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Delayed Hypothermia Following Permanent Focal Ischemia: Influence of Method and Duration

by

Darren Laree Clark

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Examining Committee

Fred Colbourne, Psychology

Teresa Krukoff, Cell Biology

Dallas Treit, Psychology

Jeremy Caplan, Psychology

Quentin Pittman, Physiology and Biophysics, University of Calgary

Abstract

Stroke is a leading cause of disability in Canada. Delayed hypothermia improves outcome in patients following cardiac arrest and reduces lesion volume in rodents after transient focal ischemia, but less is known about the effectiveness of delayed hypothermia following permanent focal ischemia. In Chapter 1, the efficacy of 12, 24 or 48 h of delayed hypothermia was evaluated one week following pMCAO. All treatments attenuated neurological deficits and brain water content, but only the 24 and 48 h treatments reduced stepping error rate and lesion volume. Thus, delayed hypothermia attenuates brain injury and functional deficits following permanent middle cerebral artery occlusion (pMCAO). Longer bouts of cooling provide superior protection; an effect that is not explained by lessened edema.

Chapter 3 describes a novel method of focal brain hypothermia in rats. A metal coil was implanted between the Temporalis muscle and adjacent skull and flushed with cold water. Focal, ipsilateral cooling was successfully produced without cooling of the opposite hemisphere or the core. One day of focal hypothermia was maintained in awake rats without significant alterations in blood pressure, heart rate or body temperature. The described simple method allows for safe inductions of focal brain hypothermia in anesthetized or conscious rats, and is ideally suited to trauma or stroke studies.

In Chapter 4, long-term efficacy of 12 and 48 h of delayed focal or systemic hypothermia was evaluated following pMCAO. Both systemic

treatments equally reduced lesion volume and skilled reaching deficits compared to normothermic controls, but only the 48 h treatment reduced neurological deficits. Conversely, 12 h of focal cooling did not significantly improve outcome, whereas 48 h of focal brain cooling attenuated functional deficits and reduced lesion volume. Thus, both delayed focal and systemic hypothermia attenuate long-term brain injury and functional deficits following pMCAO. Duration of cooling is clearly an important factor that may depend upon the method of cooling.

Overall, this data indicates that delayed and prolonged hypothermia provides substantial and persistent protection against pMCAO in the rat. Prolonged hypothermia is a promising neuroprotective therapy for acute stroke and further clinical investigation is warranted.

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TABLE OF CONTENTS Page #:

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS USED

AMPA	D,L-α-amino-3-hydroxy-5-methyl-4-isozazole proprionic acid
BWC	Brain water content
CBF	Cerebral blood flow
HR	Heart rate
ICH	Intracerebral hemorrhage
MABP	Mean arterial blood pressure
NDS	Neurological deficit score
NMDA	N-methyl-D-aspartate
pMCAO	Permanent middle cerebral artery occlusion
T_{brain}	Telemetry measured brain temperature (Chapter 2)
T_c	Core temperature
T_{core}	Telemetry measured core temperature (Chapter 3)
T_{CS}	Striatal temperature contralateral to cooling coil
T_{IC}	Cortex temperature ipsilateral to cooling coil
T_{IS}	Striatal temperature ipsilateral to cooling coil
T_{rec}	Rectal temperature
T_s	Skull temperature
TBI	Traumatic brain injury
tMCAO	Transient middle cerebral artery occlusion
TNFα	Tumor necrosis factor α
tPA	Tissue plasminogen activator

Introduction

Chapter 1

1.1 Introduction

This thesis describes the effectiveness of therapeutic hypothermia to protect against brain injury resulting from permanent focal ischemia in rat. The influence of both duration and method of hypothermia are considered. A novel method to cool the brain is introduced and its therapeutic value is evaluated. In the introductory sections that follow, background on relevant topics is provided to rationalize the work. It is essential to discuss stroke epidemiology and etiology, experimental models of stroke, the relevance of functional and long-term assessment, an overview of therapeutic interventions, including pharmacology and therapeutic hypothermia.

1.2 Stroke

1.2.1 Epidemiology

According to The Heart and Stroke Foundation, stroke, also known as cerebral vascular accident, refers to a sudden loss of brain function that is caused by the interruption of flow of blood to the brain (ischemic stroke) or the rupture of blood vessels in the brain (hemorrhagic stroke). About 80% of all strokes are ischemic in nature. Stroke is the leading cause of disability and the third leading cause of death in Canada. More than 50 000 people experience a stroke each year in Canada. That is one stroke every 10 minutes. Fifteen percent of stroke victims will die - the risk of stroke related mortality rises sharply after age 60 and is far greater in females over the age of 80 than in males. Mortality due to stroke has

decreased over time; a pattern that is likely a result of improved acute care practices, including the use of dedicated stroke units.

Of the 85% of stroke patients that survive, only 10% recover completely. That means 75% of stroke victims are left to live with disabilities and of those 50% suffer moderate to severe impairments. Deficits experienced after stroke include depression, anxiety, aphasia, memory loss, incontinence, paralysis and neglect. Many patients require care for the remainder of their lives, which places strain on family and caregivers.

Stroke is also associated with a large economic price. The Heart and Stroke Foundation states that the price tag for stroke on the Canadian economy is about $2.7 billion dollars per year when factors such as lost wages and decreased productivity are considered along with healthcare costs. The acute care cost for a single stroke victim averages $27, 500 dollars (Heart and Stroke Foundation of Canada, 2009). That figure is compounded with any long-term care the patient requires. The cost on society is large and serves as motivation to find ways to reduce the impact of stroke. Developments in neuroprotection may be one way to achieve that goal.

1.2.2 Focal Ischemic Stroke

Stroke may be one of two types, hemorrhagic or ischemic. Hemorrhage occurs when bleeding happens in the brain and comprises about 15-20% of all strokes. The other 80-85% of strokes are ischemic in nature. Ischemic stroke primarily arises in patients in one of two ways: when a blockage forms locally in a cerebral vessel (thrombolic stroke) or when a clot formed elsewhere in the body

travels and becomes lodged in a blood vessel in the brain (embolic stroke). In both cases, cerebral blood flow (CBF) is reduced below the threshold required to sustain neuronal survival. A complex series of pathological events are initiated by reduced CBF.

1.2.3 Pathophysiology

1.2.3.1 Ischemic Core

Neuronal cell death occurs through different mechanisms and at different rates depending upon the severity of blood flow reduction in that area. In the center of the ischemic area, referred to as the core, CBF reduction is the most severe. Energy failure and loss of ionic gradients lead to excitotoxicity and rapid necrotic cell death (Martin, et al., 1994).

Brain tissue consumes a relatively large amount of oxygen and glucose, compared to the rest of the body. Focal ischemia drastically reduces blood flow, particularly in the core region, and so delivery of these substrates is restricted. Energy depletion leads to the impairment of actively maintained ionic gradients and thus, to the loss of resting membrane potential. Consequently, both neurons and glia depolarize leading to voltage gated Ca^{++} channel activation which then results in flooding of the extracellular space with excitatory amino acids, such as glutamate. This is referred to as anoxic depolarization (Dirnagl, et al., 1999). Intracellular Ca^{++} is further increased by activation of both NMDA and non-NMDA glutamate receptor activity (Park, et al., 1989). Monovalent ions such as Na^{+} and Cl^{-} move into the cell through glutamate mediated receptors; water follows these ions resulting in cellular edema. As ATP stores are depleted, energy

dependent processes fail leading to a breakdown of cellular organization. Calcium activated enzymes lead to free radical production, and membrane breakdown. Within minutes, cells in the core undergo permanent anoxic depolarization that cannot recover. Cells are destroyed by protein degradation and lysis that results from the breakdown of membrane potential (Siesjo, 1992).

1.2.3.2 The Ischemic Penumbra

Not all regions of the ischemic territory experience permanent anoxic depolarization and rapid cell death. The penumbra refers to the area of tissue surrounding the core region where blood flow is restricted but energy metabolism is partially preserved. Neurons in this area depolarize but maintain the ability to repolarize. If untreated, ongoing excitotoxicity and toxic secondary processes will lead to delayed cell death and growth of the lesion (Siesjo, 1992).

Repolarized cells in the penumbra can experience repeated depolarization as glutamate accumulates in the extracellular space from previous firing. Cells will eventually die if the frequency of depolarizations increases. Excitotoxicity initiates secondary signaling pathways within the cell that can lead to delayed cell death (Hossmann, 1994). Oxidative stress, calcium overload, mitochondrial stress and DNA damage have all been implicated in delayed neuronal cell death often referred to as apoptosis (Dirnagl, et al., 1999), though morphological studies have failed to find evidence of apoptotic cell death following stroke (Colbourne and Auer, 2009, Garcia, et al., 1995, Garcia, et al., 1995, Garcia, et al., 1993). Calcium activated second messenger systems also trigger the expression of several proinflammatory genes. Inflammatory mediators, such as tumor necrosis

factor α (TNFα) and interleukins are released and induce the expression of adhesion molecules on the surface of endothelial cells. These adhesion molecules interact with neutrophils and encourage passage through the vessel wall into the brain tissue. Later macrophages and leukocytes follow across the blood brain barrier. Microglia are also activated and migrate towards the injury site. Inflammatory cell infiltration is an important adaptive reaction to injury but can also contribute to ischemic injury through slowing of red blood cell passage through micro vessels and the production of toxic substances, such as reactive oxygen species (Dirnagl, et al., 1999, Garcia, et al., 1994).

Anoxic permanent depolarization, recurrent depolarization, delayed cell death and inflammation all contribute to ischemic injury depending on proximity to ischemic core and collateral flow. The processes that contribute to cell death in the core occur within minutes; whereas cell death in the penumbra may occur over a much longer time scale such as hours or even days. Treatments aimed at saving brain tissue following stroke target these later mechanisms.

1.3 Experimental Models of Focal Ischemia

There are many experimental models of focal ischemia in animals; see Traystman 2003 for a recent review (Traystman, 2003). In general, models involve the occlusion of large cerebral arteries in small animals. The middle cerebral artery (MCA) is commonly targeted as MCA territory strokes are common in humans. This discussion will be limited to rat models, as that is the topic of this thesis. The embolic stroke model involves the injection of clots or

artificial spheres into the middle cerebral artery to achieve occlusion (Futrell, et al., 1989, Kaneko, et al., 1985). This model can produce quite variable lesion sizes but is thought to be the most similar to human embolic stroke. Focal ischemia can also be achieved through the use of injected photothrombotic dyes (Futrell, et al., 1988) or the local application of a vasoconstrictor, such as endothelin-1 (Robinson, et al., 1991). The most commonly used rodent model is the intraluminal suture model. This method involves inserting a blunted piece of filament into the internal carotid artery either directly or via the external carotid artery (Longa, et al., 1989). The suture is advanced until the tip occludes the middle cerebral artery where the artery branches. The suture may be left in place to achieve a permanent occlusion (permanent MCA occlusion; pMCAO) or removed to allow reperfusion (transient MCA occlusion; tMCAO). This method is widely used in experimental studies but can be variable because of incomplete blockage or accidental hemorrhage due to vessel puncture.

Focal ischemia may also be achieved through direct exposure and occlusion of the MCA through a small craniotomy (Tamura, et al., 1981). The artery may be occluded proximally, before the lenticulostriate branches, or distally, sparing the sub cortical structures. Transient MCAO involves temporary occlusion of the MCA by placement of a non-traumatic clip or suture. Permanent MCAO may be achieved by cautery or permanent ligation of the MCA. Unilateral occlusion of the MCA is often accompanied by unilateral or bilateral common carotid occlusion, which further reduces the CBF in the MCA territory. Permanent focal ischemia is not necessarily accompanied by permanent reduction

of regional cerebral blood flow. Regional blood flow increases to 30-50% of baseline values within 3 hours of permanent occlusion (Rha and Saver, 2007, Yanamoto, et al., 2001).

The model chosen for the studies in this thesis is distal permanent focal ischemia via coagulopathy in rat, a method shown to produce a consistent and large infarct in Sprague-Dawley rats (Duverger and MacKenzie, 1988). This model produces a relatively consistent injury profile and yields substantial functional deficits (Yamamoto, et al., 1988, Yanamoto, et al., 2001).

1.3.1 Histological Injury

The MCA supplies a large portion of multifunctional cortical areas and deep structures of the brain. These areas are mostly involved in the processing and assimilation of motor and sensory information but can also include the perirhinal cortex which is involved in cognitive and emotional processes (Corbett and Nurse, 1998). Specific pathology depends upon the mode and placement of the occlusion. The embolic model produces variable injury that ranges from diffuse lacunar injury in the cortex to near complete MCA territory infarction, depending upon the clot/sphere load and placement of the injection (Kaneko, et al., 1985). A proximal model such as the intraluminal suture model affects both the lateral cortex as well subcortical structures such as the caudate nucleus; whereas, a distal occlusion such as the craniotomy method mostly spares the deep structures (Barone, et al., 1991, Roof, et al., 2001). Transient ischemia generally results in less extensive injury compared to permanent ischemia but reperfusion can also be detrimental (Hashimoto, et al., 2008).

1.3.2 **Functional Impairments**

Focal ischemia leads to functional deficits in humans, such as sensory-motor disturbances, aphasia, memory problems and depression (Dam, et al., 1989, Jorgensen, et al., 1999). Such impairments have a profound impact of the lives of stroke patients and their families. As such, it is essential to understand the impact potential stroke treatments have on functional outcome. Similar to humans, behavioral deficits are evident in rodent models of acute ischemic stroke. There are many different ways to measure functional impairments following focal ischemia in rats; see Corbett and Nurse 1998 for a review (Corbett and Nurse, 1998). Areas supplied by the MCA are mostly involved in sensory and motor processing, though memory deficits have been demonstrated in rats following MCAO (Markgraf, et al., 1992, Okada, et al., 1995). Investigators have primarily relied upon measures sensitive to sensory-motor impairments to gauge behavioral outcome. Numerous tests measure gross sensory-motor deficits after stroke. Various tasks are used but some examples include: hind limb retraction when displaced, spontaneous circling, maintaining balance on a rotating rod and postural reflexes (Markgraf, et al., 1992, van der Staay, et al., 1996). These behaviors are often combined to produce a total neurological deficit score (NDS). Walking ability may be assessed while traversing a narrow beam (Okada, et al., 1995) or a horizontal ladder (Aronowski, et al., 1996). Spontaneous forelimb use asymmetry is demonstrated following pMCAO during exploration of a plexiglass cylinder (Roof, et al., 2001, Schallert, et al., 2000). Vibrissae-evoked behaviors are used to evaluate sensory neglect, for example, limb-placing on the corner of a

flat surface such as a table in response to vibrissae brush (Schallert, 2006) or ability to use vibrissae to navigate a T-maze (Hurwitz, et al., 1990).

Typically, MCA occlusion produces deficits that are measureable on the above described tasks within days of stroke but recovery occurs fairly quickly (Corbett and Nurse, 1998). Gross sensory-motor deficits also recover quickly in humans but fine motor skills often improve slower and to a lesser extent (Nowak, et al., 2007). Sensitive specialized testing is required in order to detect subtle fine motor deficits following stroke. Skilled reaching tasks, such as the staircase test (Montoya, et al., 1991), allow for evaluation of fine forelimb function involving dexterity of the digits, similar to finger control in humans. Following MCAO, rats display a chronic disability in retrieving sugar pellets from a small staircase using their forelimbs (Colbourne, et al., 2000, Grabowski, et al., 1993). Skilled reaching tasks are useful to detect long-term subtle changes in function in rodent models following stroke. In the studies included in this thesis, asymmetry, walking ability, neurological deficits and skilled reaching are evaluated. We used a battery of sensorimotor tasks to better evaluate the function of animals post-stroke.

1.4 Treating Stroke

Once a person has a stroke, efforts must be made to reduce the impact of that injury. The first approach is to minimize the injury by limiting the duration or severity of the insult. This can be achieved with the use of clot busting drugs (e.g., tissue plasminogen activator, tPA) that allow blood back into an ischemic area (Haley, 1993), the use of antiplatelet therapy (aspirin), heparin or by altering

physiological variables that may contribute to injury (i.e., reducing blood pressure increases following hemorrhage which may reduce bleeding). Unfortunately, only 10-15% of stroke victims are eligible for such strategies. The next tactic is to limit the effects of injury on brain tissue by interrupting injury processes that result from stroke (neuroprotection). Many treatments have shown promise in experimental models but to date none have successfully translated into clinical practice (Ginsberg, 2009, O'Collins, et al., 2006). The third strategy is rehabilitation, which serves to help stroke victims regain function following injury. Strategies to combat stroke may used individually or in concert to improve patient care.

The present thesis focuses on the second strategy: neuroprotection. The goal of neuroprotection is to save neurons in the penumbra by blocking detrimental events of the ischemic cascade, described in above sections. Limiting neurological injury in the ischemic penumbra should improve neurological function and reduce the impact of stroke.

Several pharmacological agents have been evaluated as putative neuroprotectants, each targeting a different component of ischemic cell death. Some notable examples include NMDA and AMPA receptor antagonists (Nellgard and Wieloch, 1992, Pulsinelli, et al., 1993, Small and Buchan, 1997, Warner, et al., 1990, Xue, et al., 1994), calcium channel blockers (Izumiyama and Kogure, 1988, Nishijo, et al., 1992, Wong and Haley, 1990) and free radical inhibition/trapping (Agardh, et al., 1991, Kiyota, et al., 1993, Li, et al., 2000). All of these and many more pharmacological agents have proven effective in animal

models, yet none have successfully passed phase III clinical trials in humans (O'Collins, et al., 2006)

1.5 Translation of Experimental Findings

Experimental stroke models allow investigators to empirically evaluate of the impact of therapies on outcome following stroke. There are several possibilities that may account for the failure of successful experimental treatment to translate into the clinic. The most obvious is that the majority of preclinical testing is done on rodents, not higher mammals. In fact, a recent update on the STAIR recommendations (STAIR update, 2009) points to reliance on rodent data as a likely factor contributing to the recent failure of clinical trials. It is possible that some fundamental difference between rodents and humans prevents translation of findings. Although there are substantial differences, many effective drug treatments for human disease were first found to be effective in rodent models (Chauhan, et al., 2005, Morrow, 2007). It is more likely the disconnect lies between methodology used in experimental stroke research and the reality of what is possible in the clinic (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). For example, the vast majority of neuroprotection studies use transient models of ischemia, but clinically more than half of stroke patients do not experience reperfusion in the acute period (Rha and Saver, 2007). Preclinical studies often rely on treatment administered during or immediately following an ischemic insult, whereas clinical trials generally employ much larger time windows often within a wide range (i.e. hours to days). Putative neuroprotectants that are successful with immediate treatment may be acting upon earlier processes

that are no longer active at later time points when stroke patients are likely to receive treatment. For example, a drug aimed at blocking excitatory neurotransmission may be extremely effective at preventing or delaying excitotoxic cell death, but may not have the same efficacy if administered after a delay, when inflammation and delayed cell death mechanisms are more likely contributing to injury. Therefore, it is important to consider the introduction of a post ischemic delay when evaluating the efficacy of neuroprotective treatment for stroke.

Also, outcome is often determined from histology alone at short survival times (DeBow, et al., 2003), which makes little sense considering the important clinical outcome is long term functional ability. A reduction in lesion size makes no difference to a stroke patient if there is no corresponding improvement in their function; a drug that solely reduces short-term histological injury is sure to fail in clinical trials.

In many cases, the optimal duration of a treatment has not been clearly established preclinically. Abbreviated treatment durations are common as is early assessment of outcome (O'Collins, et al., 2006). Therapeutic regimens which confer benefit at early time points may be simply delaying cell death (Colbourne, et al., 1999, Dietrich, et al., 1993, Dyker and Lees, 1998, Valtysson, et al., 1994) and such data could result in misleading information regarding optimal treatment duration. In global ischemia, prolonged treatment can provide permanent protection (Colbourne, et al., 1999, Colbourne, et al., 1997). Unfortunately, a drug or treatment applied for an insufficient duration may be rejected when it might

have provided benefit were it administered for a longer period of time. This is particularly important when one considers the above discussion on the influence of treatment windows and the timing of cell death mechanisms. If a therapy is not applied in time to influence earlier cell death mechanisms, protracted treatment may be necessary to confer benefit.

