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Influence of therapeutic hypothermia on neuroprotection and post-ischemic plasticity in a rat model of global ischemia

by

Gergely Silasi

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Abstract:

Blood flow to the brain may be disrupted by either a stroke (such as focal ischemia or hemorrhage) or cardiac arrest, where the whole brain becomes ischemic. Both forms of injury result in irreversible neuronal loss leading to neurological impairments and a decrease in the quality of life. Neuroprotective treatments are aimed at minimizing the damage that occurs after brain ischemia, and one of the most successful neuroprotectants developed so far is therapeutic hypothermia. Prolonged cooling has been shown to prevent CA1 neuronal death in animal models of global ischemia and the treatment also improves survival and neurological function in patients resuscitated from cardiac arrest. In contrast to the well know neuroprotective properties of hypothermia, the effect of this treatment on post-ischemic plasticity and reorganization has not been clearly investigated. This is an important issue, as plastic changes in remaining brain circuits facilitate functional improvement and recovery after ischemia. The current thesis evaluated the influence of hypothermia on several forms of post-ischemic plasticity including neurogenesis and growth factor expression in the hippocampus. A rat model of global ischemia was used to induce degeneration of hippocampal CA1 neurons, and hypothermia was applied either systemically (whole body cooling) or through unilateral brain cooling. We found that the treatment did not negatively impact post-ischemic plasticity on any of our measures even when cooling was maintained for up to 7 days. These results suggest that prolonged cooling may be a safe treatment, however additional models of ischemia should be used to assess this in future studies.

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List of abbreviations and symbols used

CA1	Cornus Ammonis area 1
ICH	Intracerebral Hemorrhage
SAH	Subarachnoid Hemorrhage
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
tPA	tissue Plasminogen Activator
MCAO	Middle Crebral Artery Occlusion
DNA	Deoxyribonucleic Acid
4VO	4-Vessel Occlusion
2VO	2-Vessel Occlusion
BDNF	Brain Derived Neurotrophic Factor
IGF-1	Insuling-Like Growth factor -1
NMDA	N-Methyl-D-aspartic Acid
CSN	Central Nervous System
MABP	Mean Arterial Blood Pressure
BrdU	Bromodeoxyuridine
H&E	Hematoxylin and Eosin
GFAP	Glial fibrilary acidic protein
Iba-1	Ionized calcium Binding Adaptor molecule 1
NeuN	Neuronal Nuclei
DAPI	4',6-diamidino-2-phenylindole
ANOVA	Analysis Of Variance
SEM	Standard Error of Mean
DG	Dentate Gyrus
HPC	Hippocampus

Chapter 1

Introduction

1. Introduction

Stroke research can be a rewarding field of study for two partially overlapping reasons. First, one may have the rare fortune of discovering or developing a treatment that provides a true benefit to those affected by the disease; or even better, prevent the disease from occurring. Second, by studying the diseased brain and the impact that various treatments have on it, one will inevitably learn new things about brain function and the mechanisms that produce behaviour. Stated differently, by studying the broken brain (after stroke) we can learn much about how the brain functions normally.

In the current thesis I will describe experiments where we applied a highly effective treatment (hypothermia) after forebrain ischemia and studied the endogenous repair processes that are initiated by the injury, as well as the effects of the treatment on these processes. In order to provide the necessary background for my experiments I will first introduce the problem of brain ischemia from both a clinical and basic science perspective. I will then discuss some of the available treatments for this disease (including hypothermia) and introduce the general hypothesis of my thesis as well as the experimental methods I used in my studies.

1.1 Stroke types

A stroke is defined as an interruption of blood flow to any part of the brain and can be caused by a number of factors. The most common cause is a

blockage in the lumen of an artery supplying the brain, resulting in focal ischemia. A blockage that is formed by a clot in a part of an artery that is already narrow is called a thrombus, whereas a clot that breaks away from another place in the vasculature and travels up to the brain to block a smaller artery is called an embolism. In the case of the latter, the ensuing event is called an embolic stroke. Ischemia may also be caused by the buildup of plaque (called atherosclerosis) in vessels either within the brain or in the neck. The plaque, which is mainly composed of fat and cholesterol, slows down blood flow and increases the chances that a clot will form in that region. Artherosclerotic plaques are most common in the ageing population, making age the number one risk factor for stroke. People under 40 years of age may also suffer a stroke, most commonly caused by a vascular abnormality called an arterial dissection. In this condition, a tear in the lining of an artery lets blood flow between the layers of the vessel, thus causing a narrowing in the lumen and impeding blood flow. In addition to focal ischemia, a stroke may result from a complete cessation of circulation, thus causing the entire brain to become ischemic. This condition, called global ischemia, is most commonly caused by cardiac arrest (discussed in section 1.4).

A less common form of stroke, which accounts for approximately 15% of all cases, is hemorrhagic stroke. An intracerebral hemorrhage (ICH) occurs when a blood vessel within the brain ruptures, whereas the bursting of a blood vessel on the brain surface produces a subarachnoid hemorrhage (SAH). In both conditions, the direct contact of blood and its components

with brain tissue produces cell death. In the case of ICH, the escaped blood dissects through the neural tissue producing significant mechanical damage and inflammation. Furthermore, in addition to the brain swelling that occurs due to the injury, the space occupying effect of the escaped blood often exacerbates the elevation of intracranial pressure, which can lead to poor outcome or death.

1.1.2 Risk factors and prevalence

Population studies have identified a number of risk factors that increase the chances of stroke. Among the non-modifiable risk factors, the single greatest effect is that of age, because the risk of stroke doubles in each successive decade after 55 years of age (Bennett, 2006). Overall, stroke is more prevalent in men than women (Brown *et al.*, 1996); and some races, including blacks and Hispanic Americans, have higher stroke incidence and mortality compared with whites (Gorelick, 1998). The major modifiable risk factors for stroke are hypertension, smoking and diabetes, whereas a poor diet, physical inactivity and alcohol consumption also increases stroke risk (Goldstein *et al.*, 2001). Due to the continued progression of cerebrovascular disease with age, as well as incomplete efficacy of preventative interventions, a large portion of stroke patients will suffer recurrent strokes. Within the first 10 years after a first-ever stroke, 43% of patients suffer a recurrent stroke (Hardie *et al.*, 2004).

1.1.3 Acute patient treatment and care

Although stroke remains a disease for which there is no treatment that can reverse the immediate brain damage, the acute care that patients receive in the hospital can have a significant impact on overall outcome. The primary concern in devising a treatment plan is to determine whether a patient is suffering an ischemic or hemorrhagic stroke. In most centers, this evaluation is done through CT or MRI imaging (Adams *et al.*, 2007). If ischemic stroke is identified within 4.5 hours of onset, the patient may be treated with reperfusion therapies (Shobha *et al.*, 2011), such as intravenous tissue plasminogen activator (tPA). This enzyme catalyzes the conversion of plasminogen to plasmin, which dissolves blood clots within vessels. Unfortunately, only a small percentage of patients reach a hospital in time to benefit from this treatment (Heuschmann *et al.*, 2003). Alternatively, some clots within vessels may be removed mechanically, by being ensnared in a coil that is advanced endovascularly and retracted; however the efficacy of such treatments have not been completely assessed in a randomized trial (Ansari *et al.*, 2011). In cases where reperfusion is not achieved, the majority of patients will progressively deteriorate, due to neurological complications caused by ischemia. In one third of these patients the worsening is caused by brain swelling, while parenchymal hemorrhage accounts for another 10% of cases where neurological symptoms worsen (Weimar *et al.*, 2005). Ischemia-induced swelling can be monitored through CT scans as midline structures, such as the pineal gland, are shifted by the injury. In severe cases, decompressive craniotomy is performed, where a piece of the skull is

temporarily removed to accommodate tissue expansion (Delashaw *et al.*, 1990; Staykov and Gupta, 2011). In cases where the skull is left intact, patients can develop an increase in intracranial pressure, which may be managed with hyperventilation, osmotic diuretics, or elevating the head to help with venous drainage (Schwab *et al.*, 1996). Despite these options, edema remains the leading cause of death following a major ischemic stroke (Adams *et al.*, 2007). This emphasizes the need for developing effective treatments against secondary processes that follow ischemic injury.

1.1.4 Risk factors and acute treatment following cardiac arrest

Cardiac arrest occurs when the heart is no longer able to efficiently pump blood due to a sudden change in heart rhythms. In approximately 40% of patients the arrest is caused by ventricular fibrillation or ventricular tachycardia (National Center for Biotechnology Information, 2011), which are spasm-like, fast contractions of the heart. Other major causes are coronary heart disease and non-cardiac problems, such as trauma. Major risk factors for cardiac arrest are similar to stroke, but also include previous cardiac disease and heart attacks. The average survival rate across various populations is around 8% (Sasson *et al.*, 2010), however individual survival is largely determined by the acute care that the person receives (Weisfeldt *et al.*, 2011). For example, in patients where CPR is initiated by bystanders, survival to hospital discharge was three-fold higher relative to patients who did not receive bystander CPR (Herlitz *et al.*, 1994).

An important breakthrough in the acute care of cardiac arrest patients came in 2002 when two independent studies demonstrated that hypothermia treatment initiated within the first four hours after the return of spontaneous circulation improved neurologic outcome and survival relative to normothermia (Bernard *et al.*, 2002b; HACA, 2002). These studies were based on patients that received whole-body cooling with ice packs and cooling blankets; therefore it may be possible that at least part of the benefit was due to the protective effects of hypothermia on organs other than the brain, as these were of course also ischemic. Although several animal studies had previously demonstrated that hypothermia is neuroprotective in models of cardiac arrest (discussed below), this mechanism has yet to be confirmed in human patients. Nonetheless, whole body cooling continues to be adapted as standard treatment in many centers world wide (Peberdy *et al.*, 2010), with continued positive results.

1.2 Strategies for Stroke and Cardiac Arrest Treatment

Efforts aimed at minimizing the impact of stroke and cardiac arrest can be generally divided into separate research categories that target various steps in the progression of injury or the initiation of repair processes. Each of these categories will be discussed in turn.

1.2.1 Neuroprotection

The primary goal of neuroprotective treatments is to prevent neuronal death. Although a number of pathological events precede cell death in the ischemic cascade (see section 3.1.2), many of these are reversible,

while cell death is clearly permanent. Therefore, the prevention of neuronal death is the ultimate therapeutic target that likely has the greatest impact on stroke outcome. Strategies for achieving neuroprotection focus on either restoring blood flow to the ischemic region (eg. thrombolysis or augmented collateral flow), counteracting excitotoxicity and free radical formation, decreasing the energy demands of neurons, or inhibiting apoptosis and necrosis. In most brain regions neurons begin to die within minutes of ischemia (Lipton, 1999), therefore neuroprotective strategies are most effective when initiated soon after the injury. The majority of animal studies evaluating neuroprotection in focal ischemia or hemorrhage use lesion volume assessments to infer neuroprotection. One limitation of such measures is that results may be confounded by effects on glial cells, which proliferate extensively following injury and thus may influence lesion volume. For example, treatment with minocycline, a putative neuroprotectant (Yrjanheikki *et al.*, 1999), prevents the formation of a cortical cavity in a model of focal ischemia, however the lesion area is repopulated by reactive astrocytes and not neurons (Hua and Walz, 2006). This effect may easily be misinterpreted as a decrease in neuronal death with less careful, lesion volume assessments. Direct measures of neuroprotection, such as neuronal counts, provide a more precise endpoint measure, however such analyses are not feasible in all brain regions due to irregular cell distribution or unclear borders for brain regions.

Animal models have been used to identify a large number of neuroprotectants for potential clinical application, unfortunately all of these treatments failed to work when assessed in patients (O'collins *et al.*, 2006). This trend has called into question the criteria in place for selecting effective neuroprotectants, and further suggestions have been put forth to increase the likelihood for generating translatable findings from animal models (STAIR, 1999). For example, studies with long survival times and functional assessment on a battery of behavioural tests are viewed as superior and have a greater chance of producing translatable findings. Others have argued that greater emphasis needs to be placed on evaluating the contribution of physiological factors such as temperature, blood glucose levels and blood pressure, as these factors have a greater impact on pathophysiology than pharmacological treatments that target single mechanisms (Auer, 2001).

1.2.2 Regeneration

The discovery of endogenous stem cells in the mammalian forebrain (Morshead and van der Kooy, 1992; Reynolds and Weiss, 1992) lead to the development of the new field of promoting cell replacement therapies after neurological injuries, including stroke (Parent, 2003). The two main approaches are to either promote the proliferation and differentiation of endogenous precursors or alternatively to transplant differentiated precursors into the ischemic territory and to promote integration (Kokaia and Lindvall, 2003). Unfortunately neither of these strategies has proven to be very successful in promoting functional recovery. One of the major

problems with endogenous regeneration is that stem cells are normally found within two restricted brain regions, the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricles. Therefore, in order to contribute to post-ischemic repair, cells from these regions must be recruited to the site of injury, which can be several millimeters away in a rodent brain and much longer in humans. In the uninjured brain, there is precedence for cell migration over such distances as progenitor cells originating from the ventricles migrate via the rostral migratory stream to the olfactory bulb and integrate into local circuitry (Mizrahi, 2007). This is a highly regulated process dependent on a number of signaling pathways (Lois *et al.*, 1996), and due to its complexity has not been replicated following stroke. The drive behind these efforts comes from a number of studies showing that there is some spontaneous cell migration from neurogenic zones into the site of injury (Gotts and Chesselet, 2005). For example, after middle cerebral artery occlusion progenitor cells migrate into the striatum, differentiate into medium spiny neurons and presumably integrate into remaining circuitry. However, the fact that only about 0.2% of the injured neurons are replaced by endogenous regeneration (Arvidsson *et al.*, 2002) suggests that this process has little functional significance.

The alternate approach, of transplanting progenitor cells into the site of injury, is plagued by problems related to neuronal survival (Björklund and Lindvall, 2000; Savitz *et al.*, 2002). For example, even when enriched housing was used to stimulate the survival of human stem cells transplanted into the

ischemic striatum in rats, the survival of transplanted cells at 2 months post-injury was below 1% (Hicks *et al.*, 2009). Transplanting cells into, or near the site of injury allows precise spatial control over any potential regeneration, however the hostile environment created by infiltrating immune cells makes transplantation into the injured region not practical. An alternative approach is to inject precursor cells into the circulatory system, and rely on guidance factors secreted from the injury to attract cells into the brain parenchyma. Using this approach, fluorescently labeled cells injected intra-arterially were found to accumulate in the ischemic hemisphere following stroke, whereas non-injured brain regions and other organs do not preferentially accumulate precursor cells (Pendharkar *et al.*, 2010). Taken together, these studies provide proof of principle for both spontaneous regeneration as well as the survival of transplanted cells within the adult brain. However, the fact that a very small number of cells incorporate into existing brain circuitry and the fact that the clear functional significance of these cells has not been demonstrated indicates that the translation of regenerative approaches to patient populations is still in the distant future.

1.2.3 Rewiring and rehabilitation

Perhaps the least controversial recovery mechanism that has been studied extensively in both animal models and human patients is neuronal rewiring and plasticity. Plastic reorganization is a feature of the uninjured adult brain, and facilitates many forms of learning and memory. For example, the acquisition of skilled motor behaviours in rats is mediated by plastic

changes in contralateral motor cortex (Monfils *et al.*, 2005). Similarly, the spontaneous recovery that occurs following ischemia is correlated with dendritic reorganization in remaining brain regions (Jones and Schallert, 1994). The general trend from rodent studies indicates that large unilateral lesions induce dendritic growth and synaptogenesis in the contralateral hemisphere, whereas smaller cortical lesions induce plasticity in the injured hemisphere in functionally related structures (Jones and Schallert, 1994; Jones *et al.*, 1996; Jones, 1999). Brain imaging studies in clinical patients show a similar pattern of results in that recovery is associated with increased activity in the injured hemisphere (Cramer, 2008; Ward, 2004). Rehabilitation further augments such processes, as both a general increase in motor experience as well as task-specific motor rehabilitation improve functional outcome and induce dendritic growth following stroke (Biernaskie and Corbett, 2001; Biernaskie *et al.*, 2004). Lastly, there is evidence that sprouting of axonal fibers in the cortex (Liu *et al.*, 2010), brainstem and spinal cord (Chen *et al.*, 2002; Liu *et al.*, 2007) also contribute to post-stroke recovery. These studies provide unequivocal evidence for large-scale reorganization following stroke, and in many cases such changes correlate with improved functional outcome. For this reason, there is significant clinical interest in similarly augmenting post-ischemic plasticity in stroke patients.

1.3 Animal Models

Stroke and global ischemia can be induced in animal models through a number of different methods. Clinical relevance and reproducibility are critically important factors in making comparisons among models. Further comparisons between focal and global models of ischemia indicate that the basic mechanisms leading to cell death are similar, but key differences exist in the time course and location of neuronal death.

1.3.1 Focal ischemia

The most common method for inducing focal ischemia in rodents is by either temporarily or permanently occluding blood flow through the middle cerebral artery (MCA). This can be achieved by either advancing an occluding filament through the common carotid artery up to the origin of the MCA (Longa *et al.*, 1989), or the MCA may be isolated and electrocoagulated through a small craniotomy (Yamamoto *et al.*, 1988). As the MCA supplies blood to the majority of the lateral cortex as well as the striatum and thalamus, the suture occlusion model produces large lesions affecting the majority of the targeted hemisphere. Such injury results in obvious behavioural deficits and histological damage that is easy to quantify. The clinical relevance of these measures is limited, however, as patients suffering such large strokes rarely survive their injuries. Smaller strokes produced by occluding only the distal branches of the MCA more closely mimic MCA infarction in humans while still producing measurable behavioural dysfunction and histological damage (Clark *et al.*, 2008; Gonzalez and Kolb, 2003).

Focal ischemia may also be also be induced through photothrombosis (Watson *et al.*, 1985), which involves injecting animals with a photoactive chemical (Rose Bengal) and then illuminating part of the brain to activate the compound, thus occluding the targeted vessels. This method produces consistent cortical ischemic infarcts without the need for craniotomy. Alternatively, blood vessels may be temporarily occluded by applying the potent vasoconstrictor Endothelin-1 either on the brain surface, or injecting it into the brain (Fuxe *et al.*, 1997). This results in a moderate reduction of blood flow in the affected region for approximately 24 hr, at which point blood flow returns to baseline levels (Windle *et al.*, 2006). The spontaneous reperfusion in this model approximates post-ischemic events in clinical patients, and could therefore be used to mimic the mechanisms of reperfusion injury or to evaluate neuroprotective treatments.

1.3.2 Cell death

One feature common to most focal ischemia models is that the infarct core, where blood flow is most severely impaired, is surrounded by a region of graded ischemia (Astrup *et al.*, 1981). This observation can be confirmed by both blood flow measurements during ischemia, as well as an examination of cell death at various times after ischemia. For example, MCAO occlusion produces an infarct core, where blood flow is reduced below 15% of baseline, whereas in surrounding regions, known as the penumbra, flow does not fall below 40% (Lipton, 1999). Ionic and metabolic changes are also different across these two regions as the infarct core undergoes permanent anoxic

depolarization caused by a massive depletion of ATP (Folbergrova *et al.*, 1992), whereas in the penumbra energy reserves are maintained, thus avoiding permanent depolarization (Hossmann, 1994). There are also spontaneous depolarizations within the penumbra that seem to be driven by waves of elevated extracellular potassium and glutamate (Lipton, 1999). Although these waves do not directly result in massive cell death, if ischemia persists, the repeated waves of spontaneous depolarizations will recruit the penumbra to be part of the core. Cell death first occurs in the core as early as 3 hr after ischemia, and expands into the penumbra by 24 hr (Lipton, 1999). Dying neurons become eosinophilic, have shrunken cell bodies, and may also exhibit subcellular features such as DNA fragmentation. In general, cell death in the penumbra lags behind that in the core, however by 7 days there is almost complete loss of cellular elements in the infarct region (Garcia *et al.*, 1993); a term called pan-necrosis. Thus, although there are obvious differences between core and penumbral tissue in the acute phase of focal ischemia, if interventions are not applied, the end result is identical for both regions.

1.3.3 Neuronal atrophy after focal ischemia

The majority of studies examining neuronal connections after ischemia describe changes that occur weeks or months after the injury and relate them to functional outcome. Synaptic connections, however, are also highly sensitive to ischemia, and thus should be considered in the early pathophysiology of stroke. In vivo imaging of neuronal structure shows that

dendritic branches less than 100 μm from the core of the infarct develop varicosities or swellings along the shaft, and a loss of spines during focal ischemia (Enright *et al.*, 2006). Remarkably, the sensitivity of synaptic connections is more resilient to ischemia than one would predict, as a 50% reduction in blood flow produced little change in spines and dendrites while a 90% reduction induced spine loss and dendritic beading within 10 minutes (Park *et al.*, 1996). Interestingly, following reperfusion many of the lost spines return to the exact same locations, re-forming the same functional synapses that existed before ischemia(Brown and Murphy, 2007). The mechanisms that induce these structural changes are not clear, however a recent study has shown that the spontaneous depolarizations that occur within the penumbra are temporally correlated with rapid dendritic beading and loss of spines (Risher *et al.*, 2010). This suggests that energy depletion and cell excitation may contribute to the structural changes of neurons that are observed following ischemia.

1.3.4 Rodent Models of Cardiac arrest and global ischemia

The first rodent models of global ischemia simply consisted of decapitation, as this produced reproducible ischemic cell death in the brain (Kogure *et al.*, 1981). A number of elegant models have since been developed to mimic the brain damage that occurs after cardiac arrest in patients. One commonly used method is to induce cardiac arrest through electrical fibrillation (Böttiger *et al.*, 1997). This procedure has the advantage of creating complete circulatory arrest, causing both the brain and peripheral

organs to be ischemic, however mortality is common in this model as resuscitation is not always successful. Ischemia may be restricted to the brain by occluding all four of the major arteries supplying the brain (vertebral and carotid arteries, bilaterally). This model, known as 4-Vessel Occlusion ((Schmidt-Kastner and Hossmann, 1988); 4VO), is performed in two steps. First, the vertebral arteries are permanently occluded through bilateral electrocoagulation. The following day the carotid arteries are transiently occluded, most commonly for 10-15 minutes, thus producing complete brain ischemia. Alternatively, ischemia may be further restricted to only the forebrain by combining temporary carotid artery occlusion with systemic hypotension. For this procedure, known as the 2-Vessel Occlusion ((Smith *et al.*, 1984a); 2VO), hypotension is induced by either exsanguination (removal of blood) or by increasing the level of anesthetic during surgery (Bendel *et al.*, 2005a). Once mean arterial blood pressure is lowered below 40 mmHg, the carotid arteries are bilaterally occluded, thus producing transient forebrain ischemia.

1.3.5 Cell Death and neuronal atrophy

Global ischemia models are often described as producing selective neuronal death in the CA1 of the hippocampus (Bothe *et al.*, 1986; Ordy *et al.*, 1993), however, this is a slight misnomer, as a number of other regions within the brain show significant cell death. In the rat 2VO model, CA4 (hilus) neurons are actually the first to die after brief ischemia, while CA1 and neocortical neurons become susceptible with longer ischemic periods. The

granule neurons of the dentate gyrus and pyramidal neurons of the CA3 are the least susceptible populations within the hippocampus. Outside of the hippocampus, the small to medium sized neurons of the caudate putamen are the first to die after 8-10 minutes of ischemia, while the reticular nucleus of the thalamus is also consistently damaged (Kirino, 1982; Lipton, 1999). In light of such widespread neuronal death it is more accurate to state that CA1 neurons are *highly* vulnerable to global ischemia, a pattern that closely resembles neuronal death following cardiac arrest in humans (Zola-Morgan *et al.*, 1986).

