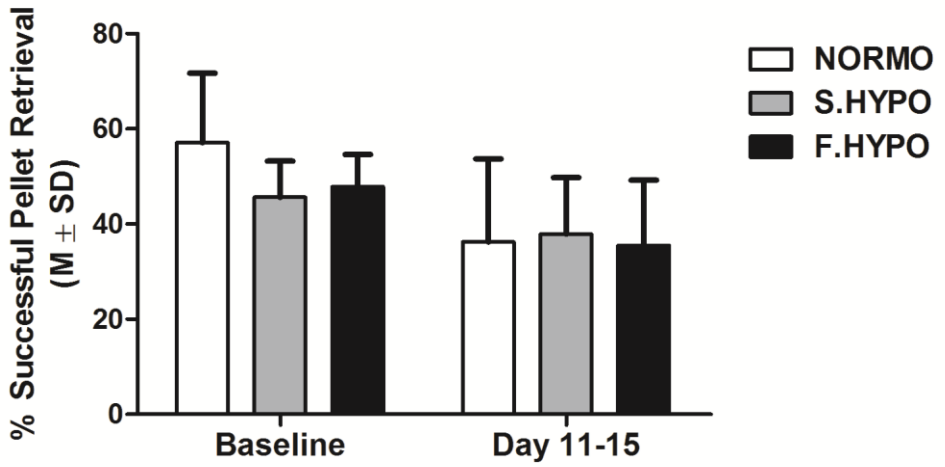

Figure A-2. Single pellet testing was performed as previously described (MacLellan et al., 2006). Percentage of successful pellet retrievals was similar among groups before (baseline) and at days 11-15 post stroke (p=0.397). There is a time effect, suggesting that the rats are impaired after the stroke (*p*=0.0018). The lack of difference in success among groups could be attributed to the lack of the sensitivity of this task to pick up a difference in such small lesion sizes.

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