EFFECTS OF PROTEIN-ENERGY MALNUTRITION ON OUTCOME FROM GLOBAL CEREBRAL ISCHEMIA

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ABSTRACT

The goal of my thesis was to elucidate the impact of protein-energy malnutrition (PEM, a condition commonly found in aging stroke patients) on outcomes from global ischemia. I first examined the hypothesis that *PEM will impair working memory in the adult gerbil as measured in the T-maze*. Gerbils were fed an adequate (12.5%) or low protein (2%; PEM) diet for 6wk. Stringent assessment of T-maze performance indicated an improvement with PEM although I was unable to reconcile whether this was increased motivation for the food reward or enhanced working memory.

The second hypothesis tested was *PEM will decrease expression of plasticity-associated hippocampal mRNA and protein expression following global ischemia in the gerbil*. The plasticity markers brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (trkB), and growth-associated protein-43 (GAP-43) were examined in the CA1 hippocampal region post-ischemia. PEM induced in gerbils for 4wk did not alter the global ischemia-induced decrease in CA1 neurons. Ischemia resulted in increased CA1 pyramidal expression of BDNF and trkB mRNA at 1, 3, and 7d post-ischemia and increased trkB protein expression at 3 and 7d. PEM further elevated the increased trkB protein detected at 7d in the fibres. Ischemia resulted in increased GAP-43 protein at 3 and 7d post-ischemia with PEM increasing this expression at 3d in the CA3 and hilar regions in addition to CA1. These findings suggest an increased stress-response and/or hyperexcitability state in the hippocampus of malnourished ischemic animals.

Since the reliability of the gerbil model of global ischemia has come into question, the third part of my thesis tested the hypothesis that *the influence of pre-existing PEM on global ischemia-induced hippocampal injury can be reliably studied with the 2-vessel occlusion rat model.* The impact of PEM on CA1 neuronal death and dendritic damage was examined. Rats received protein adequate (18%) or deficient (2%; PEM) diet for 7-8d prior to global ischemia. PEM did not worsen the decrease in CA1 neurons and dendrites observed at 7d post-ischemia. Importantly, I found that PEM altered blood glucose and acid-base balance during surgery and caused brief hypothermia post-surgically, factors which are important for consistent brain injury.

Taken together, these findings reveal (i) that nutritional care, although frequently ignored, can have robust effects on recovery mechanisms after brain ischemia; and (ii) the challenges of studying pre-existing PEM in an established rodent model of stroke.

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This thesis is dedicated to my grandmothers,
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2-VO	2-vessel occlusion
4-VO	4-vessel occlusion
AIN-93G	American Institute of Nutrition 1993 Growth diet
AIN-93M	American Institute of Nutrition 1993 Maintenance diet
AMP	Adenosine monophosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCCAO	Bilateral common carotid artery occlusion
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaMKII	Calcium/calmodulin-dependent protein kinase II
CNS	Central nervous system
CON	Control
CON-I	Control diet ischemia surgery
CON-S	Control diet sham surgery
DAPI	4’6-diamidino-2-phenylindole
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
fEPSP	Field excitatory post-synaptic potential
FOOD trial	Feed or Ordinary Food trial
GABA	Gamma-aminobutyric acid
GAP-43	Growth-associated protein-43
GSH	Glutathione
H&E	Hematoxylin and eosin
HPA axis	Hypothalamic-pituitary-adrenal axis

ICAM-1	Intracellular adhesion molecule-1
IDV	Integrated density value
IκB	Inhibitory component kappa B
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
LAR	Leukocyte common antigen-related
LSD	Least significant difference
LTD	Long-term depression
LTP	Long-term potentiation
MAP-1	Microtubule-associated protein-1
MAP-2	Microtubule-associated protein-2
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
mRNA	Messenger ribonucleic acid
N_2O	Nitrous oxide
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
NO•	Nitric oxide
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
O_2	Oxygen
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
pCREB	Phosphorylated cyclic-AMP response element binding protein
pCO_2	Partial pressure carbon dioxide
PEM	Protein-energy malnutrition
PEM-I	Protein-energy malnutrition - ischemia surgery
PEM-S	Protein-energy malnutrition - sham surgery
PKA	Protein kinase A

PKC	Protein kinase C
pO_2	Partial pressure oxygen
PSD	Post-synaptic density
ROS	Reactive oxygen species
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue plasminogen activator
trkA	Tropomyosin-related kinase A
trkB	Tropomyosin-related kinase B
trkC	Tropomyosin-related kinase C

CHAPTER 1

INTRODUCTION

1.1 Rationale

Stroke is the third leading cause of death in Canada and the leading cause of disability worldwide (Heart and Stroke Foundation. 2008). Of the victims who survive a stroke, 40-50% will be left with moderate to severe impairments (Heart and Stroke Foundation. 2008). The high proportion of patients left with impairments is in part due to the limited time frame in which the only current treatment for ischemic stroke, the thrombolytic agent tissue-plasminogen activator (t-PA), must be delivered (Bayley et al. 2008). This drug acts to break up the blood clot but is only effective if given within 4.5hr after the onset of stroke, and therefore the majority of victims are not able to receive it in time (Bayley et al. 2008). Due to this issue, research has been focused on the development of neuroprotective agents that aim to lower the susceptibility of brain tissue to damage by targeting individual mechanisms of the acute ischemic cascade in hopes of improving or reversing injury (Lee et al. 1999). A large number of animal studies have shown positive results with several neuroprotective strategies; however, these have not translated into efficacious treatments in clinical trials (reviewed in Besancon et al. 2008, Cheng et al. 2004, Dirnagl et al. 1999, Ginsberg. 2009, Lee et al. 1999). Treatment focus should therefore shift to the development of novel neuroprotective approaches and combinational therapies (Cheng et al. 2004). Optimal nutritional care should receive more consideration as part of combinational therapies since impaired nutritional status prior to stroke has been shown to worsen recovery (Davalos et al. 1996, Finestone et al. 1996, Olsen et al. 2008). Therefore the focus of this thesis is on the effect of poor nutritional status, specifically protein-energy malnutrition (PEM), on various aspects of injury and recovery following stroke.