In general, the majority of neuronal death in the CA1 occurs between 24-72 hr. post-ischemia (Kirino, 1982), however the maturation of ischemic injury can be modified by a number of factors. For example, brief ischemia produces incomplete CA1 neuronal depletion, and also delays neuronal death beyond 72 hrs after the insult (Colbourne *et al.*, 1999a). Certain pharmacological treatments, such as diazepam, also delay CA1 neuronal death; an effect that may be related to the slight hypothermia that is induced by most GABA agonists (Corbett *et al.*, 2008). Based on careful examination of ultrastructural features, the dominant mode of CA1 neuronal death is necrosis, however it may be possible that apoptotic processes are first initiated by the ischemia (Colbourne *et al.*, 1999b; Nitatori *et al.*, 1995).

In addition to neuronal death, global ischemia also alters synaptic connections within the hippocampus during the first few days after the injury.

Somewhat surprisingly, CA1 neurons undergo dendritic hypertrophy in the first 48 hr after ischemia, just prior to degeneration. This occurs through both an increase in the length of apical dendrites (Ruan *et al.*, 2006), as well as the thickness and density of postsynaptic contacts on CA1 neurons (Kovalenko *et al.*, 2006). The exact role of this hyperthrophy is unknown, however it is speculated that the ischemia-induced decrease in synaptic activity may facilitate this process, as brief periods of ischemic preconditioning, which does not induce cell death, also increases spine density on CA1 neurons (Corbett *et al.*, 2006). Taken together, these results indicate that although CA1 neurons undergo delayed neuronal death after global ischemia, the event triggers acute changes in neuronal structures thus altering hippocampal circuitry. Such changes should be considered when evaluating treatments initiated post-ischemia.

1.3.6 Behavioural deficits

Global ischemia in humans produces persistent memory impairments and an associated depletion of CA1 neurons (Zola-Morgan *et al.*, 1986). Although several rodent models can be used to induce similar CA1 depletion, the ensuing functional deficits are inconsistent among studies. For example, learning in the Morris Water Task is impaired in some studies (Green *et al.*, 1995; Hartman *et al.*, 2005; Voll *et al.*, 1989), but not others (Auer *et al.*, 1989; Gionet *et al.*, 1991). Such contradictory results are likely accounted for by differences in the post-ischemic period when the testing is performed,

task difficulty, and the level of CA1 injury at the time of testing. Other behavioural abnormalities, such as hyperactivity (Block, 1999) resolve within the first couple post-ischemic weeks, whereas some forms of spatial learning deficits persist for months after the injury (Colbourne and Corbett, 1995a; Langdon *et al.*, 2008). Although some have reported that CA1 pyramidal neuron regeneration may account for behavioural recovery in spatial navigation (Bendel *et al.*, 2005b), such claims remain controversial as most studies do not find CA1 neurogenesis. In contrast the behavioural recovery is likely supported by plasticity in remaining hippocampal circuits as well as functionally relevant cortical regions (discussed below). Increasing task difficulty enhances the chances of detecting lasting impairments, however rats may not be able to solve such tasks in the first week after global ischemia.

1.3.7 Global ischemia-induced plasticity

In addition to the early changes in CA1 neuronal morphology described above, global ischemia induces both degenerative and regenerative processes at later times. For example, layer 5 pyramidal neurons in the prefrontal cortex show a reduction in soma size as well as a decrease in dendritic length and spine density approximately 3 months after ischemia (García-Chávez *et al.*, 2008). In contrast, layer 3 pyramidal neurons of the sensorimotor cortex show a significant increase in the number of spines at a similar time (Akulinin *et al.*, 2004). The neuronal atrophy in prefrontal cortex likely

contributes to post-ischemic impairments in working memory, however the functional significance of the hypertrophy in sensory cortex is unclear. Marked synaptic reorganization also occurs within the hippocampus itself after global ischemia. Mossy fibers originating from granule dentate neurons sprout within the first post-ischemic week, forming new synapses on CA3 apical dendrites (Bernabeu and Sharp, 2000). Immunolabeling for synaptic proteins, such as synaptophysin and mint-1 can be used to indirectly estimate synapse number, and such measures have shown that the density of mossy fiber synapses is elevated by 3 days post-ischemia (Nishimura *et al.*, 2000). In addition, GAP-43, a protein mainly expressed during development, is also elevated in both hilar neurons and mossy fiber terminals after ischemia (Schmidt-Kastner *et al.*, 1997). These results suggest that ischemia stimulates the formation of new synapses through mechanisms that recapitulate developmental processes.

Another phenomenon, which mostly occurs during development but is enhanced after ischemia, is the generation of new neurons. Neurogenesis in the dentate gyrus is upregulated for approximately 12 days after global ischemia (Liu *et al.*, 1998), however the exact contribution of these newly added cells to hippocampal function remains largely unknown (Kee *et al.*, 2001). In fact, the majority of newly generated cells either differentiate into astrocytes or die within four weeks after ischemia (Sharp *et al.*, 2002), leaving a relatively small number of new neurons at long-term survival times. A likely mechanism mediating the enhanced cell genesis is the up-regulation

of growth factors such as BDNF and IGF-1 (Aberg *et al.*, 2007) in the ischemic hippocampus. In addition to enhancing neurogenesis, growth factors may also facilitate the synaptic reorganization discussed above.

1.4. Therapeutic hypothermia

1.4.1 Current Clinical Use

Based on a series of successful clinical trials in 2002, therapeutic hypothermia is now standard treatment for cardiac arrest patients as 12-24 hr of cooling (33˚C) increases survival in improves neurological outcome (Bernard *et al.*, 2002b; HACA, 2002). In addition, patients undergoing cardiac surgery with circulatory arrest are also cooled in order to minimize the brain damage that normally occurs during such surgeries (Bigelow *et al.*, 1950; Ramani, 2006). Given that written reports about the neurological benefits of hypothermia date back to the early nineteenth century (Polderman, 2004), it is somewhat surprising that a safe clinical application of the treatment was only developed relatively recently. The first scientific report describing the effects of hypothermia was published in 1945, based on a number of severe head injury patients who benefited from the treatment (Polderman, 2004). Larger clinical trials were then pursued in the 1960's and later (Rosomoff and Safar, 1965), however these studies used relatively deep hypothermia (30˚C or lower), resulting in serious side effects and complications in patient management. Due to these initial failures (Michenfelder and Milde, 1991; Michenfelder and Milde, 1992) the treatment remained abandoned until the

1980s, when animal studies showed that benefits could be attained with relatively mild hypothermia (32-35˚C), thus avoiding severe side effects (Colbourne *et al.*, 1997). These results induced a revival of hypothermia research with interests in applying the treatment to other forms of neurological injury including focal ischemia, hemorrhage, traumatic brain injury, spinal cord injury and neonatal hypoxic-ischemic encephalopathy. Clinical trials carried out so far have only shown definitive benefit for hypothermia in neonatal hypoxic-ischemic encephalopathy (Gluckman *et al.*, 2005), whereas a large clinical trial for traumatic brain injury was negative (Clifton *et al.*, 2001). Smaller trials for focal ischemia and hemorrhage have demonstrated treatment safety, but were not large enough to prove efficacy (Lyden *et al.*, 2005; Schwab *et al.*, 2001).

1.4.2 Efficacy in animal studies

The large body of literature that exist today describing the beneficial effects of hypothermia can be traced back to initial findings showing that intra-ischemic cooling in rodent models of global ischemia mitigates neuronal death in the hippocampus (Busto *et al.*, 1987b). Follow up assessments of delayed cooling found the treatment to be ineffective (Dietrich *et al.*, 1993a) as hypothermia initiated even with a 30 minute delay did not provide long-term neuroprotection (Busto *et al.*, 1989). It was later discovered that cooling duration is a critical parameter for providing long-term benefit, as longer treatments were beneficial even when initiated at a

delay (Carroll and Beek, 1992; Colbourne and Corbett, 1994b). In fact, hypothermia maintained for 48 hr provided long-term neuroprotection and functional benefit even when it was initiated 12 hr after ischemia (Colbourne *et al.*, 1999b). Therefore, hypothermia is highly neuroprotective following global ischemia, and the current challenge is to develop treatment protocols that are equally effective in other models of ischemic and heamorrhagic stroke.

In focal ischemia neuronal death occurs through similar mechanisms as after global ischemia, suggesting that therapeutic interventions may also have similar effects. Indeed, 2 days of systemic hypothermia decreased lesion volume and prevented behavioural impairments in rats that received a focal MCA occlusion (Colbourne *et al.*, 2000). A follow-up study demonstrated that treatment efficacy decreases with shorter cooling protocols, as both 12 and 24 hr of cooling were less effective relative to 48 hr of treatment (Clark *et al.*, 2008). This pattern of results matches findings from previous studies using brief cooling protocols, where little benefit was observed (Doerfler *et al.*, 2001; Moyer *et al.*, 1992). In contrast to ischemia, intracerebral hemorrhage is characterized by rapid cell death mainly caused by mechanical injury from the escaped blood (MacLellan *et al.*, 2010), however some secondary damage also occurs (Felberg *et al.*, 2002). A number of studies have found that hypothermia provides little benefit following such injury (MacLellan *et al.*, 2006; MacLellan *et al.*, 2009b) suggesting that both the timing and mechanisms of cell death are critical factors that influence hypothermic

neuroprotection.

In addition to systematically assessing the effects of various treatment durations, there is also significant interest in using animal models to compare various methods for inducing hypothermia. The majority of rodent studies induce whole-body cooling by either placing awake animals in cold rooms (Zhu *et al.*, 2005), or by simulating exposure with water misters and fans (DeBow and Colbourne, 2003a). An alternative approach is to only cool the brain, while leaving the rest of the body normothermic. This strategy offers the advantage of being less stressful for animals and also induces fewer confounding physiological changes such as elevated blood pressure, energy depletion and shivering, which may itself lead to further energy depletion (Polderman, 2009a). In a recent study, the efficacy of brain-selective cooling was compared to whole-body cooling in a model of focal ischemia. Although both cooling methods reduced lesion volume when treatment was maintained for 48 hr, only the whole body cooling method was neuroprotective after 12 hr of hypothermia (Clark *et al.*, 2009a). These findings suggest that greater benefit may be achieved with whole-body cooling, however a direct comparison between cooling protocols is problematic as brain-selective cooling induces temperature gradients within the brain which are not present during systemic cooling.

1.4.3 Mechanisms of hypothermic neuroprotection

The exact mechanisms by which therapeutic hypothermia provides

benefit after ischemia remains unknown. Although a decrease in brain metabolism certainly contributes to the beneficial effects, the level of protection offered by cooling is much greater than what would be predicted based on a decrease in metabolism alone (Milde, 1992). In fact, many studies have identified single components of the ischemic cascade that are altered by hypothermia. For example, activation of caspase enzymes is mitigated by hypothermia, thus diminishing apoptotic cell death (Xu *et al.*, 2002). Intracellular calcium accumulation, excessive glutamate release and loss of ionic balance across the cell membrane are additional pathological processes that are mitigated by hypothermia (Polderman, 2004). Lastly, the potent anti-inflammatory properties of cooling also provide significant benefit by inhibiting the release of inflammatory cytokines (Aibiki *et al.*, 1999) and decreasing immune cell proliferation at the site of injury (MacLellan *et al.*, 2006). It should be noted however, that monotherapies targeting individual mechanisms of damage are substantially less effective than hypothermia treatment. Based on this trend, T. Wieloch (personal communication) proposed that hypothermia is neuroprotective because it targets many of the components of the post-ischemic cascade simultaneously, therefore preventing cells from passing a threshold for irreversible cell damage.

It is important to note however, that not knowing the exact mechanisms of hypothermic neuroprotection should not hinder the progress of assessing the safety and efficacy of this treatment in clinically relevant disease models. In fact, systematic studies evaluating various hypothermia parameters may

provide insight into the beneficial mechanisms of the treatment.

1.5 General hypothesis and aims of thesis

The current experiments are aimed at filling a gap in our knowledge about the interaction between neuroprotective hypothermia and self-repair mechanisms after brain ischemia. These experiments will draw upon our current understanding of the plastic processes that are initiated by ischemia, as well as the irrefutable protection that is offered by therapeutic hypothermia. A model of global ischemia was chosen as the neuronal degeneration and plastic reorganization are well characterized and easily quantifiable. Our specific hypotheses were that: 1) hypothermia treatment will enhance any CA1 neurogenesis that may occur after global ischemia; 2) unilateral brain cooling will be neuroprotective in only the cooled hemisphere; 3) post-ischemic plasticity, but not neuroprotection may be negatively impacted by long-term hypothermia. Our endpoint measures included histological and behavioral outcomes at both long- and short-term survival times.

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Chapter 2

Therapeutic hypothermia influences cell genesis and survival in the rat hippocampus following global ischemia.

Gergely Silasi[1] MSc. and Frederick Colbourne[1,2] PhD.

[1] Centre for Neuroscience, University of Alberta, Edmonton, AB, Canada.

[2] Department of Psychology, University of Alberta, Edmonton, AB, Canada.

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2 Introduction

Patients resuscitated from cardiac arrest, and those who undergo cardiac surgery, are often left with permanent memory impairments (Newman *et al.*, 2001). These deficits arise in part from damage to the CA1 layer of the hippocampus, a structure highly vulnerable to ischemia (Pulsinelli *et al.*, 1982; Colbourne *et al.*, 1999a). Animal models of global ischemia show that CA1 neurons typically die 2–4 days later (Pulsinelli *et al.*, 1982), leaving animals with impaired memory and poorer spatial navigation ability (Auer *et al.*, 1989). Both the neuronal death and functional deficits are preventable with therapeutic hypothermia (MacLellan *et al.*, 2009), but efficacy declines with delays to cooling (Colbourne *et al.*, 1999b), an unfortunate clinical inevitability. This is markedly offset by increasing the duration of cooling. Indeed, brief cooling (e.g., 3 hr) provides little or transient benefit whereas prolonged hypothermia (e.g., 24 – 48 hr) provides robust, persistent protection even at greater intervention delays (Colbourne and Corbett, 1994; Colbourne *et al.*, 1999b). Importantly, clinical trials used similar hypothermia treatments (e.g., 24 hr at 33˚C) to significantly improve survival and neurological outcome in out-of–hospital cardiac arrest patients (Bernard *et al.*, 2002; Group, 2002). Furthermore, hypothermia is the only neuroprotectant to be successfully translated from animal ischemia models.

Despite successfully translating therapeutic hypothermia for cardiac arrest and hypoxic-ischemic encephalopathy (Shankaran *et al.*, 2005), there is a risk of central (CNS) side effects (e.g., impaired plasticity), especially with prolonged treatment. Indeed, many stroke targets have a biphasic role in pathophysiology

and recovery (Lo, 2008). For example, NMDA receptor activation is detrimental in the acute phase of ischemia, but delayed activation is required for enrichment-induced plasticity and neurogenesis (Lo, 2008). Accordingly, broad-spectrum treatments may then fail to improve clinical outcome.

Given the current clinical indications for therapeutic hypothermia and the considerable interest in using cooling for ischemic and hemorrhagic stroke, traumatic brain injury and spinal cord damage, we sought to evaluate whether cooling affects postischemic repair mechanisms, such as neurogenesis within the hippocampus (Bendel *et al.*, 2005; Salazar-Colocho *et al.*, 2008). We first hypothesized that cooling would attenuate the level of neurogenesis when significant neuroprotection occurred. Second, we considered the possibility that neurogenesis partly contributes to the 'neuroprotection' found in the CA1 sector, as can be the case for other neuroprotective strategies (Zhao *et al.*, 2006). In other words, hypothermia may rescue some neurons while simultaneously promoting neurogenesis within the CA1 zone. Postischemic hypothermia may influence CA1 cell genesis through several mechanism. First, inflammation inhibits neurogenesis (Monje *et al.*, 2003); therefore, the anti-inflammatory properties of hypothermia may enhance it. Second, postischemic hypothermia increases hippocampal levels of the neurotrophin BDNF and its receptor TrkB (Boris-Möller *et al.*, 1998), which promotes adult neurogenesis when activated (Scharfman *et al.*, 2005). Interestingly, some (Schmidt and Reymann, 2002; Bendel *et al.*, 2005; Salazar-Colocho *et al.*, 2008), but not all studies (Colbourne and Corbett, 1995; Colbourne *et al.*, 1999a; Tonchev and Yamashima, 2006; Yamashima *et al.*, 2007;

Langdon *et al.*, 2008), observe spontaneous postischemic regeneration of CA1 pyramidal neurons via the migration of progenitor cells from the lateral ventricles. Other reports showed that greater regeneration is possible with intracerebral growth factor infusions during the first postischemic week (Nakatomi *et al.*, 2002), further suggesting that treatment-induced regeneration of CA1 neurons is possible.

Ischemia also induces proliferation within the subgranular zone of the dentate gyrus (Liu *et al.*, 1998; Kee *et al.*, 2001; Sharp *et al.*, 2002), which likely contributes to spontaneous behavioral recovery. Interestingly, this effect occurs even when CA1 cell death is prevented by preconditioning (Liu *et al.*, 1998), indicating that large-scale cell death is not required for inducing neurogenesis. Pharmacological neuroprotectants, such as cilostazol (Lee *et al.*, 2009), can also alter postischemic neurogenesis. Despite such evidence, the influence of hypothermia on postischemic neurogenesis has been largely ignored. One exception is a recent study, showing that postischemic hypothermia did not alter neurogenesis in the dentate gyrus (Lasarzik *et al.*, 2009); however, rats were cooled for only 45 minutes, which was insufficient to provide neuroprotection and therefore has little pre-clinical relevance.

Currently, we quantified cell genesis in CA1 and dentate gyrus of rats that received global ischemia followed by prolonged (48 hr), systemic hypothermia. We chose the 2-vessel occlusion (2VO) model of forebrain ischemia because it consistently damages CA1 and causes measureable behavioural dysfunction (Langdon *et al.*, 2008). We used a proven cooling protocol that provides long-term neuroprotection (Colbourne and Corbett, 1995; Colbourne *et al.*, 1999b). In

experiment 1, we quantified the level of spontaneous regeneration in the 2VO model. In experiment 2, we evaluated neuroprotection and functional outcome when treatment was systematically delayed up to 24 hr after ischemia. We then used BrdU labeling to quantify cell genesis in the hippocampus during the third (experiment 2) and second (experiment 3) postischemic weeks.

2.1 Materials and Methods

2.1.1 Subjects

Experiments were performed on 147 male Sprague-Dawley rats (Biosciences breeding colony, University of Alberta) weighing ~300g at the time of surgery. The animals were group housed in cages of 4 (except during temperature regulation, explained below). Water and food were available *ad lib*. Surgical procedures were performed aseptically, and all procedures were in accordance with the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

Rats were assigned randomly to experimental groups prior to receiving a 2VO or sham surgery. Rats in experiment 1 (Fig. 2-1A) received 2VO ischemia and were killed either 14 (n=8) or 90 days (n=12) later. Experiment 2 contained 8 groups: sham+normothermic (n=8), sham+hypothermic (n=8), 2VO+normothermic (n=10), 2VO+intraischemic hypothermic (n=12), 2VO+1 hr delayed hypothermic (n=11), 2VO+4 hr delayed hypothermic (n=12), 2VO+12 hr delayed hypothermic (n=11), 2VO+24 hr delayed hypothermic (n=10).

Experiment 3 contained 4 groups: sham+normothermic (n=4), sham+hypothermic (n=4), 2VO+12 hr delayed hypothermic (n=24), 2VO+normothermic (n=16). All portions of these experiments, including behavioural assessment, histology and surgeries (except for intraischemic hypothermia) were performed with the experimenter blinded to treatment condition.

2.1.2 Temperature measurement and control

Core temperature was continually measured in rats by implanting a telemetry core probe (model TAT10TA-F40, Transoma Medical, St. Paul, MN) 4 days prior to ischemia as previously described. Temperature was recorded through a receiver underneath the rat's home cage and the data from 24 hr preceding the 2VO surgery served as baseline. Postoperative temperature was precisely controlled (within 0.5 ˚C of target) via a servo-regulated computer system controlling a series of fans and water misters for cooling, and an infrared heating lamp for warming. Rats were housed in individual cages for this portion of the experiment, and each cage was equipped with a temperature regulation system. We used an established cooling protocol (Colbourne *et al.*, 1999b) of lowering core temperature to 33 ˚C over 1 hr, and maintaining that temperature for 24 hr, followed by another 24 hr at 35 ˚C before finally being re-warmed to 36 ˚C (re-warmed over 1 hr and maintained for an additional 24 hr). This cooling protocol was initiated with either a 1, 4, 12 or 24 hr delay after the induction of 2VO ischemia (depending on group allocation), and in all cases the animals were

maintained normothermic for the delay period (Fig. 2-1B). Rats in the intraoperative hypothermia condition were placed on a water blanket through which chilled water was circulated, thus lowering body temperature. Ischemia was initiated when the desired temperature of 33 ˚C was reached. Following the completion of all temperature regulation the rats were returned to group cages (4 per cage) for the remainder of the experiment.

2.1.3 Global Ischemia Surgical Procedures

Forebrain ischemia was induced using the 2VO model (Smith *et al.*, 1984). Rats were fasted for ~18 hr prior to surgery in order to lower blood glucose levels into a consistent range (~6-10 mmol/L). Anesthesia was induced with 4% isoflourane (mixed in 60% N_2O, balanced O_2) and maintained at 2% during surgery. Core temperature was maintained at 37 ˚C through a rectal temperature probe connected to a warm water blanket (Gaymar TP3E, NY, USA). In order to avoid a drop in brain temperature during ischemia, skull temperature was regulated through a subcutaneous thermocouple probe (model: HYPO-33-1-T-G-60-SMG-M, Omega, Stanford, Conn.) connected to an overhead infrared lamp (150W) that was directed towards the head of the animal. The tail artery was cannulated for continuous measurement of mean arterial blood pressure (PressureMAT, Pendotech, Princeton, NJ.) and to collect small blood samples (100 µl) to be analyzed with a blood gas machine (Radiometer ABL 810, Radiometer, Copenhagen; see Table 2-1). The common carotid arteries were

isolated bilaterally, and the right jugular vein was isolated and cannulated with Silastic tubing connected to a heparinized syringe. Ischemia was induced by withdrawing blood into this syringe until MABP reached ~35 mmHg, at which point both common carotid arteries were occluded for 8 minutes using vascular clamps (00400-03, Fine Science Tools, Vancouver, Canada). Blood pressure was maintained at a target of 35 mmHg for the ischemic period, at the end of which the exsanguinated blood was slowly re-infused, the catheters were withdrawn and the neck and tail incisions were sutured. The sham procedure consisted of isolating all vessels as stated above, without withdrawing blood from the jugular vein or applying the vascular clips to the carotid arteries. Immediately after surgery rats were returned to the temperature regulation station where core temperature was recorded and regulated as stated above.

2.1.4 BrdU Administration

Rats received daily intraparitoneal injections of BrdU (100 mg/kg; Sigma, Oakville, ON, Canada) for 6 days starting either on postischemic day 14 (experiment 2) or postischemic day 8 (experiment 3). The BrdU was dissolved in warm, sterile saline at a concentration of 20 mg/mL, and the solution was prepared fresh prior to injection.

