EFFECT OF POST-ISCHEMIC CALORIC RESTRICTION ON CELL DEATH AND FUNCTIONAL RECOVERY

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Barbara Rae McEwen

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ABSTRACT

Since caloric restriction (CR) can modify multiple pathways central to the ischemic cascade and enhance neuroplasticity mechanisms, we hypothesized that CR should exert protective effects following brain ischemia. Previous studies have suggested benefit when CR was administered prior to ischemia. This study investigated whether prolonged CR beginning after global ischemia would result in lasting protection as assessed by performance in the open field, as a measure of functional outcome, and hippocampal CA1 neuronal counts. Adult male Mongolian gerbils were subjected to five minute bilateral carotid artery occlusion (I) or sham surgery (S) with tympanic temperature maintained at 36.5 ± 0.2ºC during the intra-ischemic period. After screening out gerbils with incomplete ischemia, each of the two surgical groups were randomly assigned to control diet (CON) or 30% CR for the duration of the study (60d). Gerbils were tested in the open field on d3, 7, 10, 30 and 60. Ischemic animals on control diet showed a significantly higher level of activity in the open field (impaired habituation) compared to SCON gerbils on all test days ($p<0.001$). Open field activity was decreased 9% in the ICR group versus ICON gerbils on d7 ($p=0.024$), suggesting a transient neuroprotective effect. Open field activity of the SCR gerbils began increasing relative to that of SCON gerbils during the last 30 days of the study ($p=0.055$ on d60), raising the question of suitability of the open field test for long-term studies of CR and ischemia. Brain sections obtained at d60 were stained with hematoxylin & eosin. Hippocampal CA1 neuron counts were reduced 88% by ischemia ($p<0.001$), and there was no sparing effect of CR. These findings suggest that prolonged CR administered beginning after global ischemia cannot diminish brain injury or enhance long-term recovery.

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To my family, thank you for your love, support and phone calls. Finally, thank you Colin, for your friendship and love, for believing in me and encouraging me. I couldn't have done it without you.

This thesis is dedicated to my grandma Norma Heather,
who instilled in me a life-long love of books.

I have always imagined that Paradise will be a kind of library
- Jorge Luis Borges

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LIST OF ABBREVIATIONS

AIN	American Institute of Nutrition
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
BCCAO	Bilateral common carotid artery occlusion
CA1	Cornu-ammonis 1
CON	Control
CR	Caloric restriction
FOOD trial	Feed or Ordinary Diet trial
H&E	Hematoxylin and eosin
I	Ischemia surgery
ICON	Ischemic control diet
ICR	Ischemic caloric restriction
IF	Intermittent fasting
LSD	Least significant difference
MCAO	Middle cerebral artery occlusion
NFκB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
PEM	Protein energy malnutrition
ROS	Reactive oxygen species
S	Sham surgery
SCON	Sham control diet
SCR	Sham caloric restriction

SEM	Standard error of the mean
SIR2	Silent information regulator 2
SIRT1	Sirtuin 1

CHAPTER 1

INTRODUCTION

1.1 Rationale

Stroke is the third leading cause of death in developed countries and is a major cause of serious long-term disability (Rosamond et al., 2007). Attempts to develop neuroprotective agents with lasting benefit for stroke-induced brain injury have been unsuccessful (Ginsberg, 2009; Gladstone et al., 2002). Mechanisms responsible for the cell death cascade following brain ischemia include adenosine triphosphate depletion, glutamate excitotoxicity, membrane depolarization, increased intracellular calcium, oxidative stress, and inflammation (Harukuni and Bhardwaj, 2006; Dirnagl et al., 1999; Juurlink and Sweeney, 1997). The targeting of neuroprotectants to a single component of the complex ischemic cascade is believed to be one major reason for their failure in clinical trials (Ginsberg, 2009; Savitz and Fisher, 2007; Fisher and Brott, 2003). Novel methods of neuroprotection are needed and should involve combination therapy (Fisher and Brott, 2003; Fisher, 2003; Stroke Therapy Academic Industry Roundtable, 1999).

Caloric restriction (CR), in which energy intake is reduced by 30-40% while sufficient intake of other nutrients is maintained, has been extensively studied for its remarkable capacity to extend the average and maximum life span of a number of animal species (Young and Kirkland, 2007; Martin et al., 2006; Bordone and Guarente, 2005). Caloric restriction can slow age-related alterations in the brain, reduce the incidence of age-associated cardiovascular and neurodegenerative diseases, and exert beneficial

effects in a variety of neurodegenerative disorders (Ingram et al., 2007; Mattson, 2005; Mattson and Wan, 2005; Heilbronn and Ravussin, 2003).

We hypothesized that CR should protect against stroke-induced brain damage because of its potential for disrupting multiple interrelated pathophysiological events in the ischemic cascade. Caloric restriction is believed to act as a mild stressor that increases subsequent stress resistance (de Cabo et al., 2003; Heydari et al., 1996), reducing neuronal death due to excitotoxic, oxidative, or metabolic insults (Mattson, 2005; Mattson and Wan, 2005). Stress and cell survival proteins, such as heat shock protein-70 and sirtuin proteins, are induced by CR (Lin et al., 2000; Yu and Mattson, 1999). Caloric restriction can also dampen oxidative damage and the inflammatory response (Pamplona et al., 2002; Chandrasekar et al., 2001; Forster et al., 2000; Spaulding et al., 1997; Sohal et al., 1994). Core body temperature is lowered by CR (Ferguson et al., 2007; Heilbronn et al., 2006; Lane et al., 1996), which is significant because of the marked protective effects of hypothermia against ischemic brain injury (van der Worp et al., 2007; Colbourne et al., 1997). Caloric restriction may also improve outcome from stroke by enhancing neuroplasticity mechanisms important to recovery. Increased neurogenesis has been reported in response to CR (Lee et al., 2000; Lee et al., 2002) as has increased production of growth factors, such as brain-derived neurotrophic factor, that support structural plasticity (Lee et al., 2000; Lee et al., 2002; Duan et al., 2003; Duan et al., 2001).

There are few studies of CR in experimental stroke models. Decreased brain infarct volume and improved neurological deficit score at 24 hours following middle cerebral artery occlusion have been reported in rats exposed previously to a three-month

intermittent fasting regimen as an alternate method of inducing CR (Yu and Mattson, 1999). Although this study suggests that a lifestyle incorporating CR might improve outcome after stroke, it has some critical limitations when assessed against experimental stroke research standards (Stroke Therapy Academic Industry Roundtable, 1999). To demonstrate true efficacy, evidence would need to be obtained with longer-term endpoints to establish that CR was not just postponing neuronal death and with the addition of functional tasks that are sensitive for detecting persistent disability after focal ischemia (Corbett and Nurse, 1998). Caloric restriction (40%) administered before and continuing after global ischemia has also been suggested to improve ischemia-induced memory impairments (Roberge et al., 2008a; Roberge et al., 2008b), but these studies also acknowledge some methodological limitations. The clinical relevance of testing CR provided prior to stroke can also be questioned, since a chronic energy restriction of 30-40% is not likely an attainable goal for the human population (Ingram et al., 2006). Therefore, we studied CR following bilateral carotid artery occlusion in the gerbil, a well-validated model of global ischemia, assessing both hippocampal CA1 (cornu-ammonis 1) cell death and habituation in the open field.

1.2 Hypothesis

Caloric restriction provided immediately following brain ischemia will result in a lasting decrease in brain damage as assessed by performance in the open field, as a measure of functional outcome, and hippocampal CA1 neuronal counts.

1.3 Objectives

1. Assess the long-term (60d) effects of post-ischemic CR on functional outcome, as measured by the open field test.

2. Assess the long-term (60d) effects of post-ischemic CR on hippocampal CA1 neuronal counts.

CHAPTER 2

LITERATURE REVIEW

2.1 Stroke as a health issue

Stroke, a reduction in blood flow to the brain, is a major cause of death and chronic disability in North America. Ischemic strokes account for about 80% of all strokes and are caused when a blood clot causes disruption of blood flow to the brain. Approximately 20% are hemorrhagic strokes, and are caused by bleeding into the brain when a blood vessel bursts (Heart and Stroke Foundation of Canada, 2006). There are currently more than 50 000 strokes in Canada each year and incidence is anticipated to increase as the population ages (Heart and Stroke Foundation of Canada, 2006).

The effects of a stroke will vary depending on what regions of the brain are damaged and the severity of damage. Stroke survivors can experience weakness and paralysis on one or both sides of the body, ataxia, learning impairments, memory loss, agnosia, aphasia, and depression, as well as difficulties with breathing, chewing, and swallowing. These statistics illustrate the serious morbidity and mortality of stroke with 40% of stroke victims being moderately to severely impaired following stroke, and 10% of stroke victims being severely disabled and requiring long-term care (Heart and Stroke Foundation of Canada, 2006). Despite the demand for forms of treatment that will reduce stroke-induced brain injury, so far attempts to improve stroke outcome have been largely unsuccessful (Ginsberg, 2009; Gladstone et al., 2002).

Intravenous tissue plasminogen activator (tPA) is currently the only validated therapy for acute ischemic stroke (Ginsberg, 2009; Savitz and Fisher, 2007; Besancon et al., 2008). Tissue plasminogen activator is injected into the bloodstream where it can travel to the site of the clot and start to break it up. Unfortunately, tPA cannot be given to stroke patients more than three hours after the onset of their symptoms because it is no longer effective and can increase the risk of bleeding in the brain. Multiple tests are also required to verify the occurrence of ischemic rather than hemorrhagic stroke and the majority of patients are not able to receive this treatment in time. Depending on the location of the blood clot, tPA may be injected intra-arterially directly at the site of the clot as long as six hours after the onset of symptoms, although this treatment requires special equipment and technical expertise that is only available in a small number of hospitals. Other approaches being investigated to establish more rapid reperfusion include mechanical clot retrievers and intra-cranial ultrasound (Fisher and Brott, 2003).

Neuroprotective therapies are developed with the goal of impeding the cell death cascade occurring post-ischemia. These therapies are targeted at specific mechanisms involved in ischemic brain damage. Categories of neuroprotective drugs include glutamate receptor antagonists, modulators of the inhibitory transmitter gamma-aminobutyric acid, sodium and calcium channel blockers, antioxidants, anti-inflammatory and anti-apoptotic drugs, and neurotrophins (Thornhill and Corbett, 2001). Despite the success of several of these drugs in animal studies, the results have been negative in clinical trials. One of the reasons suggested for the failure of neuroprotectants in clinical trials is that the majority of therapies tested target only one part of the ischemic cascade (Ginsberg, 2009; Savitz and Fisher, 2007; Fisher and Brott, 2003). Alternate methods of

neuroprotection are needed and should involve combination therapies, such as the use of multiple drugs, to target multiple sites in the ischemic cascade, along with some form of reperfusion (Fisher, 2003). Other forms of treatment need to be investigated in addition to targeted drug therapy. Nutritional status is a modifiable factor that can influence stroke outcome (Gariballa, 2000). However, the majority of nutrition-related stroke research to date has focused on reducing risk for developing stroke. Our laboratory has been investigating how nutritional factors can also play a role in determining the extent of disability that results from stroke.

Poor nutritional status is common in stroke patients and has been related to increased mortality and reduced functional outcome (Smithard et al., 2007; Gariballa and Sinclair, 1998; Gariballa et al., 1998b; Gariballa et al., 1998a). Protein energy malnutrition (PEM), in which protein and energy status are suboptimal, exists in approximately 16% of the elderly upon admission for stroke (Davalos et al., 1996; Axelsson et al., 1988). Correlations have been made between PEM at stroke admission and increased risk of morbidity and mortality in clinical studies (Gariballa et al., 1998b; Gariballa et al., 1998a; Davalos et al., 1996). Unfortunately these studies are limited by study design and small sample size, and a causal link is more difficult to establish in clinical studies. A major international, multi-centre study that had the potential to establish causality between PEM and stroke outcome was the FOOD (Feed or Ordinary Food) Collaboration Trial. Preliminary data suggested that compromised baseline nutritional status of acute stroke patients was associated with decreased chance of survival and increased functional dependency after six months (The FOOD Trial Collaboration, 2003). However, the completed trials failed in one of the study goals,

which was to establish that poor nutritional status worsens stroke outcome, due to the lack of standardized nutritional assessments (Prosser-Loose and Paterson, 2006; Dennis et al., 2005a; Dennis et al., 2005b). Results from a study in our laboratory were the first to show that PEM impairs functional outcome in an animal model of brain ischemia as measured by the open field behavioural test (Bobyn et al., 2005). While poor nutritional status appears to negatively affect stroke outcome, little is known about nutritional factors that could exert a positive influence on stroke. This research aims to further our knowledge on the effects of nutritional status on long-term stroke outcome by investigating the impact of caloric restriction initiated after brain ischemia.

2.2 Mechanisms of ischemic brain damage and recovery

2.2.1 Mechanisms of acute brain injury

While the brain makes up only 2% of the body weight, it receives 20% of the total cardiac output and accounts for 20% of total oxygen consumption (Juurlink and Sweeney, 1997). Anoxic conditions within the centre of the infarction (ischemic core) cause necrotic cell death to occur within minutes. While the ischemic core is not salvageable, the penumbra, lying between the ischemic core and the unaffected parts of the brain, suffers milder injury that may be salvageable or that may progress to infarction; this is due to ongoing excitotoxicity and secondary processes such as spreading depression, inflammation and apoptosis (Barber et al., 2001). Thus, neuroprotective therapy is aimed at salvaging the penumbral tissue.

The cell death cascade involved in ischemic brain damage is complex and includes adenosine triphosphate (ATP) depletion, glutamate excitotoxicity, membrane depolarization, increased intracellular calcium, production of free radicals and

inflammation (Harukuni and Bhardwaj, 2006; Juurlink and Sweeney, 1997). The decrease in cerebral blood flow deprives the brain of both glucose and oxygen and leads to decreased production of ATP, resulting in anaerobic glycolysis and acidification. Energy depletion also results in the depolarization of neuronal and glial membranes, releasing glutamate into the extracellular space. Diffusion of glutamate can create spreading waves of depolarization in the penumbra, further increasing energy consumption. Excess extracellular glutamate causes overstimulation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA), and kainate-type glutamate receptors causing a dramatic increase in intracellular calcium, sodium and chloride (Hazell, 2007; Lee et al., 2000). Water passively follows the sodium and chloride resulting in edema (Lee et al., 2000).

Increased intracellular calcium concentrations are thought to be a major factor in the development of tissue damage following ischemia. Calcium accumulation causes activation of calcium dependent hydrolytic enzymes which break down membrane phospholipids, resulting in the production of reactive oxygen and nitrogen species (Hazell, 2007). Increased production of free radicals can overwhelm endogenous antioxidants and lead to oxidative stress and inflammation. The latter are important mediators in brain damage following stroke (Block, 1999).

The brain is especially susceptible to oxidative damage because it is rich in polyunsaturated fatty acids, susceptible to lipid peroxidation, and neurons have relatively low levels of antioxidants (Coyle and Puttfarcken, 1993). Reactive oxygen species (ROS) promote destruction of cellular macromolecules and initiate a number of signalling pathways that can result in cell death. Oxidative stress is particularly

pronounced during the reperfusion period. The re-establishment of blood flow brings oxygen to the site of the injury, producing superoxide, nitric oxide and peroxynitrite, that along with secondary messengers induced by calcium, can cause activation of transcription factors such as nuclear factor kappa B (NFκB). NFκB activation leads to the synthesis of a number of other pro-inflammatory cytokines (Bordone and Guarente, 2005).