Numerous studies have suggested that at least 16% of elderly acute stroke patients are protein-energy malnourished upon admission to hospital (Axelsson et al. 1988, Davalos et al. 1996, Davis et al. 2004, Gariballa and Sinclair. 1998, Martineau et al. 2005, Yoo et al. 2008,)

and that this worsens as post-stroke hospital stay increases, with 20-35% of patients affected by PEM after 1wk (Axelsson et al. 1988, Brynningsen et al. 2007, Yoo et al. 2008) and 35-49% upon admission to a rehabilitation setting (Finestone et al. 1995, Poels et al. 2006). The presence of poor nutritional status has been associated with longer hospital stay (Davalos et al. 1996), increased functional dependency, and decreased functional improvement rates post-stroke (Finestone et al. 1996); however, this relationship had only been correlative in nature. The FOOD (Feed or Ordinary Diet) Trial Collaboration was a large, multicentre, randomized study that had the potential to establish a causal relationship between PEM and worsened outcome following stroke (Dennis et al. 2005a, Dennis et al. 2005b, FOOD Collaboration Trial. 2003). Results from a prospective cohort of the trial indicated that poor baseline nutritional status was associated with decreased chance of survival and increased functional dependency six months later (FOOD Collaboration Trial. 2003). Unfortunately, due to the lack of standardized nutritional assessments across centres, the potential existed for misclassification of patients into baseline nutritional status. Therefore, these trials when completed, (Dennis et al. 2005a, b) failed to provide a definitive answer regarding the link between suboptimal nutritional status and outcome from stroke (reviewed in Prosser-Loose and Paterson. 2006). Interestingly, a recent epidemiological study in which body mass index (BMI) of 21,884 stroke patients was taken at admission, found those classified as underweight to have the highest 5yr mortality rate (Olsen et al. 2008).

Acute damage in stroke involves a vast number of mechanisms acting in concert to cause severe injury. These include decreased glucose and oxygen supply to the brain, decreased adenosine triphosphate (ATP) production, glutamate excitotoxicity, increased intracellular Ca^{2+} concentration, increased reactive oxygen species (ROS) generation, and inflammation (Lee et al. 1999). Studies in our laboratory found that mechanisms relevant to the acute ischemic cascade, such as increased oxidative stress (Bobyn et al. 2005) and inflammation (Ji et al. 2008) can be affected by PEM. This nutritional deficit was shown to cause impaired functional outcome in a gerbil model of brain ischemia as measured by the open field behavioural test, suggesting a causal relationship between PEM and stroke outcome (Bobyn et al. 2005).

In addition to affecting mechanisms related to acute injury from stroke, PEM may also influence those involved in recovery. Neuroplasticity after stroke refers to the reorganization and anatomical and chemical changes that occur in order to protect the brain and augment recovery

(Teasell et al. 2005). In particular, neurotrophins are thought to promote neuronal survival, growth, and synaptic remodeling leading to a better recovery (Johansson. 2000). Specifically, the neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (trkB) are proposed to have central roles in recovery from brain ischemia by promoting dendritic sprouting and repair of synaptic connections (Lindvall et al. 1994). The results of one study suggest that feeding a low protein diet causes a decrease in the number of hippocampal neurons expressing BDNF or trkB (Mesquita et al. 2002), raising the possibility that PEM may exert some of its deleterious effects post-stroke by inhibition of BDNF activities.

BDNF is thought to influence the expression of another protein involved in neuroplasticity, growth-associated protein-43 (GAP-43) (Schmidt-Kastner et al. 1997). Although GAP-43 is typically increased following injury in order to promote the regeneration of axons and re-establish synaptic connections (Dinocourt et al. 2006), its induction following global ischemia has received little attention. It has been suggested that while increased GAP-43 following global ischemia may indicate synaptic re-organization, another possibility is that pathophysiological mechanisms related to increased excitability are taking place (Schmidt-Kastner et al. 1997, Tagaya et al. 1995). In the second study of this thesis, the gerbil model of global ischemia, the bilateral common carotid artery occlusion (BCCAO) model, was utilized. In this experiment the effect of PEM on plasticity mechanisms following brain ischemia, specifically the expression of the neurotrophin BDNF, its receptor trkB, and GAP-43, was examined.

A previous study in our laboratory found PEM was associated with a worsened functional recovery after global ischemia (Bobyn et al. 2005). Since BDNF is associated with behaviour such as learning and memory post-ischemia (Almli et al. 2000, Gobbo and O'Mara. 2004, Kiprianova et al. 1999b), the first part of this thesis focuses on establishing whether PEM itself affects working memory. A second goal was to test if the behavioral test used to assess this, the T-maze, would be appropriate for use in the later studies on global ischemia in the context of PEM.