2.1.5 Behavioural assessment

In experiment 2, spatial learning and memory was assessed in the Morris Water Task (Auer *et al.*, 1989). A black swimming pool (1.5 m in diameter, 60 cm deep) was filled with water (21-22 ˚C) to 20 cm below the top of the wall. A clear Plexiglas platform was submerged 1.5 cm below the surface of the water, thus rendering it invisible from the rat's position. Rats were given four swim trials per day, with each trial starting from one of the four cardinal compass points along the edge of the pool. The first four testing days were performed with the platform maintained in the same location for each day. On the fifth day a probe trial was administered where the platform was removed and rats could search the pool for 30 seconds. The rats were then tested for 4 consecutive days on a moving platform version of the task where the platform was moved to a novel location within the pool on each testing day. All trials lasted a maximum of 90 s and the rats were allowed to stay on the platform for an additional 10 s. In cases when a rat did not find the platform, it was placed on it. Performance (including latency, distance traveled and swim speed) was recorded and analyzed through an overhead camera connected to a tracking system (Water 2020; HVS Image, Hampton, UK).

2.1.6 Histology

At the end of each experiment rats were transcardially perfused with PBS followed by 10% formalin. Extracted brains were post-fixed in formalin for ~24 hr, embedded in paraffin and sectioned at 6 μm on a microtome. Sections stained

with Hematoxylin and Eosin (H&E) were used to count remaining CA1 neurons. As routinely done (Colbourne and Corbett, 1995; Colbourne *et al.*, 1999b; Langdon *et al.*, 2008), the number of viable-looking neurons in the medial, middle and lateral sectors of CA1 (each 0.2 mm long) were counted on a light microscope at 3.7 mm posterior of Bregma. Cell numbers were summed across each region and brain hemisphere. Neighboring sections were used for immunolabeling in order to visualize cell genesis and to determine the phenotype of newly generated cells. Prior to incubation with the primary antibody, antigen retrieval was performed by boiling the sections in 0.1M citrate buffer (pH 6.3) for 15 minutes in a microwave. The primary antibodies used were Rat anti BrdU (1:1000; AbD Serotec, product: OBT0030), Mouse anti GFAP (1:400; Sigma, product: G 3893), Rabit anti Iba-1 (1:1000; Wako, product: 019-19741), Mouse anti NeuN (1:500; Chemicon, product: MAB377) and Rabbit anti Ki-67 (1:500; Vector, product: VP-K451). The secondary antibodies used were from Jackson Laboratories (West Grove, PA, USA), and were applied at the following concentrations: Donkey anti Rat Cy3 (1:500), Donkey anti Mouse 488 (1:500) and Donkey anti Rabbit 594 (1:500). All incubations were at room temperature, and DAPI (1:500; Sigma) was added during the final step of each procedure to visualize cell nuclei. Images were captured using an Olympus epifluorescent microscope (BX51) equipped with a CCD camera (Infinity 3; Ottawa, ON, Canada). When performing cell counts, the DAPI signal was used to confirm that the BrdU label co-localized with a cell nucleus.

2.1.7 Statistical Analyses

Comparisons were performed using either a one-way or repeated-measures ANOVA (SPSS v18 Mac). To identify group differences the Dunnett post-hoc test was performed where $p<0.05$ was considered significant. As there were no significant differences between the sham+hypo and sham+normo groups on any measures, subjects were collapsed into a single sham group.

2.2 Results

2.2.1 CA1 neurons did not spontaneously regenerate after ischemia

Previous studies suggest that CA1 neurons may spontaneously regenerate following global ischemia (Bendel *et al.*, 2005; Salazar-Colocho *et al.*, 2008). Therefore, we determined whether the neuronal depletion was permanent in our 2VO model. Rats were killed 14 or 90 days post-ischemia and the remaining intact-looking neurons were counted in H&E stained sections. Compared to sham animals (353 ± 7 cells; mean ± SEM), the number of CA1 neurons was significantly decreased in rats killed 14 (14 ± 4) or 90 days (33 ± 15) after ischemia. While both ischemia groups had far fewer cells than sham animals ($p<0.0001$), the difference between the ischemia groups was not significant ($p=0.529$).

2.2.2 Hypothermia delayed up to 12 hr after ischemia reduces CA1 injury

We systematically varied the delay of hypothermia initiation in a single experiment and assessed CA1 injury at a 6-week survival. Hypothermia initiated either during ischemia or after a delay of 1, 4 or 12 hr significantly reduced CA1 injury relative to normothermia (Fig. 2-2A; $p<0.0001$). The level of protection in the 2VO+24 hr delay hypo group was not significant, although there was a trend ($p=0.053$). Gross morphological characteristics of neurons from hypothermia treated rats were normal in terms of size and density (Fig. 2-2B, 2-2D), whereas in non-treated rats there was a near-complete absence of the CA1 cell layer (Fig. 2-2C).

2.3.3 Neuroprotective hypothermia did not induce CA1 neurogenesis

Other neuroprotectants stimulate CA1 neurogenesis (Zhao *et al.*, 2006); therefore, we investigated whether neurogenesis contributes to the apparent neuroprotective effect of hypothermia. We found that NeuN positive neurons in the CA1 region do not co-label with BrdU when it is administered during the third week after ischemia (Fig. 2-3A), thus indicating that neurogenesis does not occur during this period. Furthermore, we did not find any doublecortin positive cells migrating into CA1. However, the number of BrdU labeled nuclei was significantly increased in the 2VO+norm group relative to shams ($p<0.0001$). This increase was mitigated by neuroprotective hypothermia (intraischemic, 1hr, 4hr, 12hr delay), as all of these groups had significantly fewer BrdU labeled nuclei in CA1 relative to the 2VO+norm group ($p<0.0001$; Fig. 2-3B).

2.2.4 Neuroprotective hypothermia mitigates chronic, but not early generation of microglia in the CA1 zone

We next determined the phenotype of the newly generated cells after ischemia. Almost all of the BrdU positive nuclei co-localized with the microglial marker Iba (Fig. 2-3C). Quantification of the number of BrdU/Iba co-labeled cells showed that in groups with significant CA1 neuronal loss (2VO+norm, and 2VO+24 hr delayed hypo) there were significantly more BrdU labeled microglial cells relative to sham animals ($p=0.004$, $p=0.002$ respectively). The intraischemic, 1 hr, 4 hr and 12 hr delayed hypothermia groups were all significantly lower than the 2VO+norm group ($p<0.027$; Fig. 2-3D). Thus, following CA1 damage there is ongoing generation of microglial cells in the third postoperative week. Furthermore, hypothermia initiated up to 12 hr after the injury mitigates both the neuronal loss and chronic microglial proliferation.

In order to determine whether hypothermia treatment has a similar effect on cellular proliferation earlier after ischemic injury we performed an additional experiment where rats were injected with BrdU during the second postoperative week (experiment 3). Based on our findings from experiment 2 we restricted our analyses to a single hypothermia delay (12 hr), as this was both neuroprotective and it significantly reduced microglial proliferation. Hypothermia delayed for 12 hr was neuroprotective relative to normothermia ($p<0.0001$; Fig. 2-4A, B), replicating our results from experiment 2. There were no doublecortin positive

neurons migrating into the CA1 region and we did not find any BrdU/NeuN co-labeled cells in the CA1 (Fig. 2-4D). In addition, neither the number of BrdU labeled cells ($p=0.994$; Fig. 2-4C) nor the number of BrdU/Iba co-labeled cells ($p=0.638$; Fig. 2-4E, F) was significantly decreased by hypothermia. Taken together, our results show that hypothermia delayed for 12 hr after 2VO ischemia decreases the proliferation of microglial cells generated in the third, but not the second week after ischemic injury.

2.2.5 Hypothermia increased survival of newly-generated neurons in the dentate gyrus

Following ischemia, neurogenesis in the dentate gyrus is upregulated for the first 14 days after the injury; however, most newly formed cells die or differentiate into glial cells (Liu *et al.*, 1998). Importantly for our current experiment, ischemia-induced neurogenesis in the dentate gyrus occurs even when CA1 neurons are salvaged (Liu *et al.*, 1998). Thus, we determined whether hypothermia altered the survival of newly generated cells. In order to do this we quantified BrdU labeling in the dentate gyrus of rats receiving BrdU injections during the second postoperative week (experiment 3). Our results show that 4 weeks after ischemic injury there was a 60% increase in the number of BrdU/NeuN positive dentate gyrus neurons of rats in the 2VO+12 hr delayed hypo group relative to the 2VO+norm group ($p<0.0001$; Fig. 2-5A, C). The numerical decrease in the 2VO+norm group (relative to sham) was not significant

(p= 0.061). Ki67 labeling at the 4-week survival time indicated that the rate of cell genesis was not different among groups (p=0.414; Fig. 2-5B), suggesting that the increase in BrdU/NeuN co-labeled cells is not due to a prolonged increase in the rate of cell division.

2.2.6 Hypothermia improves functional outcome following global ischemia

Rats were tested in the Morris Water Task starting 5 weeks after ischemia to determine whether hypothermia influenced functional performance (experiment 2). During acquisition learning (fixed platform location) all groups learned the task and improved across testing days on measures of latency (Fig. 2-6A), and distance traveled (Fig. 2-6C) when finding the platform ($p<0.0001$). Averaging across all 4 training days, however, showed that the 2VO+norm group traveled farther (p=0.009; Fig. 2-6D), and also swam faster relative to the sham group (p=0.009; Fig. 2-6F), whereas the latency to find the platform was not different among groups (p=0.150; Fig. 2-6B). These results demonstrate a quantifiable but modest deficit in the non-treated group, as their behavioural pattern in solving the task was different from shams. In contrast, all hypothermia treated groups were indistinguishable from sham performance (p=0.976), indicating that the stroke-induced deficit was ameliorated by the treatment. Furthermore, all hypothermia treated groups, except for the 2VO+intraischemic hypo group (p=0.222) were also significantly better than the 2VO+norm group ($p<0.032$). Additional analyses of

performance on the probe trial as well as reversal learning (moving platform location) did not reveal any further group differences (data not shown).

2.3 Discussion

Our study, using the 2VO ischemia model, shows that prolonged mild hypothermia provides unparalleled CA1 protection with a broad therapeutic window. Contrary to our hypothesis, neurogenesis did not contribute to this neuroprotective effect in CA1. Thus, delayed hypothermia is a true and permanent neuroprotectant, at least when the treatment is prolonged. Furthermore, CA1 neurons did not spontaneously regenerate after untreated 2VO ischemia contrary to some previous work (Schmidt and Reymann, 2002; Bendel *et al.*, 2005; Salazar-Colocho *et al.*, 2008). Interestingly, hypothermia did increase the survival of newly formed neurons in the dentate gyrus, a mechanism possibly contributing to reduced functional impairments. Conversely, chronic microglial proliferation was attenuated by hypothermia possibly due to decreased neuronal death, which likely also contributes to improved behavioral performance. These findings strongly support the assertion that hypothermia, at least with the protocols tested, potently reduces ischemic brain injury while facilitating postischemic neuroplastic events such as neurogenesis.

Previous studies assume that neuroprotection is the primary mechanism by which hypothermia improves behavioral recovery after global ischemia. Our study, which is the first to examine hippocampal neurogenesis in the setting of

varying levels of hypothermic neuroprotection, strongly supports this assumption within CA1. However, ischemia stimulates neurogenesis in two regions neighboring CA1, the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles. Further, it is the cell migration from the lateral ventricles that is believed to underlie the limited spontaneous CA1 regeneration reported in some studies (Bendel *et al.*, 2005). Our results show that CA1 neurons do not spontaneously regenerate even when we used survival times matching studies showing regeneration (experiment 1; (Bendel *et al.*, 2005)). In addition, we found that regardless of treatment delay, hypothermia does not induce CA1 neurogenesis because we did not observe any BrdU labeled neurons, or doublecortin positive cells migrating into the CA1 region. Based on these observations we conclude that neuroprotection is indeed the only mechanism by which the CA1 layer is kept intact. We cannot explain why some studies report spontaneous regeneration whereas others do not, but it is important to note that the long-term outcome is similar across studies with newly-generated CA1 cells degenerating approximately 125 days post-injury (Bueters *et al.*, 2008). In contrast, hypothermia offers permanent protection for CA1 neurons (Colbourne and Corbett, 1995; MacLellan *et al.*, 2009).

Our investigation of neurogenesis in the dentate gyrus indicates that hypothermia increases the number of newly generated neurons that survive until 4 weeks post-ischemia. Many forms of injury, including global ischemia, transiently increase proliferation in the hippocampal subgranular zone (Kernie and Parent, 2010). Indeed, BrdU incorporation is increased for approximately 14 days after

global ischemia. However, most of these newly formed cells differentiate into astrocytes or die within the next 4 weeks (Kee *et al.*, 2001; Sharp *et al.*, 2002). Enhancing neuronal differentiation and survival through voluntary wheel running improves functional recovery after ischemia in rats (Luo *et al.*, 2007). Similarly, hypothermia treatment promotes increased survival of newly generated dentate gyrus neurons and improves functional performance. Perhaps this results from hypothermia preserving synaptic integrity within the hippocampus thereby allowing more of the newly generated neurons to establish synaptic connections and integrate into existing circuitry, resulting in increased survival. This interpretation also accounts for the numerical decrease in survival of BrdU labeled neurons in the 2VO+norm group (relative to shams), as the hostile environment following untreated ischemia does not promote neuronal maturation and survival. Unfortunately we are unable to determine whether the increased granule cell survival directly contributes to the improved functional outcome, as we would need to selectively ablate a portion of the newly formed cells to determine their contributions.

The behavioural performance of hypothermia-treated rats, studied previously using more sensitive tests (e.g., (Colbourne and Corbett, 1995)), further suggests that salvaging CA1 neurons preserves the functional circuitry of the hippocampus, thus eliminating any stroke-induced impairment. We found that untreated ischemic rats traveled significantly longer when locating a hidden platform in the Morris Water Task, whereas even the 2VO+12 hr delayed hypo group did not have such a deficit. Interestingly, the 24 hr delayed hypothermia

provided only a trend for neuroprotection but significant functional benefit. Perhaps the fact that ~50% of CA1 neurons remained in the 24 hr delayed hypothermia group was sufficient for successful spatial learning (Auer *et al.*, 1989). Unfortunately, the water maze protocol used is not sufficiently sensitive to hippocampal injury to conclude that variations in level of CA1 protection observed among hypothermia groups were equally beneficial. Extensive testing would be required to tease apart such differences, but this might have affected neurogenesis levels by acting as a rehabilitation treatment.

Our study is the first to demonstrate that microglial proliferation continues beyond three weeks after untreated global ischemia. Previous studies show that microglia in the hippocampus activate within minutes of reperfusion (Morioka *et al.*, 1991), and persist in CA1 for up to 9 months following global ischemia (Langdon *et al.*, 2008). The source of these microglia is still debated, as immune cells from the periphery (bone marrow) may infiltrate the brain and express microglial markers (Hanisch and Kettenmann, 2007), thus contributing to the delayed microglial reaction. Indeed, studies of focal ischemia show that the resident microglia, and not the infiltrating cells, are responsible for the initial activation and proliferation at the site of injury (Denes *et al.*, 2007). Furthermore, selectively ablating only the proliferating microglia increased infarct volume and neuronal death, suggesting that the initial (local) microglial response may be neuroprotective (Denes *et al.*, 2007). In experiment 2 we found that in all groups where hypothermia was neuroprotective the treatment also decreased chronic microglial proliferation during the third postischemic week. In contrast, microglial

proliferation during the second postischemic week was not decreased, although the treatment was still neuroprotective (experiment 3). Therefore, our results demonstrate that delayed hypothermia is neuroprotective and also mitigates the prolonged generation of microglia after global ischemia. However, the relationship between these two phenomena is not clear because hypothermia may independently and directly alter the immune response in addition to modifying it through decreased neuronal death. We suggest that hypothermia does not directly inhibit microglial proliferation, but rather, due to its neuroprotective properties, alters the cellular signaling that normally leads to prolonged microglial proliferation.

In sum, we demonstrate that CA1 neurogenesis does not contribute to the neuroprotective effect of hypothermia following global ischemia. However, cooling does attenuate the delayed generation of microglial cells in CA1, while enhancing the survival of newly generated neurons in the dentate gyrus. These finding emphasize the need to determine the effects of hypothermia beyond neuroprotection in additional stroke models in order to better understand the effects of the treatment on endogenous repair processes.

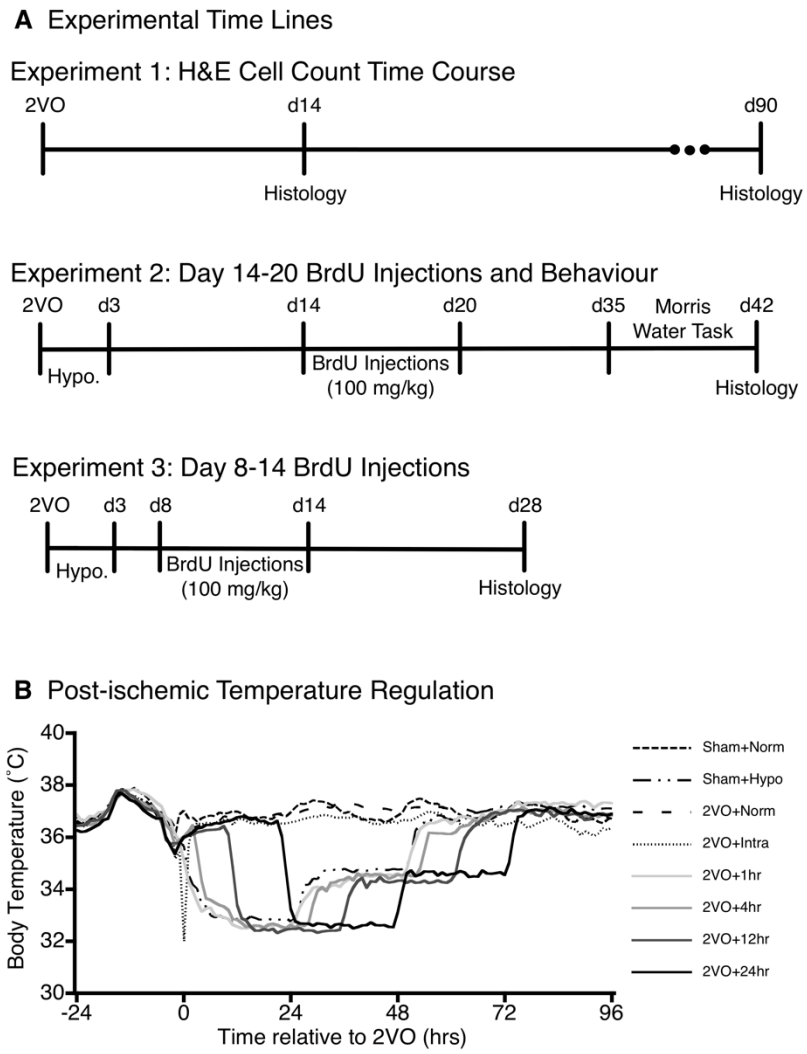

Fig. 2-1: Experimental timelines and body temperature measurements recorded by telemetry core probes. (A) Experiment 1 examined spontaneous regeneration of CA1 neurons after 2VO ischemia. Experiments 2 and 3 examined cell genesis when BrdU was injected during the third and second postischemic weeks, respectively. (B) Baseline temperature was recorded during the 24 hr preceding 2VO, whereas postischemic temperature was precisely regulated (within 0.5 ˚C) according to group allocation. Temperature recordings were collected continually every 30 seconds, and averaged over 60 minutes, to generate each data point.

Table 2-1: Physiological variables during surgery were not different among groups, except for inra-ischemic temperature, which was purposefully lowered in the 2VO+intra group. * indicates significantly different from 2VO+norm group.

Group	Temp.	MABP	pH	pCO2	pO2	Glucose	ctHb
Sham	36.6±0.2	84±3.8	7.36±0.008	41.9±1.8	128.4±3.6	6.9±0.3	16.0±0.1
2VO+Norm	36.7±0.1	34±0.2	7.36±0.007	41.0±0.8	131.7±1.8	10.3±2.2	16.2±0.1
2VO+Intra	32.0±0.3*	34±0.5	7.41±0.083	48.2±1.5	134.1±4.8	8.1±0.6	16.1±0.3
2VO+1hr	36.5±0.2	35±0.4	7.38±0.012	40.2±1.1	131.9±2.8	7.1±0.4	15.7±0.3
2VO+4hr	36.8±0.1	34±0.2	7.37±0.012	41.6±1.6	139.8±3.1	7.1±0.4	15.7±0.2
2VO+12hr	36.8±0.1	35±0.2	7.35±0.008	43.3±1.2	128.9±2.1	8.1±0.2	1601±0.1
2VO+24hr	36.8±0.1	34±0.2	7.35±0.007	44.0±1.2	130.1±2.9	7.5±0.3	15.8±0.2

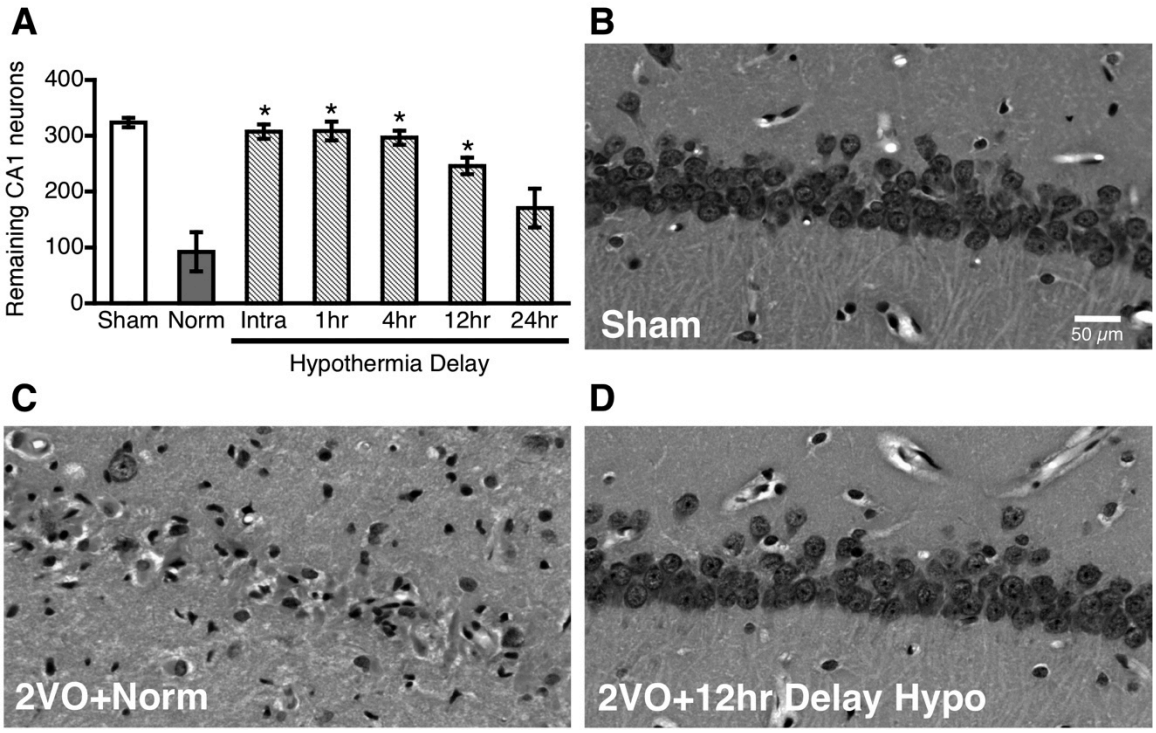

Fig. 2-2: Neuroprotective efficacy diminishes with hypothermia delay (experiment 2). (A) Normothermic ischemia significantly decreased the number of remaining neurons 6 weeks post-ischemia, relative to sham surgery. Hypothermia initiated either intra-ischemically or at a delay of 1, 4 or 12 hr was significantly neuroprotective. The 2VO+24 hr delay hypo group showed a trend for benefit, but was not significantly different from 2VO+norm ($p = 0.053$). H&E staining showed a severe depletion of CA1 pyramidal neurons in the 2VO+norm group (C) relative to shams (B). The general morphology of the pyramidal cell layer in the hypothermia treated groups (D) was not different from shams (B). * indicates significantly different from 2VO+norm group; mean ± SEM.