1.6 Hypothermia

The use of hypothermia as a therapeutic agent is a very old concept. Its origins are unknown, but accounts of remedial body and brain cooling date back centuries. Early applications were diverse; including treatment for schizophrenia, brain trauma and cancer. The first documented clinical use came about in the 1940's. Temple Fay, a physician in Philadelphia, reported extremely good outcomes in a number of trauma patients who underwent cooling (Fay, 1945, Fay, 1958). Also, dogs that underwent cardiac arrest were found to have reduced mortality when moderate hypothermia was applied immediately following resuscitation (Wolfe, 1960, Zimmerman and Spencer, 1959). Medical interest in hypothermia grew and over the next decade physicians experimented with cooling during procedures such as cardiac surgery and neurosurgery. Results of early clinical studies were unclear and some animal data even reported worse outcome in hypothermia treated groups (Steen, et al., 1980, Steen, et al., 1979). Most of these early experiments involved deep to moderate cooling (27-31°C); serious complications of treatment and frequent death of patients lead to a fairly rapid abandonment of therapeutic hypothermia. A revival of interest in intraoperative

cooling emerged from improvements in physiological monitoring and more efficient methods to induce hypothermia and re-warm patients. It became well accepted that deep intraischemic hypothermia was effective at preventing brain injury and it developed into standard procedure during operations involving interruption of blood flow.

Hypothermia did not receive renewed attention as a treatment for brain injury until the 1980's. At that point, basic researchers discovered that a *mild* form of hypothermia (32 - 35°C) could protect the brain during ischemia and trauma in animal (Colbourne, et al., 1997). Mild hypothermia results in fewer dangerous complications than severe and may be induced and maintained quite easily in most hospital settings. Interest was further bolstered by evidence that hypothermia could also reduce brain injury and improve outcome when applied *post*-injury, suggesting that it may be useful for insults occurring outside of the hospital. Subsequently, cooling was evaluated for many different types of brain injury including cardiac arrest, trauma and stroke. Hypothermia is very effective at reducing brain injury and improving function following global ischemia. Reduction of selective cell death in the hippocampus due to post-ischemic mild hypothermia was documented by multiple independent investigators (Maher and Hachinski, 1993), as long as treatment was sufficiently prolonged (Colbourne and Corbett, 1994, Colbourne and Corbett, 1995). In the early 2000's, success in animal studies combined with encouraging results from small clinical trials resulted in two large-scale randomized controlled trials that evaluated prolonged mild hypothermia following out of hospital cardiac arrest. The results were

concurrently published in 2002 and both trials reported positive and significant effects on both mortality and morbidity (Bernard, et al., 2002, The Hypothermia After Cardiac Arrest Study Group, 2002). Mild hypothermia has also been investigated in other types of brain injury. Notably, experiments showed that mild hypothermia reduced intracranial pressure and improved outcome following TBI in animals. A number of trials evaluating hypothermia in TBI patients ensued. The results of these trials were mixed. In 2001, Clifton et al. reported that mild hypothermia was not effective at improving outcome following TBI in 392 patients in a large multi-centered trial (Clifton, et al., 2001). The negative result dissuaded the medical community from viewing hypothermia as a promising treatment for TBI even though a subsequent trial with 396 patients showed improvements in mortality, morbidity and ICP (Zhi, et al., 2003). Pertinent to this thesis, there have also been investigations evaluating hypothermia as a potential therapy for acute ischemic stroke.

1.6.1 Hypothermia Therapy for Acute Ischemic Stroke

The success of hypothermia following cardiac arrest has created interest in the potential for therapeutic cooling to reduce injury following acute ischemic stroke. Clinical trials in the subject have been limited but show that hypothermia is safe and feasible in stroke patients (Georgiadis, et al., 2002, Georgiadis, et al., 2001, Kammersgaard, et al., 2000, Schwab, et al., 2001).

The majority of preclinical studies has focused on models of temporary occlusion of the middle cerebral artery. Protective effects of mild to moderate

hypothermia are demonstrated reliably in models of tMCAO (Krieger and Yenari, 2004). In general, intraischemic hypothermia (hypothermia induced during ischemia) provides more benefit than delayed hypothermia (van der Worp, et al., 2007). Delay, depth and duration are important distinctions between studies (Krieger and Yenari, 2004, Maier, et al., 1998, Maier, et al., 2001, Meloni, et al., 2009). Two notable studies are Ohta et al. (2007) and Kollmar et al. (2007). The first examined two days of mild (34-35°C) hypothermia beginning 2, 4, 6 or 8 h after 120 minutes of MCAO. Reduced brain injury was found 2 days following stroke when hypothermia was induced within the first 6 h, but not after (Ohta, et al., 2007). Kollmar et al compared differing degrees of hypothermia (32-37°C) and found only 33-34°C significantly reduced brain injury (Kollmar, et al., 2007). Duration of cooling also appears to be important in conferring permanent benefit. For instance, 16 h of mild hypothermia initiated immediately following tMCAO significantly reduced brain injury when assessed within the first two days but protection was lost when examined 3, 5 or 7 days following injury (Inamasu, et al., 2000). In contrast, prolonged hypothermia (48 hours) provides lasting benefit (up to 60 days) following tMCAO in rats (Colbourne, et al., 2000, Corbett, et al., 2000). Comparisons among studies can be problematic and few studies use multiple parameters within a single experiment. Yanamoto (1999) directly compared different durations of hypothermia. In this study, intraischemic hypothermia was initiated for 5 or 24 h. Outcome was evaluated one month later and only animals subject to the prolonged treatment showed benefit (Yanamoto, et al., 1999).

Much less work has been published in permanent occlusion models and the results are not consistent among laboratories. Prompt onset of hypothermia has reduced infarct volume by as much as 84% following pMCAO, depending upon the parameters of treatment (Baker, et al., 1991). Brief durations (1-6 h) of deep hypothermia (24°C) substantially reduced injury (27-84%) in a proximal pMCAO model (Baker, et al., 1991, Baker, et al., 1992). In contrast, brief durations (1 to 6 h) of mild to moderate hypothermia (30°C to 34.5°C) had conflicting effects in rat models of distal pMCAO (Kader, et al., 1992, Morikawa, et al., 1992, Moyer, et al., 1992, Ridenour, et al., 1992, Xue, et al., 1992). Positive results evaluated at short survival times should be interpreted with caution because it is possible that the infarcts had not been given sufficient time to mature. In a global cerebral ischemia model, brief hypothermia improved neuron survival a few days later, but this protection was no longer observed at 1 month (Dietrich, et al., 1993) suggesting cooling only delayed cell death with that paradigm. Transient benefit was also found with other neuroprotective treatments (Colbourne, et al., 1999). Yanamoto, however, found that *prolonged* mild to moderate hypothermia reduced infarct volume 2 days as well as 3 weeks after stroke (Yanamoto, et al., 2001).

Results of delayed hypothermia in pMCAO models are inconsistent. As little as 1 h of deep to moderate delayed hypothermia provided benefit in some models (Baker, et al., 1992, Onesti, et al., 1991) but not in others (Doerfler, et al., 2001, Moyer, et al., 1992). There is no experimental evidence that hypothermia ameliorates infarct volume if initiated later than 60 minutes after onset of

ischemia in permanent occlusion models. Furthermore, it remains to be seen whether delayed hypothermia provides long-term or functional benefit following permanent focal ischemia. Studies in global ischemia models indicate that *prolonged* hypothermia is protective even if cooling is delayed by several hours. Colbourne and Corbett reported protection of up to12-h delayed hypothermia, provided cooling was maintained for 48 h (Colbourne, et al., 1999). Such studies have not been conducted in the focal models. The reasons for discrepant findings are unclear, but they suggest hypothermia is less effective against permanent than transient focal ischemia although less research is available on permanent models. Few studies have assessed functional protection of hypothermia following pMCAO (Ridenour, et al., 1992, Yanamoto, et al., 2001). Ridenour et al found no benefit of 2 h of mild hypothermia following pMCAO (Ridenour, et al., 1992). Yanamoto et al demonstrated long-term improvement in neurological function with immediate prolonged hypothermia in pMCAO (Yanamoto, et al., 2001). No study has evaluated the long-term functional benefit of delayed hypothermia following pMCAO. It is predicted that *prolonged* and *delayed* hypothermia will provide both long-term functional and histological protection following pMCAO in rat.

1.6.2 Adverse Effects and Methods of Cooling

The most common method of inducing hypothermia in both experimental studies and clinical trials is whole body surface cooling (Diller and Zhu, 2009). While systemic hypothermia provides protection following many injury types

(focal and global ischemia, trauma), it is associated with serious risks. Lowering core body temperature produces many deleterious effects such as cardiovascular, pulmonary, coagulation, metabolic and immunologic complications. When core temperature is lowered below normothermia, a number of processes are initiated which attempt to counteract the cooling and return the body to homeostasis. Thermoreceptors in the skin, core organs and hypothalamus detect temperature changes and transmit the information to the preoptic area of the hypothalamus, where it is integrated. Cool temperatures invoke disinhibition of tonically suppressed thermogenic responses such as peripheral vasoconstriction and shivering which result in an increase in blood pressure and cardiac output. Also, lowering temperature reduces the number and function of neutrophils and interferes with enzymes involved in clotting (Dirkmann, et al., 2008, Schubert, 1995).

It is possible that increased risk of complications may counter some of the benefits afforded by hypothermia. Also, systemic hypothermia has an inherently slow cooling rate because of the increase in thermal resistance due to arteriovenous shunt vasoconstriction (Krieger, et al., 2001, Marion, et al., 1997, Schwab, et al., 1998). Because of the slow cooling rate, time taken to reach target temperature is necessarily longer which may compromise protection.

In patients, brain cooling is achieved through the use of different methods, including head surface cooling techniques, chilled neck collars and intravascular cooling (Diller and Zhu, 2009). In humans, surface brain cooling techniques are not effective in reducing the brain temperature quickly. More invasive techniques

such as epidural and intraparenchymal cooling devices are necessary to effectively cool the brain alone (Diller and Zhu, 2009, Wagner and Zuccarello, 2005, Wagner, et al., 2003). In animals, it is possible to surface cool the head alone while leaving the body normothermic and such brain cooling can reduce brain injury, when applied promptly (Taniguchi, et al., 2005). Unfortunately, it has been difficult to evaluate delayed or prolonged selective brain hypothermia, as all focal cooling methods in animals are only possible under constant anesthesia.

Taken together, the above discussion underlines the importance of experimental design on outcome in clinical and experimental neuroprotection studies. Thorough preclinical evaluation of putative neuroprotectants is essential. In the case where determination of clinically relevant efficacy is the primary goal, intervention delay, duration of treatment and outcome measures should all be carefully considered as to their practical relevance to that goal. The research outlined in this thesis attempts to overcome some of these confounding factors by thoroughly assessing a promising potential neuroprotectant, therapeutic hypothermia, in a model of permanent ischemia. Various durations and two different methods of cooling are considered. Both functional and long-term outcome are presented, as well as histology of the brain. It is hoped that this research will contribute to understanding whether delayed hypothermia has potential to become a clinically viable treatment strategy for acute stroke.

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Systemic Hypothermia after pMCAO in Rat[1]

Chapter 2

[1] A version of this chapter has been published as follows: Clark, D., Penner, M., Orellana-Jordan, I. and Colbourne, F. Comparison of 12, 24 and 48 hours of systemic hypothermia on outcome after permanent focal ischemia in rat. *Experimental Neurology*, **212**:386-92, 2008.

2.1 Introduction

Therapeutic hypothermia is arguably the most studied treatment for ischemic brain damage. After decades of research it is clear that hypothermia provides unrivaled neuroprotection during ischemia (Colbourne, et al., 1997, Dietrich, et al., 1996, van der Worp, et al., 2007). Delayed hypothermia is also beneficial. Notably, in the clinic mild hypothermia reduces morbidity and mortality when administered after cardiac arrest (Bernard, et al., 2002, The Hypothermia After Cardiac Arrest Study Group, 2002) and in neonates suffering from hypoxic-ischemic encephalopathy (Gluckman, et al., 2005, Shankaran, et al., 2005). Clinical studies have evaluated hypothermia for focal ischemia (Kammersgaard, et al., 2000, Schwab, et al., 2001, Schwab, et al., 1998), but they were not powered to determine efficacy.

Animal studies show that hypothermic neuroprotection depends upon: the type and severity of stroke, intervention delay, depth and duration of hypothermia, as well as the cooling and re-warming rates. An understanding of these factors was essential to the success of the aforementioned clinical trials. For instance, prolonged hypothermia (e.g., 24 h) saves more CA1 neurons in the anterior hippocampus than brief cooling after global ischemia in rodents (Colbourne and Corbett, 1995). Furthermore, lengthy hypothermia permanently reduces cell death whereas brief post-ischemic hypothermia (e.g., 3 h) only transiently protects (Colbourne and Corbett, 1994, Dietrich, et al., 1993). Although the best possible profile of hypothermia has not been established, it appears that several days of

mild hypothermia rescues the most CA1 neurons even when intervention is delayed by 12 h (Colbourne, et al., 1999, Colbourne, et al., 1999).

The effectiveness of hypothermia varies with the type (e.g., ischemia vs. hemorrhage vs. trauma) and severity of injury (e.g., temporary vs. permanent ischemia). Thus, hypothermia must be tested in each condition in order to optimize treatment protocols. In this regard, delayed hypothermia has been studied in models of temporary and permanent focal ischemia, and several have systemically varied treatment parameters. In the setting of temporary ischemia, investigators have varied the intervention delay (Karibe, et al., 1994, Maier, et al., 2001, Markarian, et al., 1996, Yanamoto, et al., 1996), depth (Kollmar, et al., 2007, Maier, et al., 1998), duration (Maier, et al., 1998, Markarian, et al., 1996, Yanamoto, et al., 1996, Zhang, et al., 1993) and re-warming rates (Berger, et al., 2007). For instance, 21 h of mild hypothermia provides greater protection than a 1 h treatment when initiated after 3 h of tMCAO in rat with a 2 day survival (Yanamoto, et al., 1996). Delayed hypothermia also persistently (e.g., 2 month survival) reduces infarct size and improves behavioral recovery after tMCAO in rat (Colbourne, et al., 2000, Corbett, et al., 2000).

Not surprisingly, hypothermia is more effective for temporary than permanent focal ischemia, especially with intervention delays (Morikawa, et al., 1992, Ren, et al., 2004, Ridenour, et al., 1992). This is particularly disconcerting as many ischemic strokes are permanent (Kassem-Moussa and Graffagnino, 2002) and intervention delays are inevitable. As with tMCAO, studies have systemically varied treatment parameters (Baker, et al., 1992, Kader, et al., 1992, Zhao, et al.,

2007). For example, one study compared 2 and 4 h of hypothermia in a rat model of permanent middle cerebral artery occlusion and they found that the 4 h duration was superior at a 2 day survival (Zhao, et al., 2007). Prolonged hypothermia has also been studied. For example, 24 h of hypothermia induced immediately after onset of pMCAO in rat reduced infarct size and improved recovery out to the 21 day survival (Yanamoto, et al., 2001).

Collectively, these studies show that hypothermia reduces injury and improves recovery in models of temporary and permanent focal ischemia. Unfortunately, it is difficult to identify optimal hypothermia protocols for clinical investigation from comparisons among animal studies. After depth of hypothermia, which is likely to be set around 33°C for fear of systemic complications, the next most important treatment parameter is the duration of hypothermia. No study has comprehensively evaluated its impact for focal ischemia using a range of durations found most effective in global ischemia. Interestingly, a recent meta-analysis found that prolonged hypothermia provided less benefit than briefer treatment in the setting of focal ischemia (van der Worp, et al., 2007), which is contrary to findings in global ischemia. We suspect this resulted from a study bias, as those experiments that used protracted hypothermia also tended to use longer survival times ($\geq$ 7 days) compared to the propensity of experiments on brief hypothermia that used brief survival times ($\leq$ 48 h), which may overestimate true benefit. Regardless, the authors urged caution in interpreting this particular finding from the meta-analysis, and recommended that further studies specifically address this question.

This study sought to evaluate the impact of treatment duration on edema, infarct size and recovery in a rat model of pMCAO. Thus, we compared 12 (HYPO-12), 24 (HYPO-24) and 48 h (HYPO-48) of systemic hypothermia in awake animals induced 1 h after onset of pMCAO to a normothermic (NORMO) ischemia group. The pMCAO insult was produced by cauterizing the distal MCA combined with bilateral carotid artery occlusion (Tamura, et al., 1981, Yamamoto, et al., 1988). Core temperature (T_c) was measured via telemetry (DeBow and Colbourne, 2003), and systemic hypothermia was induced in conscious rats (Colbourne, et al., 1996, DeBow and Colbourne, 2003). Functional recovery was evaluated with three tests. The horizontal ladder test was used to quantify walking ability (Metz and Whishaw, 2002). The cylinder task was used to measure spontaneous forelimb usage during exploration (Schallert, 2006). Finally, neurological recovery was evaluated by several simple tests (e.g., balance) that combined formed a neurological deficit scale (NDS) score (MacLellan, et al., 2006). In Experiment 1, the effects of these three treatments on infarct size and functional outcome were evaluated at 7 days after pMCAO onset. In Experiment 2, we completed a time course study of edema after pMCAO. We also determined the effects of HYPO-12 and HYPO-24 treatments on edema at 1 day, and HYPO-12 and HYPO-48 treatments on edema at 3 days (vs. NORMO groups).

2.2 Methods

Animals

All procedures were approved by the University of Alberta Biosciences Animal Care and Use Committee and conformed to Canadian guidelines. We used 124 male, Sprague-Dawley rats weighing 250 to 350 g and at approximately 10 weeks of age at the beginning of the study. Of these, 4 were excluded for various reasons (e.g., death during telemetry probe surgery, abnormal vasculature). Animals were housed individually and maintained on a 12 h light/dark cycle (07:00 – 19:00) with free access to food and water. Animals were randomly assigned to treatment groups and all procedures were done by experimenters blind to group identity. Additional details about our laboratory standard operating procedures may be obtained at: http://web.psych.ualberta.ca/~fcolbour/research.htm.

In Experiment 1, one group of rats were kept normothermic (NORMO, *N*=15) after pMCAO and three groups were treated with mild hypothermia lasting either 12 (HYPO-12, *N*=15), 24 (HYPO-24, *N*=14) or 48 h (HYPO-48, *N*=14). Rats were euthanized 7 days following stroke. In Experiment 2, brain water content (BWC) was measured from 1 to 5 days after pMCAO (days 1 to 4, *N*=5; day 5, *N*=6) and this was compared to naïve controls (*N*=7). In addition, BWC was measured 24 h after pMCAO in rats who had received HYPO-12 (*N*=6) and HYPO-24 (*N*=8) treatments. Finally, BWC was measured 3 days after pMCAO in HYPO-12 (*N*=7) and HYPO-48 groups (*N*=8).

Temperature Measurement and Control

All surgical procedures were done aseptically under isoflurane anesthesia (4% induction; 2% maintenance; 60% N_2O, balance O_2). Rats were implanted with T_c telemetry probes (model TAT10TA-F40, Transoma Medical, St. Paul, MN) 3 days prior to stroke (DeBow and Colbourne, 2003) and data from the day before stroke served as baseline. Previous studies have established that T_c and brain temperature are highly correlated and similar (i.e., <1°C difference) in non-anesthetized animals (Clark, et al., 2007, DeBow and Colbourne, 2003). Following surgery, rats had their T_c precisely servo-regulated, usually within 0.5°C of target, using fans and fine water misters for cooling and an overhead infrared lamp for warming (Colbourne, et al., 1996, DeBow and Colbourne, 2003). All rats had T_c maintained at normothermia for 1 h after stroke. The rats undergoing hypothermia began cooling 1 h after stroke onset at a rate of 2°C / h and they were kept near 33°C for 12, 24 or 48 h. Subsequently they were re-warmed at a rate of 1°C / h, after which they were kept from going below 36°C until 96 h after pMCAO. The NORMO groups were kept from falling below 36°C for 96 h post-stroke.