Due to building uncertainty regarding the reliability of the gerbil model (Laidley et al. 2005, Seal et al. 2005), the rat two-vessel occlusion (2-VO) model of global ischemia, induced by bilateral common carotid artery occlusion and hypotension, was adopted for the third study. It was crucial to determine at the onset whether PEM increased hippocampal CA1 neuronal death induced in the 2-VO model. A study in our laboratory with the gerbil model of global ischemia

had previously demonstrated that PEM induced a functional (behavioral) deficit without exacerbating CA1 neuronal death (Bobyn et al. 2005), and it was important to know if this finding would be replicated. The effect of PEM on the dendritic marker, microtubule-associated protein-2 (MAP-2), following global ischemia was also examined. The influence of PEM on serum corticosterone concentration was examined as a possible mechanism by which PEM might affect plasticity-related mechanisms after brain ischemia. Other objectives of this study were to confirm that the low protein diet previously used to induce PEM in the 11-12wk old male Mongolian gerbil (Bobyn et al. 2005, Ji et al. 2008, Prosser-Loose et al. 2007) and the 10wk old male Sprague-Dawley rat (unpublished observation) would induce moderate PEM in 30-32d old male Sprague-Dawley rats. Finally, it was also important to establish that PEM would not alter intra-ischemic blood gases, hematocrit, glucose, and blood pressure as these are key determinants of consistent hippocampal CA1 injury in the 2-VO model.

1.2 Hypotheses

The research project described in this thesis tested the following hypotheses:

1. Protein-energy malnutrition will impair working memory in the adult gerbil as measured in the T-maze.
2. Protein-energy malnutrition will decrease expression of plasticity-associated hippocampal mRNA and protein expression following global ischemia in the gerbil.
3. The influence of pre-existing protein-energy malnutrition on global ischemia-induced hippocampal injury can be reliably studied with the 2-vessel occlusion plus hypotension rat model.

1.3 Objectives

The objectives of this research were divided into three experiments as follows:

Experiment 1: CAN A REWARD-BASED BEHAVIOURAL TEST BE USED TO INVESTIGATE THE EFFECT OF PROTEIN-ENERGY MALNUTRITION ON HIPPOCAMPAL FUNCTION?

1. To investigate the effect of protein-energy malnutrition on performance in the behavioural test the T-maze, as an indicator of working memory.

2. To determine whether the food reward required for the behavioural test interferes with the induction of protein-energy malnutrition.

Experiment 2: DOES PROTEIN-ENERGY MALNUTRITION ALTER EXPRESSION OF MARKERS OF HIPPOCAMPAL PLASTICITY FOLLOWING GLOBAL ISCHEMIA IN THE GERBIL?

1. To determine the temporal expression of the plasticity markers BDNF, trkB, and GAP-43 in the vulnerable CA1 region of the hippocampus following the bilateral common carotid artery occlusion model of global ischemia in the Mongolian gerbil.
2. To determine the temporal expression of BDNF, trkB, and GAP-43 in other regions of the hippocampus important in neuroplasticity (dentate gyrus, CA3) following global ischemia in the gerbil.

Experiment 3: CAN THE INFLUENCE OF PRE-EXISTING PROTEIN-ENERGY MALNUTRITION ON OUTCOME FROM BRAIN ISCHEMIA BE RELIABLY STUDIED WITH THE RAT 2-VESSEL OCCLUSION MODEL?

Primary Objectives:

1. To determine whether PEM affects neuronal death in the CA1 region of the hippocampus following the 2-vessel occlusion (2-VO) model of global ischemia in the Sprague-Dawley rat.
2. To determine whether PEM affects dendritic damage, as assessed by MAP-2 expression, in the hippocampus following global ischemia.
3. To determine whether PEM affects post-ischemic serum corticosterone concentration.

Secondary Objectives:

1. To determine whether PEM affects physiological parameters (pH, pCO_2, pO_2, hematocrit, glucose, and blood pressure) that are key methodological determinants of consistent CA1 death in the 2-VO model.
2. To confirm that protein-energy malnutrition can be induced in the 30-32d old male Sprague-Dawley rat using a diet based on 2% protein.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Introduction

Stroke is the third leading cause of death in Canada, taking 14,000 lives, and costing the economy 2.7 billion dollars per year (Heart and Stroke Foundation. 2008). Approximately 15% of stroke victims die while 10% show complete recovery. Therefore, 75% of victims are left with some form of impairment or disability ranging from minor (25%), to moderate (40%), to severe (10%) (Heart and Stroke Foundation. 2008). Depending on what regions of the brain are damaged by a particular stroke, survivors can experience weakness and paralysis on one or both sides of the body, learning impairments, agnosia, aphasia, memory loss, vision problems, difficulties with breathing, chewing, swallowing, and speaking, ataxia, and depression (Heart and Stroke Foundation. 2008).

Of the 50,000 strokes that occur in Canada each year, 80% are ischemic, defined as a disruption of blood flow to the brain due to a clot (Heart and Stroke Foundation. 2008). Currently, the only successful treatment for ischemic stroke is the thrombolytic agent, tissue-plasminogen activator (t-PA), which acts to break up the blood clot and allow re-flow of blood, or reperfusion (Fisher and Brott. 2003). However, since this treatment is only effective if given within 4.5hr after the onset of stroke, and multiple tests are required to verify the occurrence of ischemic rather than hemorrhagic stroke, the vast majority of patients are not able to receive this treatment in time. The development of neuroprotective drugs has therefore been an important area of research. By targeting mechanisms of damage involved in stroke and thereby lowering the vulnerability of the brain tissue, it is anticipated that damage will be decreased (Lee et al. 1999). Despite the success of several of these drugs in animal studies, this has yet to be mirrored in clinical trials. In general, these drugs target a single component of the ischemic cascade, which is likely the reason for failures in the clinical setting (reviewed in Dirnagl et al. 1999). Treatment

focus should therefore shift to the development of novel neuroprotective approaches and combinational therapies, which may well involve nutritional factors (Cheng et al. 2004).