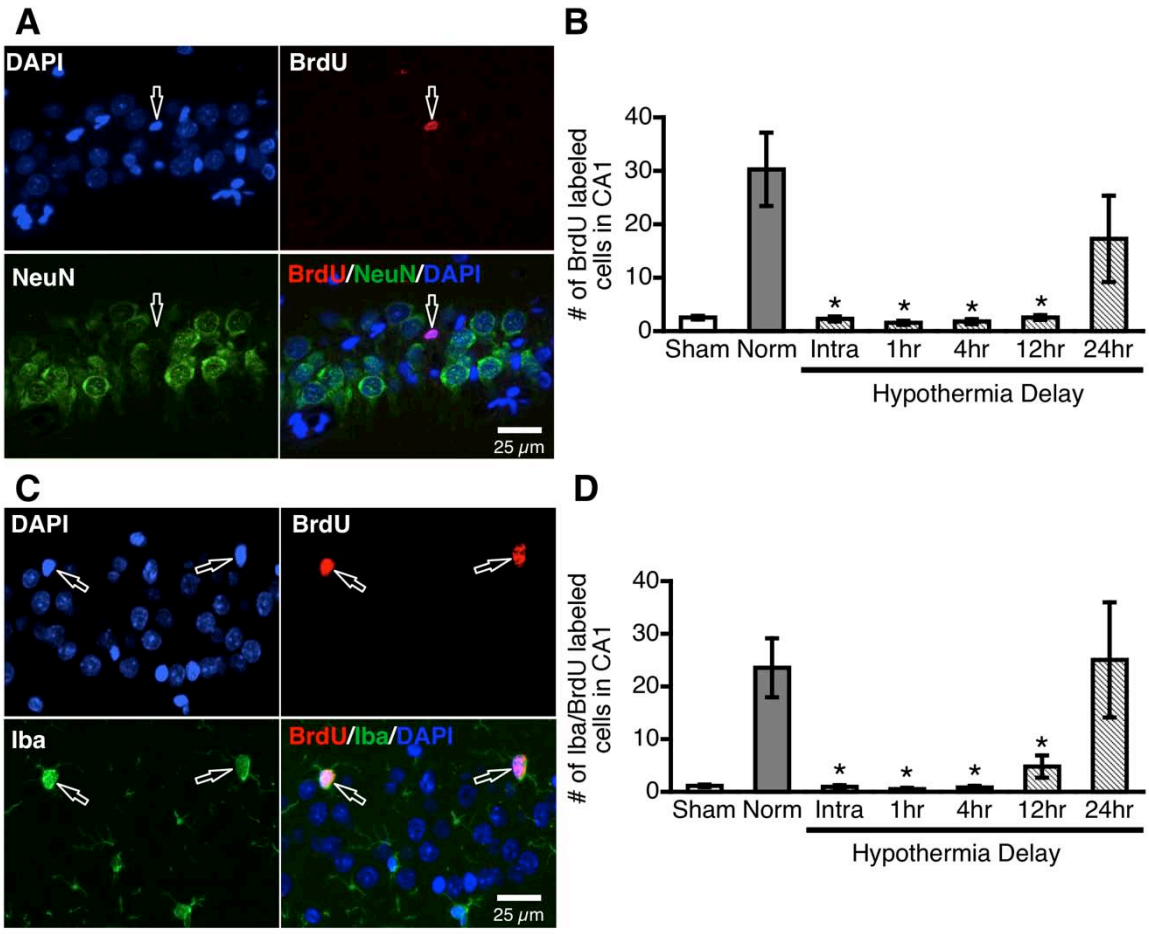

Fig. 2-3: Neuroprotective hypothermia does not induce CA1 neurogenesis, but decreases chronic microglial proliferation (experiment 2). Normothermic ischemia significantly increased the number of BrdU labeled nuclei, while neuroprotective hypothermia (intraischemic, 1, 4 and 12 hr delay) mitigated this effect (B). BrdU did not co-localize with the neuronal marker NeuN in any of the groups (A), indicating that neurogenesis does not contribute to the neuroprotective effect of hypothermia. The majority of BrdU labeled CA1 cells co-labeled with the microglial marker Iba (C), and neuroprotective hypothermia decreased the number of BrdU/Iba co-labeled cells (D). * indicates significantly different from 2VO+norm group; mean ± SEM.

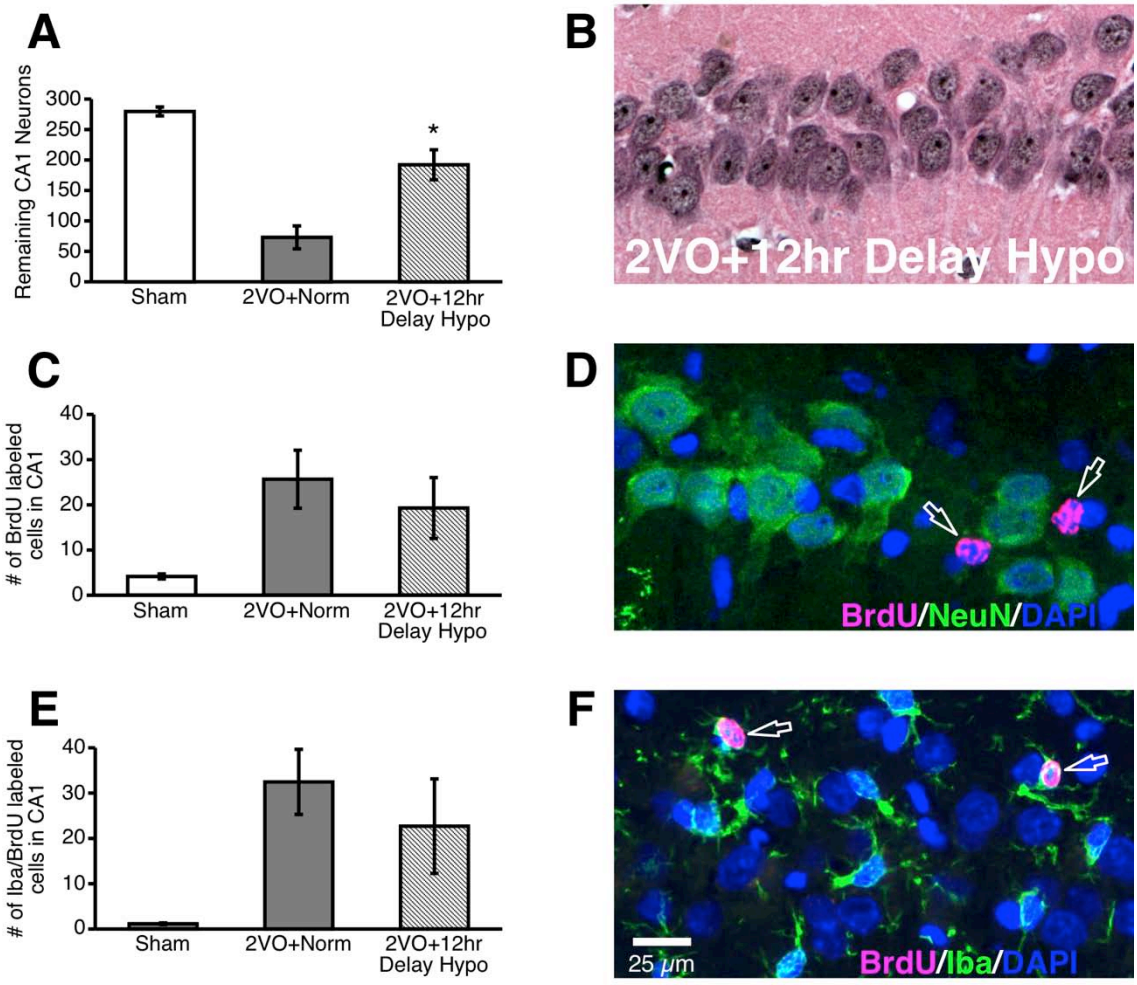

Fig. 2-4: Neuroprotective hypothermia does not decrease microglial proliferation during the second post-ischemic week (experiment 3). H&E cell counts confirmed that hypothermia delayed for 12 hr was neuroprotective (A, B). The number of BrdU labeled cells in the CA1 was not influenced by hypothermia (C), and BrdU labeled cells did not co-localize with the neuronal marker NeuN (D; arrows). There was also no difference in the number of proliferating microglia between the 2VO+norm and 2VO+12 hr delayed hypo groups (E, F). Arrows indicate BrdU/Iba co-labeled cells from a rat in the 2VO+12 hr delayed hypo group. * indicates significantly different from 2VO+norm group; mean ± SEM.

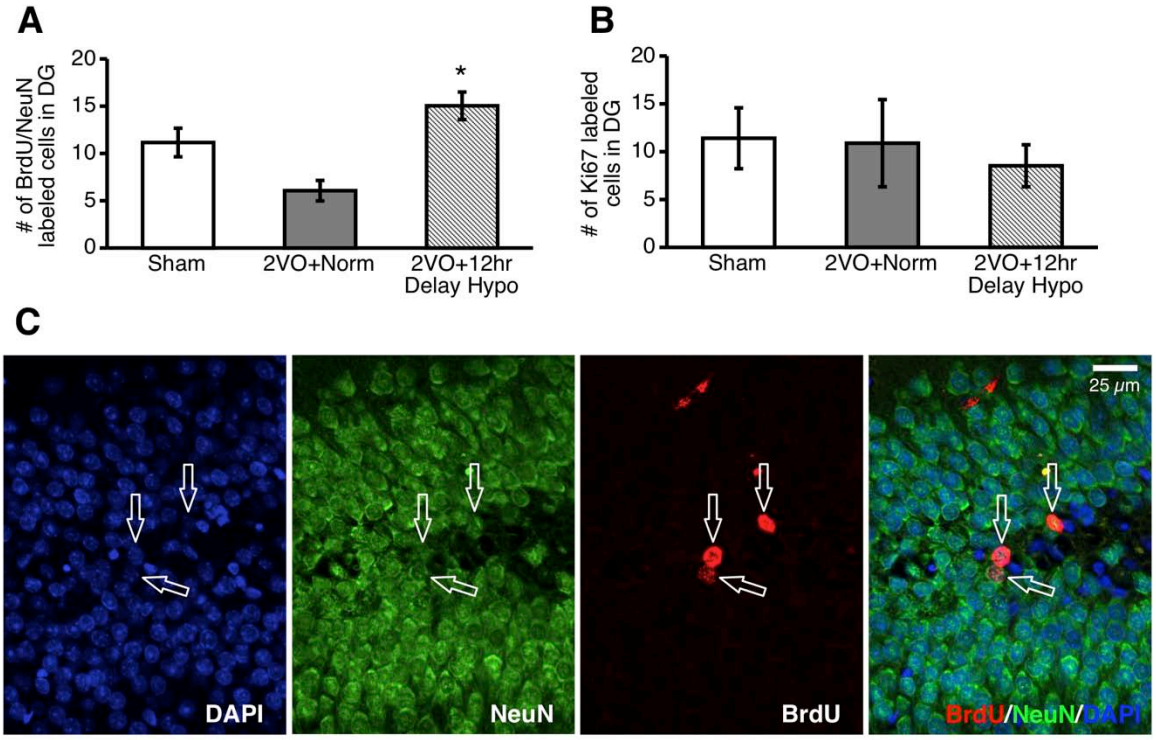

Fig. 2-5: Neuroprotective hypothermia increases the survival of newly generated neurons in the dentate gyrus (experiment 3). The 2VO+12 hr delayed hypo group had significantly more BrdU/NeuN co-labeled cells 4 weeks post-ischemia relative to the 2VO+norm group (A). The difference between the 2VO+norm and sham groups was not significant. Ki67 labeling at 4 weeks post-ischemia indicated that the rate of cell division was not different among groups (B). Arrows indicate BrdU/NeuN co-labeled cells in a rat from the 2VO+12 hr delayed hypo group (C). * indicates significantly different from 2VO+norm group; mean ± SEM.

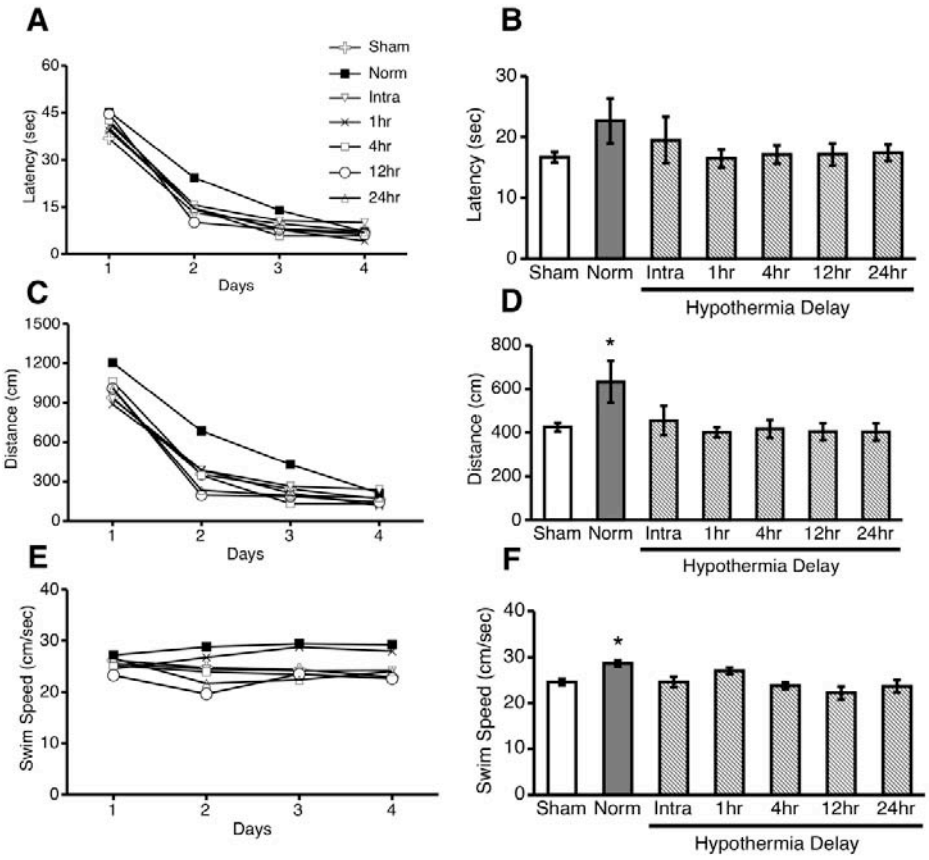

Fig. 2-6: Untreated 2VO ischemia produces a slight behavioural impairment in the Morris Water Task (experiment 2). During the 4 days of testing all groups learned the task, as both the latency (A) and distance traveled (C) decreased across testing days when finding a hidden platform. Swim speed did not differ across days (E). The mean distance traveled across all four testing days was significantly longer in the 2VO+norm group relative to shams. Furthermore, all hypothermia treated groups, except for the 2VO+intraischemic hypo group were significantly better than the 2VO+norm group (D). All other hypothermia treated groups performed at sham levels. The 2VO+norm group also swam significantly faster than the sham group (F), therefore mean latency was not significantly different from shams (B). * indicates significantly different from sham; mean ± SEM.

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Chapter 3

Unilateral brain hypothermia as a method to examine efficacy and mechanisms of neuroprotection against global ischemia.

Gergely Silasi, M.Sc.[1] and Frederick Colbourne, Ph.D.[1,2]

[1]Centre for Neuroscience, University of Alberta, Edmonton, AB, Canada.

[2]Department of Psychology, University of Alberta, Edmonton, AB, Canada.

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Therapeutic Hypothermia and Temperature Management.

3. Introduction

Rodent models of global ischemia are commonly used to study the mechanisms of ischemic damage and to evaluate neuroprotective treatments. These models can closely reproduce some aspects of the brain damage seen in patients resuscitated from cardiac arrest (Traystman, 2003). For instance, following brief ischemia there is a relatively selective depletion of hippocampal CA1 pyramidal neurons with associated memory impairments. One successful treatment developed in part from using rodent models of global ischemia is prolonged hypothermia, now used to treat some cardiac arrest patients (Bernard *et al.*, 2002a; The Hypothermia After Cardiac Arrest Study Group, 2002). Importantly, earlier work in rodents showed that mild hypothermia was not only neuroprotective when applied during ischemia (Busto *et al.*, 1987a), but also when initiated after ischemia provided that the duration of cooling was prolonged (Colbourne and Corbett, 1994a).

Many studies have examined mechanisms by which hypothermia provides benefit because it is the gold standard of neuroprotection (Polderman, 2009b; Yenari *et al.*, 2008; Zhao *et al.*, 2007). From this work it is hoped that more selective and effective therapies will emerge, and that such treatments will cause fewer side effects than hypothermia. Currently, cardiac arrest patients are cooled systemically (surface blankets, endovascular cooling, etc.), which can cause several serious side effects such as heart arrhythmias and increasing the risk of pneumonia (Polderman, 2009b). Similarity, clinical efforts in ischemic and hemorrhagic stroke patients typically use systemic cooling, which increases risk

of such side effects. One potential solution, which is more suited for stroke than for cardiac arrest, is to use localized brain hypothermia to avoid systemic complications and potentially to accelerate the rate of cooling. While this is easily achieved in rodents under anesthesia (Nurse and Corbett, 1994b; Taniguchi *et al.*, 2005b), clinical efforts with devices such as cooling helmets (Mellergard, 1992; Wang *et al.*, 2004) have yet to achieve only a modest level of cooling owing to several factors such as cranial thickness and a robust vascular supply to the scalp. As progress is being made on these and other methods (King *et al.*, 2010; Konstas *et al.*, 2007), it is appropriate to evaluate the efficacy and mechanisms of local brain hypothermia in rodent stroke models. Unfortunately, most studies examining mechanisms of action use either intra-ischemic or brief postischemic hypothermia as these are easily induced in rodents. However, when treatment is delayed, a clinical inevitability, prolonged cooling (e.g., 24 – 48 hr) is often required for substantial and permanent protection in models of global and focal ischemia (MacLellan *et al.*, 2009a). Furthermore, the mechanisms by which cooling mitigates injury likely vary with time since stroke (e.g., metabolic perturbations vs. delayed inflammation). Thus, it makes sense to use prolonged postischemic cooling to study mechanisms of injury as some have done (Colbourne *et al.*, 2003; D'Cruz *et al.*, 2002a; Florian *et al.*, 2008).

A number of methods have been used to induce and maintain prolonged hypothermia in rodents, including cold rooms (Yanamoto *et al.*, 1999), fans and water spray (Colbourne *et al.*, 1996), and various drugs (Florian *et al.*, 2008). Each of these methods has limitations and they all induce systemic cooling. Thus,

Clark and Colbourne developed a simple system for inducing focal brain cooling in fully awake rats, which avoids systemic side effects such as changes in blood pressure and the need for anesthesia while allowing for prolonged cooling (Clark and Colbourne, 2007b). The method involves placing a metal coil beneath the Temporalis muscle and adjacent to the side of skull overlying the ischemic region. The coil is cooled by flushing it with cold water while the rat is awake and mobile. Using this technique, we showed that several days of local (hemispheric) hypothermia reduces injury in a rat model of permanent middle cerebral artery occlusion (Clark *et al.*, 2009b). Some benefit was also obtained in a rat model of intracerebral hemorrhage (Fingas *et al.*, 2007).

The ability of prolonged focal brain cooling to persistently reduce hippocampal CA1 injury after global ischemia has not been evaluated. Given that this model is commonly used to study mechanisms of cell death relevant to cardiac arrest and ischemic stroke, as well as for evaluating other neuroprotectants it would be instructive to determine the effects of focal brain cooling on this form of ischemic injury. Thus, the method of Clark and Colbourne, originally developed for focal ischemia and intracerebral hemorrhage models that damage the cortex and striatum, was adapted to focally cool the hippocampus and overlying cortex in one hemisphere while the contralateral hemisphere remained normothermic. We then evaluated whether focal cooling would reduce CA1 sector injury when applied for 2 days starting 1 hr after global ischemia. We hypothesized that focal cooling would result in permanent unilateral protection, which was observed. Thus, this simple, inexpensive method is applicable for

studying hypothermic neuroprotection after global ischemia. Importantly, this approach has the advantage of providing an internal control, the contralateral, normothermic hemisphere, which makes this method highly suited to studying mechanisms of action of prolonged cooling after global ischemia.

3.1 Methods

3.1.2 Subjects and Experimental Design

Experiments were performed on 34 male Sprague-Dawley rats (Biosciences breeding colony, University of Alberta) weighing ~300 g at the time of surgery. Water and food were available *ad libitum*, except during fasting prior to ischemia. Surgical procedures were performed aseptically. All procedures were in accordance with the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

Three experiments were done. First, we determined brain temperature during focal cooling in both anesthetized and awake rats (n=5). A second experiment compared the extent of histological damage at a 1-week survival after global ischemia in normothermic rats (NORMO, n=7) versus those with focal hypothermia (HYPO, n=7). Third, we extended this experiment to include both histological and behavioral endpoints at a 1 month survival time (NORMO: n=7; HYPO: n=8). The use of a long survival time is critically important because certain hypothermia treatments only postpone injury (Dietrich *et al.*, 1993b). In

the last 2 experiments, postischemic cooling was initiated after a 1 hr delay and was maintained for 48 hr followed by gradual re-warming over 6 hr.

3.1.2 Experiment 1: Brain Temperature Measurements

A published method (Clark and Colbourne, 2007b) for focal brain cooling was modified for use in the current experiments. Instead of using the cooling coil developed in that study, we flattened a stainless steel tube (18 G) to a thickness of 1 mm and a width of 2 mm. A 6 mm length was used as it would overly the hippocampal formation. Silastic tubing (VWR Scientific, Canada, Product # 7-5224) was then attached to each end of the strip in a tight manner not allowing for water leakage (Fig. 3-1A). The dorsal (vs. lateral) placement of the cooling device is simpler and quicker to implant than the device used in our original study.