Stroke Model

All rats were subjected to pMCAO (Tamura, et al., 1981, Yamamoto, et al., 1988), but they were first food deprived for ~15 h to help ensure glucose was in a consistent range; otherwise they had free access to food. During surgery, skull temperature (T_s) was measured with a thermocouple probe (HYPO-33-1-T-G-60-SMG-M, Omega, Stanford, Conn.) and maintained near 37.2°C using an

overhead infrared lamp (175 W). The tail artery was cannulated to allow for blood gas and glucose measurements, and to measure mean arterial blood pressure (MABP). Arterial blood samples (100 μL) were taken immediately prior to ischemia and analyzed with a Radiometer ABL 810 blood gas analyzer (Radiometer, Copenhagen, Denmark). A midline neck incision was made to expose both common carotid arteries, which were permanently ligated with two strands of silk suture per artery. The neck wound was then sutured closed and Marcaine (Sanofi Canada, Markham, Ontario, Canada) was infiltrated into the wound. Next, an incision was made in the Temporalis muscle and an approximately 2 mm diameter area on the right side of the skull was thinned out with a small hand held drill. This allowed for visualization of the MCA and for gentle removal of bone. The dura covering the MCA was carefully removed and the artery was lifted onto a small hook suspended by a micromanipulator. The MCA was then cauterized (ME-102, Martin, Germany) and transected. The muscle and wound were sutured and treated with Marcaine. Rats were provided with a moist palatable food mixture (rodent chow, honey, peanut butter) for days following surgery. Body weight was recorded on the day of surgery and at euthanasia.

Behavioral Testing

In Experiment 1, a neurological deficit scale (NDS) score was measured prior to and at 7 days after stroke onset. The score was determined from 5 behaviors: hind limb retraction, contralateral forelimb flexion, bilateral forepaw grasp, traversing a narrow beam and forelimb placing. A score of 0 represented no

deficit whereas 13 represented maximum impairment (MacLellan, et al., 2006). Additionally, rats were evaluated on a horizontal ladder (Metz and Whishaw, 2002) and a forelimb use asymmetry (cylinder) task (Schallert, 2006) prior to and at 7 days post-ischemia. The ladder task evaluates foot fall rate while the rat traverses a series of parallel bars variably spaced 1 – 3 cm apart. The cylinder task measured spontaneous limb preference during exploration of a vertical Plexiglas cylinder. For the latter, we measured the number of independent wall touches with each forelimb as well as co-usage. The inclusion criterion for this test was set at 10 independent wall touches. From this, an asymmetry score (Schallert, 2006) was calculated:

(contralateral forelimb contact + ½ both) / (ipsilateral forelimb contact + contralateral forelimb contact + both) × 100.

Histology

Rats in Experiment 1 were euthanized by an intraperitoneal injection of sodium pentobarbital (~100 mg/kg) and transcardially perfused with 0.9% saline followed by 10% formalin. Frozen coronal brain sections (40 μm) were cut and later stained with cresyl violet. The Scion Image J program (Scion Corporation, Frederick, MD, USA) was used to trace and measure the area of normal tissue at 400 μm intervals extending through the lesion. The volume of tissue lost was calculated as:

Volume of tissue lost = remaining volume of normal hemisphere - remaining volume of injured hemisphere.

Volume of a hemisphere = average (area of the complete coronal section of the hemisphere - area of ventricle - area of damage) × interval between sections × number of sections.

Brain water content

All rats in Experiment 2 were briefly anesthetized with isoflurane and decapitated. Brains were quickly removed and separated into whole cortex and striatum for each hemisphere along with the cerebellum, which served as a control. After dissection, the regions were weighed and then baked (100°C) for 24 h after which the dry weight was obtained. The BWC was then calculated as previously done (Fingas, et al., 2007):

BWC = ((wet weight-dry weight) / wet weight) × 100.

Statistical Analysis

Pearson r values were computed for correlations between lesion volume and behavioral scores. Mortality was evaluated with a Chi-square test. Non-parametric statistics (Kruskal-Wallis and Mann Whitney U tests; SPSS, v. 12) were used to analyze NDS data. The edema time course data were analyzed with ANOVA with Dunnett post-hoc test. All other data were analyzed with ANOVA followed by Tukey HSD post-hoc tests. A p value of < 0.05 was considered statistically significant.

2.3 Results

Experiment 1

Eleven rats died prematurely after pMCAO presumably from brain injury. Mortality rates did not differ among treatment conditions (p=0.747). These exclusions left the group sizes at: NORMO (N=12), HYPO-12 (N=12), HYPO-24 (N=11) and HYPO-48 (N=12).

Temperature (Fig. 2.1) was regulated as stated in the Methods with little variance from the desired values. This was confirmed at the time of cooling for all animals. However, some of the T_c data was lost due to a computer hard drive failure. Physiological measurements during pMCAO did not differ significantly among groups ($p \geq 0.256$; Table 2.1). Body weight did decline ($p < 0.004$) from time of pMCAO (overall average = ~295 g) to 7 days post-stroke (~278 g), but there was no group effect (p=0.661) or interaction (p=0.195).

Baseline NDS scores were normal (group median scores = 0) with no significant differences among groups (p=0.297; data not shown). Neurological deficits were evident at 7 days post-pMCAO (Figure 2.2) at which time there was a significant difference among groups (p=0.002). Post-hoc testing showed that the NORMO group had a significantly higher NDS score (greater deficits) than the HYPO-12 (p=0.005), HYPO-24 (p=0.004), and HYPO-48 groups ($p < 0.001$). There were no significant differences between the HYPO treatments ($p \geq 0.394$).

Eight rats were excluded from the cylinder analysis due to failure to meet inclusion criterion (N=4) or a camera failure (N=4). The group sizes for this test were: NORMO (N=9), HYPO-12 (N=11), HYPO-24 (N=10), and HYPO-48

(N=9). As expected, there were no significant group differences during baseline testing (p=0.160, data not shown) during which scores averaged 51.5 % (i.e., no asymmetry). Scores were lower at 7 days after pMCAO (Figure 2.3), but the group effect at this time was not significant (p=0.388).

For the ladder analysis, one rat in the HYPO-48 group was not analyzed due to experimenter error, reducing the group size (N=11). Rats made few slips (overall average = 1.1%) with the contralateral forelimb while traversing the horizontal ladder during baseline testing, and there were no significant differences among groups (p=0.651, data not shown). Error rate was substantially greater at 7 days after pMCAO (Fig. 2.4), and there was a significant group effect (p=0.002). Post-hoc analysis revealed that the HYPO-24 (p=0 .041) and HYPO-48 (p=0 .002) groups made significantly fewer slips than the NORMO group. In addition, the HYPO-48 group made significantly fewer errors than the HYPO-12 group (p=0.034).

Untreated pMCAO caused significant cortical injury at 7 days post-insult, and occasionally the striatum sustained minor injury. Lesion volume was significantly different among groups 7 days after pMCAO (p<0.001, Fig. 2.5a). Both the HYPO-24 (p=0 .001) and HYPO-48 group (p<0.001) had significantly less injury than the NORMO group. The HYPO-48 group also had significantly less injury than the HYPO-12 group (p=0.017). Representations of the typical lesion in the NORMO and HYPO-48 groups are shown in Fig. 2.5b and 2.5c, respectively. Lesion size was significantly correlated with the NDS scores

(r=0.561, p<0.001), the cylinder data (r=-0.343, p=0.032), and the stepping error rate (r=0.553, p<0.001).

Experiment 2

In the edema experiment, naïve animals were compared to untreated NORMO groups at survival times of 1 to 5 days and to various HYPO treatments (Figure 2.6). Temperature control (Figure 2.1) and physiological variables (data not shown) were similar to Experiment 1.

There were significant differences in BWC (Figure 2.6a) between the naïve and each of the NORMO groups (days 1 – 5) for the ipsilateral cortex (p<0.001), whereas significant striatal edema only occurred on days 1 (p=0.002), 2 (p=0.003), and 3 (p=0.031). The cerebellum was unaffected (p=0.160).

A comparison of BWC among NORMO, HYPO-12 and HYPO-24 groups at 1 day after pMCAO (Figure 2.6b) revealed significant group effects for ipsilateral cortex (p=0.008) and striatum (p<0.001), but not cerebellum (p=0.193). Both HYPO-12 and HYPO-24 treatments significantly lessened edema in the ipsilateral cortex (p≤0.03) and striatum (p≤0.001), but the two cooled groups did not differ from each other in either structure (p ≥ 0.887).

A comparison of BWC among NORMO, HYPO-12 and HYPO-48 groups at 3 days after pMCAO (Figure 2.6b) revealed significant group effects for the ipsilateral striatum (p=0.003), with both HYPO groups having significantly less edema than the NORMO group (p≤0.007), but not from each other (p=1.000). There were no significant differences among groups for either the cortex or cerebellum (p≥0.540).

Table 2.1: Physiological variables (mean ± SD) measured during pMCAO surgery in Experiment 1.

	NORMO	HYPO-12	HYPO-24	HYPO-48
pH	7.405 ± 0.04	7.394 ± 0.05	7.411 ± 0.04	7.379 ± 0.04
pO_2 (mmHg)	121.3 ± 14.6	129.4 ± 16.9	120.5 ± 13.4	122.2 ± 13.7
pCO_2 (mmHg)	40.7 ± 3.8	42.0 ± 4.3	39.9 ± 3.6	42.9 ± 5.6
ctHb (g/dL)	14.9 ± 0.8	15.0 ± 1.0	15.3 ± 1.3	15.1 ± 0.9
Glucose (mmol/L)	9.1 ± 3.0	10.8 ± 6.6	9.5 ± 1.5	9.1 ± 3.0
Skull Temp. (ºC)	37.0 ± 0.5	37.1 ± 0.3	36.9 ± 0.4	37.1 ± 0.6
MABP (mmHg)	103.3 ± 12.7	103.8 ± 16.8	101.8 ± 13.8	95.0 ± 13.0

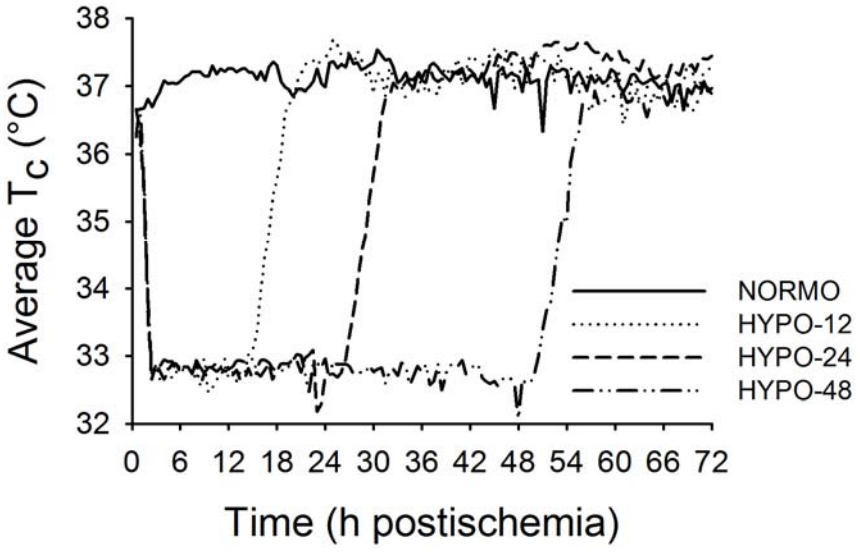

Figure 2.1: Core temperature (T_c) for 3 days after pMCAO onset reported as an average across groups in Experiments 1 and 2. Data were sampled every 30 seconds and averaged every 30 min. Temperature was regulated within a narrow range as stated in the Methods.

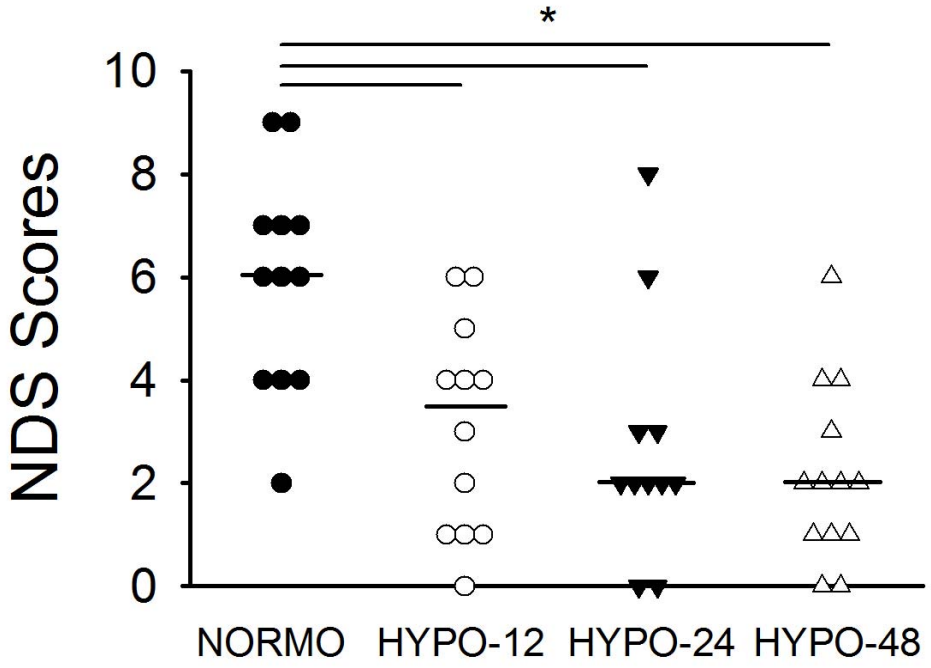

Figure 2.2: Neurological Deficit Scale (NDS) scores at 7 days after pMCAO onset in Experiment 1. All three HYPO groups had significantly lower scores (better outcome) than NORMO treated pMCAO rats. There were no significant differences among HYPO-treated groups. Bars denote group medians whereas * denote $p<0.05$.

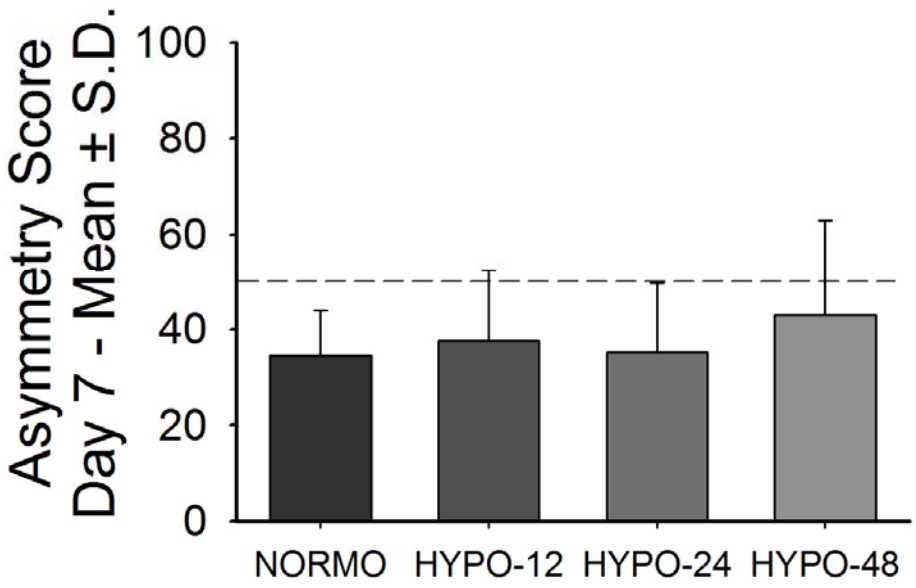

Figure 2.3: Asymmetry scores (mean ± SD) at 7 days after pMCAO onset in Experiment 1. There were no significant differences among groups.

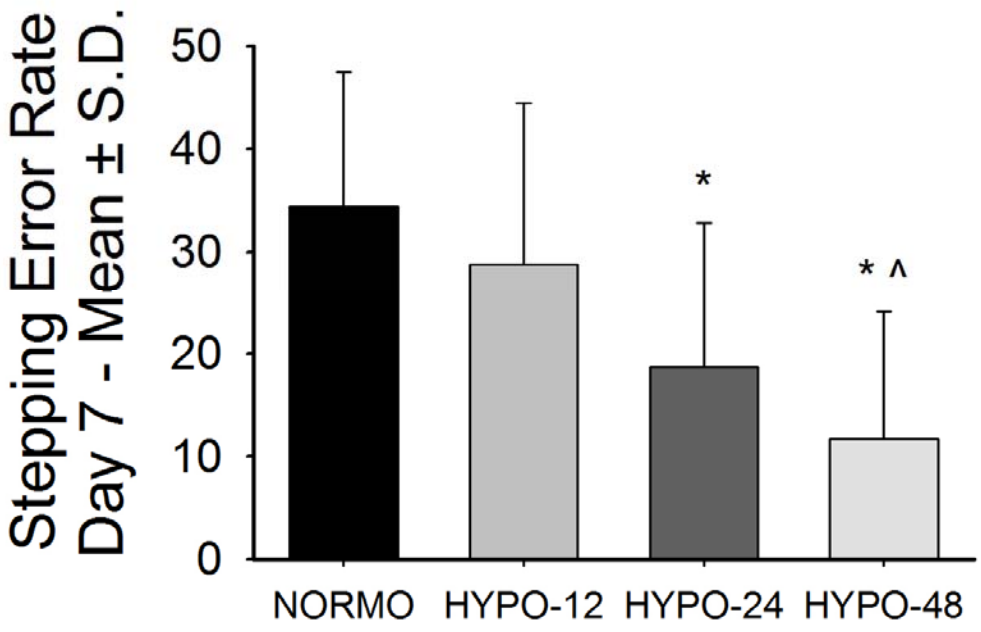

Figure 2.4: Stepping error rates (mean ± SD) at 7 days after pMCAO onset in Experiment 1. The HYPO-48 treatment significantly lessened this slip rate compared to both the NORMO and HYPO-12 groups (* $p<0.05$ vs. NORMO; ^ $p<0.05$ vs. HYPO-12).

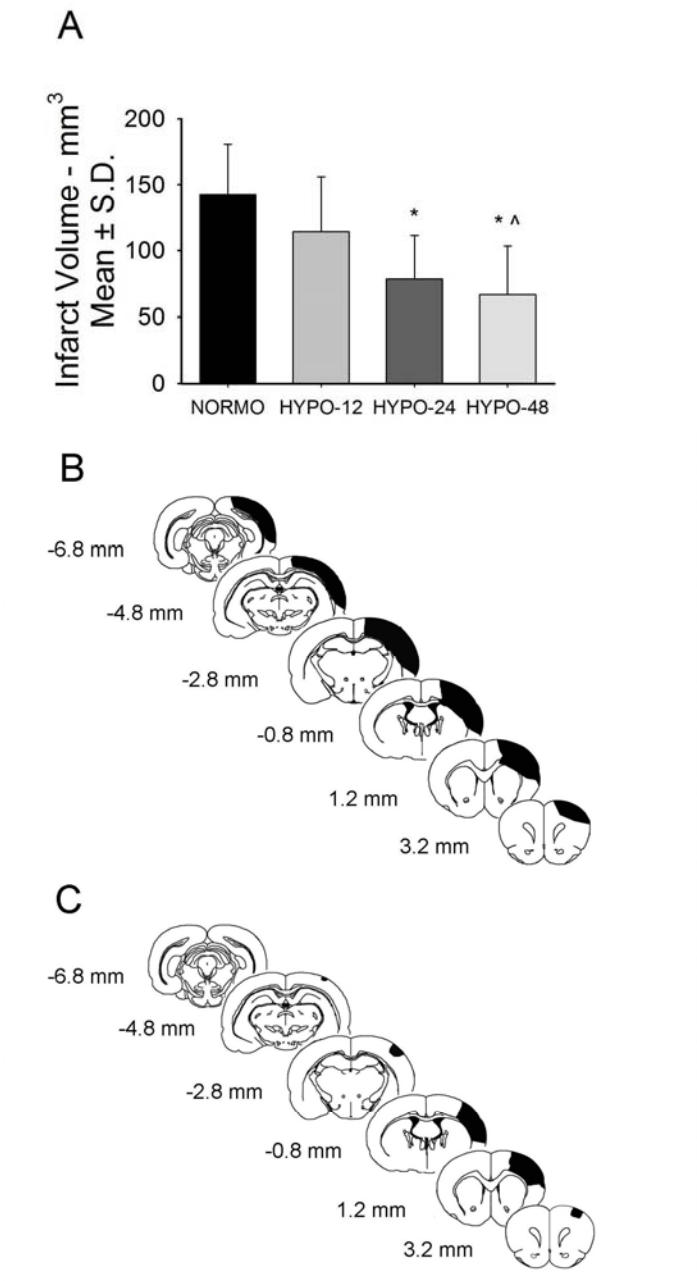

Figure 2.5: Average (± SD) volume (mm^3) of tissue lost at 7 days after stroke onset (A). An * denotes $p<0.05$ vs. NORMO, whereas ^ denotes $p<0.05$ vs. HYPO-12. Drawings illustrate the extent of injury in a typical NORMO (B) and HYPO-48 treated rat (C).