The bulk of nutrition-related stroke research focuses on risk factors contributing to the occurrence of stroke. Our laboratory is one of very few that is studying the effects that nutritional status prior to, and following, stroke may have on damage and recovery mechanisms, as well as functional outcome. Since 16% of elderly acute stroke patients are believed to be affected by protein-energy malnutrition (PEM) at admission to hospital (Choi-Kwon et al. 1998, Davalos et al. 1996, Davis et al. 2004, Finestone et al. 1996, Gariballa and Sinclair. 1998, Martineau et al. 2005, Yoo et al. 2008), our laboratory has been focusing on the effects of this nutritional deficiency on various mechanisms involved in damage and recovery from stroke, as well as functional outcome.

2.2 Mechanisms of Acute Injury in Cerebral Ischemia

Ischemic brain damage occurs as a result of energy substrate limitations as well as harmful cell-to-cell interactions and intracellular signaling (reviewed in Lee et al. 2000). The decrease of cerebral blood flow caused by ischemia leads to a decrease in glucose and oxygen transport to the brain, resulting in decreased production of ATP. In an initial attempt to spare energy, neuronal membranes hyperpolarize causing K^+ efflux, which can potentially lead to a shutdown of neural activity and unconsciousness (Lee et al. 2000). The continued lack of cellular energy substrate then causes a dramatic depolarization of neuronal and glial membranes resulting in excessive release of glutamate into the extracellular space. This neurotoxic concentration of glutamate causes over-stimulation of NMDA (N-methyl-D-aspartic acid), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and kainate-type ionotropic glutamate receptors on the pre- and post-synapse (Hazell. 2007, Lee et al. 2000). NMDA receptors have voltage-gated cation channels, and therefore are highly permeable to Ca^{2+}, causing an increase in the intracellular levels of this ion (Hazell. 2007, Lee et al. 2000). Excitotoxicity results from the excessive activation of glutamate receptors, leading to a "spreading depression" of damage from the core area to the surrounding penumbra (Lee et al. 2000). Metabolic demand is increased in these areas, and ATP is further depleted, consequently leading to further glutamate release (Lee et al. 2000). Neurons begin to swell due to influx of Na^+, Ca^{2+}, Cl^-, and H_2O (Lee et al. 2000). Further NMDA activation causes Ca^{2+} overload leading to mitochondrial failure and activation of

Ca^{2+}-dependent hydrolytic enzymes such as proteases, phospholipases, and endonucleases (Hazell. 2007). Activation of these enzymes leads to a breakdown of membrane phospholipids, producing reactive oxygen species (ROS) (Lee et al. 2000). Neurons respond to increased intracellular Ca^{2+} by increasing nitric oxide (NO•) production, which also leads to increased production of ROS (Lee et al. 2000). While the brain has several antioxidant systems, the decline in energy production causes failure of these systems and they become quickly overwhelmed, allowing excessive production of superoxide radicals, nitric oxide, hydrogen peroxide, and other reactive oxygen and nitrogen species (Hazell. 2007).

Metabotropic glutamate receptors are also affected by the decline of ATP that occurs with stroke. These G-protein coupled receptors are important for normal regulation of glutamate release and uptake via signaling through cyclic-AMP (adenosine monophosphate). Since this enzyme requires ATP for its function, ischemia causes loss of function of these receptors, leading to impaired glutamate regulation (Hazell. 2007).

Astrocytes are glial cells that are involved in removal of glutamate from the extracellular space in order to maintain normal levels of this neurotransmitter (Hazell. 2007). In order to perform this function, ATP-dependent glutamate receptors are present on the membranes of these cells. Ischemia causes a functional reversal of these receptors due to the increase in intracellular Na^{+}, thereby causing efflux of glutamate to the extracellular space, and further contributing to excitotoxicity (Hazell. 2007).

When ischemia is followed by reperfusion, other secondary damage occurs. The re-establishment of blood flow and oxygen supply brings with it an increased accumulation of inflammatory cells (Ding and Clark. 2006). Also, the Ca^{2+}-related induction of second-messenger systems and the increase in ROS causes activation of the transcription factor nuclear factor kappa B (NFκB), leading to the synthesis of pro-inflammatory molecules (Dirnagl et al. 1999). Neurons, glial cells, and endothelial cells all produce inflammatory cytokines in response to ischemia (Rodriguez-Yanez and Castillo. 2008). Specifically, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are activated early and start the inflammatory process. These cytokines lead to a prolonged activation of other cytokines, interleukin-6 (IL-6) and interleukin-8 (IL-8), causing an inflammatory response. The generation of cell adhesion molecules occurs on neutrophils and endothelial cells and includes the selectins, immunoglobulins such as intercellular adhesion molecule-1 (ICAM-1), and integrins (Ding and Clark. 2006, Rodriguez-

Yanez and Castillo. 2008). Neutrophils can then adhere to the microvasculature, causing enhanced permeability of the endothelium and disruption of the blood brain barrier via production of matrix metalloproteinases, allowing the neutrophils, followed by monocytes and macrophages, to pass through (Dirnagl et al. 1999, Ding and Clark. 2006). These cells infiltrate the brain parenchyma and occlude the microvasculature. Various toxins are produced by these cells, such as NO•, ROS, and prostanoids (Dirnagl et al. 1999). This additional tissue damage caused by reperfusion can increase the potential for further complications, including hemorrhagic transformation in which blood vessels that have been weakened by the initial ischemic attack rupture, causing hemorrhage (Sandoval and Witt. 2008).