In order to measure brain temperature at multiple points simultaneously, rats were anesthetized with isoflurane anesthesia (4% induction, 2% maintenance in 60% O_2, balance N_2O) and prepared for surgery by injecting 0.2 mL of Marcaine (Sanofi Canada, Markham, OT, Canada) subcutaneously on the skull, and shaving the top of the head. After placement in a stereotaxic frame, a midline scalp incision was made and the skull was cleared of connective tissue. Two anchor screws were threaded into the parietal bone approximately 2 mm lateral from the midline. The cooling strip was then positioned on the skull surface (Fig. 1B) adjacent to the right temporal ridge. Dental cement was applied over the anchor screws, the metal strip and a small initial length of tubing to secure the

device in place. Additional burr holes were made to allow needle-type thermocouple probes (model HYPO-33-1-T-G-60-SMG-M, Omega, Stanford, Conn.) to be stereotaxically placed into the anterior (-3.8 A-P; 1.5 M-L) and posterior (-5.3 A-P; 2.5 M-L) hippocampus of the right hemisphere as well as the contralateral hippocampus (-4.5 A-P; -1.5 M-L). Rectal temperature was maintained at 37 °C through a servo regulated warm water blanket. Following baseline temperature recordings from all 3 brain probes focal brain cooling was initiated by pumping chilled water through the cooling strip. The flow rate (115 mL/hr) matched the flow rate that would be used in subsequent experiments.

An additional two rats were used to measure hippocampal and body temperature during hypothermia in awake animals. First, a core temperature telemetry probe (model TAT10TA-F40, Transoma Medical, St. Paul, MN) was surgically implanted into the abdominal cavity as previously described (DeBow and Colbourne, 2003b). Four days later, the rats were prepared for a second surgery to implant the focal brain cooling strip and a brain temperature telemetry probe. The initial steps of this surgery were identical to above, however after the placement of the cooling strip on the skull, a single burr hole was drilled over the anterior hippocampus (-3.8 A-P; 2.5 M-L) to accommodate a 5.0 mm cannula (20 G) through which the shaft (8.0 mm long) of a telemetry thermocouple probe (XM-FH-BP, Mini-Mitter Co. Inc, Sun River, OR, USA) was lowered 3.0 mm into the brain. In order to protect the probe's electronics, it was encased in a plastic cylinder, which was secured to the skull with dental cement and three anchor screws. The silastic tubes connected to the

cooling strip were passed through a flexible spring sheath (model CIH95; 30 cm long, Instech Laboratories, Plymouth Meeting, PA, USA) to protect them from damage by the rat. Additional dental cement was applied to the head assembly to secure an initial segment of the metal sheath to the plastic cylinder containing the thermocouple. Once the dental cement had dried, anesthesia was discontinued and the rat was returned to its home cage. The metal sheath and the silastic tubes were connected to an overhead swivel (model 375/D/22, Instech Laboratories) attached to a counterbalance arm (CM375BS, Instech Laboratories) mounted on top of the rat's cage, as previously described (Clark and Colbourne, 2007b). This allowed rats to move about their cage. Brain temperature was measured continually by placing the home cage on a receiver (RPC-1, Transoma Medical) connected to a computer (ART software, v. 2.1, Transoma Medical). Signal interference from the core and brain probes prevented us from measuring both temperatures simultaneously (DeBow and Colbourne, 2003b); therefore, the core probes were only activated for short durations before, during and after focal cooling whereas brain temperature was recorded at all other times.

3.1.3 Experiment 2: Global Ischemia with 1 Week Survival

We did not measure brain temperature in this experiment in order to 1) avoid complicating and prolonging the surgical procedures, and 2) to avoid the potential loss of our brain telemetry probes, which are no longer commercially manufactured.

Three days after surgical implantation of the cooling device the rats were food deprived for ~18 hrs to lower blood glucose levels into a consistent range (~6-10 mmol/L) prior to ischemia surgery. Ischemia was induced for 10 min using the 2-vessel occlusion (2VO) model, which involved bilateral carotid artery occlusion combined with systemic hypotension to 35 mmHg (Smith *et al.*, 1984b). The later was achieved through exsanguination via the jugular vein. During surgery, core temperature was maintained at 37 °C through a rectal temperature probe connected to a warm water blanket. A 100-µL arterial blood sample was collected for blood gas analysis (Radiometer ABL 810, Radiometer, Copenhagen), which was within the normal range (PO_2: 125 – 135 mmHg; PCO_2: 35.0 – 45.0 mmHg, pH: 7.350 – 7.450; data now shown). Skull temperature, measured with a subcutaneous thermocouple probe, was also maintained at normothermia (~37°C) via an overhead infrared lamp (150W). Immediately after surgery, rats were returned to their home cage and were randomly assigned to either the NORMO or 2 day HYPO treatment starting 1 hr after ischemia. Hypothermia was induced by passing ice cold water, via the overhead swivel, through the cooling device at which point the water was ~10 °C. Rats were free to move about their cage during cooling.

At 7 days postischemia, the rats were transcardially perfused with phosphate buffered saline followed by 10% formalin. Extracted brains were post-fixed in formalin for ~24 hrs, embedded in paraffin and sectioned at 10 µm. Sections, stained with Hematoxylin and Eosin (H&E), were used to quantify the number of remaining neurons in the CA1 field of the hippocampus. Briefly, the

number of viable-looking neurons in the medial, middle and lateral sectors of CA1 (each 0.2 mm long) were counted on a light microscope at 3.7 mm posterior of Bregma (Paxinos and Watson, 1998), as previously done and illustrated (Colbourne and Corbett, 1995b). Cell numbers were summed across each region within a hemisphere. Neighboring sections were used for immunolabeling with a Rabbit anti Iba-1 primary antibody (1:1000; Wako, product: 019-19741), and Donkey anti Rabbit 594 secondary antibody (Jackson Laboratories; West Grove, PA, USA). Prior to incubation with the primary antibody, antigen retrieval was performed by boiling the sections in 0.1M citrate buffer (pH 6.3) for 15 minutes in a microwave (Tang *et al.*, 2007). All incubations were at room temperature and DAPI (1:500; Sigma) was added during incubation with the secondary antibody to visualize cell nuclei. In separate sections, Fluoro-Jade B labeling (Schmued and Hopkins, 2000) was carried out to quantify the number of degenerating neurons within the CA1 region, which was expected to provide similar data to the H&E staining. Fluoro-Jade B and Iba-1 positive cells were quantified within the CA1 pyramidal cell layer in the same regions as H&E cells counts (medial, middle and lateral sectors).

3.1.4 Experiment 3: 2VO Ischemia with 1 Month Survival

Procedures were identical to experiment 2 except that rats survived for 1 month to determine whether hypothermic neuroprotection was permanent. As well rats were tested on a modified version of the water maze task (Driscoll *et al.*,

2006) from 19 to 30 days postischemia. For the first 2 days the platform remained in the same position within the pool. For the next two days it was moved to a new location and so on for a total of 12 days of testing. Each rat was given 8 swim trials per day with each trial starting from one of the 4 cardinal compass points along the edge of the pool. A trial lasted a maximum of 90 s and the rats were allowed to stay on the platform for an additional 10 s. In cases when a rat did not find the platform it was physically placed on the platform by the experimenter. Performance (including latency, swim speed, and distance traveled) was tracked and analyzed through an overhead camera connected to a computerized tracking system.

3.1.5 Statistical Analyses

For histological measures, group differences were initially assessed using 2-way between subjects ANOVAs (SPSS v18 Mac). Treatment (HYPO vs. NORMO) and hemispheres (left vs. right) were the factors. While hemisphere may be considered a repeated measures factor, we used the more conservative approach. Due to significant interactions in these 2-way analyses, we further examined the data with 1-way ANOVAs for treatment and hemisphere comparisons. Behavioral data were analyzed using repeated-measures ANOVA. A p value of less than 0.05 was considered statistically significant.

3.2 Results

There were no exclusions or mortality in these experiments.

3.2.1 Experiment 1: Brain Temperature Measurement

Upon initiation of cooling in anesthetized rats, the ipsilateral hippocampal temperature was lowered to ~32 °C, whereas the contralateral side remained normothermic (Fig. 3-1C). Temperature then quickly stabilized around 31.6 ± 0.04 °C (mean ± SEM). This pattern was replicated in awake animals, as initiation of cooling quickly lowered hippocampal temperature to ~33.2 ± 0.05˚C (mean ± SEM), which was measured with a telemetry probe, and remained in that range for the entire cooling period (48 hr; Fig. 3-1D). Body (core) temperature, which was sampled intermittently via telemetry before, during and after hemispheric cooling remained unchanged and normothermic. Importantly, we did not observe any adverse behavioural effects of the cooling, as animals often remained engaged in the same behaviors (e.g., grooming, eating) during cooling initiation as just prior to it.

3.2.2 Experiment 2: 1 Week Outcome

The 2-way ANOVAs for H&E, Fluoro Jade B and Iba cell quantification data at a 1 week survival all revealed significant interactions ($p \leq 0.043$) and thus we compared treatments with 1-way ANOVAs. Untreated ischemia caused severe CA1 injury (Figure 3-2A and 3-3A), and as expected, there was no difference

between hemispheres within the NORMO group ($p > 0.299$). The HYPO treatment (vs. NORMO) significantly reduced cell loss as measured with H&E ($p < 0.001$, Figure 3-2A) and Fluoro Jade B ($p < 0.001$, Figure 3-3A) staining in the right hemisphere. This effect was also seen in comparing the cooled (right) hemisphere with the normothermic (left) hemisphere in the HYPO group, which were significantly different ($p = 0.014$). As expected, there was no difference ($p > 0.269$) between groups for the left hemisphere because these were normothermic in both groups. Microglial proliferation was also mitigated in the right hemisphere in the HYPO group ($p < 0.001$, Figure 3-4A) relative to NORMO. Surprisingly, there was also a significant decrease of microglial labeling in the left hemisphere in the HYPO group; however, the decrease was significantly greater in the right hemisphere ($p = 0.001$).

3.2.3 Experiment 3: 1 Month Outcome

Similar to our findings at 1 week, focal cooling increased the number of surviving H&E stained CA1 neurons in the cooled hemisphere ($p = 0.026$ vs. normothermic side), whereas the hemispheres in the NORMO group were not significantly different ($p = 0.345$, Figure 3-2B) and showed marked CA1 injury. Furthermore, only the cooled hemisphere had significantly more remaining neurons in the HYPO group ($p < 0.001$), whereas the group comparison for the normothermic hemisphere was not significant ($p < 0.252$). Fluoro Jade labeling of CA1 neurons was reduced in the cooled hemisphere of the HYPO ($p < 0.044$),

whereas there was no difference between hemispheres in the NORMO group ($p < 0.844$, Figure 3-3B). A comparison of the hemispheres across groups showed that only the cooled one had a decrease in the number of Fluoro Jade labeled CA1 cells ($p = 0.001$). In contrast to our results at 1 week, the effects of HYPO on microglia proliferation were restricted to the cooled hemisphere, as HYPO caused a significant decrease in the number of microglia in only the cooled hemisphere ($p < 0.001$, Figure 3-4B). The difference between the left and right hemispheres was only significant in the HYPO group ($p < 0.041$).

Both groups showed significant learning in the water maze task (Figure 3-5), as the latency to find the platform decreased over testing days ($p < 0.001$); however the Group × Day interaction was not significant ($p < 0.718$), indicating that the groups were not different. Additional measures of performance (distance traveled and swim speed) also indicated no significant group differences ($p \geq 0.673$).

3.3 Discussion

Our findings demonstrate that focal brain hypothermia can persistently attenuate CA1 sector injury after global ischemia in rat. Protection was observed within the cooled hemisphere, but not in the normothermic side. Selectively cooling one hemisphere would likely have significant advantages over systemic and perhaps even whole-brain hypothermia for ischemic and intracerebral hemorrhagic stroke. Although unilateral treatment is not an ideal strategy for

global ischemic insults because brain injury occurs bilaterally, patients may nonetheless benefit from brain-targeted cooling when combined with milder systemic cooling. Regardless, we are not advocating that this particular approach or this method be used clinically. Instead, there are meaningful advantages to this line of investigation in animal studies. First, the technique is inexpensive and does not require costly equipment for measuring and regulating temperature (e.g., telemetry probes). Second, the technique is simple, requiring only a quick stereotaxic surgery to implant the cooling device, which is easily made. Third, with this method one can safely cool for prolonged periods, which is required for robust and enduring protection (MacLellan *et al.*, 2009a) and similar to current clinical protocols (Polderman, 2008; Polderman, 2009b). Furthermore, focal cooling does not require sedatives, anesthetics or other drugs that may confound results especially in rodents where one is hampered by their small size and a limited ability to sample physiological variables after ischemia (e.g., blood gases). Finally, cooling one hemisphere provides for an internal control - the normothermic hemisphere. Unilateral treatment is a proven strategy to test specificity (e.g., unilateral septo-hippocampal deafferentation - (Buchan and Pulsinelli, 1990)).

As noted, one might argue that protection limited to one hemisphere is an important disadvantage of this method for global ischemia research. Certainly, with respect to evaluating behavioral outcome, the fact that only half of the hippocampus is salvaged, at best, means that it may be difficult to observe behavioral protection. In this study, we did not see any significant group effect on

water maze scores. Nonetheless, it certainly remains possible (likely) that a more demanding water maze protocol or alternative tests would detect a partial neuroprotective effect (Langdon *et al.*, 2010). Alternatively, one may conduct *in vivo* or *in vitro* electrophysiological studies to demonstrate functional preservation as previously done with systemic cooling after global ischemia (Dong *et al.*, 2001a). Such an approach would also benefit from having an internal control.

One limitation of this study is that we did not formally evaluate behavior during focal brain cooling (due to tethering to cooling lines), which is also not practical with systemic cooling methods. We did observe that rats appeared normal during cooling without overt signs of distress, which is consistent with physiological measurements taken with telemetry that show normal heart rate and blood pressure during focal cooling (Clark and Colbourne, 2007b). This is certainly not the case during systemic cooling (MacLellan *et al.*, 2004). Finally, continued treatment with analgesics, beyond that required to manage post-surgical pain (e.g., local infiltration of Marcaine during surgery), does not seem necessary with this method.

Another consideration, based upon previous findings (Clark and Colbourne, 2007b), is the occurrence of temperature gradients underneath the cooling device with cortical regions being cooled more than subcortical structures. Thus, it can be very difficult to directly compare focal and systemic hypothermia to determine the more efficacious treatment. Nonetheless, the present findings in global ischemia and our work in focal ischemia (Clark *et al.*, 2009b) and hemorrhage (Fingas *et al.*, 2007) show that both strategies are beneficial. As well,

it is important to note that the systemic complications of whole body cooling, such as elevating blood pressure by cooling with fans and water spray (MacLellan *et al.*, 2004), do not entirely account for treatment efficacy in ischemia models. Finally, it should be noted that by varying the water temperature or the flow rate to the cooling device one could manipulate the level of hypothermia achieved (Clark and Colbourne, 2007b) as well as changing cooling and re-warming rates. Such research is essential in order to determine the optimal treatment parameters and the most effective cooling method to bring forth into clinical trials.

Our first experiment in this study and the previous methods paper shows that unilateral cooling is achieved with little to no effect on the contralateral side. The lack of histological protection on the left (normothermic) side in the HYPO group further supports this claim. However, at 1 week we did find a moderate but statistically significant reduction in ischemia-induced microglia activation contralateral to cooling. Our experiments do not explain this effect, which may be due to a very modest cooling in the contralateral side. This pattern of results fits a previous study, where a hypothermia protocol insufficient for reducing neuronal death still mitigated microglial proliferation in CA1 (Drabek *et al.*, 2009). Our study with a longer survival time did not find any effects of hypothermia in the normothermic hemisphere, indicating that the reduction in microglia is transient.

We observed slightly more CA1 neurons remaining in the hypothermia treated group at the longer survival time (4 weeks in experiment 3 vs. 1 week in experiment 2). Given that these are separate experiments, this difference is likely due to chance. However, it is also possible that our “normal-looking” criterion for

counting CA1 neurons may slightly underestimate cell counts at early survival times if some of the abnormal-looking cells eventually recover. The increased number of CA1 neurons at the longer survival time is not likely due to proliferation because we recently found that CA1 neurogenesis does not occur after untreated ischemia or in systemically cooled rats in this model (Silasi and Colbourne, 2011a).

3.3.1 Conclusion

Prolonged focal cooling persistently reduces CA1 sector injury after 2VO ischemia roughly similar to that observed with systemic treatments in this and other models (MacLellan *et al.*, 2009a; van der Worp *et al.*, 2007). These results support the development and use of effective brain-selective cooling technologies for patients suffering stroke and related insults. Finally, our method to unilaterally cool the brain in global ischemia models in order to provide protection on only one side offers several methodological advantages that make this approach highly suited to further efficacy and mechanistic studies.

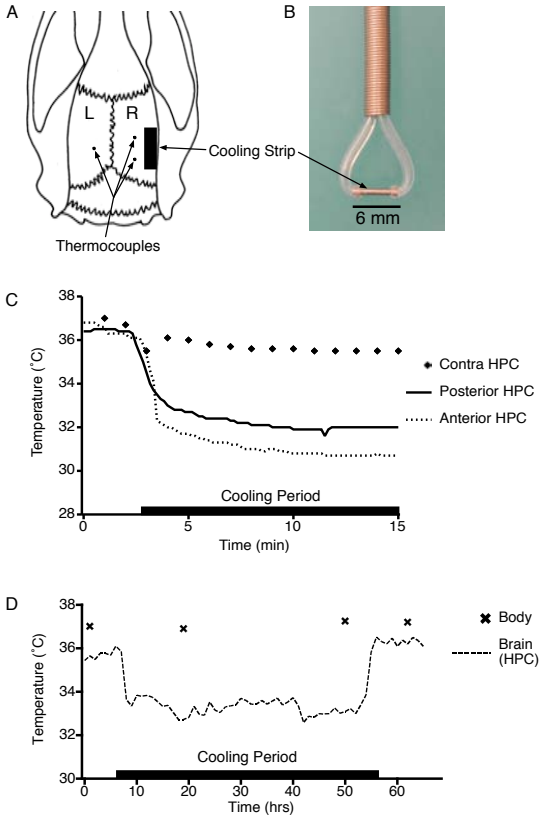

Figure 3-1: A) Illustration, modified from (Zilles, 1985), showing placement of cooling strip on the skull surface overlying the right hemisphere. Thermocouples were placed as indicated for temperature recordings shown in C. B) Photo of the cooling strip with attached tubing and protective sheath. C) Brain temperature recordings during focal brain cooling in anesthetized rats (HPC = hippocampus). Body temperature was normothermic throughout (data not shown). D) Brain and body temperature recordings (via telemetry) of an awake rat demonstrates that hypothermia persistently decreases hippocampal temperature during the cooling period, while leaving body temperature unaltered. Due to signal interference between the body and brain probes, body temperature was sampled intermittently. Otherwise, brain temperature was sampled every 30 seconds and averaged for this illustration.

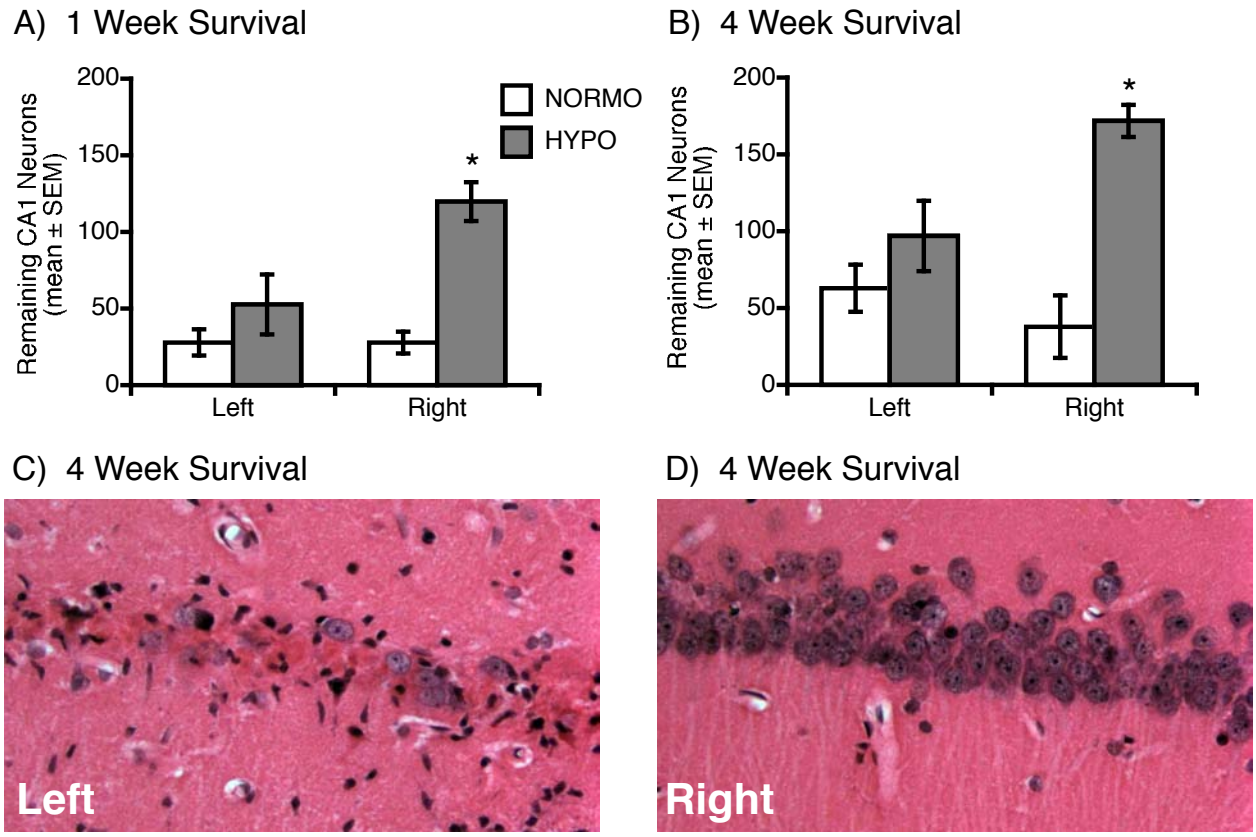

Figure 3-2: Average number of remaining healthy-looking (non-eosinophillic) CA1 sector neurons at 1 week (A) and 1 month (B) postischemia as measured in H&E stained sections. Photomicrographs of the CA1 sector at a month survival in a representative HYPO treated animal showing the left (C) and right (D) side. The CA1 zone is better preserved on the treated side of HYPO rats, whereas the contralateral (left) side is severely damaged with only a few remaining CA1 neurons. Both sides are severely damaged in NORMO rats. An * denotes significant difference compared to the right side of the same (NORMO) group.