Cytokines and other pro-inflammatory molecules are responsible for the accumulation of inflammatory cells in the brain following ischemia. Activated cytokines such as tumor necrosis factor-α and interleukin-1β can lead to tissue damage (Block, 1999). Activated microglia, astrocytes, leukocytes and endothelial cells can exacerbate tissue damage by producing cytotoxic molecules. Neutrophils are the first inflammatory cells to accumulate in the brain as soon as 30 minutes after ischemia. Neutrophils can cause tissue damage by adhering to the microvasculature and releasing ROS and proteolytic enzymes, increasing blood brain barrier permeability and leading to further infiltration of inflammatory cells, edema and hemorrhage (Barber et al., 2001; Doyle et al., 2008).

Under ischemic conditions, energy failure and disruption of cellular ion homeostasis lead to the death of neurons. Exactly how neurons die following ischemia is controversial (Colbourne et al., 1999). Cell death can exhibit features of both necrosis (plasma membrane failure and cell swelling) and apoptosis (chromatin condensation, loss of cell volume, apoptotic bodies and DNA fragmentation). This may be due to the fact that both excitotoxicity and programmed cell death are triggered by ischemia. Excitotoxic necrosis is considered the predominant mechanism of ischemic cell death in

most cases, but apoptosis may also occur. Necrosis is thought to predominate in the core acutely following ischemia, while apoptosis may predominate in the penumbra at later time intervals (Lee et al., 1999). Whether a cell undergoes apoptosis or necrosis may depend on a number of factors such as the severity of injury, neuronal maturity, the availability of trophic support and the concentration of intracellular free calcium (Lee et al., 1999). It is important to recognize that therapies designed to attenuate only necrosis may result in the promotion of apoptosis (Gladstone et al., 2002).

2.2.2 Mechanisms of recovery (brain plasticity)

While ischemia leads to tissue damage and cell death, it also results in the activation of endogenous neuroprotective mechanisms (Lee et al., 2000). Acute responses to ischemia include the release of the inhibitory neurotransmitter gamma-aminobutyric acid and a decrease in NMDA receptor function which act to reduce the excitability of circuits and deplete extracellular calcium and sodium. This results in decreased calcium entry into vulnerable neurons (Lee et al., 2000).

The inflammatory process evolved to fight infection and to aid in tissue repair. In the normal brain, the blood brain barrier blocks the infiltration of inflammatory cells from the bloodstream preventing an inflammatory response. In the ischemic brain, the blood brain barrier becomes compromised, allowing inflammatory cells to enter the brain. Despite the detrimental effects of excessive inflammation in the ischemic brain, some inflammation following stroke may be beneficial (Danton and Dietrich, 2003). Ischemia-induced activation of pro-inflammatory molecules and the subsequent inflammatory response may aid in repair and the scavenging of necrotic tissue (Danton and Dietrich, 2003). As necrotic tissue is resorbed, edema subsides, and circulation to the penumbra

can be re-established allowing for functional recovery for some neurons (Lee and van Donkelaar, 1995).

Stroke survivors typically show some degree of functional recovery although the extent of recovery is highly variable (Lee and van Donkelaar, 1995; Chen et al., 2002). Recovery in the first few days following ischemia may be due to resolution of edema and/or reperfusion of the ischemic penumbra (Chen et al., 2002). Recovery occurring in the weeks to months following ischemia is attributed to neuroplasticity (Lee and van Donkelaar, 1995; Chen et al., 2002; Bayona et al., 2005; Teasell et al., 2005). Neuroplasticity refers to the ability of the brain to reorganize itself, changing its structure following tissue damage (Di Filippo et al., 2008). Neuroplasticity is also required for the normal functioning of the central nervous system and occurs during maturation and when learning from environmental challenges. Plasticity occurring post-ischemia has been documented in both animal models and human stroke survivors (Di Filippo et al., 2008). Neuroplastic changes can occur through a number of mechanisms including the unmasking of latent synapses, the formation of new synapses as a result of neuronal sprouting, and circuit redundancy where some functions previously performed by damaged regions can be taken over by intact regions (Lee and van Donkelaar, 1995; Teasell et al., 2005). Ischemia-induced changes in neurotransmitters, neurotrophins, growth factors, and hormones also contribute to neuroplasticity (Johansson, 2000).

Neurotrophins are thought to be involved in numerous functions within the nervous system including aspects of cell proliferation, differentiation, death, survival, and plasticity (Johansson, 2000; Arevalo and Wu, 2006). Brain-derived neurotrophic factor (BDNF) plays an important role as a neuronal survival factor, during both development

and in neuronal plasticity after brain injury (Bolton et al., 2000; Nawa et al., 1997). Increased BDNF following ischemia is believed to promote dendritic sprouting and synaptic remodelling which may lead to improved recovery (Lee et al., 2002; Larsson et al., 1999; Kiprianova et al., 1999; Ferrer et al., 1998; Tsukahara et al., 1994). Stem cells located in the dentate gyrus can migrate and differentiate into neurons in the hippocampus. Brain derived neurotrophic factor has been shown to enhance neurogenesis and survival of newly generated neurons (Lee et al., 2002). This provides another potential therapeutic approach to investigate for aiding in recovery following ischemic damage (Johansson, 2000; Schmidt and Reymann, 2002; Abe, 2000).

2.3 Caloric restriction (CR)

Caloric restriction (CR) is a nutritional intervention in which energy intake is reduced by 30-40% while sufficient intake of other nutrients is maintained (Young and Kirkland, 2007). There are two basic CR paradigms: 1) controlled CR in which animals are provided a daily allotment of food that is 30-40% less than the ad libitum consumption of a control population, and 2) intermittent fasting (IF) in which animals are deprived of food for a full day and are fed *ad libitum* on intervening days (Anson et al., 2003; Mattson et al., 2004). Both methods usually result in a decrease in body weight over time although the decrease is often much more pronounced in controlled CR. Both paradigms have reported similar effects including dramatic increases in lifespan and it is assumed that both CR and IF act through similar mechanisms (Anson et al., 2003).

Energy restriction has been shown to be one of the most efficient and reproducible ways to extend average and maximum life span. In the 1930's, McCay and colleagues showed that a diet that was reduced in energy was able to extend life span in

rats (McCay et al., 1935). Since that time, CR has been shown to extend life span in yeast, worms, fruitflies, waterfleas, spiders, and fish (Martin et al., 2006; Bordone and Guarente, 2005). Studies are currently underway to investigate the effects of CR in primates and humans. One study in rhesus monkeys initiated in 1987 found improved health and increased life span in monkeys on a 30% CR diet compared to monkeys on control diet (Mattison et al., 2003). Lifespan extension appears to increase progressively as energy is reduced up to approximately 50%, beyond which further CR may be detrimental and can result in mortality (Mattson, 2005). The time of onset and duration of the CR regimen will also determine the amount by which life span is extended (Martin et al., 2006).

Although some researchers argue that increases in life span observed in rodents on CR are not likely to be observed in humans (Phelan and Rose, 2005), many studies in humans suggest a relationship between caloric restriction and good health. Energy restriction occurs naturally in some human populations although most of these populations are lacking in protein and micronutrients as well as energy, which does not meet the formal definition of CR (Heilbronn and Ravussin, 2003). One interesting case of naturally occurring CR is the centenarians of Okinawa, Japan. Studies of Okinawan centenarians have revealed that while adult Okinawans have the same protein and lipid intake as the mainland Japanese, their energy intake is approximately 20% less. Interestingly, mortality rates due to cerebrovascular disease, cancer, and heart disease on Okinawa were 59%, 69%, and 59% respectively of those for the rest of Japan (Kagawa, 1978). However, it is likely there are differences in genetics and environmental factors between these populations as well.

The Biosphere 2 in Oracle, Arizona was designed as a man-made ecosystem, containing five areas based on natural biomes, to study the natural ecosystems of the Earth and the effects of humans within them. A crew of eight people lived inside Biosphere 2 from 1991 to 1993 during which time the Biosphere was sealed off from the outside world. The air, water and food supporting the crew inside Biosphere 2 was generated and recycled within the Biosphere. The crew from Biosphere 2, underwent CR when food production inside the enclosed biosphere habitat was less than expected (Weyer et al., 2000). Findings from the Biosphere 2 volunteers confirmed that 30% CR imposed for two years could produce many of the expected physiological, hormonal, and morphological effects expected from animal studies (Walford et al., 2002).

While clinical studies have recently been initiated and some individuals have begun to voluntarily restrict themselves in the hope of extending their lives (Speakman and Hambly, 2007), CR may be a questionable long-term diet strategy. In practice, it may be very difficult to implement long-term CR in humans, as individuals who overeat are often unable to voluntarily limit their food intake despite knowledge of the considerable health hazards (Mattson, 2005). More research also needs to be done on the degree of CR necessary for the health benefits seen in rodents as this degree of restriction may not be attainable for all people.

Most animal research on CR has been done in comparison with a control group fed *ad libitum*. Under standard laboratory conditions**,** *ad libitum* food intake is excessive and exercise is limited due to the small cage size (Young and Kirkland, 2007). Thus, the control animals are more likely to represent obese individuals than non-obese individuals. This creates difficulties in interpreting the effects of CR, as it is difficult to separate the

beneficial effects of weight loss in obese individuals from potentially beneficial effects of CR in a normal weight population (Ramsey et al., 2000). More research is needed to determine the effects of CR on non-obese subjects (Bordone and Guarente, 2005).

To address these potential problems, research efforts are aimed at developing CR mimetics, compounds that can mimic the beneficial effects of CR without actually reducing caloric intake (Ingram et al., 2006; Fontan-Lozano et al., 2008). Some CR mimetics currently being studied include 2-deoxyglucose which inhibits glycolysis, metformin which enhances the action of insulin, and resveratrol which affects stress signalling pathways (Ingram et al., 2006).

2.3.1 Caloric restriction and protective mechanisms against disease

Rodents, monkeys and humans exposed to CR have decreased body temperature, heart rate, blood pressure, serum glucose and insulin levels (Anson et al., 2003; Fontana et al., 2004; Wan et al., 2003; Young et al., 1978), which have been shown to decrease the risk of diabetes and cardiovascular disease (Mattson and Wan, 2005). Caloric restriction also decreases lipid accumulation and can decrease low-density lipoprotein cholesterol levels while increasing high-density lipoprotein cholesterol levels (Roky et al., 2004). Caloric restriction also reduces the incidence of spontaneous tumors and suppresses the development and growth of cancers (Ingram et al., 2007; Mattson, 2005; Mattson and Wan, 2005; Heilbronn and Ravussin, 2003).

Caloric restriction can also impact the health of the nervous system. Caloric restriction has been shown in a number of behavioural tests to prevent age-related declines in learning, memory, and motor coordination, thus helping to maintain function later into life (Mattson, 2005; Takahashi et al., 2006; Gould et al., 1995). Caloric

restriction can decrease risk and improve existing symptoms for a number of neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease (Duan et al., 2003; Halagappa et al., 2007; Qin et al., 2006; Maswood et al., 2004; Duan and Mattson, 1999; Zhu et al., 1999). Animal models of Alzheimer's Disease have shown that animals maintained on CR exhibit reduced neuropathology and reduced learning and memory deficits (Halagappa et al., 2007; Qin et al., 2006; Wang et al., 2005). In animal models of Parkinson's Disease, IF and CR have shown protection against dysfunction and degeneration of mid-brain dopaminergic neurons (Duan and Mattson, 1999) and a reduction in behavioural deficits (Maswood et al., 2004). Intermittent fasting has also been shown to delay neurodegeneration and motor dysfunction in a mouse model of Huntington's disease, resulting in increased survival (Duan et al., 2003).

Two major mechanisms of action have been proposed for CR: 1) increased cellular stress resistance and 2) decreased free radical production and oxidative damage (Martin et al., 2006; Mattson and Wan, 2005). Organisms have evolved to respond to stressors or challenges in their environment. Mild or transient exposure to ischemia, hypoxia, or other environmental stressors inflicts stress on brain cells which respond by enhancing their ability to resist more severe stress. Preconditioning/ischemic tolerance refers to an acquired resistance to ischemia that can result from previous exposure to conditioning episodes of mild ischemia (Obrenovitch, 2008; Dirnagl et al., 2003; Farrell et al., 2001). The stress response evoked following ischemic preconditioning involves the production of heat shock proteins, antioxidant enzymes, anti-apoptotic factors, and growth factors which help to prevent tissue damage during subsequent ischemic attacks

(Corbett et al., 2006). Preconditioning is thought to be a major mechanism whereby CR can increase lifespan. Caloric restriction is thought to act as a mild stressor (Heydari et al., 1996; de Cabo et al., 2004) and results in the activation of a number of compensating mechanisms. Evidence that CR increases basal levels of glucocorticoids supports this theory (Wan et al., 2003; Patel and Finch, 2002). Increased levels of stress proteins, such as heat shock protein-70 (Yu and Mattson, 1999), neurotrophic factors such as BDNF (Lee et al., 2000; Lee et al., 2002; Duan et al., 2003; Duan et al., 2001), and increased neurogenesis in the brain (Lee et al., 2000; Lee et al., 2002) have also been reported in response to CR.

Sirtuin proteins are a type of histone deacetylase that can also increase stress resistance. The induction of sirtuins by CR is thought to be an important mechanism in life span extension (Bordone and Guarente, 2005). In yeast, life span extension by CR depends on the upregulation of the silent information regulator 2 (SIR2) gene (Lin et al., 2000; Kaeberlein et al., 1999). Caloric restriction also results in the induction of sirtuin 1 (SIRT1), the mammalian homologue of SIR2, which increases cellular stress resistance, thereby protecting against stress-induced cell death (Cohen et al., 2004; Nemoto et al., 2004). Sirtuin 1 has also been shown to downregulate NFκB, an important mediator of inflammation following ischemia (Yeung et al., 2004).

Decreased oxidative damage and inflammatory response have also been demonstrated in CR models (Pamplona et al., 2002; Chandrasekar et al., 2001; Forster et al., 2000; Spaulding et al., 1997; Sohal et al., 1994). While overeating increases oxidative stress in cells and results in oxidative damage to cellular macromolecules, CR reduces the production of ROS, resulting in decreased oxidative damage. Caloric

restriction may suppress oxidative stress through a number of mechanisms including reducing the amount of superoxide radicals produced in the mitochondria and by increasing the production of chaperone proteins and antioxidant enzymes (Mattson, 2005).

2.3.2 Caloric restriction and ischemic brain damage

Caloric restriction could be expected to exert marked protective effects in stroke because of its ability to modify multiple pathways in the ischemic cascade, as well as neuroplasticity mechanisms important to recovery. These include increasing production of stress and cell survival proteins (Lin et al., 2000; Yu and Mattson, 1999), reducing oxidative damage and the inflammatory response (Pamplona et al., 2002; Chandrasekar et al., 2001; Forster et al., 2000; Spaulding et al., 1997; Sohal et al., 1994), lowering core body temperature (Ferguson et al., 2007; Heilbronn et al., 2006; Lane et al., 1996) and increasing neurogenesis (Lee et al., 2000; Lee et al., 2002) and production of growth factors (Lee et al., 2000; Lee et al., 2002; Duan et al., 2003; Duan et al., 2001).