Neuronal death following ischemia is believed to occur via necrosis as well as apoptosis (Lee et al. 1999). Morphological features of necrotic neurons, such as plasma membrane failure and swelling of the cell body and internal organelles, are apparent following ischemia (Lee et al. 1999). Features of apoptosis are also seen in neurons and glia post-ischemia, and these include chromatin condensation, decreased cell size, and DNA fragmentation (Lee et al. 1999). Some cells exhibiting features of apoptosis have been found to also exhibit some additional features of necrosis (Lee et al. 1999). It is thought that both mechanisms of cell death are triggered in parallel leading to the mixture of morphological features (Lee et al. 1999). Several factors are involved in determining which process is principal including neuronal maturity, severity of injury, intracellular Ca^{2+} concentration, and availability of trophic support (Lee et al. 1999).

2.3 Rodent Models of Cerebral Ischemia

The goal of all ischemia models is to reduce the supply of oxygen and glucose to the brain, either permanently or temporarily, as occurs with stroke (Traystman. 2003). While rodent models, at best, mimic only ~25% of human strokes, understanding mechanisms of damage and recovery in these models will give some insight in to the human condition and allow for development of potential neuroprotective agents (Small and Buchan. 2000, Traystman. 2003). Rodent models of cerebral ischemia can be classified as either focal or global, depending on location of blood stoppage (Small and Buchan. 2000).

While there is a recent report of primates being used in Canada to model human stroke (Cook et al. 2010), most researchers are restricted to rodent models due to cost and available facilities. Commonly stroke researchers use male rats rather than females to avoid potential

estrogen effects. Unfortunately, despite the understanding that aged animals should be used to model human stroke, most studies are on young growing rodents. This is because of the cost and surgical challenges associated with using older rodents. These limitations apply to the models of brain ischemia employed in this thesis, but also allow for integration into the current large reservoir of stroke research on young adult rodents.

2.3.1 Focal Ischemia Models

Focal ischemia models involve permanent or transient occlusion of one major blood vessel, typically the middle cerebral artery (MCA) (Traystman. 2003). Occlusion of the MCA causes decreased blood flow in the striatum and cortex regions of the brain. Many variables, including duration of occlusion, exact site of occlusion on the artery, and the amount of blood flow into the area of damage from other sources will determine extent and location of damage (Traystman. 2003). Focal models, particularly those utilizing transient occlusion, are more representative of human stroke than global models (Small and Buchan. 2000). Resulting pathology includes an ischemic core of unsalvageable, necrotic tissue and the surrounding penumbra from which tissue may be rescued from damage, and to which interventions are aimed (Small and Buchan. 2000). Variations of the middle cerebral artery occlusion (MCAO) model include occlusion of the common carotid artery in conjunction with that of the MCA in order to decrease blood flow to the area of damage even further (Traystman. 2003). While typically, occlusion of the MCA involves the use of vessel clips, electrocoagulation and photochemical irradiation of the artery have also been used. The vasoconstrictor endothelin can be injected into various regions of the brain, including just adjacent to the MCA, into the forelimb region of the motor cortex, or a combination of intracortical and intrastriatal injection, in order to induce focal ischemia (Windle et al. 2006). Blood clot embolism within the carotid artery, ligature snare placement around the MCA, and intraluminal filament placement within the internal carotid in order to block MCA blood flow, are also common focal models (Traystman. 2003).

2.3.2 Global Ischemia Models

2.3.2.1 Overview

All global models of ischemia involve a brief termination of cerebral blood flow (5-15min), followed by reperfusion. These models actually produce forebrain rather than whole

brain ischemia as cessation of blood flow is not quite complete due to continued circulation to the brainstem allowing spontaneous ventilation, which helps to reduce mortality during surgery (Small and Buchan. 2000). Global ischemia produces selective death of the cornu ammonis 1 (CA1) pyramidal neurons in the hippocampal region of the brain, which is delayed occurring ~3-7d post-surgery. These neurons die in a predictable temporal-spatial manner, from mesial to lateral, and from septal to temporal regions (Small and Buchan. 2000). Global models of cerebral ischemia are more representative of human cardiac arrest than stroke (Small and Buchan. 2000). However, the advantage of global over focal models is that damage produced is much more defined and consistent, making assessment of damage and/or neuroprotection more precise and quantifiable. Also, since the mechanisms of cell death in global ischemia are similar to those occurring in focal ischemia, these models are very relevant and useful for studying cerebral ischemia (Small and Buchan. 2000). Early models of global ischemia included decapitation, neck cuff inflation, and ventricular fibrillation. In the late 1970's the rat 4-vessel occlusion (4-VO) model was developed and is still commonly used (Traystman. 2003). This model involves loose placement of clasps around each common carotid artery, followed by electrocauterization of vertebral arteries. A day later, the carotid clasps are tightened and ischemia is induced (Traystman. 2003). This model has been well validated and described. However there is a high degree of variability present in the model and success rate ranges from 50-75% (Traystman. 2003). Two-vessel occlusion (2-VO) models in the gerbil and rat were also developed in the 1970's and are often used as an alternative to the 4-VO model (Traystman. 2003). The hippocampus is considered the most important area of interest when utilizing global models of cerebral ischemia, as these models all cause selective damage to the CA1 region of the hippocampus. Damage in this region can then be assessed to ensure the model is working correctly, and to examine the effects of potential neuroprotectants. Since the hippocampus has a very important role in learning and memory, behavioral and functional effects of stroke are commonly studied using these global models (reviewed in Corbett and Nurse. 1998). While damage to the CA1 region of the hippocampus is the hallmark of global ischemia, other areas of the brain can be damaged too. These include the subiculum and the CA3 regions of the hippocampus, the neocortex and caudoputamen. Histopathological variability in global ischemia is often a result of the absence of or inadequate control of brain temperature (Traystman. 2003).