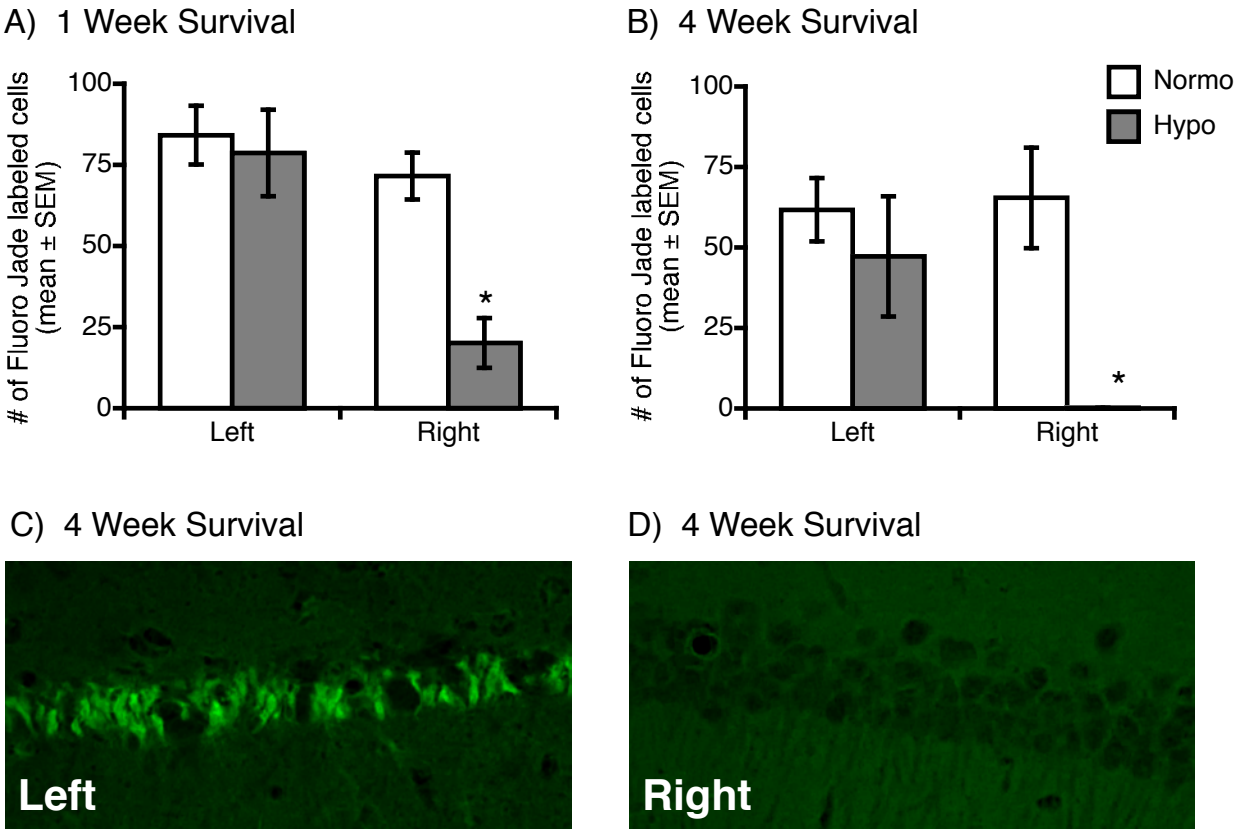

Figure 3-3: Number of Fluoro Jade positive (degenerating) neurons at 1 week (A) and 1 month (B) postischemia. The right, cooled side (C) and its contralateral, normothermic side (D) are shown for a representative HYPO animal at a 1-month survival. Note the bright band of Fluoro Jade positive cells in the CA1 zone of the normothermic side. An * denotes a significant difference compared to right side of the same (NORMO) group.

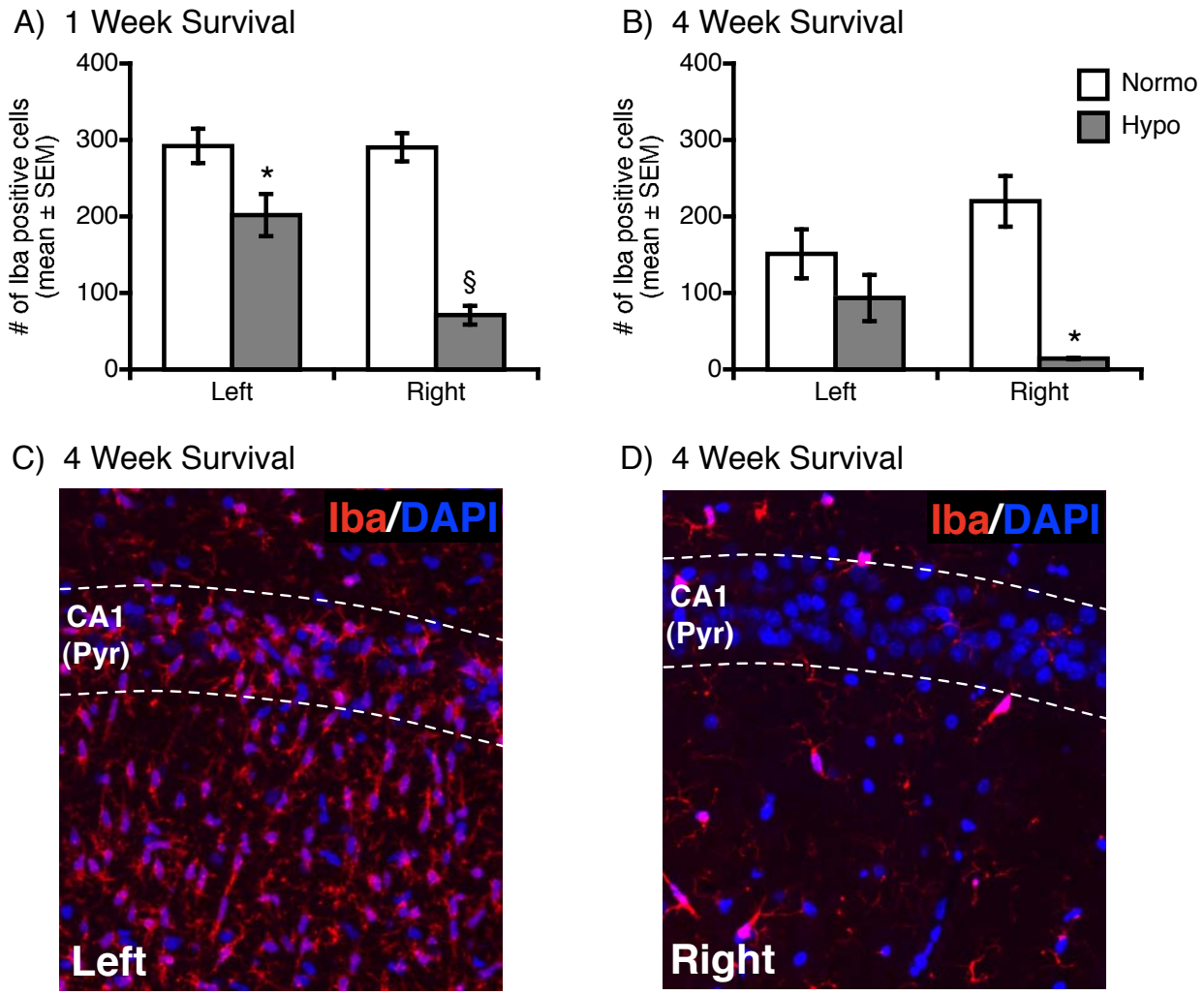

Figure 3-4: Number of Iba positive cells (microglia) at 1 week (A) and 1 month (B) postischemia. Hypothermia treatment significantly reduced this as illustrated in (D), which depicts the cooled hemisphere (Pyr = pyramidal cell layer). By contrast, the normothermic side has extensive microglial infiltration (C). An * denotes $p < 0.05$ vs. the right side of the same (NORMO) group whereas § denotes $p < 0.05$ vs. the right side of the HYPO group.

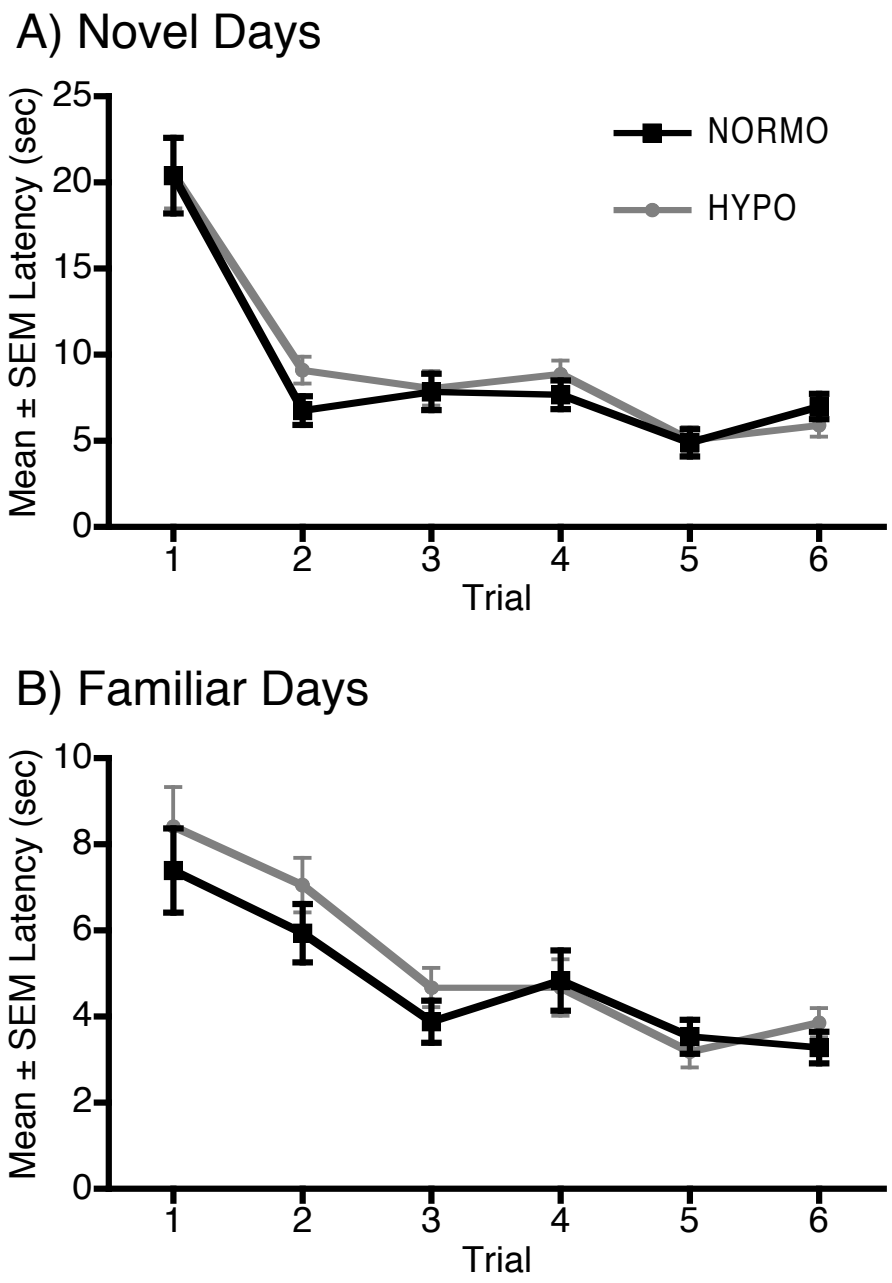

Figure 3-5: Average latencies to find the hidden platform in the water maze task for novel (A) and familiar days (B). Groups improved over time in both cases, but were not significantly different from each other. Thus, this test was not able to detect the unilateral neuroprotective effects of focal cooling.

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Chapter 4

Long-term unilateral brain cooling is neuroprotective, and does not adversely impact post-ischemic plasticity after global ischemia.

Gergely Silasi[1] MSc. and Frederick Colbourne[1,2] PhD.

[1] Centre for Neuroscience, University of Alberta, Edmonton, AB, Canada.

[2] Department of Psychology, University of Alberta, Edmonton, AB, Canada.

4. Introduction

Strategies for improving outcome following stroke or ischemia can be generally categorized into two separate efforts. First, reducing acute brain damage through neuroprotection, and second, initiating or promoting repair and recovery processes that facilitate functional improvement. This dichotomy exists partly due to the fact that neuroprotective treatments are most effective acutely after ischemia, whereas treatments that promote plasticity and regeneration are generally beneficial in the post-acute phase (days to months). It is becoming clear however, that in some cases ischemic neurodegeneration continues for days (and perhaps weeks) (Colbourne *et al.*, 1999a; Dirnagl *et al.*, 1999), whereas some forms of plasticity are initiated within minutes of the ischemic event (Sigler *et al.*, 2009). This results in a partial overlap of degenerative and regenerative processes (see Fig. 4-1A; (Lo, 2008)). Neuroprotective treatments applied in the early period following ischemia will be most effective if they selectively target neurodegenerative processes while allowing or facilitating spontaneous plasticity and regeneration.

Therapeutic hypothermia is a proven neuroprotectant in multiple models of stroke and ischemia (MacLellan *et al.*, 2009b); however in order to achieve permanent protection the treatment must be prolonged. For example, following global ischemia, 24 hrs of mild hypothermia provides permanent and substantial neuroprotection (Colbourne and Corbett, 1995a).

Importantly, a similar treatment protocol also improved functional outcome in out-of-hospital cardiac arrest patients (Bernard *et al.*, 2002b; HACA, 2002). Other forms of brain injury, such as stroke, focal ischemia, intracerebral hemorrhage or subarachnoid hemorrhage may require significantly longer cooling durations as the anti-inflammatory properties of hypothermia may be used to combat edema and inflammation (Kollmar *et al.*, 2010), which can persist for weeks (Marion and Bullock, 2009). For this reason some stroke trials are now investigating the efficacy of cooling patients for a week or longer (Gasser *et al.*, 2003), but we do not yet fully understand how such prolonged hypothermia affects endogenous repair processes that initiate within days following stroke.

Animal models of global ischemia provide a convenient system to study the effects of varying cooling duration, as the time course of degenerative (CA1 cell death) and regenerative (e.g. mossy fiber sprouting) processes have been well described. Specifically, the majority of CA1 neurons within the hippocampus die on the second and third post-ischemic days (Pulsinelli *et al.*, 1982), whereas mossy fiber sprouting is elevated on the third post-ischemic day and remains elevated until at least 7 days post-ischemia (Koh *et al.*, 1996; Nishimura *et al.*, 2000; Schmidt-Kastner *et al.*, 1997). The distribution of the trace element zinc is also influenced by ischemia, as it is involved in both neuronal death (Suh *et al.*, 2000; Suh *et al.*, 2006), and post-stroke plasticity (Nakashima and Dyck, 2009). Lastly, ischemia markedly elevates the level of neurogenesis in the subgranular zone

of the dentate gyrus (Liu *et al.*, 1998). Using this broad range of outcome measures we chose to examine how various cooling durations affect neuroprotection as well as post-ischemic plasticity, including neurogenesis, after global ischemia. Given that ~48 hr of hypothermia results in near complete neuroprotection (Colbourne and Corbett, 1995a), it may be that maintaining the treatment outside this window will actually counteract some of its neuroprotective effects. Furthermore, prolonged treatment may inhibit the expression of plasticity related proteins, which facilitate synaptic reorganization as well as neurogenesis within the dentate gyrus.

In order to examine these possibilities we used a focal cooling system (Clark and Colbourne, 2007a; Silasi and Colbourne, 2011c) that allows us to cool one hemisphere of the brain in fully awake rats. Following 2-vessel occlusion (2VO) forebrain ischemia, we maintained hypothermia for various durations (up to 7 days post-ischemia) and quantified the level of neuroprotection, the expression of plasticity-related proteins (BDNF, synaptophysin), microglial infiltration within the CA1 sector, and neurogenesis in the subgranular zone of the dentate gyrus. In a follow-up experiment, we used synchrotron Rapid Scanning X-Ray Fluorescence (RS-XRF) imaging to visualize the distribution of the trace element zinc in the hippocampus.

4.1 Methods

4.1.1 Subjects and experimental design

Experiments were performed on 69 male Sprague-Dawley rats (Biosciences breeding colony, University of Alberta) weighing ~300g at the time of ischemia. Water and food were available *ad libitum*, except during fasting prior to ischemia. Surgical procedures were performed aseptically. All procedures were in accordance with the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

In the first experiment, all rats (n=53) received 2VO ischemia followed by either sham treatment (Norm; n=19), or focal brain cooling during post-ischemic days 1 and 2 (Hyp 1-2; n=8), 3 and 4 (Hyp 3-4; n=9), 1 through 4 (Hyp 1-4; n=8), or 1 through 7 (Hyp 1-7; n=9; see Fig. 4-1B). Cooling was initiated 1 hr after the start of ischemia (for groups that start treatment on day 1) and, with the exception of the Hyp 1-7 group, rats were gradually re-warmed over 6 hr at the end of the cooling period. Rats in the Hyp 1-7 group were killed prior to the rewarming phase of the treatment.

For our second experiment, 12 rats received 2VO ischemia followed by either 2 days of hypothermia treatment (Hyp 1-2; N=6) or sham treatment (Norm; n=6). Half of the animals from each group were killed 12 hr after ischemia, and the other half were killed on post-ischemic day 7. An additional 4 naïve, weight-matched rats were included in the experiment to visualize basal zinc distribution without ischemia.

4.1.2 Global Ischemia and focal brain cooling

Rats (~300g) were surgically implanted with a focal cooling plate over the right hemisphere according to the procedures described in Chapter 3. As brain temperature was not measured in these rats, there was no guide cannula or plastic cylinder included in the head assembly. Instead, the cooling strip was positioned next to the temporal ridge, 2 anchor screws were threaded into the parietal bone, and dental cement was applied generously to secure the plate in place.

Rats were given 4 days to habituate to the tethering system before receiving forebrain ischemia using the 2VO model (Smith *et al.*, 1984a). Rats were fasted for ~18 hr prior to surgery in order to lower blood glucose levels into a consistent range (~6-10 mmol/L). Anesthesia was induced with 4% isoflourane (mixed in 60% N_2O, balanced O_2) and maintained at 2% during surgery. Core temperature was maintained at 37 ˚C through a rectal temperature probe connected to a warm water blanket (Gaymar TP3E, NY, USA). In order to avoid a drop in brain temperature during ischemia, skull temperature was regulated through a subcutaneous thermocouple probe (model: HYPO-33-1-T-G-60-SMG-M, Omega, Stanford, Conn.) connected to an overhead infrared lamp (150W) that was directed towards the head of the animal. The tail artery was cannulated for continuous measurement of mean arterial blood pressure (PressureMAT, Pendotech, Princeton, NJ.) and to collect small blood samples (100 µl) to be analyzed with a blood gas machine (Radiometer ABL 810, Radiometer, Copenhagen) for pH, PO_2, PCO_2 and glucose. The common carotid arteries were isolated bilaterally, and the right jugular vein was isolated and cannulated with

Silastic tubing connected to a heparinized syringe. Ischemia was induced by withdrawing blood into this syringe until MABP reached ~35 mmHg, at which point both common carotid arteries were occluded for 8 minutes using vascular clamps (00400-03, Fine Science Tools, Vancouver, Canada). Blood pressure was maintained at a target of 35 mmHg for the ischemic period, at the end of which the exsanguinated blood was slowly re-infused, the catheters were withdrawn and the neck and tail incisions were sutured. The sham procedure consisted of isolating all vessels as stated above, without withdrawing blood from the jugular vein or applying the vascular clips to the carotid arteries.

4.1.3 Tissue collection and Histology

4.1.3.1 Experiment 1:

One week after global ischemia, rats were administered an overdose of sodium pentobarbital and perfused transcardially with phosphate-buffered saline, followed by 10% formalin. The brains were removed and post-fixed in formalin for ~24 hr before being embedded in paraffin and sectioned at 10 μm on a rotary microtome. Hematoxylin and eosin (H&E) was used to stain one series of sections, while neighboring sections were used for immunolabeling (Silasi and Colbourne, 2011b). Prior to incubation with the primary antibody, antigen retrieval was performed by boiling the sections in 0.1M citrate buffer (pH 6.3) for 15 minutes in a microwave. Immunolabeling was performed by incubating the sections overnight in antibodies for microglia (Rabbit anti Iba-1; 1:1000; Wako, product: 019-19741), Synaptophysin (Mouse anti Synaptophysin; 1:200; Millipore, clone

SY38), BDNF (Rabbit anti BDNF; 1:500; Abcam, ab72439), doublecortin (Goat anti DCX; 1:500; Santa Cruz, C-18), and the proliferative marker Ki67 (Rabbit anti Ki-67; 1:500; Vector, product: VP-K451). The secondary antibodies used were from Jackson Laboratories (West Grove, PA, USA), and were applied at a concentration of 1:500 for 2 hr at room temperature. DAPI (1:500; Sigma) was added during the final step of each procedure to visualize cell nuclei.

4.1.3.2 Experiment 2:

Either 12 hr or 7 days after ischemia rats were anesthetized with isofluorane and decapitated. The brains were rapidly removed, frozen in chilled isopentane and stored in a -80˚ C freezer until sectioning. Cryostat sections (14 μm) were collected on glass slides for H&E staining and on Ultralene foil (EMS, Hatfield, PA, USA) for RS-XRF imaging.

4.1.4 Cell counts and densitometry analysis

In order to quantify the number of remaining intact neurons within the CA1 field, H&E stained sections were examined under a light microscope (40X). Viable looking neurons were quantified in 3 regions of interest within the CA1 cell layer as previously reported (Colbourne and Corbett, 1995a). The number Iba-1 labeled microglia were also counted in the same regions of interest under an epifluorescent microscope (Olympus model BX51). To determine if hypothermia altered neurogenesis in the dentate gyrus, the total number of DCX and Ki67 double-labeled cells was quantified in one section from each brain. Synaptophysin and BDNF expression was quantified in the mossy fiber bundle in CA3 by

performing densitometry analysis (Photoshop CS5, Macintosh) on digital images captured from an epifluorescent microscope equipped with a CCD camera (Infinity 3; Ottawa, ON, Canada). Densitometry measurements were averaged from two non-adjacent sections approximately 3.8 mm posterior from Bregma (Paxinos, 2004).

4.1.5 Rapid-scanning X-ray fluorescence synchrotron imaging

Elemental analysis was performed at the Stanford Synchrotron Radiation Lightsouce (SSRL) beamline 10-2. The energy of the exciting beam was 13 keV, and a capillary was used to focus the beam spot to 35 μm. The dwell time was 600 ms and scans progressed at a 40 μm step size. Images were acquired and analyzed through custom-made software (SMAK; courtesy of Sam Webb, beam-line scientist SSRL).

4.1.6 Statistical analyses

Group differences were assessed by performing one-way ANOVAs (SPSS v18 Mac) on each hemisphere separately. In cases of significant main effects, a Tukey's *post hoc* test was performed to identify group differences. A p value of less than 0.05 was considered statistically significant, and all data are presented as mean ± SEM.

4.2 Results

4.2.1 Focal brain cooling is neuroprotective and reduces microglial activation in the cooled hemisphere only.

Hypothermia initiated 1 hr after ischemia was neuroprotective, regardless of whether it was maintained for 2, 4 or 7 days. In contrast, hypothermia treatment on post-ischemic days 3-4 did not provide any histological benefit (Fig 4-2A). An ANOVA, followed by *post hoc* analysis indicated that the number of remaining CA1 neurons in the right (cooled) hemisphere was significantly higher in the Hyp 1-2, Hyp 1-4 and Hyp 1-7 groups relative to Norm ($P < 0.004$). There were no significant differences among groups in the left (normothermic) hemisphere ($P < 0.154$).

Untreated ischemia resulted in extensive microglial infiltration in the CA1 region, whereas neuroprotective hypothermia mitigated this process in the cooled hemisphere (Fig 4-2B). An ANOVA indicated that the Hyp 1-2, Hyp 1-4 and Hyp 1-7 groups had significantly fewer Iba-1 labeled cells within the CA1 of the right hemisphere relative to Norm ($P < 0.001$). The difference between Hyp 3-4 and Norm was not significant in the right hemisphere ($P < 0.693$), nor were there any group differences in the left hemisphere ($P < 0.103$).