One previous study has examined whether IF was protective when administered for three months prior to focal ischemia, a model in which blood flow is reduced to a specific brain region. Yu and Mattson (1999) reported that rats on an IF regimen had reduced brain infarct volume and improved functional outcome following middle cerebral artery occlusion, a model of focal ischemia in the rat (Yu and Mattson, 1999). Increased levels of the stress protein heat shock protein-70 in striatal cells of CR animals suggest the neuroprotective action of CR might be due, in part, to preconditioning. Although a cursory assessment of these data suggest that CR may be a valuable approach for improving outcome following stroke, this study has major flaws when assessed against

the standards recommended for experimental stroke research (Stroke Therapy Academic Industry Roundtable, 1999) (see section 2.4.3.1 below). One limitation is that outcome was only assessed at 24 hours so it is not known if the decrease in brain damage was transient or permanent. In addition, brain function was assessed by a crude neurological score (Hunter et al., 2000) instead of the more sensitive tests of functional outcome available for focal ischemia models such as a skilled reaching task (Gharbawie et al., 2005; Farr and Whishaw, 2002). For example, the staircase test, in which animals reach to retrieve a food pellet from a set of stairs, shows great sensitivity in detecting persistent disability months after focal ischemia, unlike simpler sensorimotor tasks (Gladstone et al., 2002; Corbett and Nurse, 1998). Caloric restriction (40%) administered before and continued after global ischemia has also been suggested to improve ischemia-induced memory impairments (Roberge et al., 2008a; Roberge et al., 2008b), however no studies have yet examined the effects of CR provided solely during the post-ischemic period.

The clinical relevance of testing CR provided prior to stroke can also be questioned since the chronic energy restriction of 30-40% that is beneficial in experimental animals is not likely an attainable goal for the human population (Ingram et al., 2006). It is of clinical interest, however, to investigate whether CR administered after the stroke could form part of a neuroprotective strategy to diminish brain damage and disability.

2.4 Animal models of stroke

Appropriate animal models are necessary for the study and treatment of human cerebral ischemia. Cerebral ischemia has been studied in large and small animal species. Small animal species (especially rodents) present a number of advantages over large

animal species including the lower cost of obtaining and maintaining animals, lower cost of procedures due to smaller size, relative genetic homogeneity, close resemblance of the cerebrovascular anatomy and physiology to that of higher species and greater acceptability (Ginsberg and Busto, 1989). Procedures in small animals can also be controlled so that injury is more reproducible and less variable than in larger species.

2.4.1 Focal ischemia

In focal ischemia, blood flow is reduced to a very specific brain region producing an ischemic core of unsalvageable tissue and the surrounding penumbra which has the potential to be saved (Traystman, 2003). Focal ischemia models are often used because of their relevance to human stroke. Focal models can involve either permanent or transient artery occlusion. The most common focal ischemia model involves occlusion of the middle cerebral artery. Middle cerebral artery occlusion (MCAO) results in a reduction in blood flow in the striatum and cortex. Focal ischemia models are more technically demanding than global ischemia models. Vessel occlusion is most often accomplished with the use of clips, but can also be accomplished by electrocoagulation and photochemical irradiation. Other focal ischemia models include blood clot embolism and intraluminal filament placement within the carotid artery and ligature snare placement around the middle cerebral artery (Traystman, 2003). Occlusion of the common carotid artery in conjunction with that of the middle cerebral artery aids in further reducing blood flow to the area. Injection of the vasoconstrictor endothelin into the cortex and striatum and adjacent to the middle cerebral artery, into the forelimb region of the motor cortex, has also been used to reduce blood flow (Windle et al., 2006).

2.4.2 Global ischemia

Global ischemia occurs when cerebral blood flow is reduced throughout the brain causing neuronal injury to selectively vulnerable areas. Global ischemia models involve a brief termination of cerebral blood flow (5-15 minutes) followed by reperfusion. While global ischemia models have been criticized for mimicking human cardiac arrest rather than focal stroke, they share many of the same pathophysiological mechanisms (Harukuni and Bhardwaj, 2006) and provide more consistent injury (McBean and Kelly, 1998). Thus, global stroke models are useful for understanding mechanisms of tissue damage and evaluating the efficacy of interventions before advancing to focal models where more variables must be controlled (Ginsberg and Busto, 1989; Small and Buchan, 2000). The three most common models of global ischemia are the four-vessel occlusion (4-VO) and two-vessel occlusion (2-VO) models in the rat and bilateral common carotid artery occlusion (BCCAO) in the gerbil.

Pulsinelli and Brierley (1979) developed the 4-VO model of reversible forebrain ischemia in rats. The 4-VO model is highly technically demanding and involves a two-step process to produce ischemia. Anterior vertebral arteries are cauterized, followed the next day by occlusion of the common carotid arteries. The 4-VO model results in extensive brain injury including damage in the striatum, hippocampus, and neocortex and commonly results in increased mortality.

The 2-VO model of ischemia developed by Eklof and Siesjo (1972) involves bilateral common carotid artery occlusion coupled with systemic hypotension to produce ischemia. Two-vessel occlusion in the rat is a much simpler surgery and produces consistent and reliable damage. Inducing hypotension during occlusion is a critical part

of the technique. The 2-VO model produces injury that is similar to the 4-VO model with damage occuring within the hippocampus, neocortex and caudoputamen as a function of occlusion time.

2.4.3 Bilateral common carotid artery occlusion in Mongolian gerbils

Until recently, the gerbil model of global ischemia, in which global forebrain ischemia is produced by BCCAO, was used extensively in stroke research. Bilateral common carotid artery occlusion requires minimal surgical intervention and has historically produced very consistent, reproducible injury to the hippocampal CA1 region (Corbett and Nurse, 1998; Small and Buchan, 2000). Consistency of the injury had been attributed to the gerbil's unique vascular anatomy as they lacked posterior communicating arteries, and therefore had an incomplete Circle of Willis (Ginsberg and Busto, 1989).

Unfortunately, recent work has discovered that cerebral vasculature in the North American commercial supply of gerbils is changing. Experiments in our laboratory (Bobyn et al., 2005; Ji et al., 2008) and others (Laidley et al., 2005; Wang et al., 2002; Breuer and Mayevsky, 1992) have shown increased variability in hippocampal damage and functional effects following BCCAO. In 2005, Laidley and colleagues compared the vasculature of gerbils from Charles River Canada (our current supplier) with those from High Oak Farms (ON, Canada). Approximately 61% of animals from Charles River were found to have a complete (22.7%) or partial (38.6%) Circle of Willis due to the presence of significant posterior communicating arteries. These animals had less severe CA1 loss as well as attenuated behavioural deficits in the open field after undergoing BCCAO compared to those with no posterior communicating arteries. Posterior

communicating arteries were also found in gerbils from High Oak Farms although to a lesser extent (2.6% with bilateral and 13.2% with unilateral); High Oak Farms is no longer a reliable commercial source of gerbils. Similar results have since been reported in gerbils from other major North American suppliers (Seal et al., 2006).

A screening technique based on activity monitoring was developed in our laboratory as a temporary solution to this problem, and this approach was used for the current study to ensure consistent forebrain ischemia. Following ischemia, animals exhibit transient locomotor hyperactivity for up to 30 hours (Corbett and Nurse, 1998). This hyperactivity is a reliable indicator of ischemic severity (Corbett et al., 1997; Mileson and Schwartz, 1991). Considering the possibility for both false positive and false negative results, a previous study in our laboratory demonstrated that the monitoring method correctly categorizes 85% of animals tested (M.I. Harmon et al., unpublished observations) although this estimate is limited by a small sample size. Although screening greatly reduces experimental variability, the disadvantage is that a much larger sample size is required in order to replace those that fail to meet the criteria.

2.4.3.1 Endpoints and standards for assessing hippocampal damage in the bilateral common carotid artery occlusion model

As with the other global ischemia models mentioned above, BCCAO in the gerbil results in neuronal cell death in several vulnerable brain structures, including the hippocampal CA1 region, the neocortex, and striatum. Cell death does not occur immediately but is delayed with most cell death occurring 3-7 days following ischemia. Delayed cell death provides a window of opportunity when intervention may reduce cell loss and associated learning and memory deficits (Colbourne and Corbett, 1994). This

delayed and selective cell death has been well characterized and is similar among all global ischemia models (Small and Buchan, 2000). The extent of brain injury is usually assessed histologically by counting viable hippocampal CA1 neurons since these neurons are highly susceptible to global ischemic injury.

Five minutes of global ischemia results in extensive loss of CA1 neurons in the hippocampus by seven days after surgery (Corbett and Nurse, 1998; Colbourne and Corbett, 1994; Nurse and Corbett, 1994). Cell death in the CA1 region following global ischemia is highly reliable and damage is more consistent than in other brain regions. The distinctive distribution and large size of CA1 neurons also makes them relatively easy to quantify (Corbett and Nurse, 1998). Histological and behavioural endpoints for the gerbil BCCAO model have been developed and are well established. Studies have shown a relationship between damage in the CA1 region of the hippocampus and deficits in spatial learning.

The results obtained with rodent models of stroke are only reliable when experiments are carried out according to the current recommendations for quality preclinical stroke research (Gladstone et al., 2002; Stroke Therapy Academic Industry Roundtable, 1999). While histological endpoints, such as cell counts and infarct volume, are frequently used to assess the efficacy of potential therapeutic interventions, it is important that functional endpoints are also assessed (Stroke Therapy Academic Industry Roundtable, 1999; Corbett and Nurse, 1998; Colbourne and Corbett, 1995). Neurons that have a normal histological appearance can be functionally abnormal (Corbett and Nurse, 1998), and lesion size does not always correlate with functional impairment (Stroke Therapy Academic Industry Roundtable, 1999). Functional recovery is the major

endpoint in clinical trials and can persist for months after ischemia. Therefore it is important that functional endpoints be assessed in combination with histological endpoints (Gladstone et al., 2002; Corbett and Nurse, 1998).

Behavioural tests such as the open field test, T-maze and radial arm maze have demonstrated that gerbils exposed to global ischemia show profound learning deficits (habituation and working memory impairments) similar to humans (Mileson and Schwartz, 1991; Colbourne and Corbett, 1995). These behavioural tests are used to assess functional outcome and have been shown to be reliably sensitive to ischemia (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Colbourne et al., 1998a). The open field test is a useful functional test for global ischemia research because it is sensitive to differences in CA1 injury and can differentiate groups treated with beneficial therapies from non-treated ischemic controls. Other benefits of the open field test are that test sessions are relatively short, it is not labour intensive, and functional deficits can persist for months (Corbett and Nurse, 1998).

Immediately following stroke surgery, gerbils progress through a series of changes in their activity patterns. As the gerbils recover consciousness they enter a quiet period during which they often exhibit a characteristic hunching (Corbett and Nurse, 1998). This quiet period is followed by a period of heightened locomotor activity, which as indicated above, can be used as an indicator of ischemic severity (Mileson and Schwartz, 1991). This period lasts for 24-48 hours after surgery, after which activity levels return to normal.

Following the initial hyperactivity phase, a more chronic form of heightened locomotion occurs that can only be detected when animals are placed in a novel

environment. When placed in an open field, ischemic gerbils display significantly elevated locomotion compared to sham-operated gerbils, which has been interpreted as an inability to habituate to a novel environment (Corbett and Nurse, 1998). Sham-operated animals will gradually habituate to the novel environment, resulting in a decrease in activity, while animals exposed to global ischemia will continue to exhibit high activity levels. This is believed to be due to a spatial learning deficit (Colbourne et al., 1998b; Wang and Corbett, 1990). If testing is repeated regularly, ischemic animals will gradually habituate and activity levels will decrease; differences in activity levels can be seen for months if testing is intermittent (Corbett and Nurse, 1998). Ischemia-induced hyperactivity in novel environments has been found to correlate with CA1 cell death in the hippocampus (Corbett and Nurse, 1998; Mileson and Schwartz, 1991; Nurse and Corbett, 1994).

Some investigators suggest that anxiety also influences open field activity following ischemia (Plamondon and Khan, 2005). An unfamiliar environment may be mildly anxiogenic as animals may consider it potentially dangerous. Upon exposure to a novel environment animals may initially exhibit decreased exploratory behaviour, increased heart rate, urination, defecation, and plasma corticosterone levels (Palanza, 2001). It has been suggested that ischemia results in increased activity because it causes disinhibition to explore novel environments (Plamondon and Khan, 2005). Rodents are known to prefer the periphery of the open field to the centre, a behaviour known as thigmotaxis. Increased time spent in the periphery of the open field may indicate increased anxiety while increased time spent in the centre of the open field is considered anxiolytic (Calabrese, 2008; Prut and Belzung, 2003). Thus, it is possible that measuring

activity in the centre relative to that in the periphery of the open field might yield additional information about functional outcome after global ischemia. This approach has, however, never been validated in a global ischemia model.

The T-maze is another useful behavioural test that can be used to detect deficits in working memory following global ischemia in the gerbil (Corbett and Nurse, 1998). Animals are tested in two trials. Information learned in the first trial must be remembered in the second trial in order for the animal to be successful. The Win-Shift strategy is the most commonly used application of the T-maze. On the first trial, the animal is allowed access to one arm of the maze to receive a food reward. On the second trial, both arms of the maze are accessible, but the animal will only receive a reward if it enters the opposite arm. In the Win-Stay strategy, the animal has to choose the same arm that it entered on the previous trial in order to receive the reward. This strategy is much more difficult for the gerbil to learn as revisiting of the same arm is contrary to the gerbil's normal foraging behaviour (Babcock and Graham-Goodwin, 1997). Delaying the time between trials also increases the difficulty of the test (Corbett and Nurse, 1998). Following ischemia, impairments in working memory are indicated by decreased choice accuracy and increased time to reach criterion (Corbett and Nurse, 1998; Farrell et al., 2001; Babcock and Graham-Goodwin, 1997). One disadvantage of this test is that the use of food rewards may confound results in nutritional studies (Prosser-Loose et al., 2007).

The radial arm maze has also been used to detect reference and working memory deficits in the gerbil following global ischemia although it has been noted that these deficits recover over time (Corbett and Nurse, 1998; Block, 1999). The radial arm maze

presents the same disadvantage as the T-maze for nutritional studies as it also involves the use of food rewards. A second major disadvantage of both the T-maze and radial arm maze is that they are highly time consuming and labour intensive compared to the open field test (Corbett and Nurse, 1998). Because the open field test does not require extensive training and test sessions are short, it is a valuable tool for studies with multiple groups or large numbers of animals. Thus the open field test was chosen for use in this study.

In addition to the need discussed above of employing functional testing as well as histological assessment, animal studies should include long-term endpoints, as some treatments have been found to only postpone cell death (Corbett and Nurse, 1998). Neuroprotection observed in many animal studies have, for the most part, been based on experiments that used short survival times (1-7 days) with histology (cells counts or infarct volumes) as the only endpoint. Studies with longer survival times should be better indicators of whether the effects of interventions are truly neuroprotective (Stroke Therapy Academic Industry Roundtable, 1999).