2.3.2.2 The Hippocampus

2.3.2.2.1 Location and Categorization

The hippocampal region includes two sets of cortical structures, the hippocampal formation, and the parahippocampal region. As shown in Figure 2.1, in the rat, the hippocampal formation is a C-shaped, elongated structure, extending from the basal forebrain into the temporal lobe with the parahippocampus lying caudally and ventrally (Witter and Amaral. 2004). There are several schools of thought regarding criteria for categorizing an area as part of the hippocampal formation. In the most common categorization, regions included have a three-layered structure and unidirectional connectivity (Witter and Amaral. 2004). The hippocampal formation is composed of three distinct regions. As illustrated in Figure 2.2, the first region, the hippocampus proper, is subdivided into three fields, the cornu ammonis 1, 2, and 3 (CA1, CA2 and CA3). The dentate gyrus (DG) and the subiculum make up the other two regions (Witter and Amaral. 2004). Connectivity within the hippocampus is unidirectional, a feature that is unique among cortical structures where connections are usually reciprocal (Witter and Amaral. 2004). Granule cells within the dentate gyrus project their axons, called mossy fibres, to the CA3 field. Pyramidal cells of the CA3 region project their axons, named the Schaffer collaterals, to the CA1 region, the pyramidal cells of which send their axons to the subiculum (Witter and Amaral. 2004).

Areas belonging to the parahippocampal region typically have more than three layers and share a reciprocal connectivity with various regions of the hippocampal formation. This region consists of the entorhinal, perirhinal, and postrhinal cortices, as well as the presubiculum and parasubiculum (Witter and Amaral. 2004).

2.3.2.2.2 Hippocampal Regions and Cell Types

2.3.2.2.2.1 The Cornu Ammonis Regions

The hippocampus proper is divided into the CA1, CA2, and CA3 regions, based on cell size and connectivity. The main cell layer in all three regions is made up of excitatory pyramidal cells, which transmit the neurotransmitter glutamate (and are therefore called glutamatergic). Various other cell types, including basket cells and interneurons, are present (Witter and Amaral. 2004). In the CA3 region, the pyramidal cells are large and receive inputs from the mossy fibre