4.2.2 Hypothermia does not alter the rate of neurogenesis in the dentate gyrus.

In order to quantify the effect of hypothermia on neurogenesis in the hippocampus, the number of Ki67 positive cells that also expressed DCX was quantified in the dentate gyrus of each hemisphere (Fig. 4-3A). An ANOVA indicated that there were no group differences in either the cooled (right; $P <$

0.210) or normothermic (left; P < 0.974) hemispheres in the number of Ki67/DCX co-labeled cells (Fig. 4-3B). We did not find any co-labeled cells in other regions of the hippocmpaus, such as the CA fields.

4.2.3 BDNF and Synaptophysin expression within the hippocampus remains unaltered by prolonged hypothermia treatment.

Following global ischemia, the mossy fibers originating from dentate granule cells undergo significant sprouting and increase the expression of plasticity related proteins such as BDNF (Boris-Möller *et al.*, 1998) and synaptophysin (Nishimura *et al.*, 2000). We quantified the expression of these proteins in the mossy fiber supra-pyramidal bundle in the CA3 region in order to determine whether hypothermia alters the plasticity following global ischemia. We found that both proteins were selectively expressed in the mossy fiber bundle (Fig. 4-4B, D), however hypothermia did not alter the level of BDNF (P < 0.631; Fig. 4-4A) or synaptophysin (P < 0.427; Fig. 4-4C) expression.

4.2.4 Hippocampal zinc distribution is maintained following treatment with neuroprotective hypothermia.

Synchrotron RS-XRF imaging was used to visualize zinc distribution within the hippocampus in normothermic and hypothermia treated rats. In contrast to potassium, which was homogeneously distributed throughout the hippocampus and neocortex, zinc signal was restricted to the hillus and the mossy fibers within the hippocampi of naïve rats (Fig. 4-5 A, B). This pattern

of distribution remained unaltered after untreated global ischemia, or hypothermia treatment as both hemispheres showed similar zinc distribution at the 12 hr and 7 day survival times (Fig. 4-5C-J).

4.3 Discussion

In the current experiment we evaluated neurodegeneration as well as a number of markers of synaptic plasticity, when hypothermia was maintained for various durations following global ischemia in rats. We confirm our earlier finding that 2 days of focal brain cooling is sufficient to salvage most CA1 pyramidal neurons (Silasi and Colbourne, 2011c), however we also show that protracted cooling (up to 7 days) does not counteract the initial neuroprotective effect of the treatment. Importantly, we found that hypothermia does not alter the expression of plasticity related proteins (BDNF and Synaptophysin), the rate of neurogenesis within the dentate gyrus, or the distribution of the trace element zinc, thus suggesting that the treatment does not impede post-ischemic plasticity.

Currently the use of therapeutic hypothermia is restricted to cardiac arrest patients mainly due to a lack of knowledge about effective treatment parameters (eg. duration) for other conditions such as focal ischemia or hemorrhage (van der Worp *et al.*, 2010). As hypothermia mitigates edema and inflammation in animal models of stroke there is significant interest in cooling stroke patients to similarly combat edema (Kollmar *et al.*, 2010). In contrast to animal models, edema in the human brain resolves relatively

slowly, thus necessitating hypothermia treatments that may last a week or longer. Furthermore, animal studies have shown that when hypothermia is delayed, which is an unfortunate clinical inevitability, longer treatment durations are required to achieve neuroprotection (Colbourne and Corbett, 1994b). This presents a potential challenge as endogenous repair mechanisms, such as synaptic reorganization, may also occur during a similar timeframe (Lo, 2008). The results of our current experiment suggest that in the setting of global ischemia, which is used to predict treatment efficacy in cardiac arrest and ischemic stroke, prolonged hypothermia does not inhibit post-ischemic plasticity even when treatment is maintained for up to one week.

Enhanced synaptophysin labeling within the mossy fibers has been previously used to visualize the significant plastic changes that occur in the first seven days after global ischemia (Nishimura *et al.*, 2000). Although the exact function of synaptophysin is unknown, the protein is found within presynaptic vesicle membranes and is used as a marker for functional synapses (Hinz *et al.*, 2001; Li *et al.*, 2002). Other synaptic proteins, such as synapsin-1, are also selectively elevated in mossy fibers after global ischemia (Bernabeu and Sharp, 2000), suggesting that the injury does not globally increase protein expression, but rather only targets pathways that undergo structural reorganization. Furthermore, mossy fiber plasticity is observed following other injury models, such as trauma and epilepsy (Li *et al.*, 2002),

indicating that multiple forms of injury may initiate a common mechanism that leads to mossy fiber sprouting.

One possible mechanism that may mediate injury-induced plasticity across injury models is the upregulation of neurotrophins, such as BDNF. The mossy fiber terminals have the highest level of BDNF expression of any region within the CNS (Conner *et al.*, 1997), however due to a disparity between mRNA and protein expression after ischemia the exact changes that occur following injury have been difficult to determine. For example, one study found that BDNF mRNA increases 3 h after ischemia and returns to sham levels by 3 days, whereas protein expression was decreased 3 h after ischemia and returned to normal levels by 7 days (Lee *et al.*, 2002). Another study found that protein levels were elevated 24 h after global ischemia and that hypothermia administered during this early period augmented BDNF expression at 24 h but not 12 h after ischemia (D'cruz *et al.*, 2002b). Our current study shows that more prolonged hypothermia treatment protocols do not alter BDNF expression 7 days after ischemia relative to normothermic ischemia.

Because our hypothermia treatment was restricted to one hemisphere we were also able to compare the levels of BDNF protein expression between salvaged and unprotected regions within the same animal. We found that neuroprotection did not affect BDNF levels, suggesting that CA1 neuronal death does not independently influence BDNF expression. This finding is

somewhat surprising given that hypothermia significantly reduced the number of infiltrating microglial cells within the CA1, and microglia produce BDNF following injury (Lai and Todd, 2008). Furthermore, other studies found that CA1 neuroprotection by glutamate receptor antagonists prevents the stroke-induced elevation of Synapsin-1 that is normally observed in mossy fiber terminals after global ischemia (Bernabeu and Sharp, 2000). Based on these data one would predict that in our current experiment the ischemia-induced elevation of BDNF would only occur in the non-treated hemisphere, as hypothermic neuroprotection would prevent the initiation of plasticity related signaling. In contrast, we found no differences in BDNF levels between hemispheres in any of the groups. Previous studies found that cooling initiated after global ischemia augmented BDNF expression (D'cruz *et al.*, 2002b), while intraischemic cooling had no effect (Boris-Möller *et al.*, 1998) in the hippocampus. These results are at odds with our current data, and may be explained by a difference in survival times as quantification in the previous studies was performed at 12-24 hr, in contrast to our 1 week survival time. It may be that our hypothermia treatment also increases BDNF expression early on after injury, however we have not quantified this.

Neurotrophin expression in the hippocampus also modulates the rate of neurogenesis in the dentate gyrus therefore we evaluated if hypothermia altered the rate of post-ischemic neurogenesis. We found no significant difference in the number of Ki67/DCX co-labeled cells between normothermic and any of the hypothermia treated groups 7 days after

ischemia. This result is in line with our finding of no significant difference in BDNF expression among groups. Furthermore, other studies have shown that the level of post-ischemic neurogenesis remains elevated in animals where CA1 neuronal death is prevented through preconditioning (Liu *et al.*, 1998). Taken together, these findings indicate that downstream processes mediated by BDNF signaling, such as neurogenesis, are not hindered by any of our hypothermia treatment protocols.

In order to complement our immunohistochemical quantification of plasticity related proteins in the hippocampus, in a follow-up experiment we used synchrotron RS-XRF imaging to visualize zinc distribution after global ischemia. Several studies have demonstrated that during the acute phase of ischemia the translocation of zinc from mossy fiber terminals to vulnerable neurons in CA4 and CA1 is correlated with neurotoxicity in those neurons (Frederickson *et al.*, 2006; Koh *et al.*, 1996). However, synaptic plasticity at mossy fiber terminals is dependent on the presence of zinc (Nakashima and Dyck, 2009), therefore a depletion of zinc from this region may hinder post-ischemic plasticity. Our results show that 7 days after ischemia there are large amounts of zinc remaining in the mossy fiber terminals in both hypothermia treated and normothermic animals. Based on this finding we conclude that the availability of zinc in mossy fiber terminals is not a limiting factor for plasticity after ischemic injury or neuroprotective hypothermia.

Unfortunately, a causal link between post-ischemic plasticity and functional recovery has not been established as it is difficult to selectively block such plastic changes in the brain (Whishaw *et al.*, 2008). Furthermore, behavioural impariments are extremely difficult to detect after unilateral brain injury due to compensatory pathways from the uninjured hemisphere. For these reasons, in the current study we did not examine behavioural outcome following hypothermia treatment. Others have shown that behavioural deficits (such as hyperactivity) present in the early post-ischemic period become difficult to detect, or disappear completely during the second post-ischemic week (Block and Schwarz, 1996; Karasawa *et al.*, 1994), suggesting that mossy fiber sprouting may in fact contribute to recovery. In sum, our results demonstrate that focal brain cooling does not hinder post-ischemic plasticity in the hippocampus, even when the treatment is maintained for up to a week. Animal models will continue to be a valuable tool in guiding and refining hypothermia treatment protocols for clinical application.

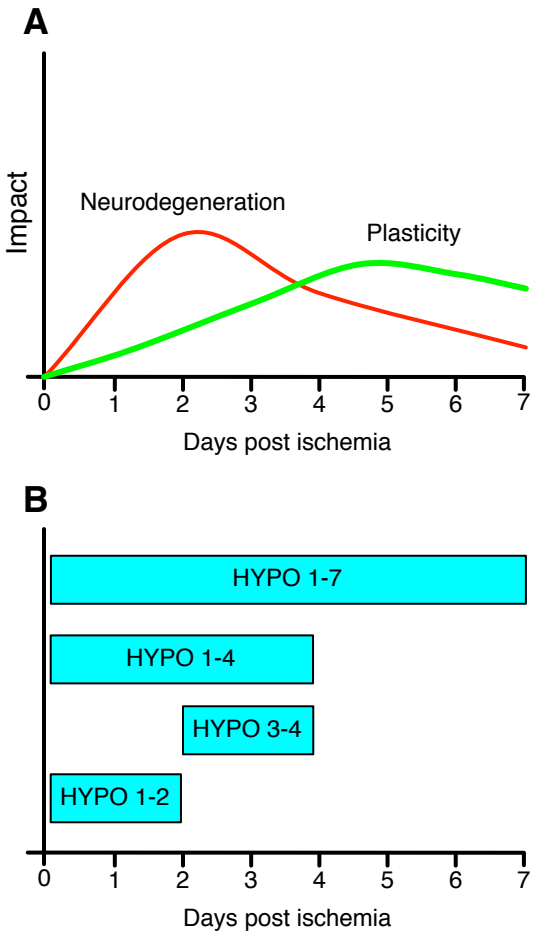

Figure 4-1. Following ischemic injury, there is a partial overlap of neurodegeneration and the initiation of plasticity and repair processes (A). In experiment 1, hypothermia treatment was applied either for the first 2 days (HYPO 1-2), days 3 and 4 (HYPO 3-4), the first 4 days (HYPO 1-4) or the first 7 days (HYPO 1-7) after ischemia. In groups where hypothermia started on the first day, the treatment was initiated 1 hr after the start of ischemia (B).

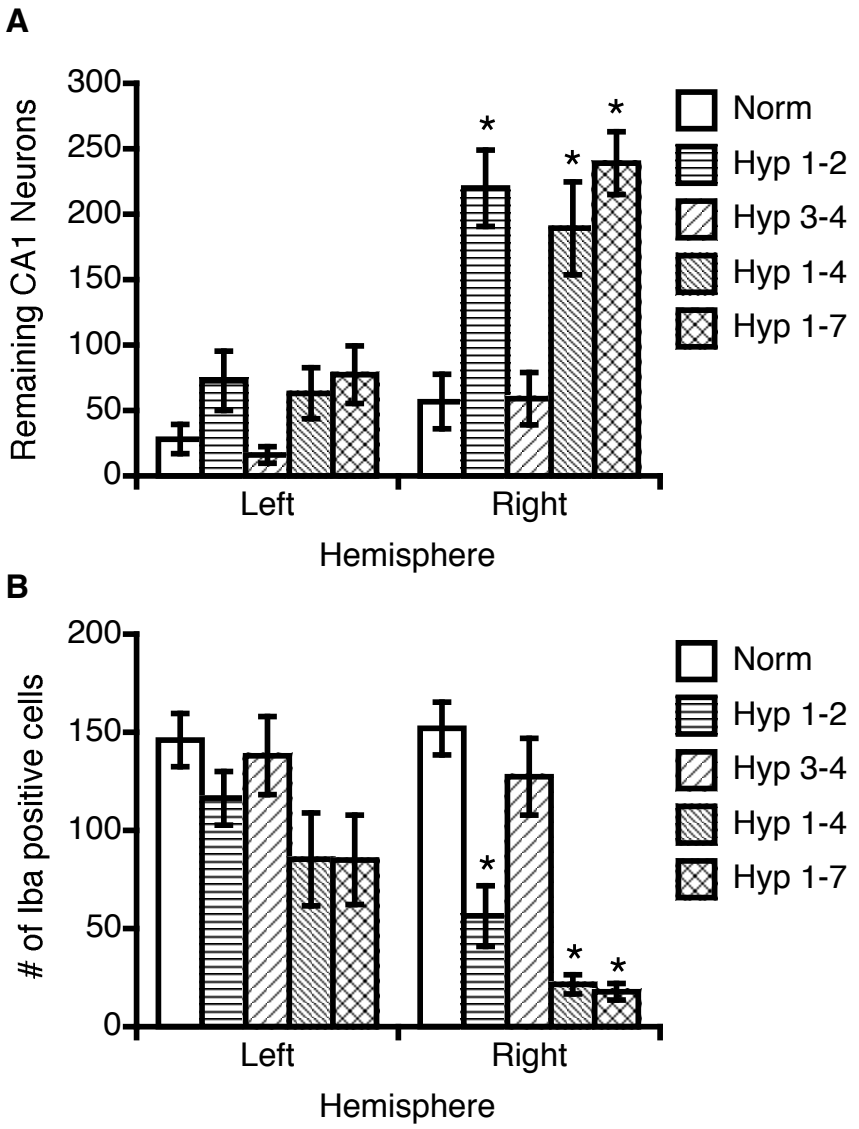

Figure 4-2. Hypothermia initiated on the first post-ischemic day was neuroprotective in the cooled (right) hemisphere only. There were significantly more remaining CA1 neurons in the Hyp 1-2, Hyp 1-4 and Hyp 1-7 groups relative to normothermic animals (A). Neuroprotective hypothermia decreased the number of Iba positive microglia in the CA1 region in the cooled hemisphere, as the Hyp 1-2, Hyp 1-4 and Hyp 1-7 groups had significantly fewer Iba positive cells relative to the normothermic group (B). * indicates significantly different from Norm group; mean ± SEM.

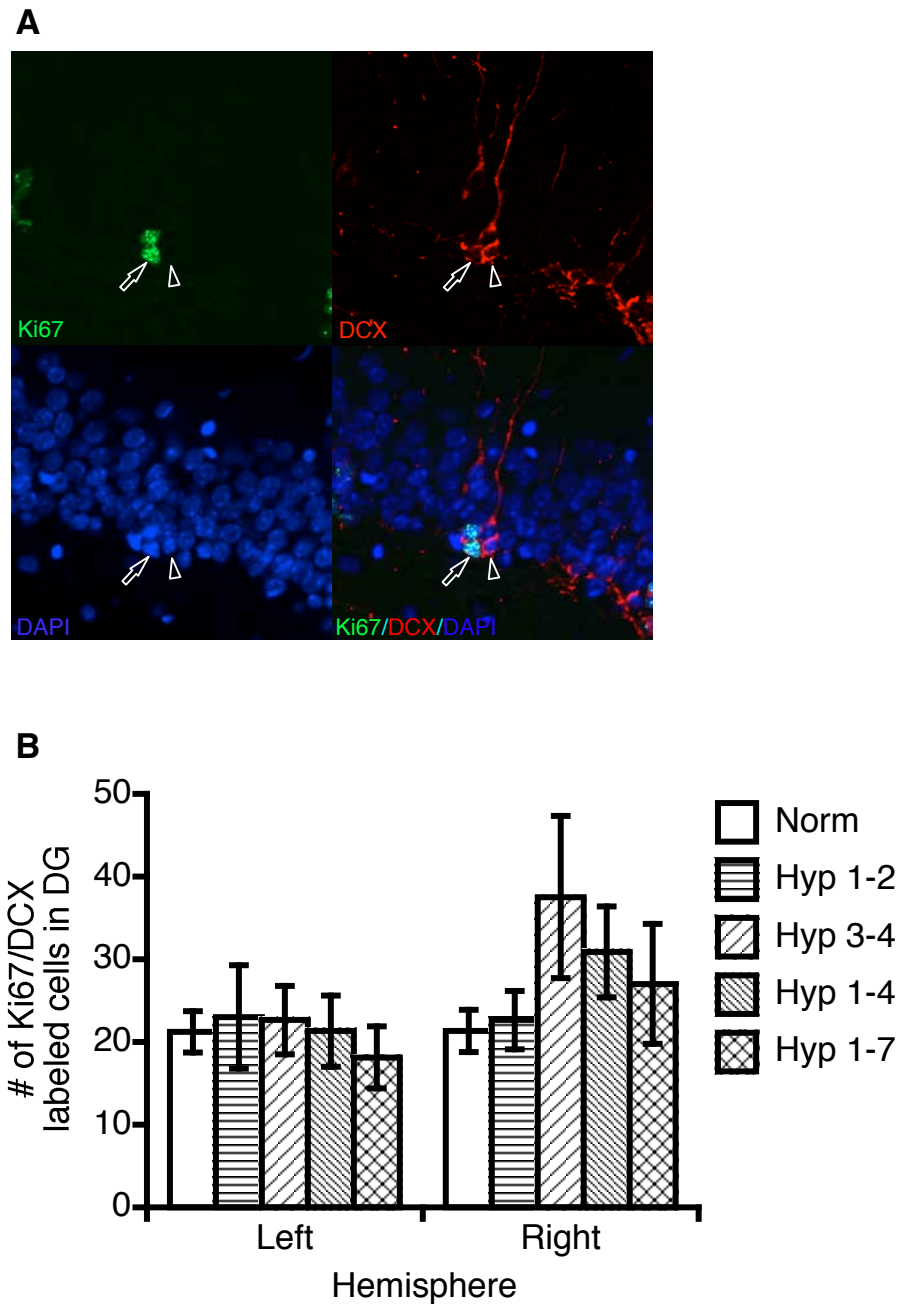

Figure 4-3. Hypothermia treatment does not alter the rate of neurogenesis in the dentate gyrus folowing global ischemia. The number of Ki67 positive nuclei (arrow) that also expressed the immature marker DCX (arrowhead; A) did not differ among groups in either the cooled (right) or normothermic (left) hemispheres (B). Mean ± SEM.

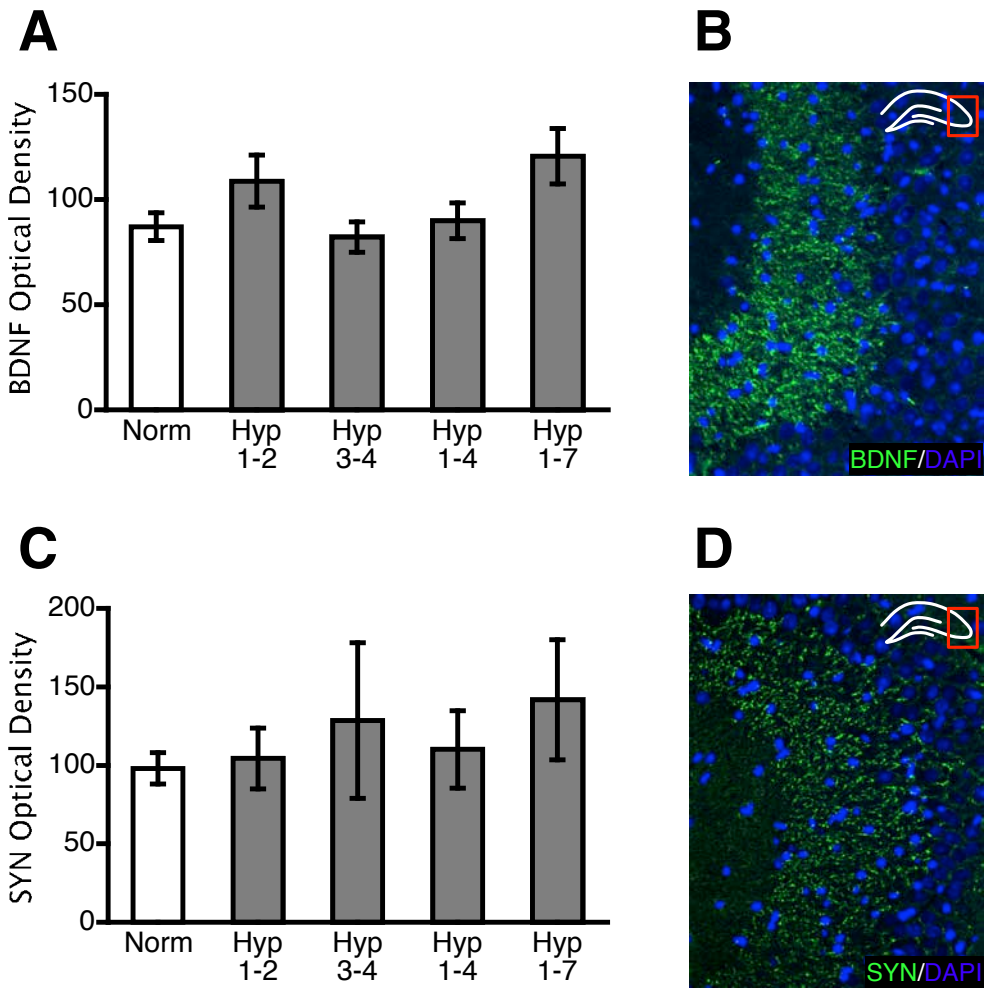

Figure 4-4. Hypothermia treatment did not alter the expression of BDNF (A) and synaptophysin (C) in the mossy fiber bundle of the hippocampus. Expression of both proteins was largely restricted to the mossy fiber terminals near CA3 (B, D), and was mostly absent from the pyramidal cell layers. Quantification of optical density was performed in the regions indicated in the insets (B, D), and the results are expressed as a per cent expression in the cooled (right) hemisphere relative to the normothermic (left) hemisphere. Mean ± SEM.