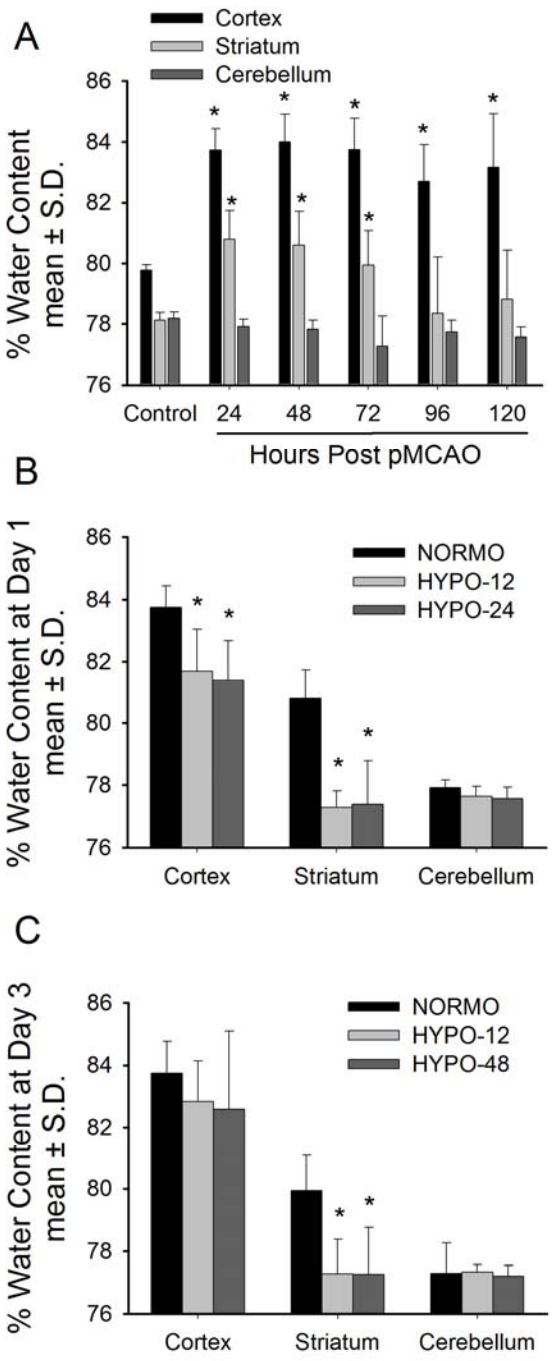

Figure 2.6: Brain water content (mean ± SD) from 1 to 5 days after pMCAO versus naïve control rats (A). Water content at 1 day (B) and 3 days (C) after pMCAO in NORMO and HYPO treated rats. An * denotes $p<0.05$ vs. controls (A) or NORMO groups (B and C).

2.4 Discussion

In this study we used a severe model of pMCAO, which involved permanent bilateral carotid artery occlusion combined with distal MCA cauterization, to cause substantial cortical infarction. We initiated systemic hypothermia treatment (vs. NORMO) beginning 1 h after onset of ischemia and continued cooling at 33°C for 12, 24 or 48 h. Our primary findings are that hypothermia reduces infarct size and improves functional recovery at 7 days after pMCAO in a duration-dependent manner with 24 and 48-h treatments providing the best outcome. Therefore, these findings contradict those of a recent meta-analysis (van der Worp, et al., 2007), and they strongly support the use of protracted cooling, whenever tolerable, in clinical studies for ischemic stroke.

Previous studies have investigated the importance of treatment duration for transient (Maier, et al., 1998, Markarian, et al., 1996, Yanamoto, et al., 1996, Zhang, et al., 1993) and permanent focal ischemic insults (Zhao, et al., 2007). However, most used relatively brief durations (e.g., 4 h) that are clearly less effective in treating global ischemia, and sub-optimal according to the present findings. We also predict that longer durations of hypothermia will become substantially more important following greater intervention delays after stroke. Indeed, this has been shown in global ischemia where relatively brief durations of hypothermia (e.g., 12 - 24 h) provide benefit if induced within a few h of ischemia, but not later, whereas 48 h of cooling is persistently effective even at 6 to 12 h delays (Colbourne and Corbett, 1995, Colbourne, et al., 1999, Colbourne, et al., 1999). Comparing the present findings to earlier studies (Doerfler, et al.,

2001, Moyer, et al., 1992) leads to the same conclusion because the use of brief hypothermia in those studies did not improve outcome when delayed after pMCAO. Presently, we showed that 48-h of hypothermia was significantly better than 12-h treatment in reducing lesion size and improving walking ability, but 48-h was not statistically better than 24-h hypothermia treatment. Thus, until further research is done we recommend at least 24 h of cooling for this type of stroke. However, it is possible that even longer durations of hypothermia will prove more efficacious in some situations. Indeed, clinical studies in traumatic brain injury indicate that 5 days is more effective than 2 days of cooling (Jiang, et al., 2006).

Cerebral edema was significantly reduced by 12, 24 and 48 h of hypothermia and there were no duration effects at the times sampled. Thus while hypothermia lessens edema, this effect alone cannot explain the superior protection of 48-h over 12-h treatment. The reduction in edema with hypothermia was also somewhat less than anticipated. Indeed, it did not reach statistical significance in the cortex at 3 days after pMCAO. This may be because cooling had ended before that time and edema simply returned after the loss of symptomatic benefit, and / or it may have been stimulated by re-warming. In this study, we re-warmed at a rate of 1°C / h, which is slower than the rates thought to be harmful (Berger, et al., 2007, Nakamura, et al., 1999, Ueda, et al., 2004). However, those studies used brief hypothermia, and it is possible that optimal re-warming rates will vary with the duration of cooling. Indeed, we predict that rapid re-warming will have a comparably less harmful effect after brief than after prolonged hypothermia owing largely to greater systemic cardiovascular effects

with more prolonged hypothermia. Finally, systemic and brain-selective cooling reduces edema after intracerebral hemorrhage in rats (Fingas, et al., 2007, MacLellan, et al., 2006), but in these studies this effect did not detectably improve functional or histological outcome. Cerebral edema is clearly dangerous following stroke in humans, but it may not carry the same risk in some rodent models including the craniotomy model presently used. Therefore, we do not recommend that neuroprotection studies rely upon edema as sole endpoint.

Behavioral testing is integral to evaluating putative stroke therapeutics and has been recommended by numerous reviewers (Corbett and Nurse, 1998, Stroke Therapy Academic Industry Roundtable (STAIR), 1999). Thus, we used three tests to better gauge outcome. Three different outcomes were found: 1) no treatment effect in the cylinder task, 2) every treatment was equally effective with the NDS, and 3) a duration-dependent effect occurred with the ladder task. The cylinder task has been repeatedly shown to be sensitive to motor system injury (Schallert, 2006), as presently found with the pMCAO model. A possible explanation for the lack of a significant treatment effect is that the severe injury caused by pMCAO resulted in bilateral impairments, which would reduce asymmetry scores, as occurs transiently after large striatal hemorrhagic lesions (MacLellan, et al., 2006). Thus, the results of this test are confounded when bilateral deficits occur as this lessens asymmetry thereby underestimating the real impairment. Further, a neuroprotective treatment, such as HYPO-48, may improve both ipsi- and contralateral limb use, which may then falsely appear as ineffective with this test. Conversely, the hypothermia-induced neuroprotection

may have simply been below the threshold needed to markedly alter the rat's limb preference for spontaneous exploration, in contrast to its improved ability to use that limb in a task that requires limb use, as previously found with a rehabilitation treatment (Nguyen, et al., 2008).

The NDS was sensitive to injury and protection by all 3 hypothermia protocols (vs. NORMO) with no differences among cooled groups. This is surprising given the remarkable differences in brain injury. However, a recent striatal hemorrhage study also found this test does not always distinguish among variations in lesion size (MacLellan, et al., 2006). Thus the NDS is an effective lesion detector, but not particularly sensitive to variations in cortical or striatal injury. Conversely, the horizontal ladder showed a dose-dependent effect of hypothermia duration in line with the lesion volume results that show significant protection only with 24 and 48-h treatments. The different outcomes among these tests highlight the need to use multiple tests in stroke studies. Furthermore, we recommend that future hypothermia studies incorporate tests of sensory, motor and cognitive function. Finally, it is possible that hypothermia had persistent effects on subsequent behavioral testing, and this may have differed among the three hypothermia treatments, thereby confounding the testing. While behavior is affected during cooling, the pattern of behavioral results found presently suggests that there were no lasting carry over effects. Furthermore, two days of mild hypothermia does not affect subsequent performance in the staircase (skilled reaching), cylinder, and ladder tests from 7 to 60 days after a sham pMCAO insult (MacLellan and Colbourne, unpublished data).

Besides edema, this study did not determine how hypothermia reduced injury, which has been extensively studied with intra-ischemic cooling and to a lesser extent with brief post-ischemic hypothermia. Based upon our present findings we strongly recommend that future mechanistic studies include the use of prolonged hypothermia, which likely affect mechanisms not targeted by brief, early hypothermia. In the present setting of pMCAO, it is expected that prolonged hypothermia preserves tissue over a period long enough for collateral blood flow to be established, such as through the posterior communicating arteries.

In summary, our study used a clinically-relevant model of pMCAO to study 3 durations of systemic hypothermia on lesion size and functional recovery. Thus, we have complied with the STAIR recommendations of completing dose-response work, using a permanent ischemia model, assessing functional recovery and using longer survival times. Primarily, we report that delayed hypothermia significantly lessens infarction and improves walking ability in a duration-dependent manner. Our findings along with existing literature strongly support the continuing clinical investigation of protracted hypothermia (e.g., 48 h) for focal cerebral ischemia. Nonetheless, further animal experimentation is also recommended. Specifically, it is possible that alternative cooling protocols will be more efficacious depending upon the type and severity of stroke, the intervention delay and the method of cooling (e.g., brain-selective vs. systemic).

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Local Brain Hypothermia in Rat[1]

Chapter 3

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3.1 Introduction

Hypothermia has been studied extensively as a treatment for stroke. Early studies established that cooling during global or focal cerebral ischemia greatly diminishes cell death and promotes functional recovery (for comprehensive reviews see (Ginsberg, et al., 1992, Maher and Hachinski, 1993, Thornhill and Corbett, 2001, Wagner and Zuccarello, 2005)). Although initially controversial (Dietrich, et al., 1993), delayed postischemic hypothermia also reduces cell death and improves functional recovery (Carroll and Beek, 1992, Colbourne and Corbett, 1995, Colbourne, et al., 2000, Maier, et al., 1998, Yanamoto, et al., 1996); therefore, hypothermia has great potential as a clinical therapy. Indeed, recent studies show that systemic hypothermia improves survival and recovery in out-of-hospital cardiac arrest victims (Bernard, et al., 2002, The Hypothermia After Cardiac Arrest Study Group, 2002). Overall, it appears that hypothermia is the gold standard of neuroprotection for ischemic cell death.

Systemic hypothermia, which is most commonly used, has several side effects that limit its use and perhaps its efficacy. For instance, systemic hypothermia causes shivering, increases the risk of infection and results in cardiovascular complications (Schubert, 1995, Schwab, et al., 2001). Such side effects may be avoided through the use of local brain cooling, an old idea that is currently being advocated by many investigators (Wagner and Zuccarello, 2005).

A variety of methods have been used to induce hypothermia in rodents. These techniques fall into several categories including: 1) focal vs. systemic

cooling, 2) anesthetized vs. non-anesthetized cooling, 3) invasive vs. non-invasive, and 4) short-term (e.g., < 12 h) vs. prolonged cooling. Besides having clinical relevance, one must consider practical limitations with these methods (e.g., feasibility, safety, cost). Systemic cooling can be easily produced in anesthetized or conscious animals. For instance, animals can be placed on a cooling blanket or in a cold room. Hypothermia can also be induced pharmacologically (Babcock, et al., 1993, Dowden, et al., 1999); although maintaining sufficient prolonged cooling while avoiding side effects has not yet been achieved. Local brain cooling can be produced in anesthetized animals with a cooling blanket (Nurse and Corbett, 1994) or coil (Taniguchi, et al., 2005) wrapped around the head while body temperature is maintained. Intravascular cooling of the ischemic territory has also been used successfully (Ding, et al., 2004).

Studies have established that protracted cooling provides superior protection in global (Colbourne and Corbett, 1994) and likely focal ischemia resulting from middle cerebral artery occlusion (Colbourne, et al., 2000, Maier, et al., 1998, Yanamoto, et al., 1996). Long-term cooling (e.g., 24 h) cannot be easily achieved in anesthetized rodents due to excessive anesthetic-induced mortality, which is likely due to anesthetic overdose under hypothermic conditions. Therefore, studies investigating prolonged cooling have used exposure techniques (e.g., fan and water spray) in conscious, mobile rodents (Colbourne, et al., 1996).

In this study we developed a method to selectively lower brain temperature (T_{brain}) in otherwise normothermic rats. The method involves placing

a metal coil next to the skull. The coil is attached to a cold water source either directly, as used in anesthetized rats, or through an overhead swivel in conscious rats. The latter allows for considerable animal mobility. Three experiments were conducted. First, we tested whether focal hypothermia could be quickly produced in anesthetized rats. Thus, we measured T_{brain} in three locations along with rectal temperature (T_{rec}). The second study used T_{brain} and core temperature (T_{core}) telemetry probes to test whether prolonged (24 h) local brain cooling was feasible in conscious rats. The third experiment used telemetry probes to assess body and brain temperature along with mean arterial blood pressure (MABP) and heart rate (HR). We report that local cooling can be easily and safely produced in anesthetized or conscious rodents. Accordingly, our method provides a simple and inexpensive way to induce focal brain hypothermia. This technique is, therefore, ideally suited for stroke (i.e., focal ischemia, intracerebral hemorrhage – ICH) and traumatic brain injury studies.

3.2 Methods

Animals

In total, eight male Sprague-Dawley rats (Ellerslie, Edmonton, Alberta, Canada) were used. Rats weighed between 350 and 500 g and were approximately 4 – 6 months old at the time of cooling. Rats were housed individually with free access to food and water. All surgical procedures were done aseptically and all were in accordance with the Canadian Council on Animal Care guidelines. This

study was approved by the Biological Sciences Animal Policy and Welfare Committee at the University of Alberta.

Cooling Coil

A 3 cm length of hypodermic tubing (20 G) was bent into a spiral pattern measuring 8 mm diameter and 2 mm thick (Figure 3.1). This was achieved by bending a 20 G needle around an immobilized post. This coil was then connected to 0.76 mm internal diameter silastic tubing (Dow Corning Corp., Midland, MI, USA) through which water could be perfused. The sterilized coil was then surgically placed under the Temporalis muscle on the right side of the skull (see below).

Experiment 1: Focal Brain Cooling in Anesthetized Rats

Rats (N=3) were anesthetized with isoflurane (4% induction; 1.5 – 2% maintenance in 70% N_20 and 30% O_2) and placed in a stereotaxic frame. A rectal temperature probe estimated body temperature (T_{rec}) that was servo-regulated by a heating pad placed under the animal. The scalp was shaved and treated with Betadine, and an incision was made along the midline of the scalp. The Temporalis muscle was then gently separated from the underlying skull thereby forming a pocket on the right side. The muscle was not cut. In this space we placed the cooling coil that was attached via silastic tubing to a cold water reservoir placed ~2 m above the animal (i.e., gravity perfusion). A segment of the tubing close to the rat was placed in an ice bath to cool the water entering the coil thereby facilitating brain cooling. Flow was initially off. Two burr holes were then made in the skull. One was situated contralateral to the coil at 3 mm left of

and 1 mm anterior to Bregma. A thermocouple probe (HYP1-30-1/2-T-G-60-SMP-M, Omega, Stamford, CT, USA) was stereotaxically lowered 5 mm below the skull surface through this burr hole so that temperature in the contralateral striatum was measured (T_{CS}). The second burr hole was made over the ipsilateral striatum (3 mm lateral of Bregma, 1 mm anterior). Two thermocouple probes were inserted into this hole – one lowered directly down 5 mm into the ipsilateral striatum (T_{IS}), and one was inserted at 27° (from vertical) to a distance of 5.59 mm, which placed it in the ipsilateral cortex (T_{IC}) underlying the cooling coil.

After baseline temperature measurements, local brain cooling was achieved when cooled water was allowed to flow through the coil (~180 ml / h) for 60 minutes and then stopped. Temperature was recorded every 5 minutes during cooling and for 10 minutes of re-warming. After cooling, animals were quickly euthanized and thermocouple placement was confirmed by histology (fresh-frozen; sectioned at 40 μm).

Experiment 2: Focal Brain Cooling in Conscious Rats

Brain and Core Probe Implantation

Rats (N=3) were anesthetized with sodium pentobarbital (65 mg/kg i.p.; Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada). After shaving and treatment with Betadine, a 2 cm incision was made in the abdominal wall. Sterilized telemetry probes (model TA10TA-F40; Transoma Medical, St. Paul, MN, U.S.A.) that measured core temperature (T_{core}) were then inserted into the peritoneal cavity and the wound closed as previously described (DeBow and Colbourne, 2003). Animals were then placed in a stereotaxic frame as described

above. First, a small burr hole was made 2 mm lateral and 1 mm anterior to Bregma on the right side. Three surrounding burr holes were also made into which small metal screws (Model MX-080-2; Small Parts, Miami Lakes, FL, U.S.A.) were placed. Second, a 5 mm long guide cannula (20 G) was placed into the centre hole, but not into the brain, and secured with dental cement. Third, we placed the cooling coil between the right Temporalis muscle and the skull. Fourth, we secured a ~2.5 cm length of a plastic cylinder (5 cc syringe barrel) surrounding and centered on the guide cannula. This was secured with dental cement as described and illustrated previously (Colbourne, et al., 1996). The head cap assembly subsequently permitted the safe insertion of a T_{brain} telemetry probe thermocouple shaft through the guide cannula. The bottom of the 5 cc syringe barrel also held the cooling coil in place, which was further secured by dental cement. Fifth, the wound was closed and the silastic tubing attached to the cooling coil was fed through a flexible wire sheath (model CIH95; 30cm long, Instech Laboratories, Plymouth Meeting, PA, USA). This end of this sheath was taped securely to the 5 cc syringe barrel to avoid movement of and stress on the cooling coil. The other end of the metal sheath and the silicon tubing was then attached to a swivel (model 375/D/22, Instech Laboratories, Plymouth Meeting, PA, USA) and counterbalance arm (CM375BS, Instech Laboratories) mounted to the top of the rat's cage. Rats then recovered from Somnotol anesthesia in their home cages, which rested upon RPC-1 receivers (Transoma Medical), while T_{core} was recorded every 30 seconds via telemetry (A.R.T. v. 2.2; Transoma Medical). Somnotol was

used instead of isoflurane as animals remain immobile for hours allowing time for the dental cement to harden sufficiently.

After a monitoring period of two days, the rats were briefly anesthetized (~ 5 min of isoflurane) and a telemetry probe (model VM-FH-BP, Mini-Mitter Co. Inc, Sun River, OR, USA) was inserted to measure temperature of the ipsilateral (to coil) striatum (T_{IS}) at a depth of 4 mm from the skull surface. Due to signal interference and size restrictions it was not possible to measure with multiple brain probes. Likewise, it was not possible to simultaneously measure T_{core} and T_{IS} via telemetry. Accordingly, T_{IS} was measured for a baseline period of one day while the core probe was turned off (activation / deactivation via magnetic switch). However, it was possible to measure T_{core} accurately when the brain probe was on (see below) as the A.R.T. software treated the brain probe's signal as noise and excluded it to analyze only the T_{core} signal. We have previously used this method to near-simultaneously (within 30 sec) measure T_{core} and T_{brain} with telemetry (DeBow and Colbourne, 2003).

Focal Brain Cooling in Awake Rats

Two days following coil implantation, water was allowed to flow from a reservoir (~ 2 m above animal) through the coil as described above. Cooling continued for 24 h. The rate of flow was ~100 ml / h. This was lower than in the previous experiment due to the flow restriction imposed by the 22 G swivel, which was needed to allow animal mobility. Striatal temperature (T_{IS}) was continually monitored (every 30 seconds) during cooling and T_{core} was intermittently sampled (every 4 h) by briefly activating the implanted core probe.