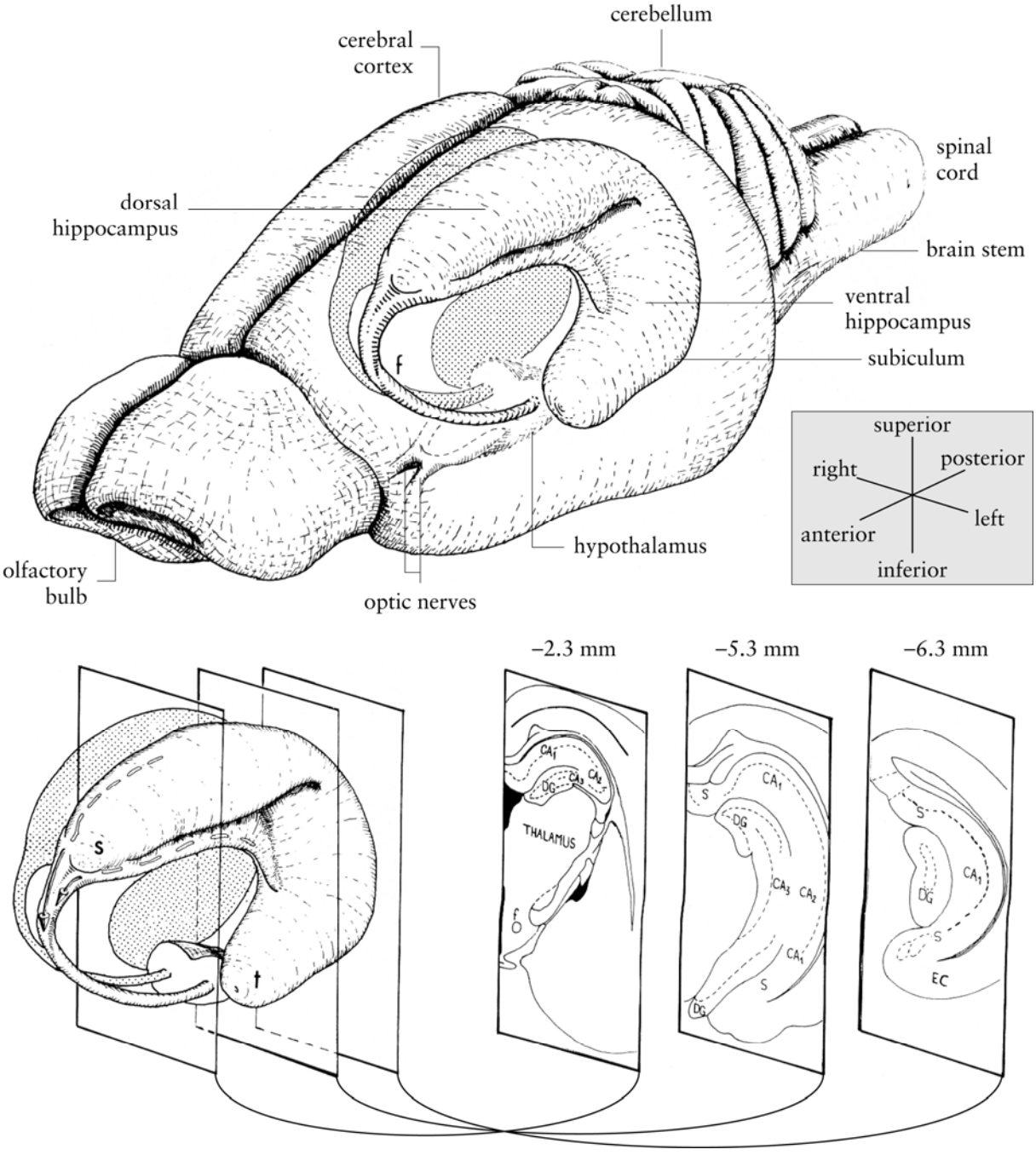

Figure 2.1. Diagram of the hippocampus showing its location within the rat brain. Coronal sections show the anteroposterior organization of the hippocampus. CA1, CA2, CA3: cornu ammonis fields 1–3; DG: dentate gyrus; EC: entorhinal cortex; f: fornix; s: septal pole of the hippocampus; S: subiculum; t: temporal pole of the hippocampus. (From: Cheung TH, Cardinal RN. BMC Neurosci. 2005;6:36).

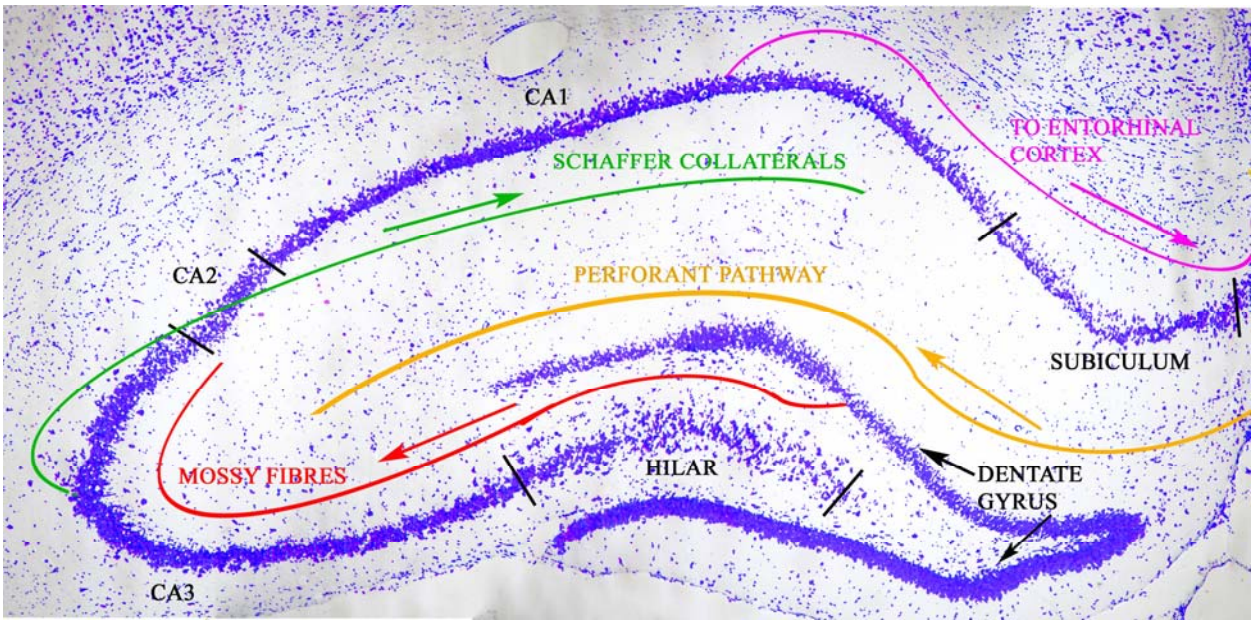

Figure 2.2. Diagram illustrating regions and connections in the rodent hippocampus.

axons of the granule cell layer. The pyramidal cells of the CA1 region are relatively small and do not receive mossy fibre axons. The CA2 region is more difficult to define as it lies as a narrow, blurred border between the CA1 and CA3 regions. The pyramidal cells within the CA2 region tend to be large similar to those within the CA3, but do not receive mossy fibre inputs (Witter and Amaral. 2004).

Above the principal pyramidal cell layer is the relatively cell-free stratum oriens, above which lies the fibrous alveus. A narrow layer just adjacent to the CA3 pyramidal cells contains the mossy fibre axons originating from the granule cells and is called the stratum lucidum. The stratum radiatum lies just below the CA1/CA2 pyramidal cell layer and next to the stratum lucidum. This region consists of projections from the CA3 to the CA1 dendrites, called the Schaffer collaterals. Below this region lies the stratum lacunosum-moleculare, which contains axons from the entorhinal cortex, making up the perforant pathway (Witter and Amaral. 2004).

2.3.2.2.2.2 The Subiculum

The subiculum borders the CA1 region and is defined by the widening and diffusing of the pyramidal cell layer. The main cell type of this region is the pyramidal cell, which tends to be

larger than those within the CA1 region. Several other cell types are also present in the region including interneurons, many of which are GABAergic. The subiculum receives inputs from CA1 axons as well as from the parahippocampal region, but has no interconnections with the CA2, CA3 or the dentate gyrus. The subiculum gives rise to minor projections to the CA1 and the parahippocampal region, as well as projections to non-hippocampal cortical structures (Witter and Amaral. 2004).