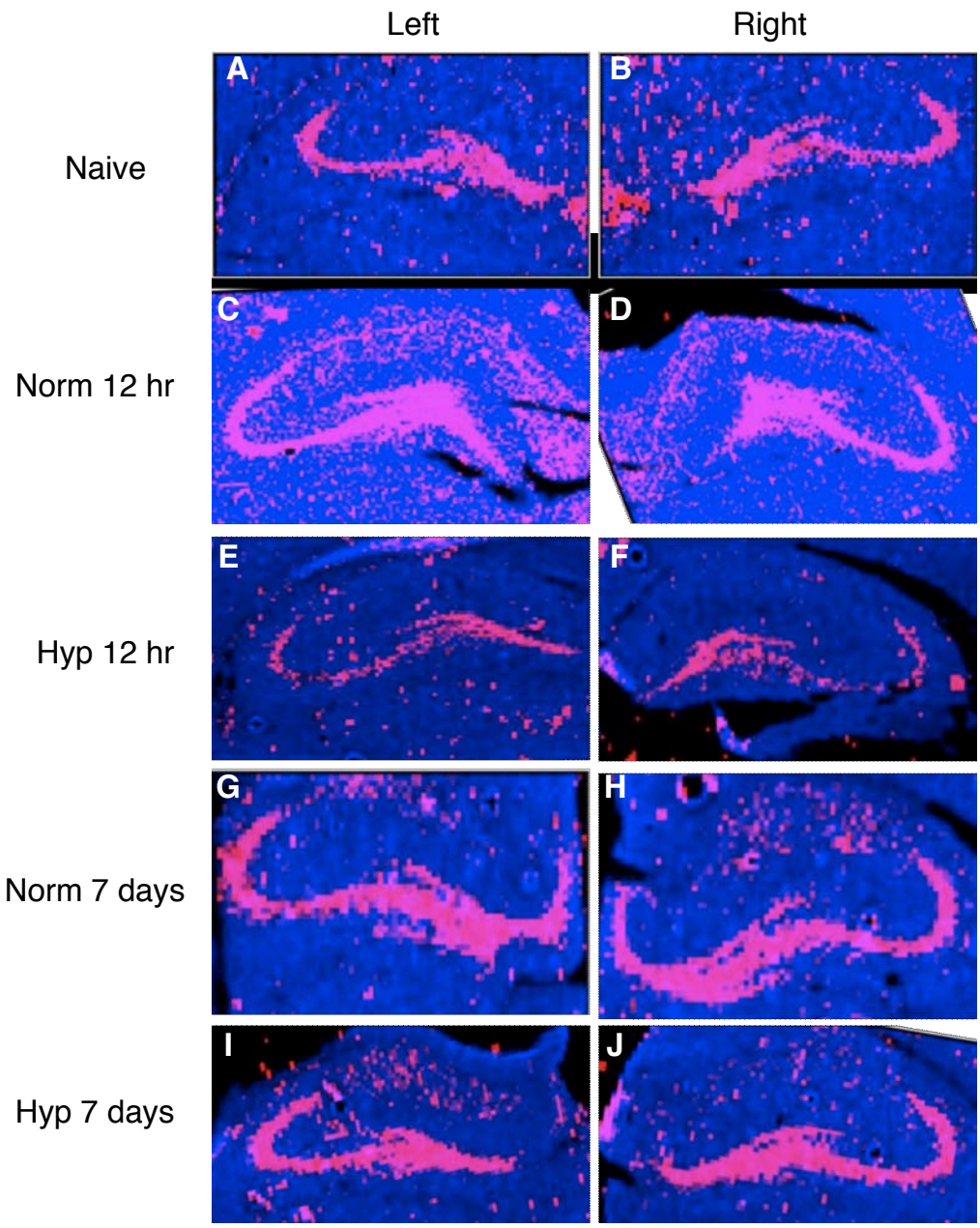

Figure 4-5. Distribution of Zn (pink) and K (blue) in the hippocampus of naïve rats (A, B) and rats killed either 12 hr (C-F), or 7 days (G-J) after global ischemia. In hypothermic rats, only the right hemisphere was cooled. The pattern of Zn distribution was not altered by injury, nor by hypothermia treatment as there are no obvious differences between hemispheres in treated animals.

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Chapter 5

General Discussion

5. General Discussion

Brain ischemia, resulting from stroke or cardiac arrest, is a major cause of disability worldwide, and the ensuing brain damage drastically alters the quality of life for those impacted by the disease. One of the most significant discoveries in brain research has been the identification endogenous repair mechanisms that support recovery after brain injury through circuit reorganization and synaptic plasticity (Murphy and Corbett, 2009). Both animal and human studies have correlated such plastic reorganization with limited functional recovery after brain ischemia (Warraich and Kleim, 2010), suggesting that the injury-induced anatomical changes are functionally significant. Given that spontaneous reorganization results in only partial recovery, there is continued interest in developing treatments that minimize ischemic brain injury, while facilitating endogenous repair processes. Animal studies have identified hypothermia as one of the most effective neuroprotective treatments for brain ischemia (MacLellan *et al.*, 2009b), however the effects on endogenous repair processes have been under-investigated. Due to the significant clinical interest in adapting therapeutic hypothermia for various forms of ischemic and traumatic brain injury (Polderman, 2004), it is important to assess the interaction of this treatment with endogenous repair processes.

The current set of experiments examined the influence of therapeutic hypothermia on hippocampal plasticity following global ischemic injury in

rats. In the first experiment, a systemic cooling paradigm was used to study post-ischemic cell genesis and the influence of hypothermia treatment on this process. In the second experiment, a brain selective cooling method was adapted to assess the level of neuroprotection offered by focal brain cooling after global ischemia. Lastly, the onset and duration of focal brain cooling were examined as factors that could potentially influence post-ischemic plasticity. The main finding of the current thesis is that neuroprotective hypothermia does not impede on post-ischemic plastic processes in a model of global ischemia. More specifically we show that: 1) CA1 neurogenesis does not contribute to the neuroprotective effects of systemic hypothermia, however the treatment facilitates post-ischemic plasticity in the dentate gyrus by enhancing the survival of newly generated neurons; 2) neuroprotection following global ischemia can be restricted to one hemisphere through unilateral brain cooling, thus allowing comparisons to be made to ongoing degenerative processes in the contralateral (untreated) hemisphere; 3) prolonged hypothermia (up to 7 days) is neuroprotective, but does not alter post-ischemic plasticity after global ischemia. In the following discussion I will emphasize the relevance of our work in the context of previous animal research and current clinical interests. In addition I will discuss the limitations of our experiments.

5.1 Neuroprotective effects of hypothermia

Several studies have reported that CA1 pyramidal neurons may regenerate after global ischemia either spontaneously or after treatment with growth factors (Bendel *et al.*, 2005b; Nakatomi *et al.*, 2002). Such claims are based on the finding that ischemia is a potent stimulus for cell proliferation (Liu *et al.*, 1998), therefore raising the possibility of augmenting regeneration through pharmacological or physiological treatments. We hypothesized that if CA1 neurogenesis occurs after global ischemia, this process would be enhanced by delayed hypothermia. Our rationale was that the period of untreated ischemia (prior to initiating hypothermia) would promote neurogenesis in precursor populations in the hippocampus, while the hypothermia treatment would provide a favorable environment for neuronal differentiation and integration by lowering immune cell infiltration and the secretion of toxic cytokines. Our results showed that the neuroprotective efficacy of hypothermia decreased with increasing treatment delays, however none of the treatment protocols promoted CA1 neurogenesis, nor did we observe CA1 neurogenesis in untreated animals. In addition to demonstrating the lack of a neurogenic gradient with increasingly delayed hypothermia, our experiment systematically assessed neuroprotection when hypothermia was initiated at various delays; similar to dose-response curves performed for pharmacological agents (e.g. (Sydserff *et al.*, 2002)) . A previous study evaluated hypothermic neuroprotection when treatment initiation was varied systematically (Carroll and Beek, 1992),

however no benefit was observed after a 3 hr delay as the cooling duration was only 6 hr; insufficient to provide long-term benefit.

We next wanted to investigate how cooling duration influenced neuroprotection, however modulating this variable using systemic cooling paradigms is not feasible, because rats do not tolerate this form of cooling for more than ~3 days. In order to overcome this limitation, we induced unilateral hypothermia after global ischemia using a focal brain cooling system where longer treatment durations are possible (Clark and Colbourne, 2007a). Intra-ischemic cooling of the head is known to be neuroprotective in rats (Nurse and Corbett, 1994a; Taniguchi *et al.*, 2005a) however, our current study is the first to demonstrate that delayed unilateral hypothermia is neuroprotective in the cooled hemisphere only, whereas the normothermic, contralateral hemisphere undergoes ischemic damage.

Our experiments were not designed for statistical comparisons between systemic and focal cooling protocols, however there are general conclusions that can be made regarding the method of cooling and stroke outcome. First, we found that both systemic and focal brain cooling protocols inhibit immune cell infiltration at the site of injury, as the treatments decreased the number of Iba labeled microglial cells in the CA1 region. This is partly mediated by the neuroprotective effect of hypothermia, as a decrease in the level of injury would recruit fewer immune cells to the region. Alternatively, hypothermia may directly inhibit microglial infiltration by

diminishing inflammatory cytokines (Webster *et al.*, 2009). Indeed, following intracerebral hemorrhage, hypothermia initiated 4 hr after the injury significantly decreased the number of iron positive immune cells in the peri-hematoma (MacLellan *et al.*, 2006), while hypothermia initiated immediately after global ischemia had a similar effect (Cao *et al.*, 2008). The fact that microglial infiltration was diminished by delayed cooling in our current experiment suggests that the treatment may actively inhibit microglial activation, as increased microglial staining is evident within 20 minutes after global ischemia (Morioka *et al.*, 1991), well before hypothermia was initiated.

Our finding that the level of neuroprotection offered by systemic and brain-selective cooling is similar across our experiments suggests that the decrease in brain temperature is likely the mechanism mediating this effect. Although the physiological changes induced by systemic cooling (eg. increased blood pressure; (MacLellan *et al.*, 2009b)) may alter stroke outcome, the fact that a similar level of protection is observed in only the cooled hemisphere of focally cooled rats suggests that the physiological changes induced by systemic cooling are not important. Furthermore, the normothermic hemisphere of focally cooled animals allowed us examine any side effects of our tethering system that may have influenced cell survival following global ischemia, but found no significant effects. In sum, our results confirm that the level of hypothermic neuroprotection following global ischemia is determined by the delay in the onset of treatment and not the method of cooling (whole-body vs. brain-selective).

5.2 Effects of hypothermia on post-ischemic plasticity

Given that ischemic injury initiates a number of plastic processes that may contribute to recovery (Murphy and Corbett, 2009), we evaluated whether hypothermia alters some of these processes. Post-ischemic plasticity may be facilitated by a change in either the number or function of synaptic connections, and such modifications can be achieved either through synaptic rewiring of existing neurons or the addition of new neurons. In the first experiment, using systemic cooling, we found that neuroprotective hypothermia increased the survival of newly generated dentate granule cells after global ischemia. This effect is at least in part mediated by the anti-inflammatory properties of hypothermia, as inflammation inhibits neuronal precursor division and differentiation (Monje *et al.*, 2003). In contrast to a previous report stating that hypothermia decreases cell proliferation in the hippocampus (Kanagawa *et al.*, 2006), our subsequent experiment using focal brain cooling (experiment 3) showed that the treatment does not alter neurogenesis in the dentate gyrus. We found that the number of Ki67/DCX co-labeled neurons was not altered in any of the hypothermia treated groups 1 week after global ischemia. The previous report was carried out in healthy neonatal rats, which were cooled to 30˚C, whereas our results were from adult rats with ischemic injury, cooled to 33˚C. These differences in the age of the animals, the cooling depth and the presence of brain injury may explain the difference in results. Further support for our finding that hypothermia does not impede injury-induced neurogenesis comes from a recent study

showing that brain cooling increased the number of BrdU/NeuN co-labeled cells after traumatic brain injury (Kuo *et al.*, 2010).

In addition to cell genesis, synaptic reorganization and sprouting are major components of post-ischemic plasticity (Nudo, 2006). We used synaptophysin labeling to estimate synaptic density in the mossy fiber bundle, as this region undergoes significant sprouting following injury (Nishimura *et al.*, 2000). In addition, we quantified the level of BDNF expression, as neurotrophin signaling is an upstream mechanism that mediates both synaptic reorganization (Gómez-Palacio-Schjetnan and Escobar, 2008) and neurogenesis (Takahashi *et al.*, 1999) within the hippocampus. We found that hypothermia did not alter the density of synaptophysin or BDNF labeling in the mossy fiber bundle, suggesting that the treatment does not interfere with post-ischemic plastic processes. This result is somewhat surprising given that the level of ischemic damage was clearly greater when hypothermia was delayed for 2 days versus other treatment groups where neuroprotection was nearly complete. One would expect that a greater level of injury would also induce a greater response in plasticity promoting factors, such as BDNF, however our data do not follow this trend. It may be that our 1 week survival time missed any transient changes in BDNF expression that occurred within the first post-ischemic week. Although BDNF has been implicated in facilitating plasticity in the hippocampus after global ischemia (Kiprianova *et al.*, 1999), the expression profile of the protein has been difficult to determine, and the exact

timecourse of BDNF elevation remains unknown. Alternatively, related trophic molecules such as NT-3 and NGF may have a greater role in facilitating post-ischemic plasticity (Chen *et al.*, 1999); therefore the effects of hypothermia on these signaling pathways should also be investigated.

5.3 Assessing the safety of long-term hypothermia

Prior to evaluating the efficacy of therapeutic hypothermia in other clinical conditions such as focal ischemia or traumatic brain injury, animal models should be used to establish the safety of such treatment protocols. Initial studies have shown that briefly cooling the rat brain to ~30˚C slows down synaptic transmission, however, spatial learning abilities remain intact even during the cooling period (Moser and Andersen, 1994). In order to provide benefit for stroke patients, in some cases hypothermia will have to be maintained for days or even weeks (Kollmar *et al.*, 2010), but there have been no animal studies thus far evaluating the safety of such prolonged treatments. The current experiments demonstrate that focal brain cooling maintained for up to a week does not alter the post-ischemic expression of the synaptic marker synaptophysin or the neurotrophin BDNF. In a subsequent study we carried out a more rigorous evaluation of long-term focal brain cooling by quantifying dendritic complexity and spine density in uninjured animals that were cooled for 3 weeks (Appendix 1). In order to identify any persistent effects of this treatment on neuronal morphology, rats were killed 3 days after rewarming and the brains were processed for Golgi-

Cox staining. We found that neuronal morphology in layer 5 of the motor cortex was indistinguishable between the normothermic and hypothermic groups. Specifically, Scholl analysis (a measure of dendritic length), as well as spine density measurements of the basilar dendrites revealed no significant difference between cooled and normothermic animals, indicating that even this long period of cooling does not induce synaptic atrophy in the rat brain. This duration of cooling may be necessary for some forms of injury where edema persists for several weeks (Marion and Bullock, 2009). Taken together, the current set of experiments provide multiple lines of evidence indicating that therapeutic hypothermia can be safely applied for up to a week following global ischemia, whereas three weeks of cooling in the uninjured brain does not result in dendritic atrophy or changes in spine density.

5.4 Functional significance of treatment

Many studies have shown that hypothermic neuroprotection after ischemic injury is generally associated with improved functional outcome. For example, in models of global ischemia, learning deficits were prevented even at 6 month survival times (Colbourne *et al.*, 2000; Maier *et al.*, 2001). Similarly, motor deficits resulting from MCAO injury are also diminished with neuroprotective cooling (Clark *et al.*, 2009). Our first study, using systemic cooling showed significant amelioration of spatial navigation deficits in hypothermia treated groups. Unexpectedly however, the behavioural benefit was significant even when hypothermia was delayed for 12 hr, at which point

it was no longer neuroprotective for CA1 neurons. One possible explanation may be that the behavioural deficits are not causally linked to CA1 cell depletion, but rather other regions of cell death (hillus, cortex, striatum) may contribute to our observed functional impairment. We did not quantify the level of injury in these regions, however it is possible that hypothermia decreased cell death in regions other than CA1, which may account for the functional benefit across all groups.

We also assessed spatial learning following global ischemia and unilateral, brain-selective hypothermia (Chapter 3), however we did not see a difference between the treated and untreated groups. This may be due to a lack of deficit in the untreated group, as their performance continued to improve over testing days to levels comparable to uninjured rats from previous studies (Silasi and Colbourne, 2008). Unfortunately we did not include a sham group in our current experiment. In addition, given the lack of clear behavioural sparing in unilaterally treated rats (Chapter 3) we were unable to evaluate the behavioural effects of various cooling durations (Chapter 4). Including extensive behavioural testing would have confounded our measures of plasticity, as testing itself may act as a form of rehabilitative training, and thus induce plasticity. In sum, the current experiments demonstrate that neuroprotective hypothermia improves behavioural outcome following global ischemia. However, we were unable to determine whether the effects of hypothermia on post-ischemic plasticity (eg. enhanced

survival of newly generated neurons; Chapter 2) influenced behavioural outcome after global ischemia.

5.5 Clinical relevance of neuroprotection in animal models of global ischemia

Given that therapeutic hypothermia is already a proven treatment for cardiac arrest, (Bernard *et al.*, 2002b; HACA, 2002) it may be argued that animal models should be aimed at identifying effective treatment protocols for other forms of strokes, such as focal ischemia. Nonetheless, there are several advantages of global ischemia models that make it a practical choice for both mechanistic and efficacy studies evaluating neuroprotection. First, the timing and location of neuronal degeneration within the hippocampus is well characterized after global ischemia, thus allowing neuroprotective treatments to be precisely evaluated through neuronal counts (most commonly in the CA1 cell layer). Such cell counts are difficult to perform in models of focal ischemia as the exact location of injury differs among rats; therefore lesion volume assessment is commonly used to infer neuroprotection. Second, the delayed neuronal death in CA1 may be used as a model for the delayed cell death that occurs in penumbral regions around focal ischemia (Dereski *et al.*, 1993). As lesion volume expansion is a significant clinical problem (Baird *et al.*, 1997), a better understanding of treatments that prevent delayed ischemic cell death would be relevant in treating focal ischemia in patients.

5.6 Limitations and future directions

The current thesis has several limitations that could be addressed with further experiments. First, additional functional measures could be applied to complement our current behavioural measures. For example, electrophysiological recordings of evoked or spontaneous activity in the hippocampus could be used to assess circuit integrity *in vivo* (Barth and Mody, 2011). Similarly, acute slice preparations could be used to compare excitatory post-synaptic currents in individual CA1 neurons from protected and non-injured animals (Dong *et al.*, 2001b). This approach would benefit greatly from our unilateral treatment model as the electrophysiological recordings could be compared between the two hemispheres to determine the effects of the treatment on post-ischemic plasticity.

Alternatively, additional histological measures would further strengthen our current findings. We used synaptophysin labeling as an indirect measure of synaptic density, however direct synaptic counts on electromicrographs could be used to confirm our results. Spine density analysis (through Golgi-Cox staining) could also have also been performed to estimate synaptic density. We did not undertake these experiments as both the ultrastructural and the spine density analyses are incompatible with H&E staining (to assess neuroprotection) and immunolabeling. In addition, we could also use more sensitive measures, such as ELISA or Western blots, to quantify growth factor expression after ischemia as small group differences

may not be detectable with our densitometry measures on histological sections.

5.7 Conclusion

The current set of experiments evaluated the effects of whole body and brain-selective cooling paradigms on neuroprotection and post-ischemic plasticity in the rat brain. Our findings confirm that the delay in cooling onset is a critical factor in determining neuroprotective efficacy, whereas cooling duration (up to 1 week) does not negatively impact post ischemic plasticity. Based on these data we suggest that the safety of protracted cooling be evaluated in models of ischemia that more closely approximate the clinical condition where such treatments may be necessary.

5.8 References

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Appendix 1:

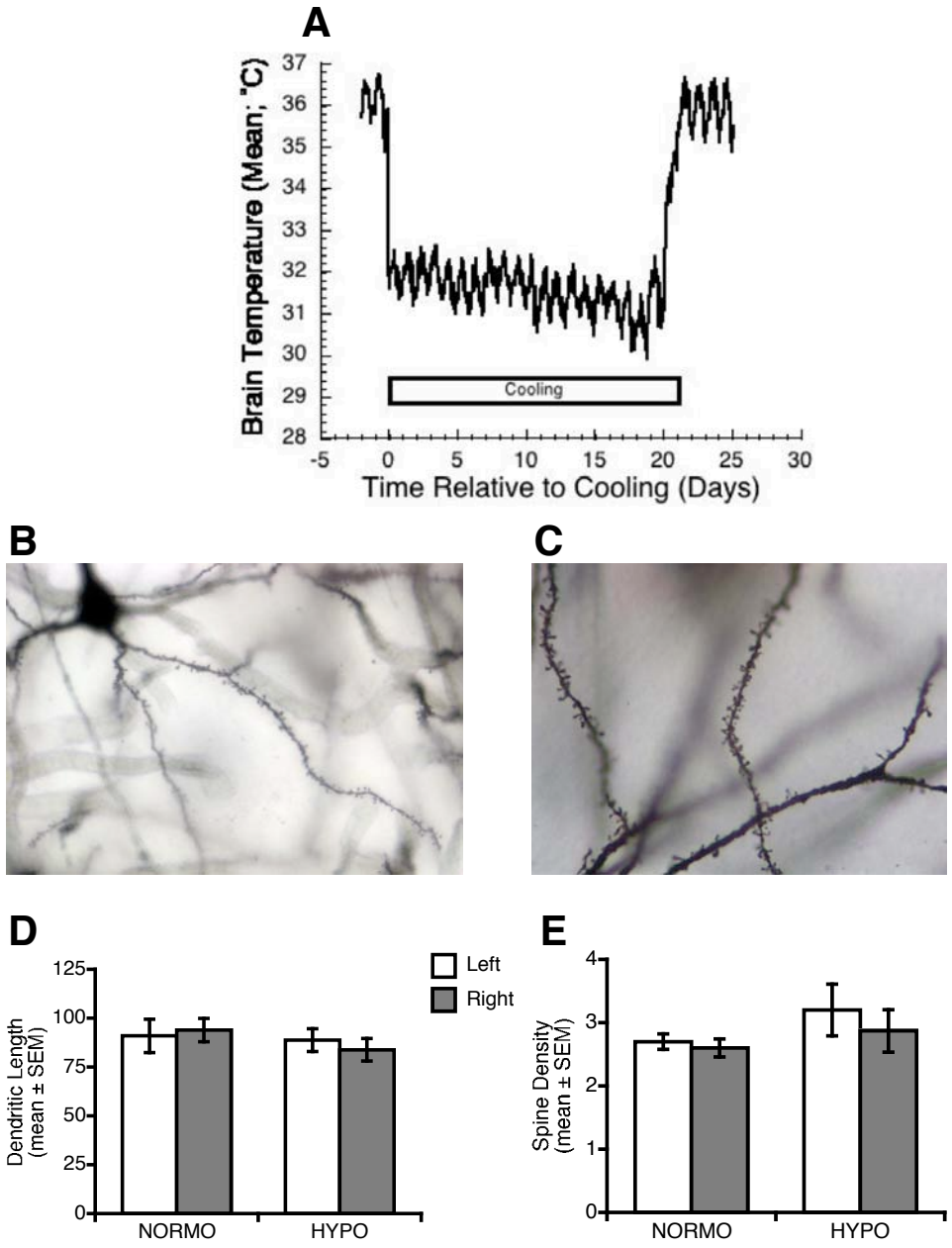

Figure 1. Focal brain cooling for 3 weeks does not alter dendritic morphology or spine density in motor cortex layer 5 neurons. Brain temperature was monitored through a Telemetry thermocouple, which was implanted into layer 5 of the right motor cortex (cooled hemisphere). During the cooling period brain temperature was persistently decreased to ~32˚C (A). Three

days after re-warming rats were killed, and the brains prepared for Golgi-Cox staining to visualize dendritic processes (B) and spines (C). Quantification of dendritic length through Scholl analysis indicated that there was no significant difference between the cooled (right) and normothermic (left) hemispheres (D). Hypothermia also did not have any lasting effects on spine density, as this measure did not differ between the right and left hemispheres (E). These data are part of a larger study in preparation by A. Auriat, G. Silasi, M. Penner and F. Colbourne; *An evaluation of the safety of long-term therapeutic hypothermia.*