Rats remained free to move about throughout these procedures. Water flow was turned off at the end a 24 h period, but T_{IS} was monitored for an additional 24 h. Rats were euthanized a day following cooling with an overdose of Somnotol and transcardially perfused with 0.9% saline and then 10% neutral buffered formalin. Forty-micrometer coronal sections were taken with a cryostat every 400 μm starting at +1.7 mm to bregma and extending back to −4.8 mm to bregma. Sections were stained with hematoxylin and eosin and examined by an experienced investigator for signs of tissue injury (FC).

Experiment 3: Blood pressure and heart rate measurements during cooling

In Experiment 3 rats (N=2) were first implanted with TML2 C50-PXT telemetry probe (Transoma Medical) under isoflurane anesthesia (~ 45 min duration). In addition to measuring T_{core}, this probe has two leads, which are tunneled under the skin, to measure heart rate (HR). A catheter, which was inserted into the descending aorta, measured mean arterial blood pressure (MABP). The catheter does not obstruct blood flow. One week later rats were operated upon again (Somnotol anesthesia) to implant the guide cannula and cooling coil as described for Experiment 2. However, in this case the TA10TA-F40 probe was not implanted because the C-50 PXT probes measure T_{core}. After a one day recovery period the brain probe was inserted under brief isoflurane anesthesia and the C50-PXT probe was turned off to allow T_{brain} recordings. Brain temperature was recorded for 24 h at which time the C50-PXT probe was turned on and cooling was initiated. Brain temperature was measured every 4 h by intermittently turning off the C50-PXT probe for < 1 min. In this study a larger

bore swivel was used (375/D/20, Instech Laboratories, Plymouth Meeting, PA, USA) which allowed a flow rate of ~150 ml/h. The temperature of the water was ~11°C at the entry point into the coil.

Data Analysis

All data are presented as mean ± standard deviation (SD). Using SPSS (v.12) software, data was analyzed using ANOVA. A p value of < 0.05 was considered statistically significant.

3.3 Results

Experiment 1

Focal hypothermia was quickly (within minutes) produced in the ipsilateral hemisphere during the cooling protocol (Figure 3.2). The average T_{IS} during cooling (31.3°C ± 0.6) was 5°C below baseline (36.3°C ± 0.3), which was statistically significant ($p<0.01$). The average T_{IC} during cooling (26.7°C ± 0.8) was more than 9°C cooler than baseline (36.2°C ± 0.6; $p<0.01$). While the T_{CS} was statistically ($p<0.05$) lower during cooling than baseline the difference amounted to only 0.6°C (36.1°C ± 0.3 vs. 36.7°C ± 0.2) and thus T_{CS} was well within the normothermic range. The average T_{rec} during the focal cooling did not significantly change (37.7°C ± 0.3 vs. 36.9°C ± 0.3; $p>0.05$). Thermocouple probe placement was found to be accurate in all animals (data not shown).

Experiment 2

Figure 2.3 shows the average T_{core} and T_{brain} of rats during baseline and the 24 h cooling period. During cooling the T_{core} was only intermittently recorded due to technical limitations with telemetry probes as noted in the Methods. Cooling was induced within a few minutes and the 24-h average T_{IS} during cooling (34.7°C ± 0.2) was significantly lower than baseline (37.1°C ± 0.1; $p<0.01$). The T_{core} during cooling (37.4°C ± 0.2) was not significantly different than baseline (37.5°C ± 0.2; $p>0.05$).

Examination of several coronal sections for these animals revealed no signs of pathology (e.g., acidophilic neurons) aside from the direct injury caused by the brain probe insertion (data not shown).

Rats tolerated the cooling setup well (e.g., no loss of weight; data not shown). Some rats initially scratched at the head cap assembly but this stopped within a few hours. Some rats also initially scratched during cooling; however, this behavior quickly ceased (within a few minutes).

Experiment 3

Physiological variables (i.e., HR, MAPB, T_{core}, T_{IS}) during baseline and cooling periods for Experiment 3 are shown in Figure 4. The T_{core} (Figure 3.4a) did not significantly change during the 24 h focal cooling (37.6°C ± 0.5 vs. baseline value of 37.7°C ± 0.1; $p>0.05$). The T_{IS}, however, was significantly lower (33.4°C ± 0.6 during cooling vs. baseline average of 37.2 ± 0.4; $p<0.05$). Cooling did not significantly affect MABP (Figure 3.4b; 114.5 mmHg ± 1.4 vs. baseline average of 117.7 mmHg ± 5.5; $p>0.05$). Similarly, HR (beats / min) was

not significantly altered during cooling (Figure 3.4c; 347.7 ± 34.8 vs. baseline of 361.5 ± 40.6; $p>0.05$).

A comparison of the average T_{IS} during cooling revealed that significantly ($p<0.05$) greater cooling was achieved in Experiment 3 (20 G swivel; ~150 ml / h flow rate) than Experiment 2 (22 G swivel; ~100 ml / h flow rate). Therefore, a higher flow rate caused greater cooling. The temperature reduction was even greater in the first Experiment due to the lack of a swivel (e.g., minimizing heat transfer) and / or a higher flow rate (~180 ml / h), which was due to the lack of a swivel. We did not use the swivel as the rats were anesthetized in Experiment 1.

Figure 3.1: Cooling coil (mm scale bar below) with attached tubing through which cold water is flushed. The coil is placed underneath the Temporalis muscle with the flattest face adjacent to the skull. In this case the coil was constructed out of a 20 G needle.

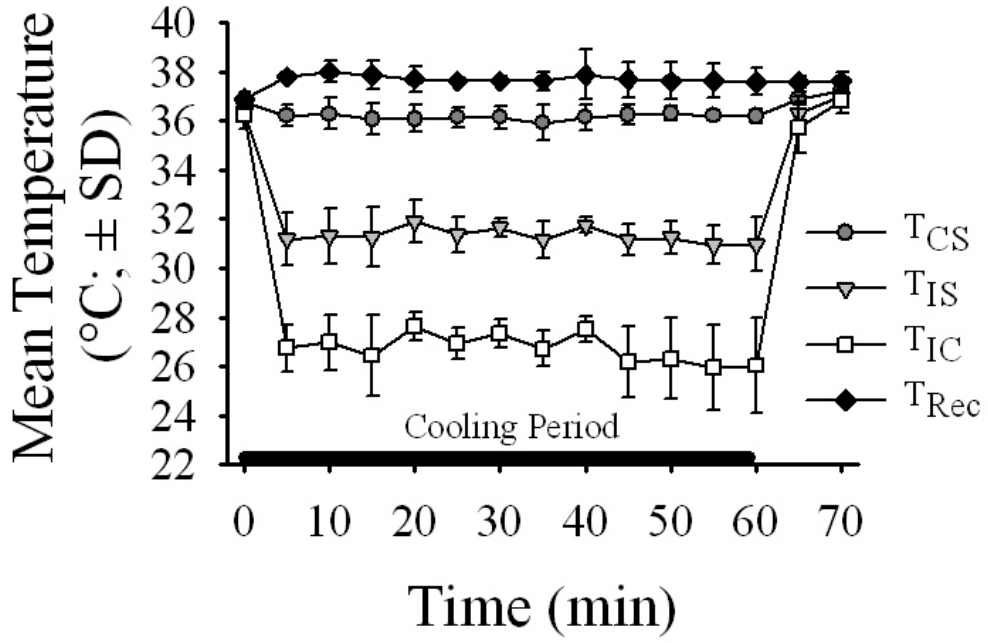

Figure 3.2: Temperature (contralateral striatum – T_{CS}; ipsilateral striatum – T_{IS}; ipsilateral cortex – T_{IC}; rectum – T_{rec}) before, during and after induction of cooling (black bar on x-axis) in anesthetized rats. Ipsilateral cooling was significant whereas T_{rec} and T_{CS} remained normothermic.

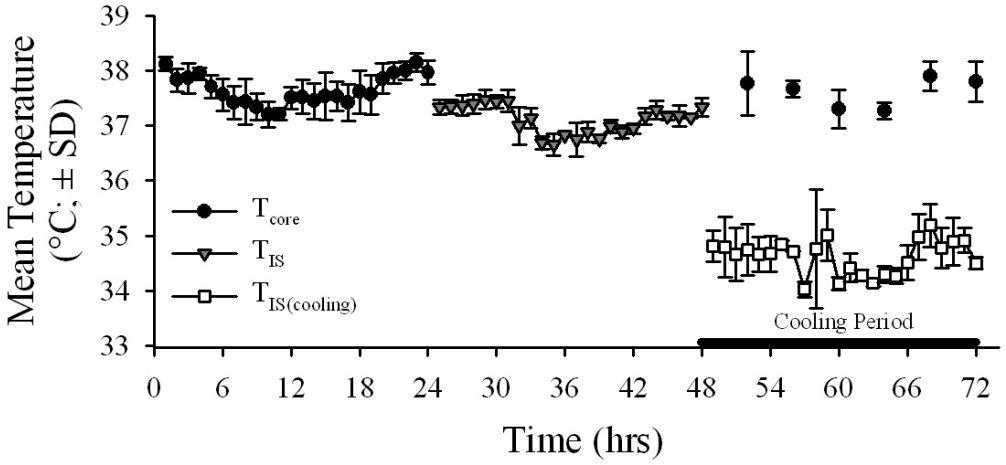

Figure 3.3: Body and brain temperature during baseline readings (0 – 48 h) and during cooling (black bar from 48 – 72 h). The ipsilateral striatal temperature (T_{IS}) was significantly reduced during cooling whereas body temperature (T_{core}) remained normothermic.

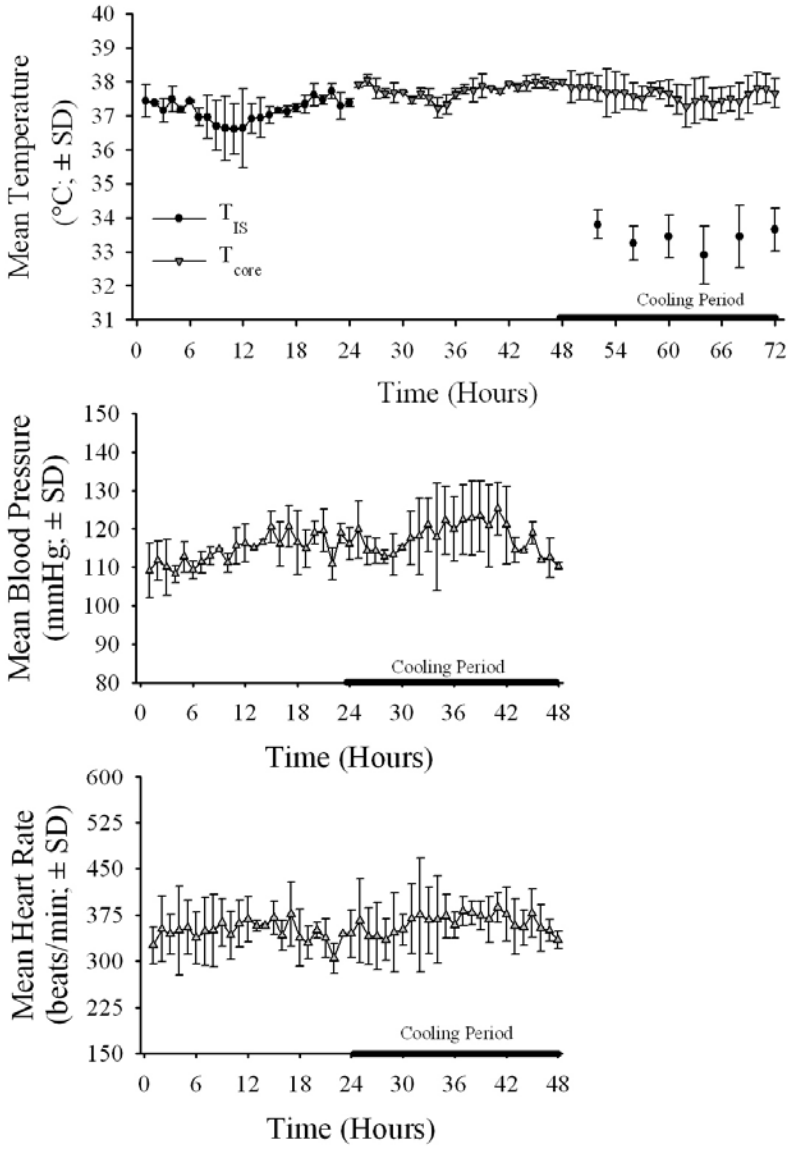

Figure 3.4: Temperature (ipsilateral striatum – T_{IS}; body temperature – T_{core}; top graph), MABP (middle graph) and HR (bottom graph) during baseline and cooling (black bar) periods. Significant local hypothermia occurred in otherwise normothermic rats. Heart rate and MABP did not substantially change during cooling.

3.4 Discussion

Herein we describe a simple, safe and effective method to induce local brain hypothermia in anesthetized or awake rats. The technique involves surgically placing a small, metal, coiled tube adjacent to the skull and subsequently flushing the coil with cold water. Our first experiment demonstrated that local cooling of one hemisphere is possible without cooling either the contralateral hemisphere (T_{CS}) or the body (T_{rec}) when the animal is anesthetized. Second, we showed that prolonged mild hypothermia is easily produced in conscious rats and that local brain cooling for one day did not affect T_{core}. Third, we showed that local brain cooling did not change HR or MABP. Furthermore, we did not note any obvious brain injury from local cooling, which is in line with earlier observations that mild hypothermia does not cause structural alterations in hippocampal neurons (Colbourne, et al., 1999). Given that therapeutic levels of local brain hypothermia are safely achieved, our cooling method is ideally suited to studies of focal brain injury such as occurs with MCAO, ICH and traumatic brain injury.

Our method has several advantages over current techniques to induce systemic hypothermia. First, our method allows for a greater reduction in T_{brain} than that safely achieved with whole-body cooling where complications can occur at any hypothermic temperature, but especially those below 32°C (Schubert, 1995). Mild systemic cooling (e.g., 33°C) with water spray and fans significantly alters MABP and HR in rats (Colbourne, et al., 2003, MacLellan, et al., 2004) and

results in loss of body weight (Colbourne and Corbett, 1995). Our local cooling method avoided these complications. Importantly, side effects may impact efficacy as found recently in an ICH model in rats (MacLellan, et al., 2004). Accordingly, we predict that, at least in some situations, local cooling would be superior to systemic hypothermia given an equivalent brain target temperature. Second, our present method achieved a stable level of cooling (vs. baseline temperature) without the need for any adjustment; although it is recognized that this may not always be the case and variability may be underestimated because we used small group sizes. Conversely, induced hypothermia through use of water spray and fans requires constant servo-regulation thus necessitating expensive telemetry recording equipment and a sophisticated control system (Colbourne, et al., 1996, DeBow and Colbourne, 2003).

Focal cooling, however, could be done without invasive and expensive temperature recordings once an ideal cold water flow rate has been established. In this case, investigators are strongly encouraged to determine the ideal cooling parameters for their particular setup as it would vary depending upon water temperature, flow rate and coil design among other factors. We recommend the 20 G swivel (vs. 22 G) for producing hypothermia in conscious animals owing to the greater cooling with gravity-induced water flow, which could be replaced and augmented with forced perfusion. The amount of cooling would also depend upon the metabolic activity of underlying tissue (e.g., greater cooling during ischemia) as well as generalized temperature responses to the insult. For instance, many (Abraham, et al., 2002, Reglodi, et al., 2000, Zhao, et al., 1994), but not all

(Corbett, et al., 2000), MCAO studies report that spontaneous systemic hyperthermia occurs. Accordingly, this would make it more difficult to achieve a target temperature with our local cooling method. Nonetheless, this problem can be circumvented by increased cold water perfusion. Using a servo-controlled cold water flow system analogous to our computerized systemic-cooling apparatus (Colbourne, et al., 1996) would offer additional flexibility, but also increase system complexity and cost.

Our technique also offers advantages over current local cooling methods such as using a cooling blanket (Nurse and Corbett, 1994) or coil wrapped around the head of anesthetized animals (Taniguchi, et al., 2005). First, our method does not result in cooling of the entire head, which means that less or no systemic cooling will occur. Second, other local cooling methods require anesthesia, which potentially confounds the use of hypothermia and limits the duration of cooling obtainable. Cooling under anesthesia also increases costs and severely limits the number of animals that can be studied concurrently. With our system many animals can be simultaneously cooled at a low cost. Third, our method is minimally invasive as it does not require either vessel catheterization or intracranial placement of a cooling device as is required with some other methods. Fourth, local cooling can be done in mobile, conscious rodents allowing for simple behaviors (e.g., activity levels, spontaneous rotation, paw usage) and physiology to be easily monitored throughout cooling. Fifth, various depths and durations of hypothermia can be easily induced by simply changing the cold water temperature or flow rate. Indeed, this was demonstrated in comparing

Experiments 2 and 3. A significantly greater drop in T_{IS} occurred in Experiment 3 due to using a larger bore swivel, which allowed for a greater flow rate.

The described method is also limited in its scope. The coil is best suited to single-hemisphere injuries such as MCAO, ICH or focal traumatic brain injury. A gradient of cooling also exists with cortical structures undergoing cooling to a greater extent than subcortical structures. Thus, in MCAO models (e.g., intraluminal suture occlusion) one might protect the cortex better than the striatum in part due to temperature gradients. Other local cooling methods, such as a cooling blanket wrapped around the head, likely also suffer from this limitation. Therefore, temperature gradients must be considered in such studies and should be determined in each situation (e.g., model, species). Our method is not entirely suitable for global ischemia studies as only one hemisphere would be protected; although, this might be considered an advantage if an internal control was desired (i.e., a normothermic hemisphere). Likewise, focal cooling to moderate or severe hypothermia levels (e.g., 20°C) would likely increase the risk of systemic hypothermia. Such deep levels may only be feasible if either external heating was provided to the rest of the body or if a combination of local deep hypothermia and mild systemic cooling was acceptable as done recently in neonatal pigs (Tooley, et al., 2003). Nonetheless, we have demonstrated that prolonged mild hypothermia, in the therapeutic range for global (Colbourne and Corbett, 1995, Hickey, et al., 2000) and focal ischemia (Colbourne, et al., 2000, Maier, et al., 1998, Yanamoto, et al., 1996) and ICH (MacLellan, et al., 2004), can be safely achieved without systemic problems.

We did not assess this technique in either the gerbil or mouse, which are common stroke models. We expect that similar results can be achieved with a smaller coil suitable for these species, but further study is needed to test efficacy, safety and practicality. It should be noted that we are not advocating this method for larger animals (e.g., pigs) including humans. Owing to thicker skulls and larger brains, it is highly unlikely that the current method would be sufficiently effective nor would it be better than devices placed under the skull. Finally, we did not vary either the rate of cooling or rewarming. Our method induces rapid hypothermia which is followed by rapid rewarming upon cessation of water flow, which was gravity powered. If slower rates are desired the water flow rate could be slowed, however, this would have to be actively monitored and regulated (e.g., infusion pump, flow regulator).

In summary, the described method offers investigators a way to study focal brain hypothermia that is effectual, simple, cost-effective and safe in rats. Importantly, this method allows for more flexibility than is available with current methods of cooling. This will permit researchers to better identify the ideal hypothermia treatment (e.g., depth and duration of cooling) as well as uncovering its mechanisms of action. We hope that this simple method will encourage more investigators to study therapeutic (mild and prolonged) hypothermia, which, at present, is arguably the best neuroprotectant.