Specific physiological variables must also be carefully controlled to ensure that the model induces consistent brain damage. In particular, brain temperature is a critically important variable and should be maintained in a constant normothermic range as it can have striking effects on stroke outcome (Small and Buchan, 2000). Hyperthermia can exacerbate ischemic damage while hypothermia can provide robust protection against ischemic damage (van der Worp et al., 2007; Colbourne et al., 1997). Ignoring this key information is one of the reasons thought to account for the failure of neuroprotective treatments in clinical studies.

2.5 Summary

Based on evidence that CR could modify pathways in the ischemic cascade as well as enhance neuroplasticity mechanisms important to recovery, we investigated whether CR provided solely after brain ischemia could decrease brain injury and improve functional recovery. Previous studies have suggested some benefit when a CR regimen was initiated prior to ischemia, but to date no studies have examined the effects of CR solely after ischemia. The gerbil BCCAO model was used to address this question as it is very well-characterized. Histological and behavioural endpoints have been well established for the gerbil BCCAO model and studies have shown a relationship between damage in the CA1 region of the hippocampus and deficits in spatial learning. Due to the emerging problem of changing brain vasculature in the gerbil that has threatened the reliability of this model, we have utilized a method of hyperactivity screening to ensure consistent forebrain ischemia.

CHAPTER 3

MATERIALS AND METHODS

3.1. Acclimation

Adult male Mongolian gerbils (*Meriones unguiculates*, Charles River Canada, QC, Canada), age 11 to 12 weeks, were acclimated for ten days at 22ºC with a 12 hour light/dark cycle in shoebox cages with free access to food and water. Animals were group housed for three days and were then separated into individual cages for the remaining acclimation and experimental period. Regular rat chow was provided for the first three days while the animals were group housed, and purified control diet (see section 3.1.4) was provided for the remaining seven days. All animal care and procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply. Following acclimation, animals were assigned to sham surgery or global ischemia, induced by BCCAO.

3.2 Bilateral common carotid artery occlusion

Anaesthesia was induced with 4.0% isoflurane and 1L/min oxygen and was then reduced down to 2.5-3.0% isoflurane and 1L/min oxygen for the duration of the surgery. Both common carotid arteries were isolated through a ventral midline incision. A surgical silk thread was passed under both arteries allowing them to be gently lifted and isolated. Once the arteries were isolated, they were occluded for five minutes with

microaneurysm clips. Occlusion and reperfusion were visually verified, as this is an ischemia-reperfusion model. During occlusion, brain temperature was estimated via a tympanic probe (Barnant Type T Digi-Sense Thermometer) and maintained at 36.5±0.2ºC by wrapping a Mul-T-Pad® water heated blanket (Global Medical Products, Inc., Burlington, ON , Canada) around the gerbil's head. A rectal probe was used to monitor body temperature, which was maintained with a homeothermic blanket (Harvard Apparatus Canada, St. Laurent, QC, Canada). The incision was then closed and local anaesthetic (Xylocaine) applied. Sham surgery was identical to ischemia surgery with the exception of artery occlusion. Following surgery, gerbils were placed in individual cages and observed for two hours. A heat lamp was placed over part of the cage to keep the gerbil warm but with the opportunity for them to move away from the heat when mobile. Animals were then returned to cages to be monitored for activity. Any animals showing seizures (n=1) were excluded from the study.

3.3 Screening for complete ischemia

After a two hour recovery period, gerbils were monitored for a 20 hour period to determine the success of ischemia surgery. Five grams of purified control diet (see section 3.1.4) and water *ad libitum* were provided during this period. Ischemic animals are known to exhibit heightened activity for up to 30 hr post-ischemia (Corbett and Nurse, 1998) and this hyperactivity can be used as a tool to screen out incomplete forebrain ischemia.

Gerbils were monitored by an Opto-M3 Activity Meter (Columbus Instruments, Columbus, OH, USA) using infrared beam interruption in two-dimensions. The accumulated interruption counts were recorded every 15 minutes. The following criteria

were used to identify animals with severe forebrain ischemia: 1) an activity level greater than three standard deviations above the mean activity level of a pool of laboratory sham animals (n=30) and 2) hyperactivity had to be sustained for the entire 20 hour monitoring period (M.I. Harmon et al., unpublished observations).

3.4 Experimental diets

Following activity monitoring, each of the two surgical groups were randomly divided into control (CON) or caloric restriction (CR) groups, generating four experimental groups; 1) sham - control diet (SCON), 2) sham - caloric restriction (SCR), 3) ischemia - control diet (ICON) and 4) ischemia - caloric restriction (ICR). The experimental design is shown in Figure 3.1. Control and CR diets (Dyets Inc. Bethlehem, PA, USA) were based on the AIN-93M rodent diet (Reeves et al., 1993), but did not contain the antioxidant tert-butylhydroquinone because of its potential to confound studies involving oxidative stress and inflammation (Table 3.1). The two diets were isocaloric, but CR groups were fed 30% less food each day than their respective CON groups to create 30% energy restriction. The diet fed to the CR groups was formulated to ensure that intake of essential nutrients (protein, fat, vitamins and minerals), considering the 30% reduction in food intake, was the same as that of the CON group.

The CON group had free access to the control diet. The amount of CR diet to be supplied to the SCR and ICR groups was calculated separately throughout the experiment since food intake can differ between ischemic and sham gerbils following their respective surgeries (Bobyn et al., 2005). For the first three days following surgery, SCR and ICR animals were fed 30% less than the average food intake observed in a previous experiment of group-housed SCON and ICON gerbils, respectively. Beginning on day

four following surgery, SCR and ICR gerbils were fed 30% less than the actual average intake consumed in the current study by the respective control group during the previous day. Because of the experimental design in which animals were continually added to the experiment, these estimates of food intake were based on an increasing sample size as the study progressed. Daily food intake and weekly body weight were recorded over the 60 day study period.

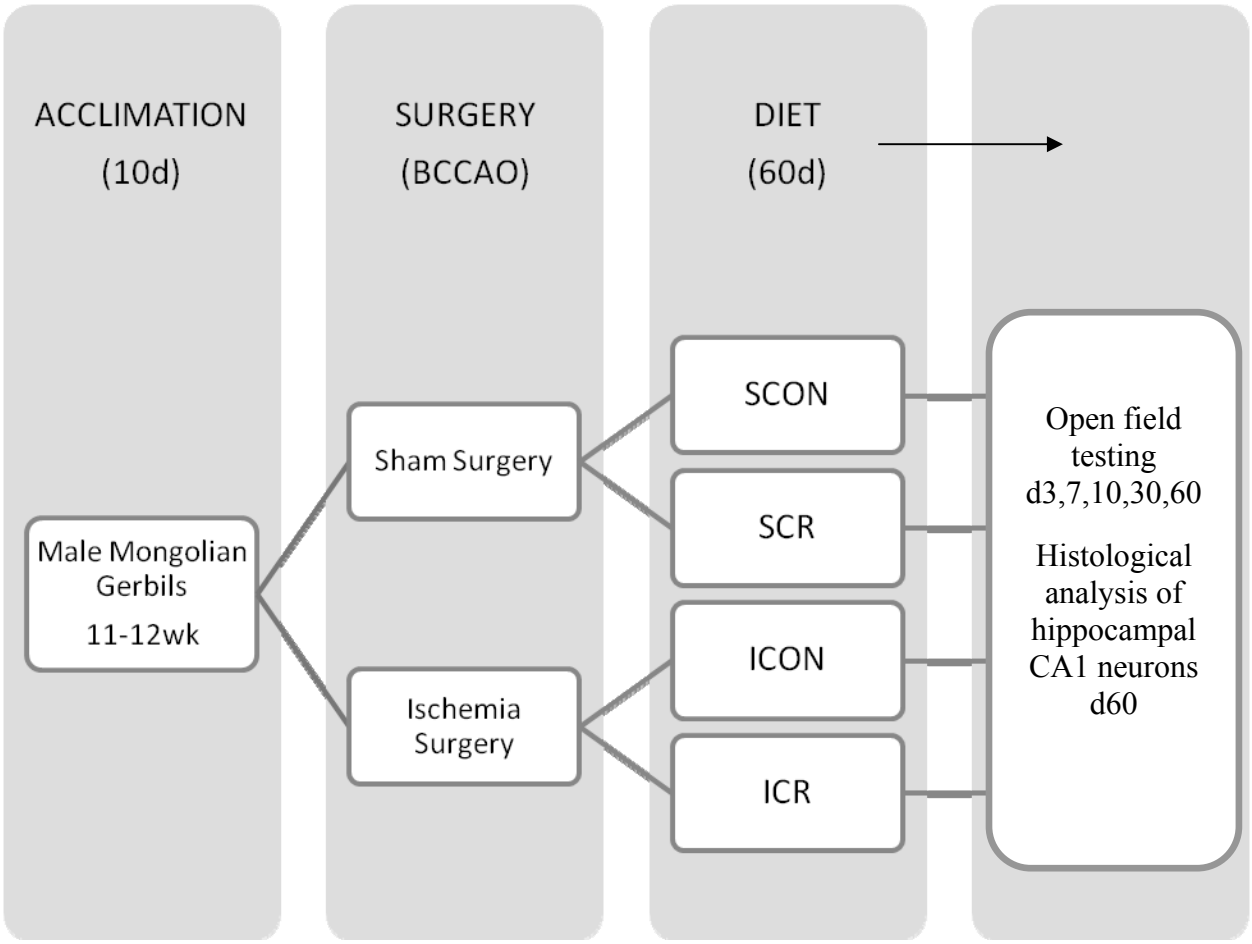

Figure 3.1 Experimental design showing time intervals for all experimental procedures. BCCAO: bilateral common carotid artery occlusion. SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

Table 3.1 Experimental diets

Component	Control (CON) g/kg	Caloric Restriction (CR) g/kg
Vitamin Free Casein	140	199
L-Cystine	1.8	2.6
Sucrose	100	142
Cornstarch	465.7	341.8
DYETROSE	155	119
Soybean Oil [a]	40	57
Cellulose	50	71
Mineral Mix [b]	35	50
Vitamin Mix [c]	10	14
Choline Bitartrate	2.5	3.6

[a] Soybean oil without tert-butylhydroquinone.
[b] AIN-93M mineral mix (Reeves et al., 1993).
[c] AIN-93M vitamin mix (Reeves et al., 1993).

3.4.1 Dietary and euthanasia interventions

Following surgery, it was noticed that some CR animals (both sham and ischemic) were losing weight too rapidly, were losing an excessive amount of weight or were showing signs of weakness. This concern was discussed with one of the University veterinarians. A humane endpoint for euthanasia was identified as well as an experimental intervention strategy to be used if needed. Animals were monitored daily, and gerbils were humanely killed if they showed weakness, poor grooming or other signs of poor health. A decision was made that if 10% of the calorically restricted animals (7/72) had to be euthanized, the following intervention strategy was to be invoked: any animal that lost >35% of body weight, showed a period of excessive rate of weight loss

(>16% in one week) or showed signs of poor grooming or weakness was to be fed 5% more diet each day until the end of the study.

3.5 Behavioural testing

To test for chronic habituation impairments, gerbils were placed in an open field (75 X 75 X 75 cm) on d3, 7, 10, 30 and 60 post-ischemia (Colbourne and Corbett, 1995). The open field was located in a secluded room, and environmental cues (e.g. experimenter, shelving, lighting) were kept constant during testing. Once in the open field, gerbils were monitored by video tape and analyzed (EthoVision Basic, Noldus Information Technology) to determine total distance travelled and preferred location of activity (i.e. periphery vs. centre). The periphery or outer zone was designated as the outside 12 cm of the open field. Each test session lasted 10 minutes.

3.6 Histology

Following open field testing on day 60, gerbils were anaesthetized and perfused transcardially with heparinized saline (4 min at 12 mL/min), followed by 10% neutral buffered formalin (8 min at 12 mL/min). To minimize the occurrence of dark neuron artifact (Cammermeyer, 1962), intact heads were fixed in formalin for 24 hours. Brains were then gently removed and stored in formalin for at least 24 hours prior to paraffin embedding. Brains were cut into 6 μm sections and stained with hematoxylin and eosin (H&E). Slides were blinded to prevent assessment bias, and brain damage was assessed histologically by counting hippocampal CA1 neurons. Cells were counted if they looked viable, were non-eosinophilic, and had a defined cell membrane and nucleus. Neurons were counted bilaterally at 400x magnification in three sections representing the entire anterior-posterior axis of the hippocampus. Neurons were counted in medial, middle, and

lateral sectors (sector length = 0.2mm) of the hippocampal CA1 region at -1.7 and -2.2 mm relative to bregma and in a single sector (middle CA1) at -2.7 mm from bregma using a 200μm square (10X10) microscope grid using a method modified slightly from that of (Colbourne and Corbett, 1995) (Figure 3.2). Neuron counts were summed over left and right hemispheres. Treatment effects were analyzed for each of the 3 hippocampal regions and for the total neuron counts summed for all 3 sectors.

3.7 Statistical analysis

All data are presented as mean ± SEM. Food intake for each week (or portion of a week) and hippocampal CA1 cell counts were analyzed by two-factor ANOVA. Body weight and open field data were analyzed by three-factor repeated measures ANOVA. Two factor ANOVA followed by a posthoc test (least significant difference) was used for body weight and open field analysis on individual days when appropriate. A probability value of ≤ 0.05 was considered to be statistically significant. The SPSS 16 for Windows (SPSS Inc., Chicago IL) version was used for all analysis.

To determine if the 5% extra diet that was fed to 3/17 SCR gerbils and 4/14 ICR gerbils confounded the experimental results, all data (with exception of food intake) were analyzed twice: 1) One analysis included all animals for as long as data could be obtained (option a) and 2) the second analysis included only animals that completed the study as planned (excluding animals that required intervention or died prematurely) (option b).

Open field data were analyzed by three-factor repeated measures ANOVA to identify any treatment by diet by day interactions. This analysis cannot be applied to any animals with missing data, that is, those gerbils that did not complete all five open field test sessions (those that had to be euthanized or simply missed a test**)** therefore, these

animals were excluded from the analysis. Sample sizes for the three-factor repeated measures ANOVA are as follows: option a: SCON, n=12**,** SCR, n=13**,** ICON, n=13**,** ICR, n=12**;** option b: SCON, n=12**,** SCR, n=10, ICON, n=13**,** ICR, n=8. Because of the importance of testing whether the intervention influenced results, these animals were included in a subsequent 2-factor ANOVA used to evaluate effects on individual days. This larger sample size is presented in Figure 4.3 and Figure C.1. Since the results from statistical analysis did not differ between the two options for any endpoint, only the results from option a are presented in the main body of the thesis. Note that option a has a higher sample size, therefore increasing statistical power. The exception is for food intake, for which data presented are from option b analysis. It was not possible to use option a for food intake data due to incomplete data from the time intervals analyzed. All data analysis from option b are available in appendices B-D.

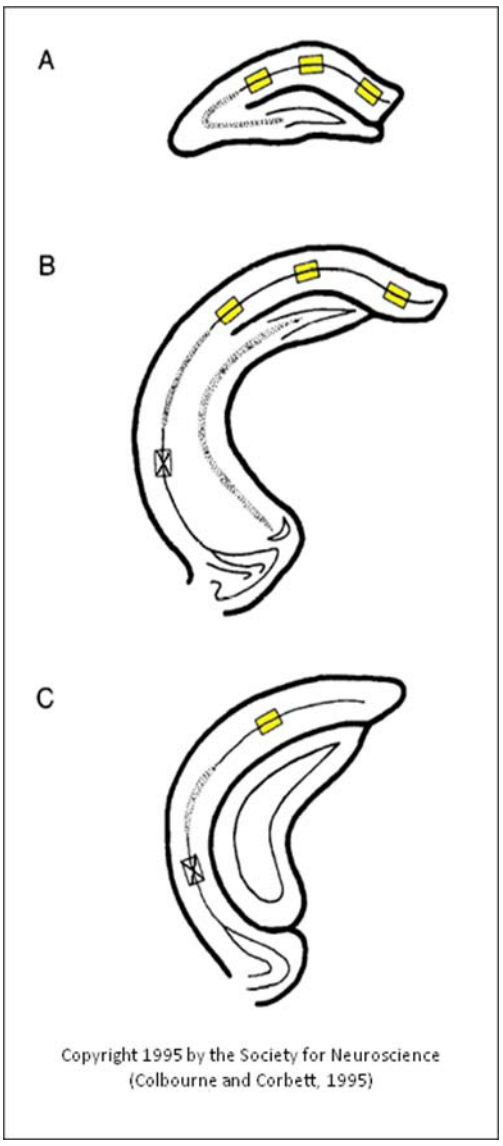

Figure 3.2 Hippocampal CA1 regions selected for histological assessment. Level A, ~-1.7mm relative to bregma; Level B, ~-2.2mm relative to bregma; Level C, ~-2.7mm relative to bregma. Permission was obtained from The Journal of Neuroscience to reprint this figure.

CHAPTER 4

RESULTS

4.1 Surgical outcome

All animals met criteria set for brain temperature during the ischemic period. One animal was excluded from the study because of subsequent seizures. Three animals died during surgery, two due to ruptured arteries, and one due to cessation of breathing. Thirty-two out of a total of 66 (48.5%) animals that underwent bilateral carotid artery occlusion did not meet the criteria for complete forebrain ischemia based on activity monitoring and were excluded from the study. Figure 4.1 shows representative activity patterns for 20 hr following ischemia or sham surgery. Activity patterns observed in ischemic animals can be divided into three categories 1) complete ischemia, in which the pattern of persistent hyperactivity exhibited met the established criteria, 2) incomplete ischemia in which low activity was observed, and 3) incomplete ischemia, in which gerbils exhibited initial hyperactivity that was not sustained over the monitoring period.

4.2 Dietary and euthanasia interventions

After 10% of the calorically restricted animals had to be euthanized due to weakness, poor grooming or other signs of poor health, the planned dietary intervention became necessary. As noted in section 3.14, any gerbil that showed excessive weight loss (>35%), a period of excessive rate of weight loss with poor grooming or weakness, or only the clinical signs were fed 5% more diet than calculated for each day until the end

of the study. This intervention strategy was required in 3/17 SCR gerbils and 4/14 ICR gerbils. This prevented any further deaths or abnormal clinical signs, with the exception of one animal (ICR) for which intervention was likely too late.

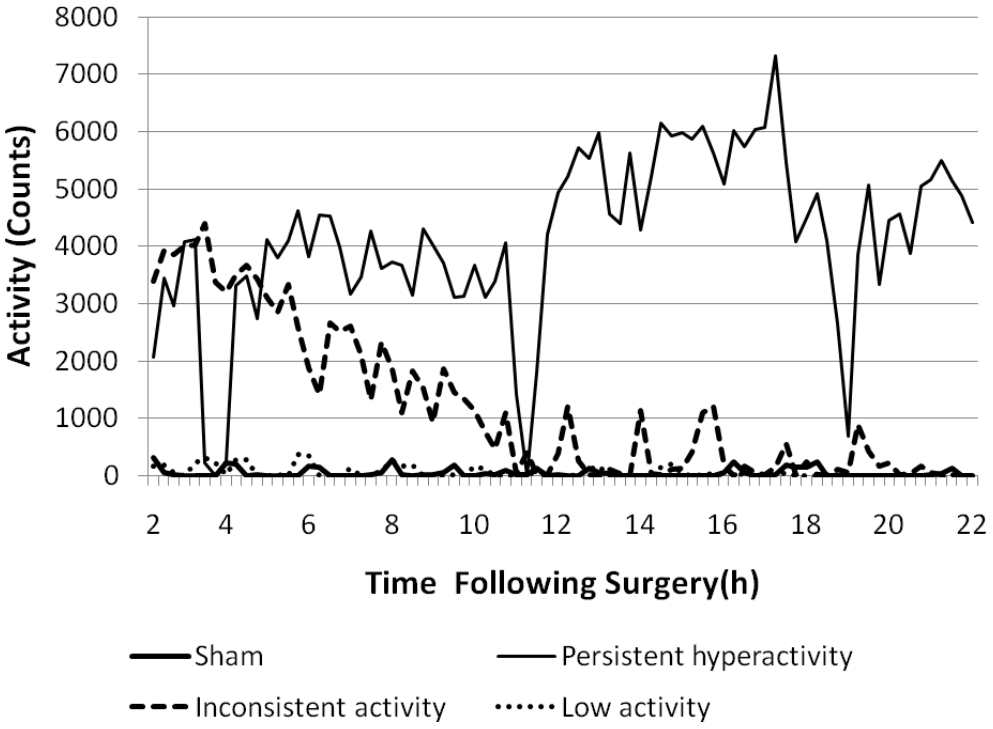

Figure 4.1 Patterns of activity identified in the period following ischemia or sham surgery. Representative patterns of activity are shown for: —— sham surgery; —— complete forebrain ischemia identified by persistent hyperactivity; ····· incomplete forebrain ischemia identified by low activity; – – – incomplete forebrain ischemia identified by initial hyperactivity that was not sustained throughout the 20 hr testing period.

4.3 Food intake and body weight

Food intake following surgery was divided into the following time periods: d1, d2, d3, d4-7, d8-14, d15-21, d22-28, d29-35, d36-42, d42-49, d50-56, and d57-60. Days

1, 2 and 3 were analyzed separately because food allotment for the SCR and ICR groups was calculated differently on d1-3 than for the rest of the study (see section 3.1.4). and because the pattern of food intake varied within this time interval.

Throughout the 60 day study, and as expected according to treatment, the food intake of CR gerbils was significantly less than that of animals fed the CON diet ($p<0.001$) (Table 4.1). Ischemia significantly depressed food intake on d1 ($p=0.023$) and significantly increased food intake over d4-7 ($p=0.017$), d8-14 ($p<0.001$) and d15-21 ($p=0.003$). The effect of ischemia on food intake disappeared by d22-28 ($p > 0.05$). There was no significant interaction between surgery and diet at any of the timepoints tested ($p>0.05$). Table 4.1 also shows that CR gerbils were underfed during the first three days of the study when their food allotment was being based on data from a previous study. Average energy reduction during the first three days of the study was 41%, 56%, and 40% for SCR animals and 71%, 55% and 55% for ICR animals. Between d4 and the end of the study, when food allotment in the CR groups was calculated relative to actual intake of the respective CON group, average energy reduction was at the desired level, being 31.3% for the SCR group and 29.9% for the ICR group.

Figure 4.2 presents the mean (±SEM) body weight for each group throughout the experiment. Three-factor repeated measures ANOVA revealed a significant effect of diet ($p<0.001$, between subject effects) and week, ($p<0.001$, within-subject effects) as well as a significant diet by week interaction ($p<0.001$, within subject effects). The interactions of surgery by week ($p=0.606$) and diet by surgery by week ($p=0.677$) were not significant. Mean initial body weight (±SEM) was not statistically different among groups upon entry into the study, but from d7 until perfusion (d60), mean body weight of

CR gerbils was significantly lower than that of gerbils on the CON diet ($p<0.001$, between-subject effects). Body weight data from option b are shown in Appendix A (Fig. A.1).

Table 4.1 Food intake throughout the post-surgical period

	Food intake (g)			
Days following surgery	SCON	SCR*	ICON	ICR*
1	4.2±0.3	2.4±0.1	3.8±0.5#	1.0±0.1#
2	4.3±0.2	1.9±0.0	4.4±0.4	2.0±0.0
3	4.9±0.3	2.9±0.0	5.4±0.6	2.4±0.0
4-7	17.5±0.8	12.2±0.1	20.3±1.1#	13.7±0.5#
8-14	30.8±1.0	20.6±0.1	34.6±1.0#	24.2±0.7#
15-21	30.9±1.0	20.0±0.3	33.2±1.1#	23.4±0.2#
22-28	31.6±1.0	20.8±0.3	31.4±1.0	21.6±0.1
29-35	31.3±0.9	20.9±0.3	30.8±1.0	20.2±0.2
36-42	31.3±1.1	21.3±0.1	32.2±0.9	23.5±0.3
43-49	32.1±1.2	22.0±0.1	32.2±0.8	22.5±0.1
50-56	32.5±1.5	22.4±0.2	31.9±1.1	21.5±0.2
57-60	13.9±0.6	10.0±0.1	13.7±0.5	9.7±0.1

Data are expressed as mean ±SEM; SCON, n=14; SCR, n=10; ICON, n=15; ICR, n=10. Data were analyzed by two-factor ANOVA. *Food intake was significantly decreased at all timepoints in CR animals as compared to CON animals ($p<0.001$). #Ischemia independently decreased food intake on d1 ($p=0.023$) and increased food intake during d4-7 ($p = 0.017$), d8-14 ($p<0.001$) and d15-21 ($p=0.003$). SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

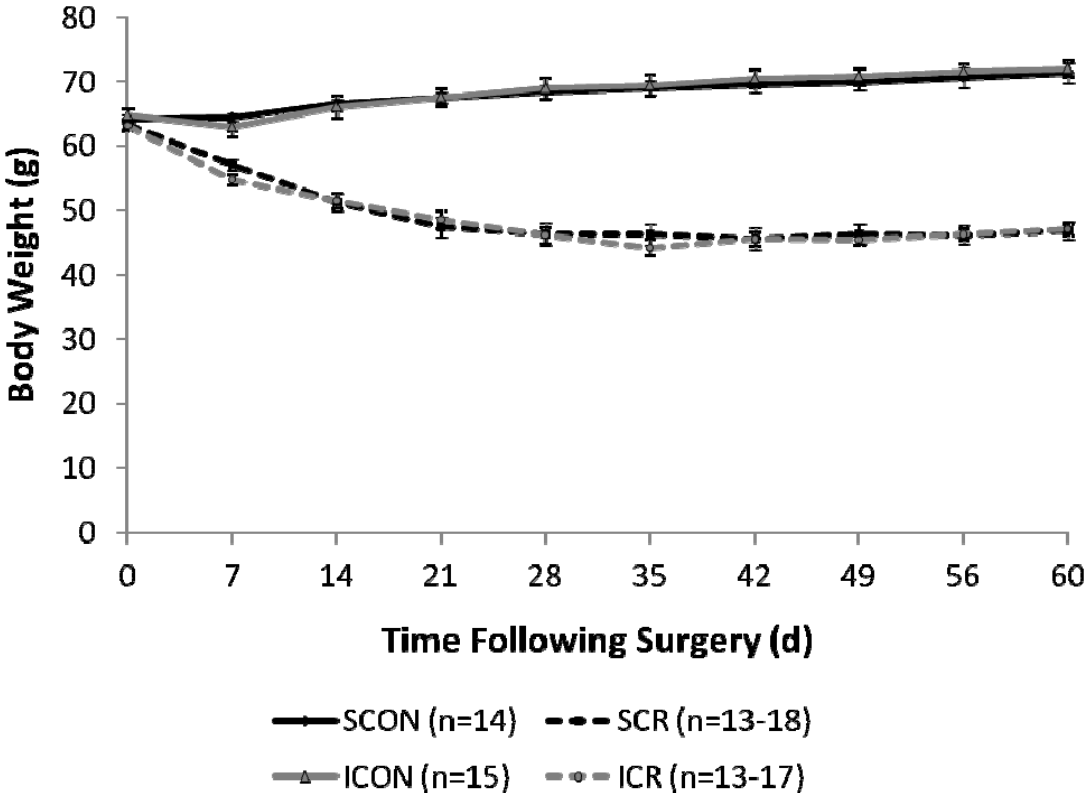

Figure 4.2 Mean (±SEM) body weight throughout the post-surgical period: Analysis by option a. Initial body weight on the day of surgery (shown as d0) was not significantly different among groups, but from d7 onwards, CR gerbils weighed significantly less than gerbils on the CON diet ($p<0.001$), as determined by two-factor ANOVA and LSD post hoc tests. SCON: sham surgery with control diet; SCR: sham surgery with CR; ICON: ischemic surgery with control diet; ICR: ischemic surgery with CR.

4.4 Open field activity

Appendix B shows the pattern of activity in the open field on each test day. Data analysis from the open field is presented in Figure 4.3. Three-factor repeated measures ANOVA revealed a significant effect of surgery ($p<0.001$, between-subject effects) and day ($p<0.001$, within-subject effects), as well as a significant surgery by day interaction ($p<0.001$, within-subjects effects). Diet ($p=0.984$), interactions of diet by day ($p=0.138$) and surgery by diet by day ($p=0.924$) were not significant.

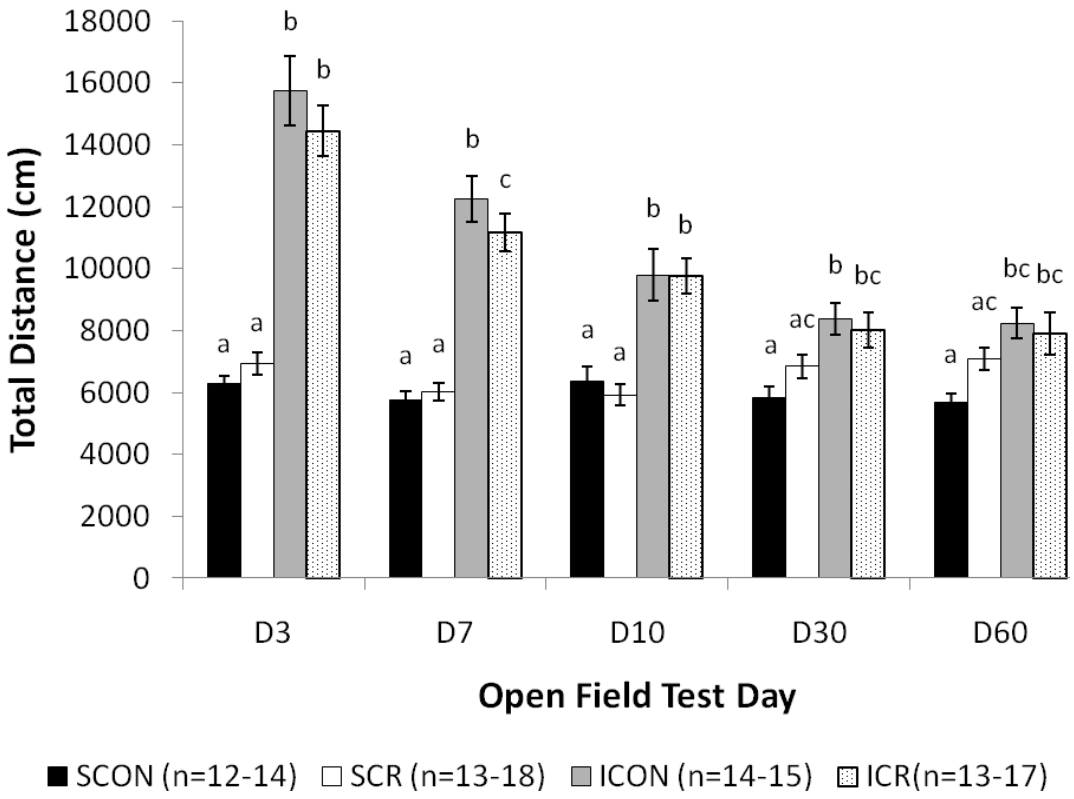

Figure 4.3 Total distance (mean ± SEM) travelled in the open field on d3, 7, 10, 30, and 60. Three-factor repeated measures ANOVA demonstrated a significant effect of surgery ($p<0.001$, between-subject effects) and day ($p<0.001$, within-subject effects), as well as a significant surgery by day interaction ($p<0.001$, within-subjects effects). Diet ($p=0.984$), interactions of diet by day ($p=0.138$) and surgery by diet by day ($p=0.924$) were not significant. Different letters indicate significant differences among groups within a day by two-factor ANOVA followed by LSD post-hoc tests ($p<0.05$). Sample size varied within a group by day because of death, euthanasia or a missed test. SCON: sham surgery with control diet; SCR: sham surgery with CR; ICON: ischemic surgery with control diet; ICR: ischemic surgery with CR.