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Systemic and Focal Hypothermia after pMCAO[1]

Chapter 4

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4.1 Introduction

The potential for hypothermia to reduce brain injury has been broadly studied (MacLellan, et al., 2009, Nagel, et al., 2008, Polderman, et al., 2008). Hypothermia has been investigated in different injury types, but most extensively in ischemic brain injury. Neuroprotection afforded by hypothermia depends upon many factors, such as the type of injury, severity of injury, onset delay and duration of hypothermia (Krieger and Yenari, 2004, Meloni, et al., 2009, van der Worp, et al., 2007). For instance, in rodent models of global ischemia, brief intraischemic hypothermia drastically reduces brain injury and improves behavioral recovery (Ginsberg, et al., 1992) but prolonged hypothermia is needed to obtain permanent benefit after an intervention delay is introduced (Colbourne and Corbett, 1995, Dietrich, et al., 1993, Iwai, et al., 1993) or injury is severe (Colbourne and Corbett, 1994). Clinical trials based on such findings show that prolonged mild hypothermia improves outcome in patients following out of hospital cardiac arrest (Bernard, et al., 2002, The Hypothermia After Cardiac Arrest Study Group, 2002). Given the similar mechanisms of injury, hypothermia would likely improve outcome following acute ischemic stroke. Small scale clinical trials have determined hypothermia to be safe and feasible in stroke patients (Georgiadis, et al., 2002, Georgiadis, et al., 2001, Kammersgaard, et al., 2000, Schwab, et al., 2001). It is not yet known whether hypothermia provides benefit in acute ischemic stroke patients as there have been no randomized trials large enough to determine efficacy. Although hypothermia has been evaluated as

a treatment for stroke in many experimental models (van der Worp, et al., 2007), the ideal parameters of hypothermic therapy have yet to be determined.

Intraischemic hypothermia provides significant and lasting protection in models of transient focal ischemia (Maier, et al., 1998, van der Worp, et al., 2007, Yanamoto, et al., 2001). Delayed hypothermia also reduces brain injury and functional deficits following transient focal ischemia (Colbourne, et al., 2000, Maier, et al., 1998, Maier, et al., 2001, Yanamoto, et al., 1996). Few studies have evaluated hypothermia in permanent models, despite the fact that only a minority of clinical strokes are acutely re-canalized (Rha and Saver, 2007). Results are inconsistent in the studies that have been done (Krieger and Yenari, 2004). For instance, Kader et al reported that 1 h of immediate cooling significantly reduced brain injury at 24 h but Ridenour et al found that 2 h of prompt hypothermia failed to provide benefit 96 h following permanent focal ischemia in rat (Kader, et al., 1992, Ridenour, et al., 1992). Also, brief hypothermia was found only to transiently reduce lesion volume when applied 1 h following permanent middle cerebral artery occlusion (pMCAO) (Doerfler, et al., 2001), yet our recent study showed that delayed hypothermia reduced both functional and histological deficits one week following pMCAO when cooling was sufficiently prolonged (Clark, et al., 2008). Evidence is lacking that cooling provides functional and long-term benefit in models of permanent ischemia and further studies are needed.

The standard method of inducing hypothermia in both experimental studies and clinical trials is whole body surface cooling (Diller and Zhu, 2009). While systemic hypothermia provides benefit, there are serious risks associated

with hypothermia. Lowering core body temperature alters immune reactivity, metabolic activity, blood clotting ability and cardiac output/regularity (Dirkmann, et al., 2008, Polderman, 2008, Schubert, 1995). Alternate cooling methods have been investigated in an attempt to avoid some of these dangerous side effects. In patients, brain cooling has been attempted through the use of head surface cooling techniques, chilled neck collars and intravascular cooling (Diller and Zhu, 2009). Non-invasive (surface) brain cooling techniques in humans are not effective in reducing the brain temperature quickly and often result in some reduction of core temperature. More invasive techniques such as epidural and intraparenchymal cooling devices are currently being developed and tested (Diller and Zhu, 2009, Wagner and Zuccarello, 2005). Such techniques reduce brain temperature quickly and epidural cooling reduces edema in swine (Wagner, et al., 2003). The necessary invasive nature of direct brain cooling is a major concern and it likely would not be practical in many clinical scenarios. In animals, it is possible to cool the head alone, through the surface, while leaving the body normothermic. Selective brain hypothermia is effective at reducing brain injury following ischemia (Taniguchi, et al., 2005). Unfortunately, it has been difficult to evaluate delayed or prolonged selective brain hypothermia, as all previous methods involve constant anesthesia.

We recently developed a method of regionally cooling the brain that does not induce systemic hypothermia, thus avoiding some of the side-effects caused by core cooling. Our method of focal hypothermia (FH) results in mild to moderate local cooling (~32-33°C) but does not cause significant blood pressure

or heart rate changes (Clark and Colbourne, 2007). Adverse effects of systemic hypothermia would likely be controlled in patients cooled in the clinic (Polderman, 2008). Thus, focal hypothermia in rats might be a better model of prolonged systemic cooling in humans than conventional hypothermia non-anesthetized rodent methods (Colbourne, et al., 1996). This method reduced edema but not lesion volume following intracerebral hemorrhage (Fingas, et al., 2007). The efficacy of this cooling technique in ischemic stroke has yet to be assessed.

In the current study, we investigated the effects of two durations (12 and 48 h) of both systemic and focal hypothermia induced 1 h after onset of pMCAO in rats. We compared two different durations of hypothermia to match the range used clinically (Polderman, 2008); systemic hypothermia as well as focal cooling were used to evaluate potential differences in efficacy between the two methods. Behavioral recovery was evaluated with a battery of functional tests including the horizontal ladder test measuring walking ability (Metz and Whishaw, 2002), the cylinder test of spontaneous forelimb usage asymmetry (Schallert, 2006) and the staircase test that evaluates skilled reaching (Montoya, et al., 1991). Histological injury was assessed 32 days following injury. Infarct volume and functional ability of rats were evaluated at long survival times as benefit at early time points may only be transient (Colbourne, et al., 1999, Doerfler, et al., 2001, Valtysson, et al., 1994) and of little clinical importance.

4.2 Methods

Subjects

One hundred and forty six male, young-adult, Sprague-Dawley rats were used. They were housed individually on a diurnal light cycle (on time: 07:00 – 19:00 h) with free access to food, except as described below, and water. These experiments were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Two experiments were conducted to evaluate the efficacy of prolonged hypothermia initiated 1 h after pMCAO. The first used whole-body hypothermia whereas the second used a brain-selective cooling technique. Both experiments compared 12 and 48-h hypothermia treatments to a normothermic control group. Lesion volume and several behavioral endpoints were evaluated as illustrated in Figure 1. Experiment 1 used 84 rats of which 8 were excluded before treatment randomization because of technical problems (e.g., surgical error). This left Ns of 27 in the 12-h systemic hypothermia group (SH-12), 25 in the 48-h systemic hypothermia group (SH-48), and 24 in the normothermic control group (NOR (SH)). Experiment 2 used 62 rats of which 9 were excluded for similar technical reasons prior to randomization. First, a small pilot study (N = 6), which measured core (T_c) and brain temperature (T_b) via telemetry, was done to establish and illustrate a focal hypothermia cooling protocol. Of these 6 rats, 3 were cooled for 48 h while 3 were kept normothermic. This information guided our main efficacy experiment, where 13 rats were subjected to 12 h of focal hypothermia (FH-12),

16 were subjected to 48 h of focal cooling (FH-48), and 18 rats served as a normothermic control group (NOR (FH)).

Anesthesia/Surgery

All surgeries were performed under isoflurane inhalant anesthetic (4% induction; 2% maintenance; 60% N_2O, balance O_2). Aseptic techniques were used to reduce the chance of infection and wounds were treated with local anesthetic (Marcaine; Sanofi Canada, Markham, Ontario, Canada) to reduce pain following surgery. All rats received subcutaneous saline injections (5 ml) post-surgery to aid in recovery.

Core Probe Implantation

Each rat in Experiments 1 and 2 was surgically implanted with T_c probe 3 days before pMCAO. As described previously (DeBow and Colbourne, 2003), rats were anesthetized while a sterilized probe (model TAT10TA-F40, Transoma Medical, St. Paul, MN) was implanted into the abdominal cavity. The wound was sutured closed. The accuracy of these probes was determined by comparison to a laboratory standard. We used the 24 h period before stroke as our baseline. In awake animals, T_c correlates well with brain temperature (DeBow and Colbourne, 2003).

Permanent Middle Cerebral Artery Occlusion

All rats were subjected to pMCAO via electrocauterization of the distal MCA (Clark, et al., 2008, Tamura, et al., 1981, Yamamoto, et al., 1988). Briefly, to induce the stroke we first occluded both common carotid arteries (two strands of silk suture per artery), which was accessed by a ventral neck incision. Next a

craniotomy (~2 mm diameter) was made dorsal to the temporal ridge on the right side of the skull. The dura was cut to allow the MCA to be elevated from the brain and then electrocauterized (bipolar cautery forceps, ME-102, Martin, Germany) and transected. The wounds were closed. For at least a week following surgery, the rats were given moist palatable food mixture (rodent chow, honey, peanut butter). Notably, rats were food deprived for ~15 h prior to surgery to help keep glucose within a narrow range. Skull temperature (T_s; thermocouple probe model HYPO-33-1-T-G-60-SMG-M, Omega, Stanford, Conn.) was maintained near a target temperature of 37.2°C using a combination of heating pad and overhead infrared lamp (175 W). Blood gas and glucose measurements were taken via the tail artery catheter, which was also used to measure mean arterial blood pressure (MABP). Arterial blood samples (100 μL) were taken immediately prior to ischemia and analyzed with a Radiometer ABL 810 blood gas analyzer (Radiometer, Copenhagen, Denmark). Body weight was recorded on the day of pMCAO surgery, seven days later and at euthanasia.

Systemic Hypothermia

After probe implantation, the rats in Experiment 1 were placed on a telemetry receiver (RPC-1, Transoma Medical) interfaced to a computer running A.R.T. data collection software (v. 2.2, Transoma Medical). Following pMCAO surgery, a servo-regulated system that used fans and fine water misters to cool and infrared lamps to heat was used to precisely control T_c (Colbourne, et al., 1996, DeBow and Colbourne, 2003). All rats were maintained at normothermia for 1 h after stroke onset. At that time rats randomized to receive HYPO began to be

cooled at a rate of 2°C / h to 33°C, which was maintained for 12 (SH-12) or 48 h (SH-48). Afterwards animals were warmed by 1°C / h. These rats were then maintained above 36°C until 96 h after pMCAO, which was the treatment for the NOR group.

Focal Brain Hypothermia

In Experiment 2, all rats were surgically implanted with a cooling strip immediately following pMCAO, which was modified from our published method (Clark and Colbourne, 2007). Briefly, the Temporalis muscle was retracted to allow for the placement of a 7 mm long x 3 mm wide stainless steel strip against the skull. The strip was secured with dental cement and stainless steel screws. In a subset of rats a telemetry brain probe was also implanted (model VM-FH-BP, Mini-Mitter Co. Inc, Sun River, OR, USA) secured to the skull inside a protective plastic cylinder (Clark and Colbourne, 2007, Colbourne, et al., 1996, DeBow and Colbourne, 2003). Cortical brain temperature (T_b) was measured at 0.5 mm anterior and 3.5 mm lateral to Bregma at 3 mm below the skull surface. Following anesthesia, T_b was measured every 30 s whereas T_c was sampled once per day (noon) for three days. Simultaneous measurement of T_{brain} and T_c with these telemetry probes is not possible owing to signal interference. These animals were not included in any the other analysis, due to the injury induced by the brain probe. All animals were tethered to an overhead swivel (model 1 375/D/20; Instech Solomon, Plymouth Meeting, PA) that was connected to an overhead water source. In rats that underwent FH, gravity fed cold water was allowed to pass through the strip starting 1 h after pMCAO onset. Cooling and re-warming

rates were controlled with a peristaltic pump to approximate those used for SH. The NOR group was tethered but no water was allowed to flow through the cooling strip. All animals remained tethered until 2 days, at which point all rats were returned to their normal cages.

Behavioral Testing

Neurological Deficit Scale

In both Experiments, a neurological deficit scale (NDS) score was measured prior to and at 7 days after stroke onset. The score was determined from 5 behaviors that assess motor and sensory function: hind limb retraction, contralateral forelimb flexion, bilateral forepaw grasp, traversing a narrow beam and forelimb placing. Scores can range from 0 (no deficits) to a maximum of 13 (Clark, et al., 2008).

Horizontal Ladder

Rats were evaluated on a horizontal ladder task (Metz and Whishaw, 2002) prior to and at 7 and 28 days post-ischemia. This test is used to determine the rat's ability to traverse a series of parallel bars variably spaced 1 – 3 cm apart. Error rate was determined as the percentage of slips (foot falls below the level of the bars) made while traversing the middle 0.5 m segment of the apparatus. This test is sensitive to pMCAO-induced injury (Clark, et al., 2008).

Montoya Staircase Reaching Task

Skilled reaching was evaluated in Experiments 1 and 2 with the Montoya staircase test (Montoya, et al., 1991), which is sensitive to MCAO-induced cortical injury (Colbourne, et al., 2000). First rats were food deprived to 90% of

their free-feeding weight and trained in the staircase over 40 trials (2 trials per day, 5 days per week) prior to pMCAO. Rats had to at least obtain 9 pellets (45 mg each; Bio-Serv, Frenchtown, NJ, U.S.A.) per side out of a possible 21 by the last 5 consecutive days during training or they were excluded from the analysis. Rats were returned to ad lib feeding following training which ended 3 days before pMCAO. Skilled reaching, under food deprivation, was assessed over 10 trials on days 28 –32 following pMCAO onset.

Histology

Rats were euthanized by an intraperitoneal injection of sodium pentobarbital (~100 mg/kg) and transcardially perfused with 0.9% saline followed by 10% formalin. Brains were frozen and 50 μm coronal sections were obtained and later stained with cresol violet. The Scion Image J program (Corporation, Frederick, MD, USA) was used to measure the area of normal tissue at 400 μm intervals extending through the entire brain. The volume of tissue lost was calculated as:

Volume of tissue lost = remaining volume of normal hemisphere - remaining volume of injured hemisphere.

Volume of a hemisphere = average (area of the complete coronal section of the hemisphere - area of ventricle - area of damage) × interval between sections × number of sections.

Statistics

A p value of < 0.05 was considered statistically significant. The NDS scores were analyzed with Kruskal-Wallis tests, and, if significant, follow up comparisons were done with the Mann Whitney U test (SPSS, v. 12). All other data were analyzed via one-way analysis of variance (ANOVA) with LSD post-hoc tests if needed. Mortality was evaluated with a Chi-square test.

4.3 Results

Experiment 1:

Exclusions and protocol violations:

In Experiment 1, early death occurred in all three groups (NOR, N=5; SH-12 N=3; SH-48, N=5), presumably due to brain injury. Mortality did not differ among groups (p=0.587). Five rats were excluded from Montoya because they failed to reach criterion (NOR, N=2; SH-12, N=2; SH-48, N=1). Experimental error resulted in a failure to collect NDS data for the first 43 rats entered into Experiment 1. Data shown are from the remaining rats which were all randomized together. Hard drive failure resulted in loss of logged temperature data, therefore only 40 rats are represented here (Figure 4.1a), but all rats were servo-regulated in a similar fashion.

Physiological variables:

Physiological measurements (pH, etc.) taken during surgery are presented in Table 4.1. One way ANOVAs on these showed that there were no group effects on all measures ($p \geq 0.221$) except T_s ($p=0.024$). However, differences between

groups were 0.3 °C at most. Indeed, a correlation between T_s and infarct size yielded $r^2 = 0.104$ ($p = 0.010$), which indicates that very little (10%) of the variability in infarct size can be accounted for by T_s. Body weight on the day of surgery and 7 and 32 days later was not significantly different among groups ($p \geq 0.100$; Table 4.1). Core temperature profile is presented in Figure 4.1a.

Behavioral tests:

The staircase data (number of pellets consumed) were analyzed for the ipsi- (Figure 4.2a) and contralateral-to-stroke forelimb (Figure 4.2b). Initially a repeated-measures ANOVA using within-subjects contrasts was used to analyze all of the data, which included the baseline average, in order to determine whether scores after stroke were significantly lower than baseline performance. For both limbs this was true over all test days ($p \leq 0.007$). As expected, baseline scores analyzed by 1-way ANOVAs were not significantly different for each limb ($p \geq 0.292$). Post-stroke scores for each limb were then analyzed with repeated measures ANOVAs, which revealed significant day effects ($p < 0.001$) due to improved scores over test days, non-significant interactions ($p \geq 0.202$), and significant group main effects ($p \leq 0.022$). Post-hoc tests showed that both SH groups were retrieved significantly more pellets than the NOR (SH) control group ($p \leq 0.027$). The SH-12 and SH-48 groups were not different ($p \geq 0.598$).

A repeated-measures ANOVA on the stepping error rate with the contralateral-to-stroke forelimb in the ladder test (Figure 4.3a) showed a significant day effect ($p < 0.001$) with error rates being significantly higher on both test days compared to baseline ($p < 0.001$; within-subjects contrasts). However,

neither the group main effect ($p=0.236$) nor the interaction ($p=0.116$) was significant. Baseline scores were not different among groups (1-way ANOVA: $p=0.137$). A repeated-measures ANOVA on the post-stroke data also showed a non-significant group effect ($p=0.168$) and a non-significant interaction ($p=0.379$) with the day effect being significant ($p<0.001$). Thus, stepping error rates significantly increased after stroke, but this was not significantly attenuated by SH treatment.

The NDS scores were not significantly ($p=0.096$, Kruskal-Wallis test) different among groups at baseline (data not shown) as most scores were 0 (no deficit). Post-stroke scores were significantly higher than baseline ($p<0.001$; Wilcoxon Signed Ranks Test) and there was a significant difference among groups at 7 days postischemia ($p=0.019$, Figure 4.5a). Here the SH-48 group were significantly better than the NOR (SH) group ($p=0.009$, Mann-Whitney test) whereas the other comparisons were non-significant ($p\geq0.064$). Thus, only the SH-48 group significantly lessened neurological impairment on the NDS.

Histology:

Lesion volume Figure 4.4a was significantly different among groups ($p=0.006$; 1-way ANOVA). Post-hoc testing showed that the NOR (SH) control group (Figure 4.1c) had a significantly larger lesion than the SH-12 ($p=0.005$) and SH-48 ($p=0.004$; Figure 4.1d) groups, which were not different ($p=0.897$). Thus both SH treatments equally lessened tissue loss.

Experiment 2:

Pilot experiment:

Average brain temperature of rats undergoing 48 hours of cooling (FH-48) or strip placement alone (NOR (FH)) is presented in Figure 4.1b. Core temperature was measured daily and averaged 37.1 and 37.5 °C in the control and cooled groups, respectively.

Main Study:

Exclusions and protocol violations:

In Experiment 2, premature death was also observed in all three groups (NOR, 3; SH-12, 2; SH-48, 1). Mortality did not differ between groups (p=0.353). One rat was excluded from Montoya because they failed to reach criterion (SH-48, N=1).

Physiological variables:

Physiological measurements taken during surgery are presented in Table 4.1. One way ANOVAs on these showed that there were no group effects on all measures ($p \geq 0.059$) except T_s ($p=0.048$). However, differences between groups were <0.4 °C – a biologically insignificant difference. Furthermore, T_s over this small range was not significantly related to infarct size ($r^2 = 0.008$, $p = 0.582$).

Behavioral tests:

The staircase data (number of pellets consumed) was analyzed for the ipsi- (Figure 4.2c) and contralateral-to-stroke forelimb (Figure 4.2d). Initially a repeated-measures ANOVA using within-subjects contrasts was used to analyze all of the data for each limb separately, which included the baseline average.

These ANOVAs determined whether scores after stroke were significantly lower than baseline performance, which for both limbs was true over all test days ($p<0.001$). As expected, 1-way ANOVAs on baseline scores were non-significant for each limb ($p\geq0.108$). Post-stroke scores for each limb were then analyzed with repeated measures ANOVAs, which revealed significant day effects ($p<0.001$) due to improved scores over test days, significant group main effects ($p\leq0.002$), and non-significant interactions ($p\geq0.203$). Post-hoc tests showed that both FH-48 rats retrieved significantly more pellets than the NOR (FH) and FH-12 groups ($p\leq0.007$), which were not significantly different ($p\geq0.269$). Thus, only the FH-48 treatment significantly improved skilled reaching ability, which occurred with both limbs.

A repeated-measures ANOVA on the stepping error rate with the contralateral-to-stroke forelimb in the ladder test (Figure 4.3b) showed a significant day effect ($p<0.001$) with error rates being significantly higher on both test days compared to baseline ($p<0.001$; within-subjects contrasts). The group main effect ($p=0.002$) and the interaction ($p=0.010$) were significant. Baseline scores were not different among groups (1-way ANOVA: $p=0.739$). A repeated-measures ANOVA on the post-stroke data also showed a significant group effect ($p=0.002$) and a non-significant interaction ($p=0.548$) with the day effect being significant ($p<0.001$). Post-hoc testing showed the FH-48 treatment to significantly reduce stepping error rate ($p<0.001$), while the FH-12 treatment did not significant improve outcome ($p=0.081$).Thus, stepping error rates significantly increased after stroke, and this was attenuated by FH-48 treatment.