Sham animals showed habituation on all test days. Although both ischemic groups showed some habituation over time, ICON animals showed a significantly higher level of activity (impaired habituation**)** as compared to SCON animals on all test days ($p<0.001$). ICR and SCR animals were significantly different on d3, d7, and d10 ($p<0.001$) but not on d30 and d60 ($p>0.05$). This was partly due to an increase in total distance traveled by

the SCR gerbils on these days. There was a trend for SCR activity to increase relative to that of the SCON group by d60 ($p=0.055$). Open field activity was significantly lower in the ICR group as compared to ICON gerbils on d7 only ($p=0.024$).

Figure 4.4 shows the percent of the total distance spent in the outer zone of the open field. Ischemic groups spent significantly more time in the outer zone than sham groups as indicated by three-factor repeated measures ANOVA ($p<0.001$, between-subjects effects). Percent distance spent in the outer zone was unaffected by dietary treatment ($p=0.664$, between-subject effects). There were no significant effects of day ($p=0.159$), or the interactions diet by day ($p=0.309$), surgery by day ($p=0.177$) or surgery by diet by day ($p=0.845$). Open field data from option b are presented in Appendix C.

4.5 Hippocampal CA1 cell counts

Table 4.2 shows the effects of diet and ischemia on hippocampal CA1 neuronal cell counts at day 60. Gerbils exposed to global ischemia had significantly fewer hippocampal CA1 neurons at each level and totalled than sham gerbils, irrespective of diet ($p<0.001$). There was no significant independent effect of diet on histological outcome nor was there an interaction between surgery and diet. Five minutes of global ischemia resulted in a mean (± SEM) total loss of 87.1±0.9% and 89.2±1.1 % of CA1 neurons in gerbils fed control diet and exposed to CR respectively. Hippocampal CA1 neuronal cell count data for option b are presented in Appendix D. Table 4.3 shows the extent of hippocampal neuron loss in individual gerbils exposed to global ischemia as well as the extent to which neuronal damage is consistent across both sides of the hippocampus. Percent CA1 neuronal loss was calculated relative to the mean neuronal number of the combined sham groups. Sham groups were combined as there was no

effect of diet on their CA1 neuronal counts. All animals exposed to global ischemia which were included in the study on the basis of hyperactivity monitoring had extensive neuronal loss (77-96%) on both sides of the hippocampus with no evidence of unilateral damage. The pattern of neuronal damage for option b data is shown in Appendix D.

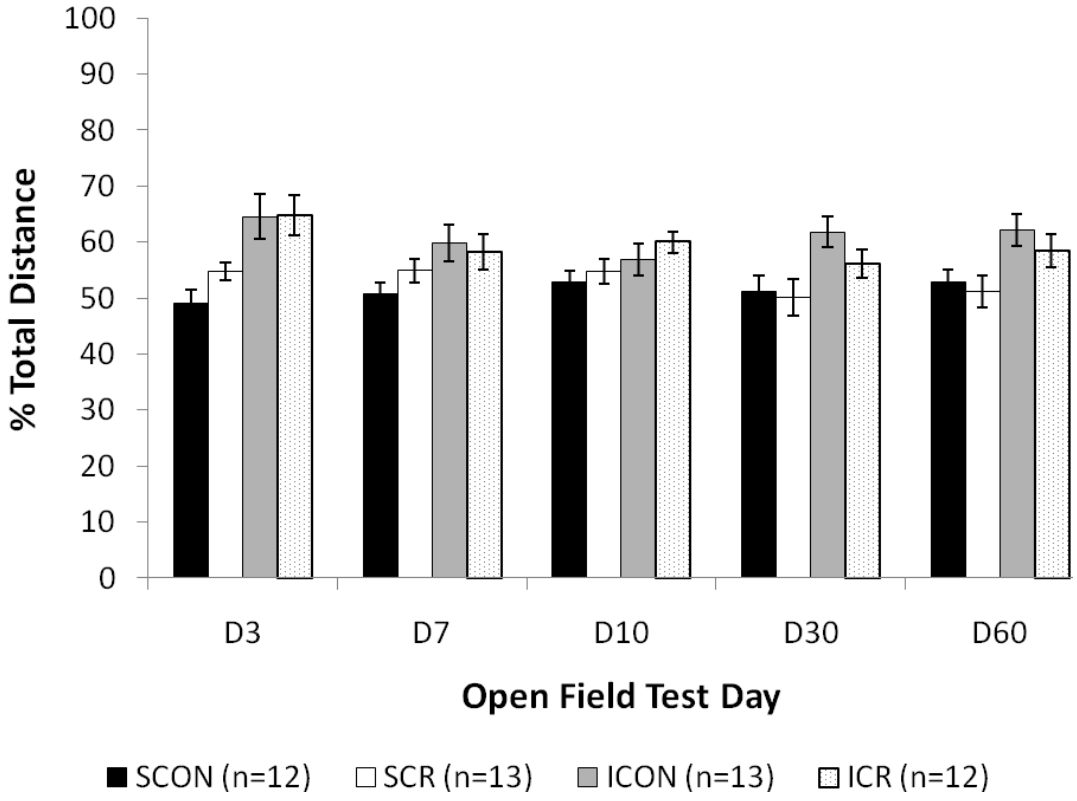

Figure 4.4 Percent of distance travelled spent in the outer zone of the open field on d3, 7, 10, 30, and 60. Ischemic groups spent significantly more time in the outer zone than sham groups as indicated by three-factor repeated measures ANOVA ($p<0.001$). Diet ($p=0.664$), day ($p=0.159$), interactions of diet by day ($p=0.309$), surgery by day ($p=0.177$) or surgery by diet by day ($p=0.845$) were not significant. SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

Table 4.2 Caloric restriction does not alter ischemia-induced death of hippocampal CA1 neurons at d60: Analysis by option a.

	SCON	SCR	ICON*	ICR*
Level A	261.1±5.6	264.2±3.0	31.6±2.8	26.5±3.3
Level B	260.3±5.6	269.8±4.4	33.4±2.5	27.6±2.8
Level C	90.8±1.9	90.0±2.0	14.9±1.1	12.7±1.2
Total	612.1±12.0	623.9±7.7	79.9±5.4	66.8±6.5

Mean (±SEM) cell counts were taken across the anterior-posterior axis of the hippocampus (Level A, ~ -1.7mm relative to bregma; level B, ~-2.2mm relative to bregma; level C, ~-2.7mm relative to bregma); SCON, n=14; SCR, n=13; ICON, n=15; ICR, n=15. Data were analyzed by two-factor ANOVA. *Ischemia independently decreased the number of surviving cells at all hippocampal levels ($p<0.001$). SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

Table 4.3 Extent and reliability of hippocampal CA1 neuron loss between left and right hemispheres following five min of global ischemia: Analysis by option a.

Experimental Group	Left hemisphere	Right hemisphere
(gerbil #)	[neuron counts (% loss)]	
ICON (1)	52 (83)	26 (92)
(2)	29 (91)	36 (88)
(3)	37 (88)	34 (89)
(4)	34 (89)	25 (92)
(5)	37 (88)	33 (89)
(6)	31 (90)	31 (90)
(7)	51 (84)	56 (82)
(8)	44 (86)	44 (86)
(9)	34 (89)	24 (92)
(10)	81 (74)	52 (83)
(11)	36 (88)	35 (89)
(12)	53 (83)	55 (82)
(13)	42 (87)	36 (88)
(14)	42 (87)	32 (90)
(15)	39 (87)	38 (88)
ICR (1)	58 (81)	70 (77)
(2)	12 (96)	21 (93)
(3)	24 (92)	21 (93)
(4)	24 (92)	26 (92)
(5)	29 (91)	28 (91)
(6)	23 (93)	36 (88)
(7)	41 (87)	42 (86)
(8)	49 (84)	37 (88)
(9)	34 (89)	26 (92)
(10)	30 (90)	42 (86)
(11)	25 (92)	44 (86)
(12)	32 (90)	24 (92)
(13)	38 (88)	32 (90)

Percent neuronal loss was calculated relative to mean neuronal number in combined sham groups (SCON and SCR). ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

CHAPTER 5

DISCUSSION

This study is the first to assess whether CR provided solely after brain ischemia can provide a lasting decrease in brain damage. Using BCCAO in the gerbil, I demonstrated that CR did not alter long-term activity in the open field test or hippocampal CA1 neuron cell counts, which are two commonly used and well-validated measures of brain damage in this model. Exposure to brain ischemia caused a significant increase in the distance travelled in the open field on all test days indicative of a spatial learning deficit. CR did not ameliorate this deficit on days 3, 10, 30 or 60. Open field activity was significantly lower in the ICR group as compared to ICON gerbils on d7, which might indicate a transient protective effect. An interesting trend toward increased activity in the calorically restricted sham group relative to that of the control diet fed sham group by d60 suggests that the open field may have limitations for chronic studies of CR. There was also no protective effect of CR on hippocampal CA1 neuron death.

In order to undertake this study, it was necessary to employ a screening method to identify animals with severe forebrain ischemia as it has been shown that commercially available gerbils now show a high incidence of posterior communicating arteries that increase variability of ischemic damage (Laidley et al., 2005; Seal et al., 2006). Hippocampal CA1 neuron cell counts at day 60 revealed that hyperactivity screening correctly identified severe ischemia in 100% of cases. Animals that underwent global ischemia and were included in the study on the basis of hyperactivity monitoring had

extensive neuronal loss (77-96%) on both sides of the hippocampus with no evidence of unilateral damage. This level of damage is much less variable than that of similar recent studies completed without a screening procedure (Bobyn et al., 2005) and more similar to the extent of damage seen in studies prior to the problem of changing brain vasculature in the gerbil (Colbourne and Corbett, 1995) While no animals that were included in the study on the basis of screening were identified by histology as having incomplete damage (false positive results), histology was not completed on animals removed from the study based on screening. Therefore, it is not possible to report on the number of false negative results. The screening accuracy and the extent and consistency of damage observed in the ischemic animals yields confidence in the screening process, although the large number of unusable animals (48.5%) makes the gerbil model of global ischemia undesirable, especially for large scale studies.

It appears that 60 days of post-ischemic CR had no protective effect on CA1 neuronal death. As neurons can appear viable on simple histological assessment but be functionally abnormal (Corbett and Nurse, 1998), open field testing was also employed to assess hippocampal function. Damage to the hippocampal CA1 region is associated with spatial learning and memory impairments, which can be accurately detected in this model of global ischemia by the open field test (Corbett and Nurse, 1998; Corbett et al., 1997; Colbourne et al., 1998a; Colbourne et al., 1998b).

Gerbils exposed to sham surgery showed the expected decline in total activity, indicative of habituation, whereas ischemic animals exhibited persistently elevated activity, indicating learning impairment (Colbourne and Corbett, 1995; Colbourne et al., 1998a ; Colbourne et al., 1998b). Although habituation improved with time after global

ischemia, as previously reported (Corbett and Nurse, 1998; Colbourne and Corbett, 1995), the activity of gerbils exposed to ischemia and control diet did remain significantly elevated over that of the matched sham group throughout the testing period. The functional assessment was in agreement with the histological findings in that CR did not ameliorate the ischemia-induced spatial learning deficit exhibited in the open field on days 3, 10, 30 or 60. The significantly lower activity of the ICR group relative to that of ICON gerbils on day 7 may suggest a transitory protective effect of CR. If this interpretation is correct, it would illustrate the importance of long-term testing in experimental stroke models to identify treatments that have only transient beneficial effects. Many promising therapeutic interventions that appear to be neuroprotective when assessed at early timepoints (1-7 days) following ischemia do not provide lasting benefit but only postpone cell death (Corbett and Nurse, 1998). Although the possibility cannot be excluded, it is thought that the additional 5% diet required in a small number of CR animals to address the problem of post-surgical morbidity and mortality did not confound the results of this study. This interpretation is based on the fact that excluding these gerbils from behavioural and histological analysis yielded the same results. The limitation of this conclusion, however is that this is not an exact comparison because of differences in statistical power between options a and b.

The timing of CR is likely a key explanation for the findings of this study. Although CR administered beginning one day following brain ischemia might have been too late to affect the ischemic cascade, it was predicted that this dietary treatment would be able to moderate ongoing secondary processes such as inflammation and apoptosis. The capacity of CR to increase stress resistance pathways (de Cabo et al., 2003; Heydari

et al., 1996) prior to the ischemic cascade may have been essential for CR to exert neuroprotection, and this mechanism was precluded by the study design. Such mechanisms are reminiscent of those described for ischemic preconditioning (Obrenovitch, 2008). Chronic CR has previously been shown capable of inducing heat shock protein-70 in the striatum (Yu and Mattson, 1999).

While post-ischemic CR may be too late to influence early events accounting for CA1 neuronal death, 30% CR was continued up until day 60, providing ample opportunity to improve functional recovery by enhancing neuroplasticity mechanisms. Considerable functional recovery can take place in both animal models of ischemia and in human stroke patients (Corbett and Nurse, 1998; Lee and van Donkelaar, 1995). Despite this, no long-term benefit from CR was observed in open field performance. No direct measurements of neural plasticity mechanisms were investigated, which would have aided interpretation of the behavioural data.

The increased distance travelled by CR sham animals on day 60, relative to sham animals fed control diet, raises the possibility that interpretation of open field results have been confounded by the presence of CR. The open field test has been used extensively in the gerbil model of global ischemia because of its sensitivity to detect differences in CA1 injury and identify beneficial treatments (Corbett et al., 1997; Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Colbourne et al., 1998b). Increased open field activity in this model of global ischemia generally reflects decreased habituation (Wang and Corbett, 1990; Babcock et al., 1993). However, this does not exclude the possibility of other influences on the test (Plamondon and Khan, 2005).