The NDS scores were not significantly ($p=0.985$, Kruskal-Wallis test) different among groups at baseline (data not shown) and most scores were 0 (no deficit). Post-stroke scores on day 7 were significantly higher than baseline ($p<0.001$; Wilcoxon Signed Ranks Test) and significantly different among groups ($p=0.033$, Figure 4.5b). Specifically, the FH-48 group was significantly better than the NOR (FH) group ($p=0.009$, Mann-Whitney test) whereas the other comparisons were non-significant ($p\geq0.148$). Thus, only the FH-48 group significantly lessened neurological impairment on the NDS.

Histology:

Lesion volume (Figure 4.4b) was significantly different among groups ($p=0.031$; 1-way ANOVA). Post-hoc analysis showed that the FH-48 group had a significantly smaller lesion than the NOR (FH) ($p=0.014$) and FH-12 ($p=0.042$) groups, which were not different ($p=0.787$). Thus, only the 48-h FH treatment significantly lessened tissue loss.

Table 4.1: Physiological variables (T_s – °C; pCO2, pO2 and MABP – mmHg; ctHb – g/dL; Glu – mmol/L; weight – g). Only T_s was slightly, but significantly, different among groups in Experiments 1 and 2.

	Experiment 1			**Experiment 2**		
	NOR (SH)	**SH-12**	**SH-48**	**NOR (FH)**	**FH-12**	**FH-48**
T_s	36.8 ± 0.08	36.5 ± 0.09	36.7 ± 0.08	36.4 ± 0.08	36.8 ± 0.16	36.4 ± 0.09
pH	7.422 ± 0.006	7.420 ± 0.006	7.417 ± 0.006	7.438 ± 0.010	7.430 ± 0.010	7.424 ± 0.011
pCO_2	40.3 ± 0.7	40.7 ± 0.7	38.4 ± 1.9	36.7 ± 1.3	38.9 ± 0.8	38.3 ± 1.1
pO_2	131.8 ± 4.3	128.3 ± 3.8	127.6 ± 3.4	123.0 ± 4.5	129.0 ± 3.3	126.4 ± 3.7
ctHb	16.3 ± 0.1	16.4 ± 0.2	16.3 ± 0.1	16.3 ± 0.2	16.2 ± 0.4	16.1 ± 0.4
Glu	10.5 ± 0.6	9.9 ± 0.4	9.4 ± 0.3	8.7 ± 0.3	9.6 ± 0.5	9.3 ± 0.4
MABP	104.0 ± 1.9	103.1 ± 2.1	103.7 ± 2.4	98.1 ± 2.1	103.6 ± 2.5	106.5 ± 2.8
Surgery weight	368.4 ± 6.9	374.0 ± 6.9	368.9 ± 5.7	374.2 ± 11.7	395.4 ± 15.7	384.6 ± 11.6
Day 7 weight	377.1 ± 10.9	366.5 ± 11.2	344.1 ± 9.0	380.5 ± 11.4	395.7 ± 9.2	375.2 ± 14.8
Day 32 weight	447.9 ± 6.8	445.4 ± 13.4	452.7 ± 6.3	457.9 ± 10.7	477.0 ± 9.0	455.4 ± 16.5

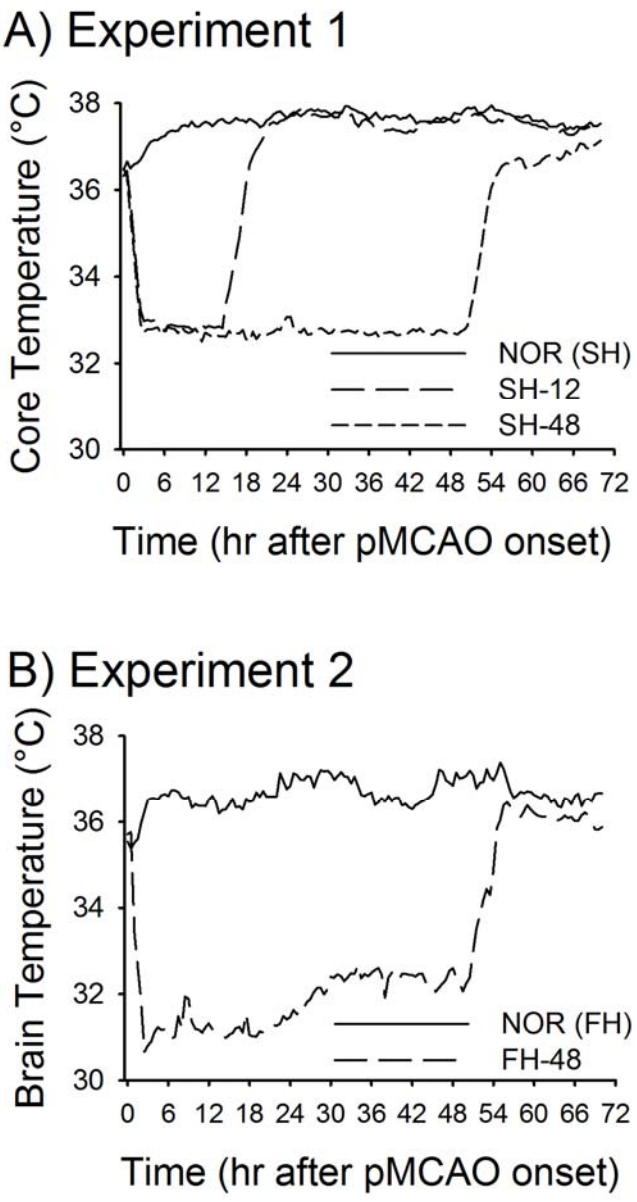

Figure 4.1: A) Body temperatures are depicted for NOR (SH), SH-12 and SH-48 for three days following stroke B) Brain temperature is depicted for NOR (FH) and FH-48 for three days following stroke.

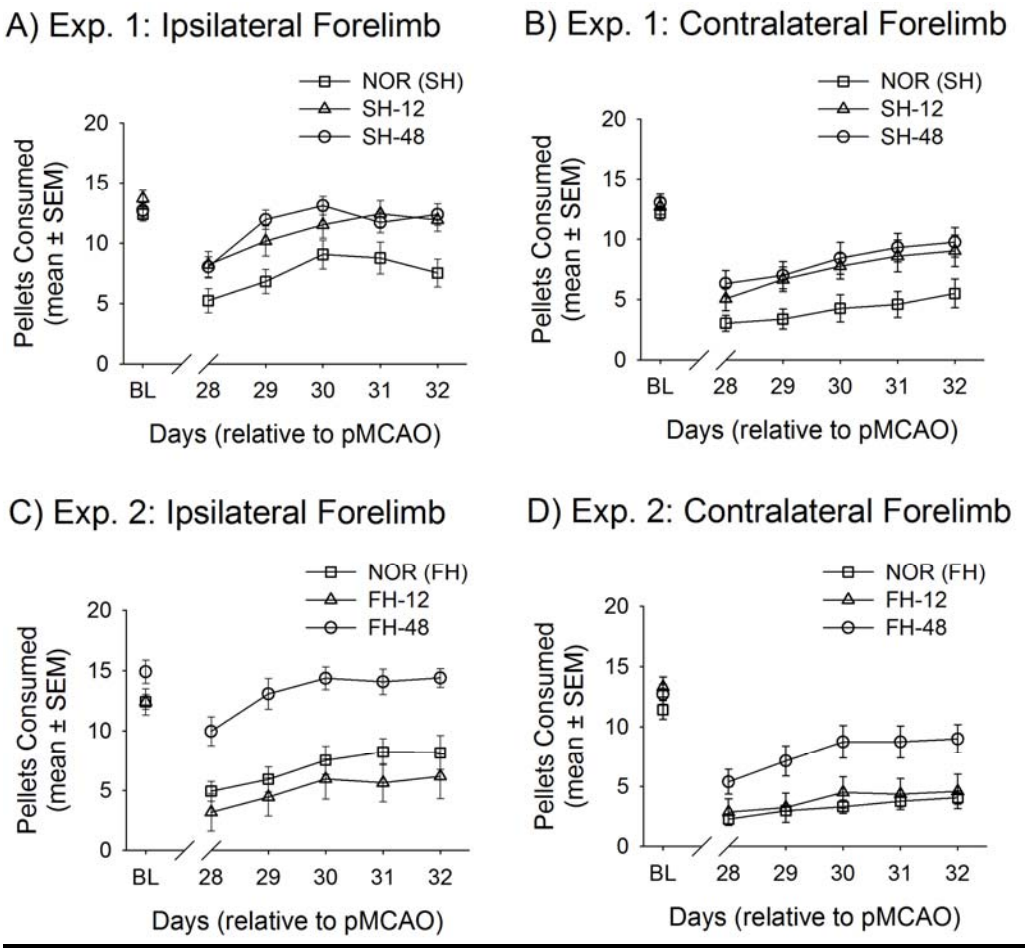

Figure 4.2: Mean number of pellets consumed in the staircase test with the ipsilateral (A and C) and contralateral-to-stroke (B and D) forelimbs for Experiment 1 (A and B) and 2 (C and D). Both SH-12 and SH-48 improved skilled reaching success in Experiment 1, whereas only the FH-48 treatment improved success in Experiment 2.

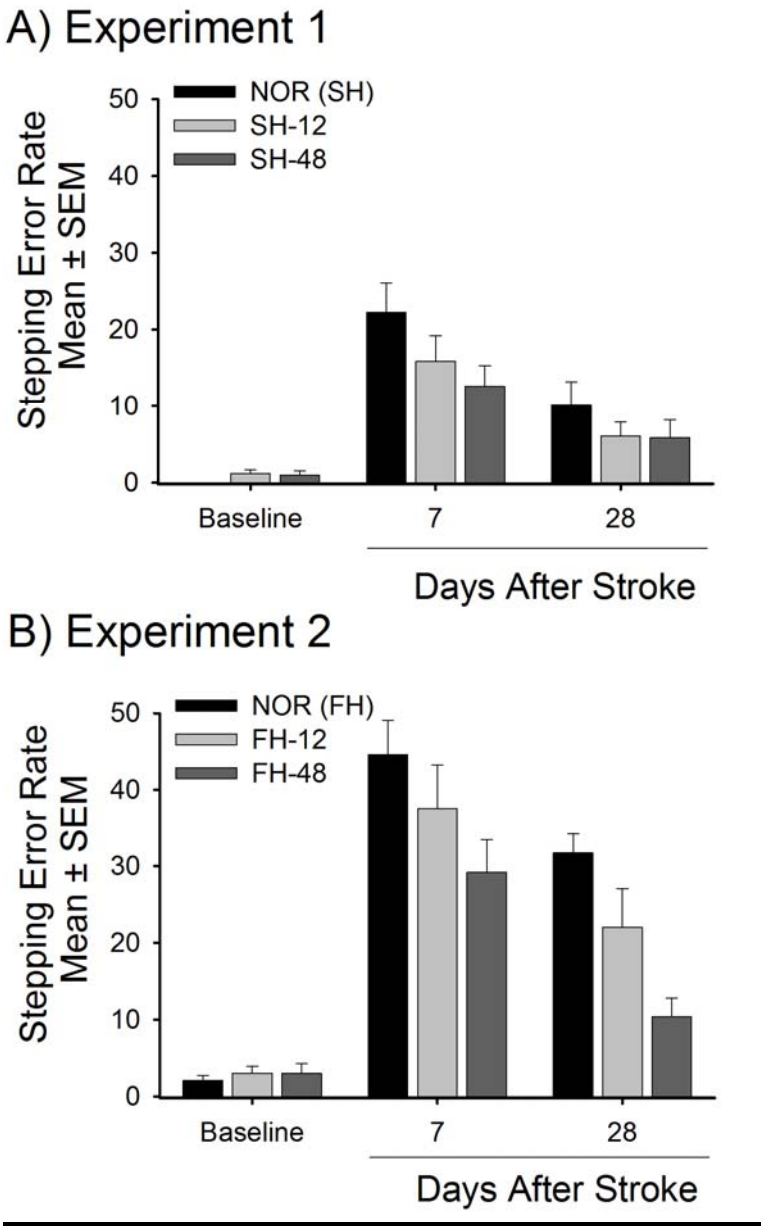

Figure 4.3: Stepping error rate in the horizontal ladder test on the day of baseline assessment and at 7 and 28 days after pMCAO in Experiment 1 (A) and 2 (B). Error rates significantly increased after stroke, but, despite trends, these were not significantly lessened by SH-12, SH-48 or FH-12 treatment. Only the FH-48 treatment significantly lowered the error rate.

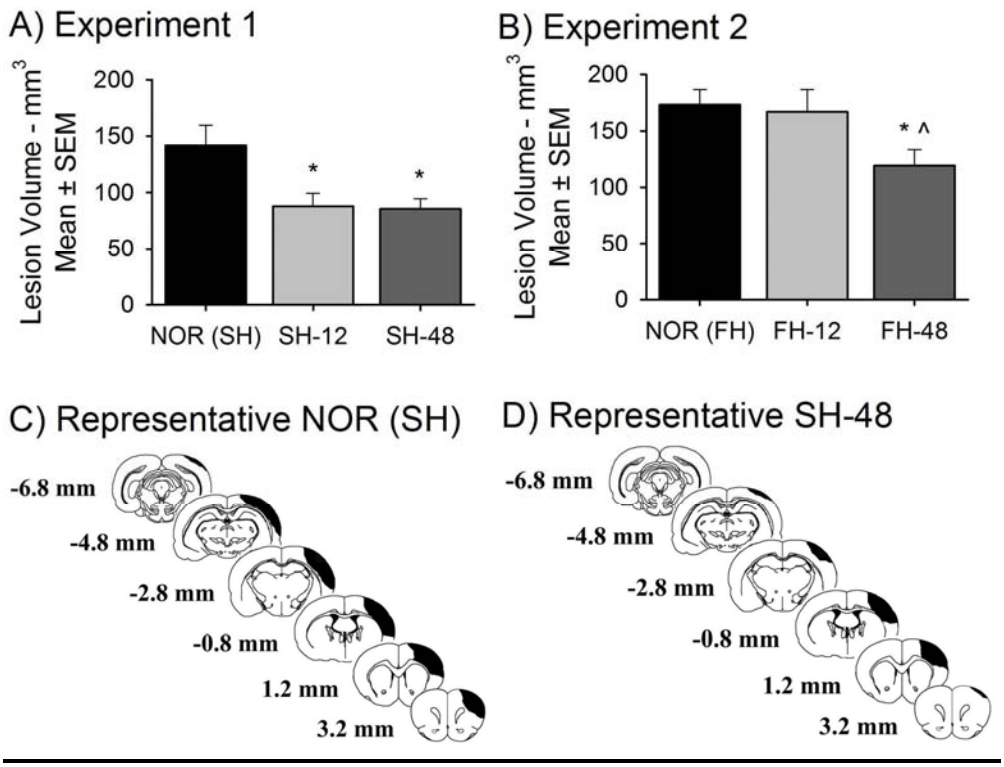

Figure 4.4: Lesion volume was measured at 32 days after pMCAO in Experiment 1 (A) and 2 (B). The SH-12 and SH-48 treatments significantly and equally reduced tissue loss whereas only the FH-48 treatment significantly reduced tissue loss. The typical extent of injury after untreated stroke in Experiment 1 is illustrated in C whereas D illustrates the average animal protected by 48 hr of hypothermia treatment (black region denotes necrotic tissue and lesion cavity).

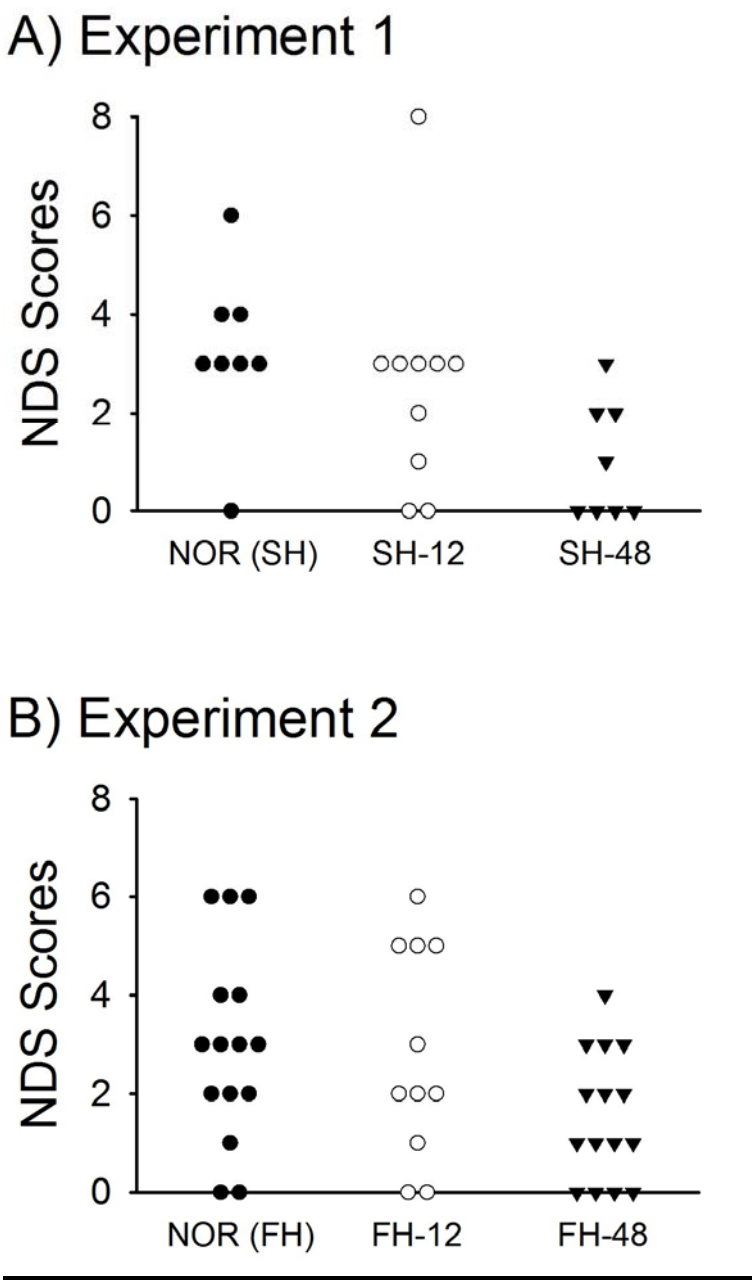

Figure 4.5: Neurological deficits were evaluated at baseline and 7 days after pMCAO in Experiment 1 (A) and 2 (B). Deficits significantly increased following stroke but only the SH-48 and FH-48 groups significantly reduced deficit scores.

4.4 Discussion

In this study both prolonged systemic and focal hypothermia initiated one hour following onset of pMCAO persistently reduced brain injury and functional deficits in rats. Both 12 and 48 h of systemic hypothermia reduced brain injury and improved skilled reaching following permanent focal ischemia but only 48 hours of systemic hypothermia significantly reduced neurological deficits. Focal brain cooling was easily induced and maintained in rats for 48 h without inducing core hypothermia. Two days of brain cooling clearly attenuated brain injury, neurological deficits, reduced reaching deficits and improved walking ability on a horizontal ladder walking test. The briefer duration (12 h) was not sufficient to provide any benefit. This study is the first to evaluate prolonged focal brain hypothermia following ischemia in conscious rats and our results support this method as an effective, safe and simple alternative to systemic cooling.

The results of Experiment 1 largely agree with our previous study where we evaluated 12, 24 and 48 h of systemic hypothermia 7 days following pMCAO (Clark, et al., 2008). In that study, 48 h of cooling reduced lesion volume, edema, neurological deficits and improved walking ability following pMCAO. Cooling for 12 h also reduced neurological deficits and edema following stroke in that study but did not significantly reduce lesion volume, despite a trend in that direction. It is possible that we could have detected a significant histological benefit with 12 h of cooling in that study if we had used larger group sizes. Differences between the two studies in severity of injury may also explain the apparent disparity. One month following stroke, the lesion volume of untreated rats in the current study averaged

~140 mm^3, which is slightly smaller than the average found for untreated rats in our previous study one week after injury (~145 mm^3). Given that infarct size increases over time in this model (Yanamoto, et al., 2001), it is possible that injury in our earlier study was somewhat more severe and perhaps the briefer cooling was sufficient to protect against a less severe injury, as is the case in global ischemia (Chopp, et al., 1991, Colbourne and Corbett, 1994).

This study clearly demonstrates that delayed hypothermia provides long-lasting benefit following permanent ischemia. This finding is in keeping with some previous work (Baker, et al., 1992, Kader, et al., 1992, Park, et al., 1998) but contrasts other data (Doerfler, et al., 2001, Moyer, et al., 1992) which find no lasting benefit of hypothermia when delayed. Few studies have examined long durations of hypothermia following pMCAO. In models of global ischemia, duration of cooling is a crucial factor of hypothermic protection (Colbourne, et al., 1997). The results of our previous study suggest that is also true in permanent ischemia (Clark, et al., 2008).

Currently, we report that 48 h of focal cooling significantly reduced brain injury, errors in ladder walking and skilled reaching, whereas 12 h of focal cooling did not significantly impact either functional outcome or brain injury. Additionally, we found that both 12 and 48 h of systemic hypothermia reduced lesion volume and skilled reaching but only 2 days of cooling reduced neurological deficits. Excluding our previous work, (Clark, et al., 2008) all earlier studies used brief hypothermia. No clear patterns emerge from the small variation of brief cooling (Krieger and Yenari, 2004). For instance, 1 h of delayed hypothermia reduced brain injury 24 h following stroke in one study (Kader, et al., 1992) but not another (Moyer, et al., 1992). The

discrepancy between studies is difficult to explain. Perhaps other factors that differed among the various studies (e.g. delay, depth, strain) are more important than treatment duration when brief cooling is used and such factors are responsible for the inconsistency in findings. Overall, our results support our hypothesis that duration of cooling is a crucial factor in hypothermic protection, but further studies should be done in order to investigate the interaction of treatment duration with other factors such as model, strain, lesion size, depth and delay.

We found 48 h of focal brain hypothermia reduced brain injury and behavioral deficits following stroke. Focal cooling provided a similar level of protection to systemic hypothermia when maintained for two days but 12 h of focal cooling provided no benefit. It is not possible to make direct comparisons between systemic and focal cooling, as animals were not randomized together and factors differed between the two studies (e.g. brain temperature profiles), but the data suggests that systemic may provide superior protection to focal hypothermia. It is possible that one or more of the systemic side effects of hypothermia may be contributing to protection. For instance, the induction of systemic hypothermia in awake animals results in transient elevations of blood pressure (MacLellan, et al., 2004), which may improve cerebral blood flow; whereas, focal cooling does not significantly affect blood pressure (Clark and Colbourne, 2007). Cooling through the surface of the skull creates a gradient of temperature within the brain. This is problematic as a gradient of protection might also be expected when injury is widespread. The model of stroke used in this study produces an extensive cortical injury and parts of the ischemic region more distal to the cooling device would be

warmer than closer portions, which may have reduced protection in distal areas. Further work is needed to compare focal and systemic cooling protocols after ischemic to characterize differences in precise temperature, side effects and protection.

Hypothermia only selectively improved performance on functional tasks. Inconsistencies among functional tests within a study are likely due to the attributes of each test as well as the nature of brain areas salvaged by a treatment. In this study we found skilled reaching ability predicted histological injury indicating that brain tissue rescued by hypothermia contributes to performance on that task, as previously reported (Colbourne, et al., 2000). Stepping error rate showed a similar pattern to skilled reaching and histology with focal hypothermia but neither 12 or 24 h of systemic hypothermia improved stepping, despite improvements in skilled reaching and reduced lesion volume. Only small stepping deficits were detected in Experiment 1 and that may explain why significant improvement wasn't detected. Neurological deficits were reduced with 48 but not 12 h of both systemic and focal hypothermia. The diversity in behavioral outcome underlines the importance of utilizing a battery of functional tests, as opposed to one single measure.

In conclusion, we used a clinically relevant model of focal ischemia (pMCAO) to evaluate the long-term efficacy of two methods of therapeutic hypothermia. We evaluated two durations of cooling and used multiple functional endpoints as well as histological outcome. We showed that hypothermia is effective at reducing long-term brain injury and functional deficits in rats. In the

case of focal hypothermia, we showed this effect is dose-dependant. Taken together, our results strongly support prolonged hypothermia as a neuroprotective therapy for stroke and encourage continued clinical investigation. We also recommend further innovation of new clinical methods of focal cooling. Further animal research is also needed to clarify the interaction between delay, duration, severity and method of cooling.

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General Discussion

Chapter 5

5.1 Introduction

The central aim of this thesis was to investigate the efficacy of prolonged hypothermia after permanent focal ischemia. In Chapter 2, we compared three durations of mild systemic hypothermia and showed that delayed hypothermia reduces infarct size and improves functional recovery at 7 days after pMCAO in a duration-dependent manner with 24 and 48-h treatments providing the best outcome. Given the side effects of systemic cooling, we developed a simple, safe and effective method to induce local brain hypothermia in anesthetized or awake rat (Chapter 3). With these methods, we found that both prolonged systemic and focal hypothermia initiated one hour following pMCAO persistently reduced brain injury and functional deficits in rats (Chapter 4). There are three major conclusions of this thesis. First, delayed and prolonged hypothermia improves both functional and histological outcome following pMCAO. Second, prolonged local brain hypothermia is protective against acute focal ischemia and may be achieved easily, safely and reliably without inducing peripheral hypothermia or the systemic complications of hypothermia. Third, duration of hypothermia is important and prolonged hypothermia may be required to provide protection following more severe acute ischemia, or when focal cooling is used.

5.2 Systemic Hypothermia Following pMCAO

In Chapter 2, we used a clinically relevant model of permanent focal ischemia (pMCAO) and compared three durations (12, 24 and 48 h) of systemic hypothermia initiated one hour post-occlusion. We found that delayed

hypothermia improved functional outcome and reduced brain injury in a duration dependant manner. Length of cooling has previously been assessed, mostly in temporary models of focal and global ischemia. Transient focal ischemia models a scenario where blood flow is restored to the ischemic area, either by tPA or other mechanisms. Transient models are used in the vast majority of preclinical investigations of potential neuroprotective agents for stroke (DeBow, et al., 2003, Stroke Therapy Academic Industry Roundtable (STAIR), 1999), despite the fact that over half of clinical strokes are not acutely re-canalized (Rha and Saver, 2007). Benefit is more easily obtained in such models and this may explain its preferred use. There are striking differences in outcome between studies using transient versus permanent models of stroke (Krieger and Yenari, 2004). For instance, Ridenour et al found that brief mild hypothermia reduced brain injury (47%) and neurological deficits during transient focal ischemia but the same treatment had no effect during permanent focal ischemia (Ridenour, et al., 1992). These results led the authors to report that mild hypothermia is useful against transient but not permanent focal ischemia, a conclusion clearly disputed by our current results.

Other studies have also failed to find benefit of mild brief hypothermia in permanent models (Doerfler, et al., 2001, Moyer, et al., 1992), suggesting longer durations may be needed to protect against permanent focal ischemia. Indeed, these studies found duration to be a key factor determining outcome (Chapter 2, 4). Brief therapeutic hypothermia is commonly used as prolonged treatment requires sophisticated monitoring and temperature control (Colbourne, et al.,

1996, DeBow, et al., 2003). Often, cooling is induced under anesthesia, which limits the duration of hypothermia that can be applied safely. Therefore, as the majority of prior work has been done in temporary ischemia, very few studies have examined prolonged cooling following pMCAO. One study found 24 h of immediate post-ischemic hypothermia improved blood flow, reduced neurological deficits and lesion volume, even when outcome was assessed 21 days later (Yanamoto, et al., 2001). In contrast, Campbell et al showed no benefit of 24 h of mild hypothermia initiated 2 h following occlusion (Campbell, et al., 2008). Notably, a fast rate of re-warming was used in that study, which can exacerbate injury (Berger, et al., 2007). The introduction of a post-ischemic delay influences efficacy of hypothermia following global ischemia (Colbourne and Corbett, 1995, Colbourne, et al., 1999) and tMCAO (Maier, et al., 2001). Treatment duration also influences efficacy in transient models using prompt onset (Maier, et al., 1998) or delayed hypothermia (Zhang, et al., 1993). The results of our current study suggest prolonged cooling is also necessary to confer benefit following delayed cooling following pMCAO, though further work is needed to determine how duration interacts with re-warming rates, depth and delay of treatment.

In Chapter 2, we examined functional and histological outcome 7 days following injury. Although we do not consider one week to be a long-term outcome, it is substantially longer than survival times used in most studies (Krieger and Yenari, 2004). Indeed, histological outcome at short survival times is often presented as the sole endpoint (DeBow, et al., 2003, Krieger and Yenari, 2004). Positive results obtained from such studies must be interpreted with

caution as early histological benefit may be transient (Doerfler, et al., 2001, Valtysson, et al., 1994). Also, we used a battery of behavioral tests to determine functional outcome. Such a strategy is wise, as individual measures may not provide a clear indication of recovery. In a model of hemorrhagic striatal injury, certain tests were unable to differentiate between large differences in injury whereas others (i.e. skilled reaching) were quite sensitive (MacLellan, et al., 2006). In Chapter 2, two days of cooling had no effect on forelimb use asymmetry but did reduce neurological deficits, stepping error and lesion volume. These results underline the importance of using multiple tests when assessing functional outcome. In Chapter 2, we assessed only gross sensory and motor deficits. It would be wise to expand upon this approach and include other tests that are sensitive to more subtle changes in behavior (e.g. skilled reaching).

5.3 Focal Brain Cooling in Conscious Rats

In Chapter 3, we described a simple method to induce local brain hypothermia in rats. The technique involves placing a small, metal coil against the skull and flushing the coil with chilled water. We produced local cooling confined to one side of the brain, leaving the opposite hemisphere and core temperature normothermic (see Figure 3.1). We also maintained prolonged focal brain hypothermia (24 h) in conscious rats (see Figure 3.2) and one day of focal hypothermia did not significantly affect body weight, heart rate or blood pressure (Figure 3.3) nor did we identify any signs of brain injury. Thus, we demonstrated

that therapeutic levels of focal cooling are feasible in rats and would be suitable for use in many focal injury models, such as MCAO, ICH or trauma.

Local head cooling did not produce core temperature decreases or biologically significant changes in blood pressure or heart rate. That finding makes sense as peripheral cutaneous and core thermoreceptors would not be activated by local brain cooling and presumably local hypothalamic receptors, being a midline structure, would only be cooled slightly. The preoptic area integrates information from all parts of the body when determining output; therefore, focal brain cooling would not result in a significant level of thermogenic output responses, such as shivering and vasoconstriction, which produce blood pressure and heart rate increases when the body is systemically cooled.

This new technique offers advantages over previously described local cooling methods such as a coil (Taniguchi, et al., 2005) or cooling blanket wrapped around the entire head of anesthetized animals (Nurse and Corbett, 1994). Cooling of the entire head can lead to systemic cooling which may result in systemic complications. Other local cooling methods also require anesthesia, which limits the duration of cooling that is feasible. Our method is minimally invasive and can be done in freely moving, conscious rodents. Importantly, various depths and durations of hypothermia can be easily induced simply by changing the flow rate or temperature of the circulating water. Indeed, very prolonged hypothermia is possible with this method. Focal cooling was successfully induced with the same method described in Chapter 3 and

maintained for up to 6 days following ICH (Fingas, et al., 2007, Fingas, et al., 2009) and for 3 weeks in normal animals (Auriat, et al., 2009). Cell death can continue to occur over days (Valtysson, et al., 1994) and it may prove extremely useful to assess the effect of cooling on pathological processes contributing to injury at later time points.

5.4 Long-term Functional Efficacy of Hypothermia

In Chapter 2, we showed that hypothermia reduces brain injury and improves function at one week, but did not assess whether our treatment also provided long-term protection or improved fine motor control. In Chapter 4, we found both prolonged systemic and focal hypothermia provided long-term histological protection and functional benefit following pMCAO. Notably, we demonstrated that both methods of cooling significantly improved skilled reaching a month following injury. The importance of both histological and functional long-term assessment in stroke research has been firmly asserted by the STAIR report (Stroke Therapy Academic Industry Roundtable (STAIR), 1999) and other expert reviews (Corbett and Nurse, 1998). Several reports have indicated the necessity of evaluating animals for longer periods because early findings of efficacy may not indicate similar protection later on (Coimbra, et al., 1996, Colbourne, et al., 1999, Valtysson, et al., 1994). Despite this, very few stroke neuroprotection studies have used long survival times (DeBow, et al., 2003, Meloni, et al., 2009), most likely owing to the onerous nature of such experiments. This is particularly true in the case of focal ischemia models, where studies evaluating long-term outcome are extremely sparse (Colbourne, et al., 2000,

Corbett, et al., 2000, Maier, et al., 2001, Yanamoto, et al., 1999, Yanamoto, et al., 2001). Only one prior study has characterized continuing benefit of hypothermia in a model of permanent focal ischemia. Yanamoto et al evaluated neurological deficits and lesion volume 21 days following pMCAO in rats treated with prolonged (24 h) mild hypothermia initiated at the time of occlusion (Yanamoto, et al., 2001). Ours was the first study to show sustained protection of delayed hypothermia in a permanent model.

In Chapter 4, we also demonstrated that delayed *focal* cooling is efficacious at reducing long-term brain injury and functional deficits following pMCAO. This provides proof of principle that the focal brain cooling described in Chapter 3 is a valid method of therapeutic hypothermia. The success of focal cooling to protect against a relatively severe injury (pMCAO) when initiated after a delay suggests it may also be useful in other models of focal injury, such as trauma. No adverse effects of focal cooling were observed over the month long recovery, indicating the method is suitable for studies requiring long-term survival. In Chapter 2, prolonged duration of cooling was vital to the protective effect of delayed hypothermia. The importance of cooling duration was again demonstrated in Chapter 4. Two days of focal cooling reduced brain injury, NDS, skilled reaching and stepping error but 12 h had no effect on any measure. Both 12 and 48 h of systemic hypothermia successfully reduced lesion volume and skilled reaching but only two days of cooling significantly reduced neurological deficits. Protracted treatment may be necessary to confer benefit, especially when therapy is delayed.

5.5 Limitations

Cortical injury was reduced by delayed and prolonged hypothermia but the effect on other areas of the brain affected in human stroke (i.e. striatum, white matter) is not yet known. For example, Corbett et al found prolonged hypothermia substantially reduced cortical injury following proximal tMCAO but striatal injury was only marginally reduced (Corbett, et al., 2000). Delay to treatment is an important factor and clinical intervention delays are typically much longer and more varied than those used in experimental studies (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). A conservative but clinically relevant intervention time (1 h) was chosen for these studies, but variations in delay were not evaluated. Behavioral protection was not uniformly observed in these studies. Furthermore, functional outcome dissociated from histological findings in a number of cases. Typical histological methods can miss subtle changes in the brain that may lead to injury (i.e. dendritic atrophy; scattered necrosis) or protection (i.e. plasticity; trophic environment). Such covert mechanisms could contribute to functional performance on behavioral tasks (Corbett and Nurse, 1998). Also, the sensitivity of an individual test depends upon factor such as injury location, motivation and stress, therefore multiple outcome measure are recommended to achieve a more powerful assessment of neuroprotection (Corbett and Nurse, 1998, Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

The maximum duration of cooling evaluated was 48 h. It is possible that longer periods of cooling may provide even greater protection. Conversely, it is also possible that adverse side effects of protracted cooling may negate some of

its benefit. The maximum duration of systemic cooling is limited (~2 days), but focal cooling is possible for longer durations (Fingas, et al., 2007, Fingas, et al., 2009). The described focal cooling method removes heat from the surface of the skull and therefore creates a temperature gradient within the brain. Hence, it is impossible to absolutely control the temperature of the entire target area. Also, if the target area is sub cortical, overcooling of cortical tissue would occur (Fingas, et al., 2007, Fingas, et al., 2009). Ramifications of such cortical overcooling are not known.

Both systemic and focal hypothermia were evaluated in Chapter 4, but no direct comparisons between the methods were made. It would be interesting and valuable to directly contrast the efficacy of focal and systemic cooling. Unfortunately, the existence of gradients prevents the accurate matching of target temperature, making it impossible to validly compare the two. Lastly, the method of focal cooling presented is not directly relatable to clinical practice. Various clinical methods exist to focally cool the brain (Diller and Zhu, 2009), but skull thickness, cerebral blood flow and brain size and composition would likely prevent the use of a localized trans-cranial cooling device for hemispheric cooling in patients. The described method is recommended for use in rodent models and would not likely directly relate to those used in the clinic.

Only young male rats were used in this thesis. Gender and age differences are important considerations for the investigation of a therapeutic agent for stroke (Petrea, et al., 2009, Popa-Wagner, et al., 2007, Popa-Wagner, et al., 2007). Notably, female rats subject to tMCAO tend to have a smaller lesion volume

compared to males or ovariectomized females (Alkayed, et al., 2000). Although work in global ischemia shows that post-ischemic hypothermia is effective in elderly and female rodents (Corbett, et al., 1997), it cannot be assumed that therapeutic findings in young, male rats would translate to female and/or aged rats. These factors were not assessed in the described studies. Finally, location and severity of injury would likely affect the efficacy of neuroprotective therapies such as hypothermia. We assessed outcome in only one model of stroke (distal pMCAO) and therefore cannot make conclusions about the effectiveness of delayed and prolonged hypothermia in other injury models. Notably, a weakness of the described method (vs. suture method) is the presence of a craniotomy which may influence important injury mechanisms (e.g. intracranial pressure). Finally, a major limitation of this work is that is done in rodents models which cannot be directly related to humans. Factors such as treatment duration in rat models are not necessarily predictive of such boundaries in humans; however determination of ideal parameters may be useful to determine such bounds in a relative fashion.

5.6 Future Directions

Numerous questions related to this thesis, many raised in the preceding section, deserve further investigation. The data described herein demonstrates the importance of factors such as model, duration and intervention delay to the therapeutic effect of hypothermia. Basic parametric studies are needed to clearly define the ideal treatment paradigm necessary to bring about the best outcome in

different stroke models. Such studies should compare factors within experiments to allow for direct comparisons. Some studies of this nature have been done (Kollmar, et al., 2007, Maier, et al., 1998) but the ideal parameters of therapeutic hypothermia following stroke are still unknown.

Many studies have investigated the mechanisms of action by which hypothermia protects the brain, the vast majority of which evaluate intra-ischemic hypothermia (Colbourne, et al., 1997). Most of the described mechanisms (i.e. excitotoxicity) occur very early in the injury process. The efficacy of delayed hypothermia and the influence of prolonged durations indicate that injury mechanisms are occurring over many hours/days and hypothermia must be acting upon those later processes. Future studies need to investigate the mechanisms of delayed and prolonged hypothermia, as they are likely different from those underlying the effect of intraischemic cooling.

This thesis introduced a new method of focal cooling in rats and demonstrated that it is effective at reducing brain injury and improving function following pMCAO. Future research should focus on further characterizing this model. Studies should determine how focal hypothermia affects physiological parameters such as CBF and corticosterone levels (stress). Three weeks of focal cooling has been achieved with this method (Auriat, et al., 2009). What is the effect of very long (i.e. weeks) durations of local cooling, with respect to behavior, neuronal morphology, core temperature, body weight, HR and MABP?

5.7 Conclusions

The data in this thesis convincingly demonstrates that delayed and prolonged post-ischemic hypothermia can significantly reduce brain injury and improve functional ability following permanent ischemia and that the protection is sustained. Furthermore, cooling can improve outcome whether it is induced systemically or focally. The findings also indicate that prolonged hypothermia (24 to 48 h) may be necessary to confer benefit in some cases. In fact, this may explain the inconsistency in previous studies that used briefer cooling.

Evidence is lacking from clinical trials evaluating therapeutic hypothermia for acute stroke. The level and persistence of protection demonstrated herein is sufficient to encourage further clinical investigation of prolonged hypothermia following stroke. Nonetheless, it is not known how useful hypothermia may be at much longer delays, nor do we know if very prolonged hypothermia (i.e. 3-7 days) would add further protection or harm following stroke. It would be prudent to investigate the limits of hypothermia therapy so one may understand how to optimize therapy. As it stands, hypothermia is likely the most promising neuroprotectant for acute stroke and it is hoped that eventually stroke patients will benefit from therapeutic cooling, as cardiac arrest patients have.

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