It is possible that hunger from chronic CR interfered with interpretation by increasing motivation to forage. However, the post-ischemic pattern of open field activity in both control and CR gerbils is very similar to that previously reported in other long-term studies with this global ischemia model (Farrell et al., 2001; Corbett et al., 1997; Colbourne and Corbett, 1995), suggesting that the open field test results are valid even in the presence of CR. Ideally, multiple behavioural tests should be used for more accurate assessment of memory deficits. While the T-maze and radial arm maze are very sensitive for detecting impaired reference memory and working memory following global ischemia in the gerbil (Corbett and Nurse, 1998; Mileson and Schwartz, 1991; Colbourne and Corbett, 1995), these tests require the use of food rewards and might also be confounded by increased motivation in chronically hungry animals. Future research on CR may benefit from the use of behavioural tests that do not require the use of food rewards such as the Morris water maze (Corbett and Nurse, 1998; Langdon et al., 2008), object recognition testing (Plamondon et al., 2008; Plamondon et al., 2006; Yukie et al., 2006) and operant conditioning (Spencer et al., 2008; Jing et al., 2008; Maia et al., 2004). The Morris water maze has been shown to detect impairments in reference memory and working memory following global ischemia, but is not suitable for the gerbil, a desert species unsuited to swimming (Corbett and Nurse, 1998; Langdon et al., 2008). The latter two other possibilities show promise in global ischemia studies although they have not been as well-characterized.

While total activity measured as distance travelled is the most commonly used validated endpoint for the open field test in global ischemia studies (Corbett and Nurse, 1998), it is also possible to examine time spent in the periphery of the open field versus

the centre. This is an endpoint more commonly used for assessing anxiety (Palanza, 2001; Prut and Belzung, 2003). Ischemic groups spent significantly more time in the periphery of the open field than sham groups, which could indicate increased anxiety (Calabrese, 2008). Percent distance spent in the outer zone was not affected by dietary treatment. Analyzing zone data for the open field did not appear to provide any additional information than the standard measure of activity in this study. In fact, more subtle changes were detected analyzing distance travelled in the entire open field, such as the difference between SCON and SCR gerbils on day 60, which was not apparent from examining travel in the periphery.

Another limitation of this study was the unexpected underfeeding during the first 3 days following ischemia (40-74% energy restriction) that resulted from extrapolating food intake data obtained previously from group-housed gerbils to the current study with singly-housed gerbils. Imposing 40-74% CR at the same time as the neuroendocrine and cytokine-mediated stress response to surgery (Lowry and Perez, 2005) may have been a factor in the morbidity and mortality observed in a small proportion of calorically restricted gerbils. This severe underfeeding would have occurred in parallel with the elevations in resting metabolic rate, energy requirement, gluconeogenesis, and lean body mass breakdown that typically occur as part of the classic metabolic response to surgery (Lowry and Perez, 2005). No adverse effects were reported in a recent study of energy restriction administered immediately following surgical induction of spinal cord injury in the rat (Plunet et al., 2008). These investigators employed an IF regimen, an alternate form of CR in which animals are fasted and fed ad libitum on alternating days. As this form of energy restriction causes less weight loss than the 30-40% CR regimens (Anson

et al., 2003; Goodrick et al., 1983), it may be a better way to study CR in the surgical models needed to invoke brain ischemia.

In contrast to our results with post-ischemic CR, previous studies indicate that CR started prior to brain ischemia might be beneficial (Yu and Mattson, 1999; Roberge et al., 2008a; Roberge et al., 2008b). The decrease in infarct volume induced by CR after focal ischemia has not yet been shown to extend beyond 24 hours (Yu and Mattson, 1999). This timepoint is too early following ischemia to know whether the treatment is truly neuroprotective or is only postponing cell death (Corbett and Nurse, 1998). However, two studies in which 40% CR was provided for 30 days before and either 30 (Roberge et al., 2008a) or 70 days (Roberge et al., 2008b) after global ischemia demonstrated improvements in working memory in the radial arm maze. Although the potential confounding influence of hunger was acknowledged, the comparable performance between CR and control-fed sham rats suggests that the radial arm maze results are valid (Roberge et al., 2008a; Roberge et al., 2008b). The mechanisms responsible for the improved recovery remain to be identified. Although CR may have exerted beneficial effects both before and after ischemia in these studies, the interpretation is somewhat confounded as CR was postponed for five days after ischemia. Presumably the latter was done to avoid complications with the combination of CR and surgical stress, such as was encountered in the present study. Although potential effects of CR on intra- and/or post-ischemic brain temperature were not ruled out in these studies (Roberge et al., 2008a; Roberge et al., 2008b), any hypothermia that may have occurred was not of sufficient magnitude to reduce hippocampal neuron death. While intra-ischemic brain temperature was strictly controlled in our study, post-ischemic brain temperature was not and is a

limitation of this study as well. The finding that CR did not affect neuron survival suggests that the CR-induced reduction in memory deficit was caused by enhanced function of either the remaining hippocampal neurons or those in other brain regions.

Further studies linking functional outcome with cell death and neural plasticity mechanisms with different paradigms and timing of CR will be insightful for understanding neuronal death and recovery processes following brain ischemia. For example, although there are few studies in which CR and IF have been directly compared, some data indicate that results may vary with the caloric restriction paradigm; it has been suggested that cellular stress resistance pathways may be activated to different degrees (Mattson and Wan, 2005). Whether or not CR can permanently decrease neuronal death after brain ischemia, there is increasing evidence that CR can positively influence hippocampal plasticity (Stranahan and Mattson, 2008; Gillette-Guyonnet and Vellas, 2008), which suggests that the study of these mechanisms after brain ischemia is warranted.

In conclusion, 30% CR provided after global ischemia does not improve long-term functional outcome in the open field test or hippocampal CA1 neuronal degeneration. Thus, within the confines of this CR paradigm, CR did not prove to be an effective means of improving outcome following global ischemia. Evidence from other studies of functional improvement with combined pre- and post-ischemic CR, however, suggests that it remains a valuable tool to elucidate mechanisms by which the brain protects itself against ischemic injury. Future research should examine other paradigms of CR. Few studies have directly compared CR and IF (Anson et al., 2003). While both paradigms produce similar results**,** they may act through different mechanisms.

Intermittent fasting may be a better way to study CR in models of brain ischemia as it results in less dramatic weight loss than controlled CR, possibly reducing the risk for morbidity and mortality. It is also important that future studies attempt to employ multiple behavioural tests. The use of multiple behavioural tests can provide a more accurate assessment of functional deficits following stroke and the effects of potential therapeutic treatments. It may be especially valuable to characterize functional tests that do not require the use of food rewards as it is possible that these rewards may confound nutritional studies. Due to the considerable functional recovery that can occur following stroke it would also be beneficial to examine The effects of CR on neuroplasticity mechanisms such as synaptic reorganization (e.g. dendritic morphology) or neurogenesis. A better understanding of the effects and mechanisms of CR could lead to new approaches for improving brain damage following stroke including the development of CR mimetics.

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APPENDIX A

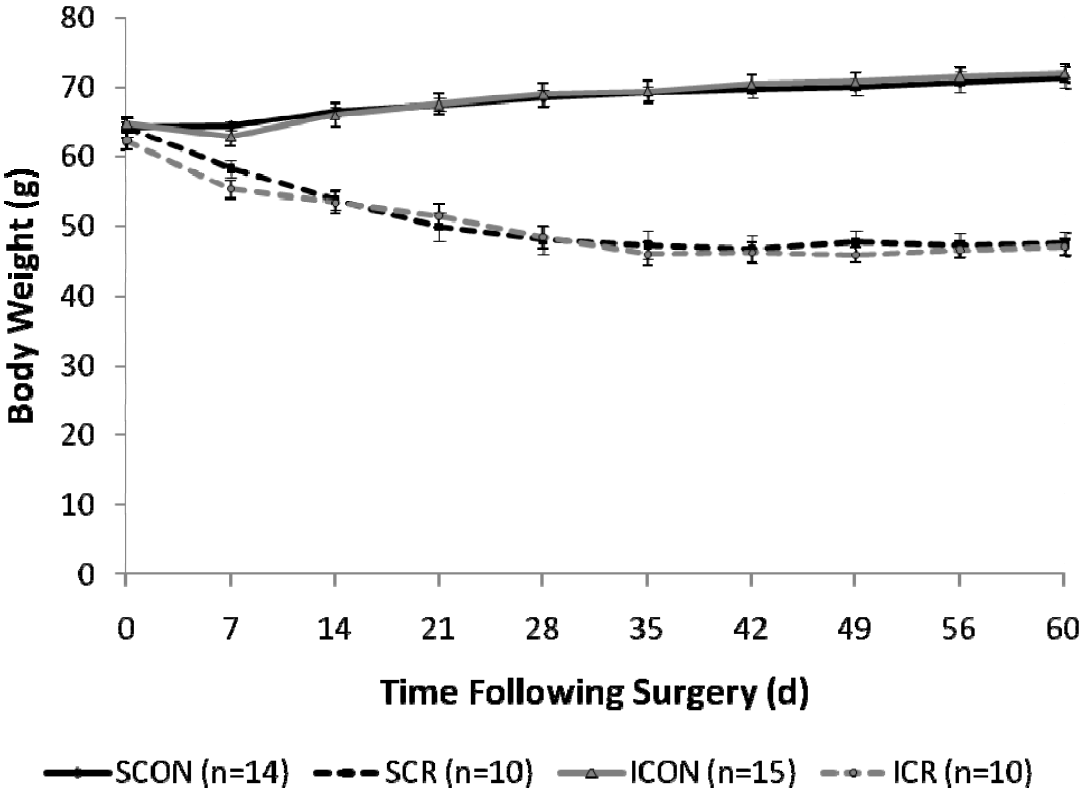


Figure A.1 Mean (±SEM) body weight throughout the post-surgical period: Analysis by option b. Initial body weight on the day of surgery (shown as d0) was not significantly different among groups, but from d7 onwards, CR gerbils weighed significantly less than gerbils on the CON diet ($p<0.001$), as determined by two-factor ANOVA and LSD post hoc tests. SCON: sham surgery with control diet; SCR: sham surgery with CR; ICON: ischemic surgery with control diet; ICR: ischemic surgery with CR.

APPENDIX B

Figure B.1

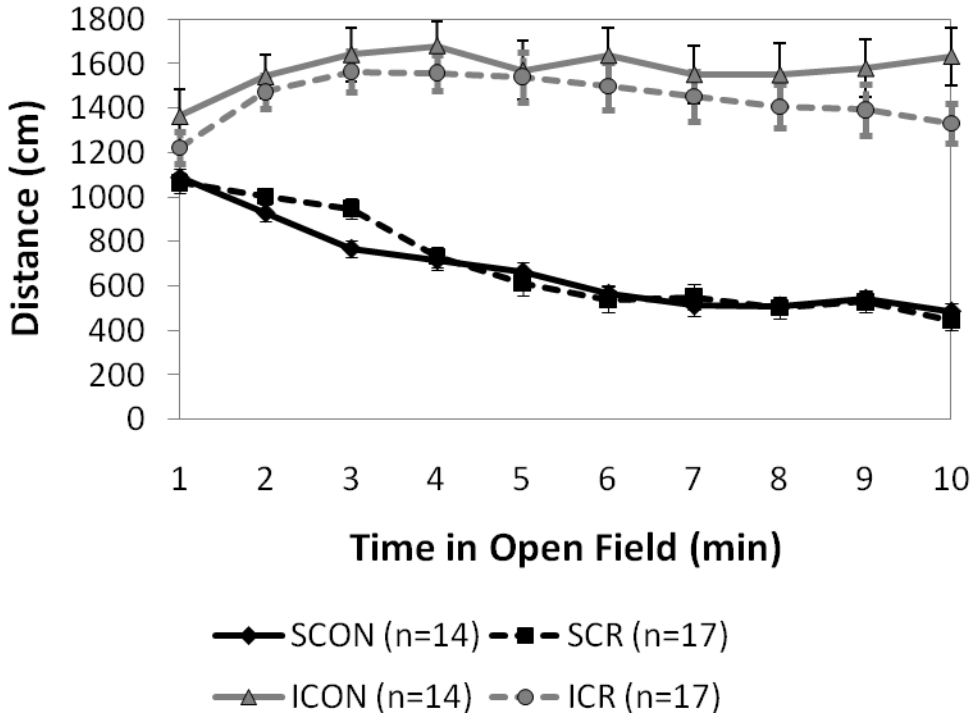
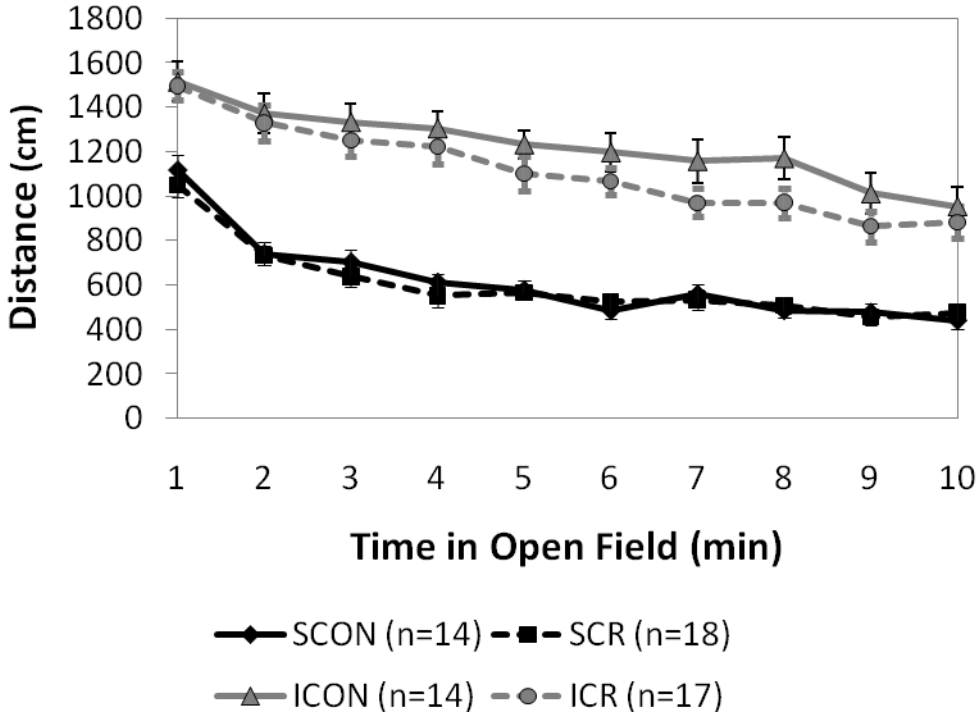


Figure B.2



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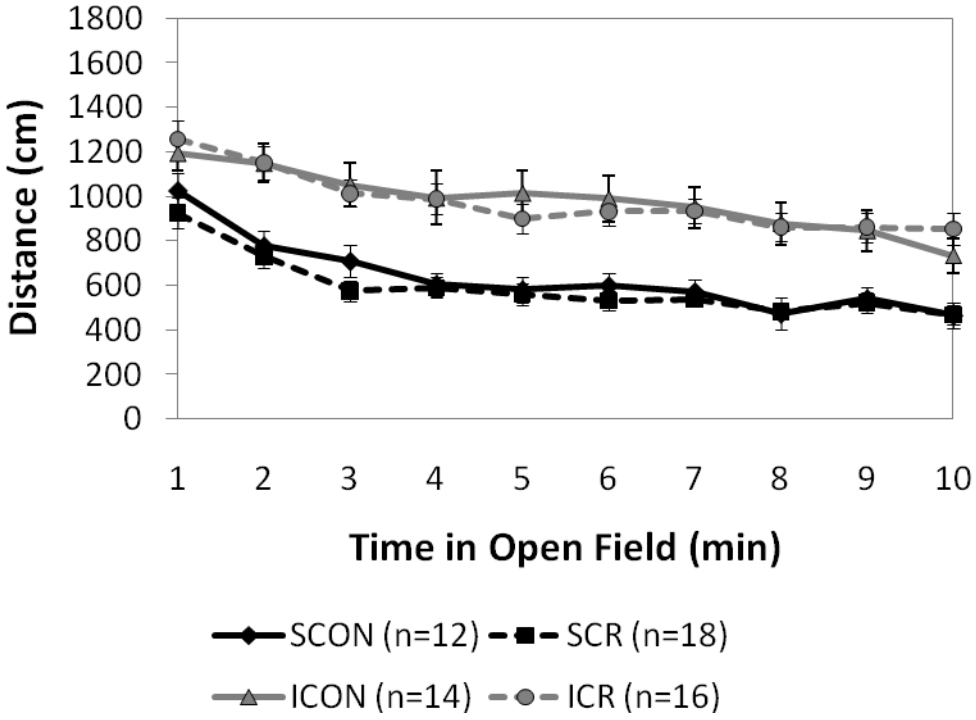

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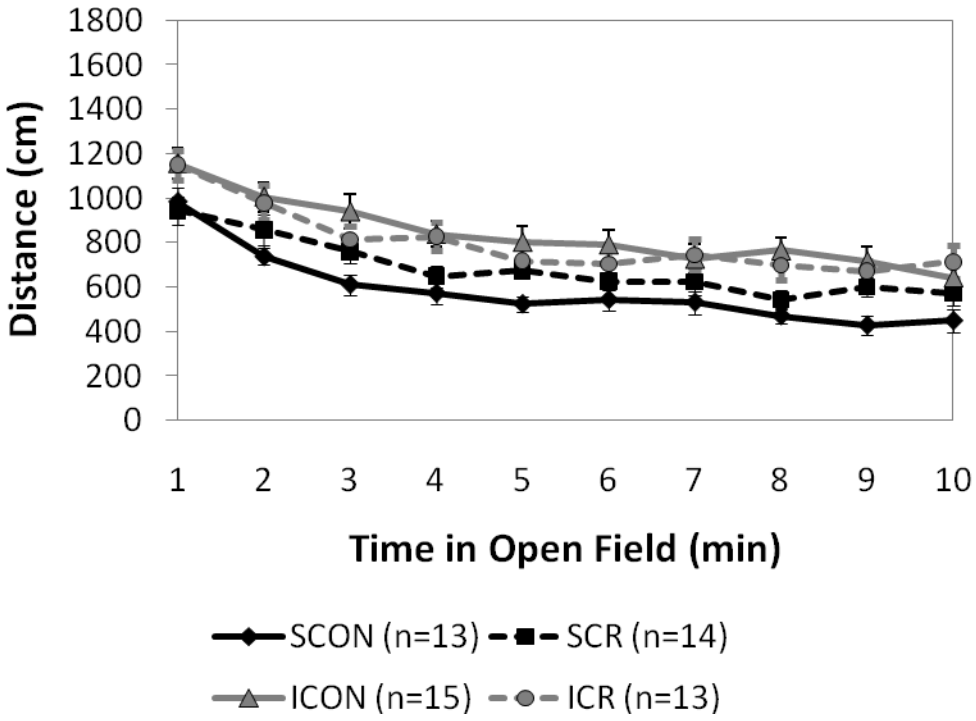

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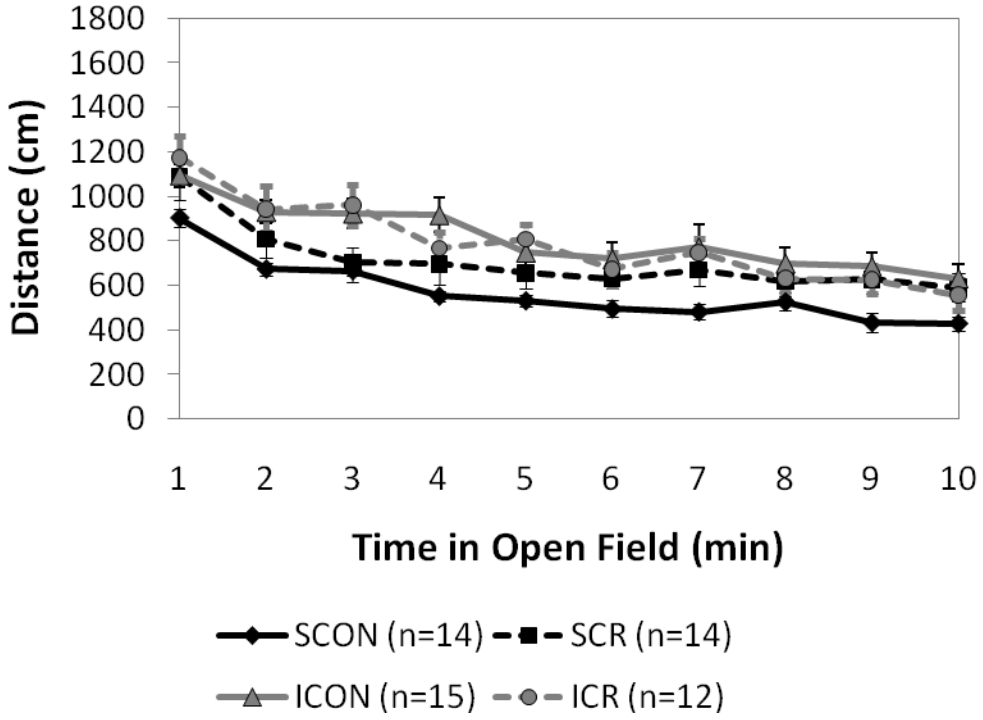

Figure B Pattern of habituation in the open field by the four experimental groups (option a). Figures B1-B5 show mean (±SEM) total distance travelled on d 3, 7, 10, 30, and 60, respectively. SCON: sham surgery with control diet; SCR: sham surgery with CR; ICON: ischemic surgery with control diet; ICR: ischemic surgery with CR.

APPENDIX C

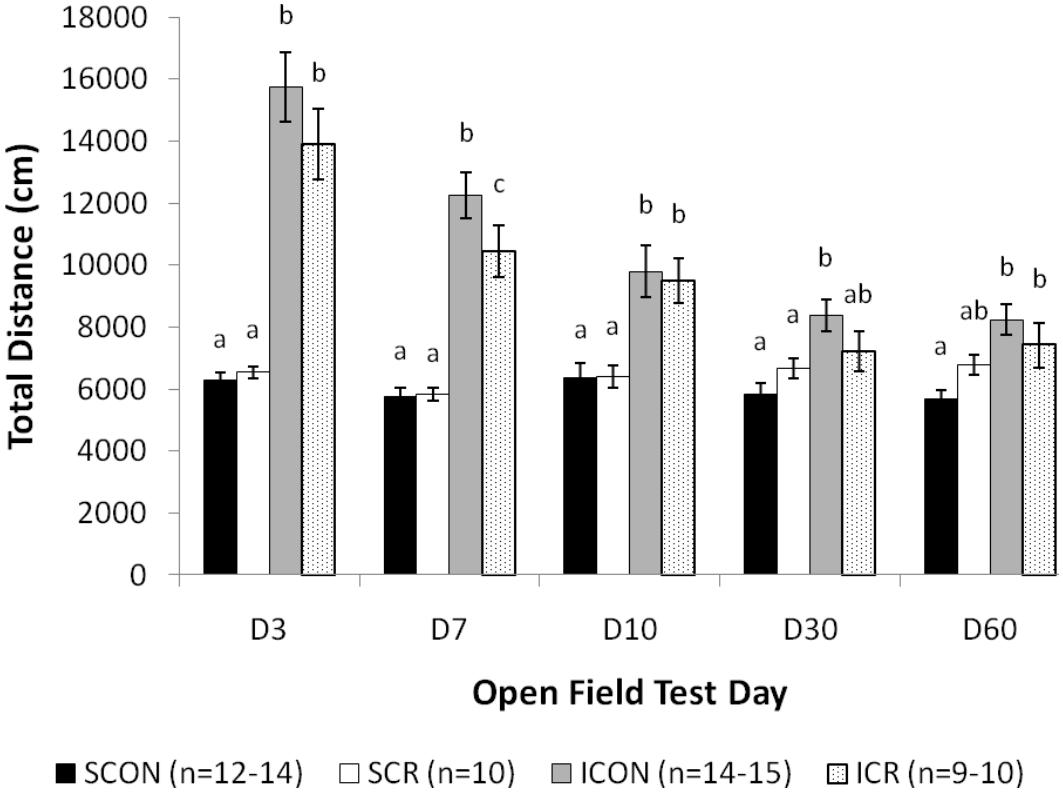

Figure C.1 Total distance (mean ± SEM) travelled in the open field on d3, 7, 10, 30, and 60: Analysis by option b. Three-factor repeated measures ANOVA demonstrated a significant effect of surgery ($p<0.001$, between-subject effects) and day ($p<0.001$, within-subject effects), as well as a significant surgery by day interaction ($p<0.001$, within-subjects effects). Diet ($p=0.767$), interactions of diet by day ($p=0.209$) and surgery by diet by day ($p=0.477$) were not significant. Different letters indicate significant differences among groups within a day by two-factor ANOVA followed by LSD post-hoc tests ($p<0.05$). Sample size varied within a group by day because of missed tests. SCON: sham surgery with control diet; SCR: sham surgery with CR; ICON: ischemic surgery with control diet; ICR: ischemic surgery with CR.

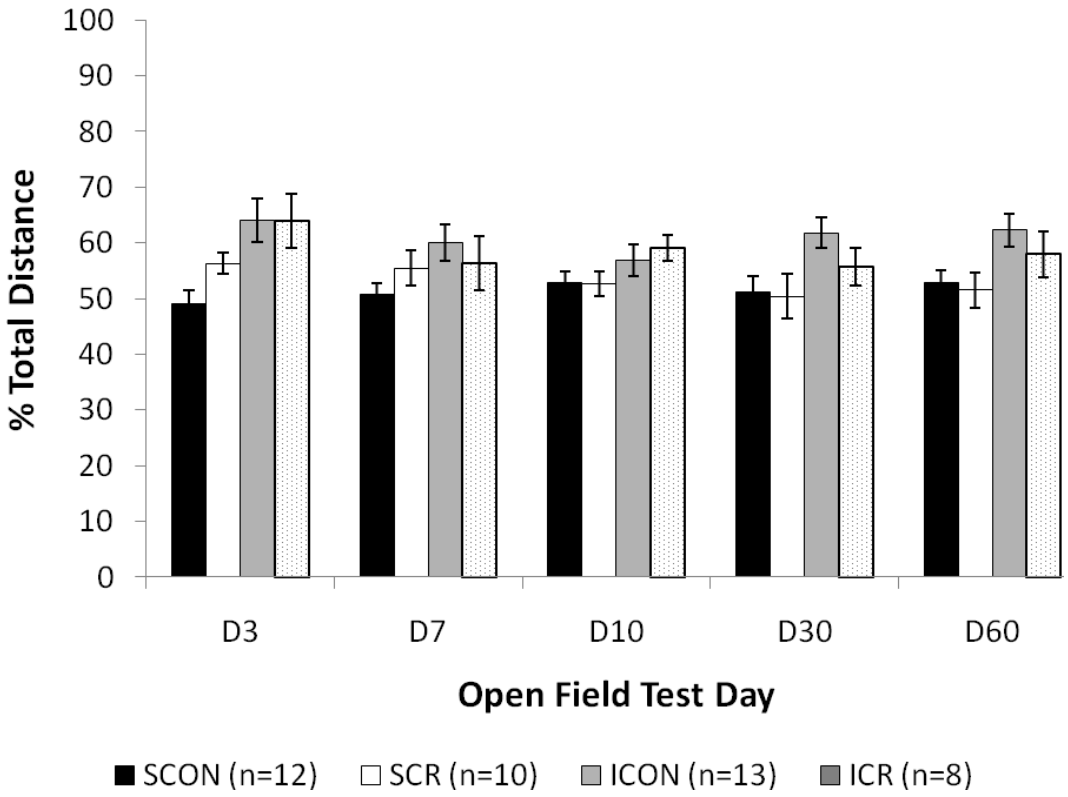

Figure C.2 Percent of distance travelled spent in the outer zone of the open field on d3, 7, 10, 30, and 60: Analysis by option b. Ischemic groups spent significantly more time in the outer zone than sham groups as indicated by three-factor repeated measures ANOVA ($p<0.001$). Diet ($p=0.839$), day ($p=0.230$), interactions of diet by day ($p=0.489$), surgery by day ($p=0.471$) or surgery by diet by day ($p=0.777$) were not significant. SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

APPENDIX D

Table D.1 Caloric restriction does not alter ischemia-induced death of hippocampal CA1 neurons at d60: Analysis by option b

	SCON	SCR	ICON*	ICR*
Level A	261.1±5.6	263.3±3.0	31.6±2.8	27.0±4.2
Level B	260.3±5.6	267.1±5.2	33.4±2.5	29.0±3.5
Level C	90.8±1.9	89.7±2.7	14.9±1.1	13.6±1.4
Total	612.1±12.0	620.1±8.9	79.9±5.4	69.6±8.4

Mean (±SEM) cell counts were taken across the anterior-posterior axis of the hippocampus (Level A, ~ -1.7mm relative to bregma; level B, ~-2.2mm relative to bregma; level C, ~-2.7mm relative to bregma); SCON, n=14; SCR, n=10; ICON, n=15; ICR, n=10. Data were analyzed by two-factor ANOVA. *Ischemia independently decreased the number of surviving cells at all hippocampal levels ($p<0.001$). SCON: sham animals with control diet; SCR: sham animals with CR; ICON: ischemic animals with control diet; ICR: ischemic animals with CR.

Table D.2 Extent and reliability of hippocampal CA1 neuron loss between left and right hemispheres following five min of global ischemia: Analysis by option b.

Experimental Group (gerbil #)	Left hemisphere [neuron counts (% loss)]	Right hemisphere [neuron counts (% loss)]
ICON (1)	52 (83)	26 (91)
(2)	29 (91)	36 (88)
(3)	37 (88)	34 (89)
(4)	34 (89)	25 (92)
(5)	37 (88)	33 (89)
(6)	31 (90)	31 (90)
(7)	51 (84)	56 (82)
(8)	44 (86)	44 (86)
(9)	34 (89)	24 (92)
(10)	81 (74)	52 (83)
(11)	36 (88)	35 (89)
(12)	53 (83)	55 (82)
(13)	42 (87)	36 (88)
(14)	42 (87)	32 (90)
(15)	39 (87)	38 (88)
ICR (1)	58 (81)	70(77)
(2)	12 (96)	21 (93)
(3)	24 (92)	21 (93)
(4)	24 (92)	26 (91)
(5)	41 (87)	42 (86)
(6)	49 (84)	37 (88)
(7)	34 (89)	26 (91)
(8)	30 (90)	42 (86)
(9)	25 (92)	44 (86)
(10)	38 (88)	32 (89)

Percent neuronal loss was calculated relative to mean neuronal number in combined sham groups (SCON and SCR).