2.3.2.2.2.3 The Dentate Gyrus

The dentate gyrus is made up of three regions. The granule cell layer is the most obvious as the granule cells are tightly packed and form a distinctive V-shape. Basket cells, which transmit the neurotransmitter gamma-aminobutyric acid (and are therefore called GABAergic) are also present within the granule layer and are thought to modulate the activity of the glutamatergic granule cells. The granule cells project unmyelinated axons, called mossy fibres, to the CA3 region (Witter and Amaral. 2004).

The molecular layer is mostly made up of dendrites from neurons in the granule cell region and some axon terminals from other regions. While this layer is relatively cell-free, a few neuronal cell types are present which are mostly GABAergic (Witter and Amaral. 2004).

Enclosed within the granule cell region is the third region, the polymorphic or hilar region. This region is made up of many cell types including the mossy cells, the fibres of which, for the most part, remain within the polymorph region (Witter and Amaral. 2004).

2.3.2.2.2.3.1 Neurogenesis in the Dentate Gyrus

It is important to point out that the subgranular zone (SGZ) of the dentate gyrus is one of two sites (the other being the subventricular zone of the lateral ventricle) in the adult mammalian brain known to actively generate new neurons (Ming and Song. 2005). Stem cells in the SGZ differentiate into immature neurons and are integrated into the granule cell layer of the dentate gyrus, where they extend axons to the hilus and CA3 regions, and dendrites to the molecular layer where they receive input from the entorhinal cortex (Ming and Song. 2005). Neurogenesis in the SGZ can be upregulated by several factors including enriched environment, exercise, hippocampal-dependent learning, estrogen, and anti-depressants (reviewed in Lee and Son. 2009). Global ischemia increases neurogenesis occurring in the SGZ. These new cells mature

into neurons located in the granule cell layer and are thought to indicate an adaptive response to the insult and may be involved in the promotion of functional recovery (Liu et al. 1998, Kee et al. 2001, Salazar-Colocho et al. 2008).

2.3.2.3 The Gerbil Bilateral Common Carotid Artery Occlusion Model

2.3.2.3.1 Overview

The most simple and common 2-vessel occlusion model used, until recently, was the bilateral common carotid artery occlusion (BCCAO) in the gerbil. This species has historically lacked posterior communicating arteries, resulting in an incomplete Circle of Willis, thereby avoiding collateral blood flow during occlusion of the common carotid arteries and resulting in almost zero cerebral blood flow (Traystman. 2003). Damage produced from 5min of carotid artery occlusion was therefore very consistent, especially to the CA1 area of the hippocampus (Colbourne and Corbett. 1995, Small and Buchan. 2000).

2.3.2.3.2 Assessment of Injury

Histological and behavioural endpoints for this model have been developed and well established. Brain sections are stained and hippocampal CA1 neurons are counted at various levels throughout the hippocampus. Historically, 5min of ischemic insult with normothermic brain temperature had resulted in ~98% loss of CA1 neurons in the dorsal hippocampus by 7d after surgery (Colbourne and Corbett. 1994, Corbett and Nurse. 1998, Nurse and Corbett. 1994).

Behavioral endpoints and electrophysiological recordings on hippocampal slices were also used to assess functional damage in this model, allowing for a more powerful assessment of brain injury than relying solely on histological assessment (Corbett and Nurse. 1998). While CA1 cell death can take several days to develop, behavioural deficits were evident immediately after ischemia and could be used to verify that the surgery did induce insult effectively (Corbett and Nurse. 1998). Immediately following surgery, gerbils would go through a brief period of immobility during which they recovered consciousness. Animals typically exhibited a hunched posture during this time, which could last for a few hours following surgery (Corbett and Nurse. 1998). This quiet period was followed by an acute period of hyperactivity that lasted for about 24-48hr post-surgery. During this time animals exhibited heightened locomotor activity and might have also stopped typical behaviours such as paper shredding and nest building (Corbett

and Nurse. 1998). After 1-2d, activity returned to normal levels and animals resumed normal activities. Following this phase, a more chronic form of heightened locomotion occurred that could only be detected when the animals were placed in a novel environment, such as the open field (Corbett and Nurse. 1998). Ischemic animals showed significantly elevated locomotion, thought to indicate an inability to habituate, compared to sham animals, and this was found to correlate with CA1 cell death (Corbett and Nurse. 1998, Mileson and Schwartz. 1991, Nurse and Corbett. 1994). This test was useful since it did not require much training, was sensitive to differences in CA1 injury, could involve short test sessions and could detect differences for months after ischemia. If testing was done on a daily basis, ischemic animals would eventually habituate and activity scores would decrease faster than if testing was done after longer intervals (Corbett and Nurse. 1998).

The T-maze is a behavioural test that had been used to detect deficits in working memory following global ischemia in the gerbil (Corbett and Nurse. 1998). Working memory refers to the retention of information gathered on one trial, required for success on the next trial. The T-maze apparatus consists of a stem at the end of which is a removable door defining the start area. Two side arms extend from the stem and removable doors are also present at the entrance to each arm. All strategies in the T-maze involve an initial adaptation phase in which sunflower seeds are distributed throughout the maze, eventually becoming localized to the ends of the arms. The animal is allowed to explore the maze freely for 5min during this phase and eat the seeds. A forced trial and a choice trial are involved in the testing phase. In the win-shift strategy, the animal is first forced to choose one arm and receive the reward by blocking the other with the removable door. In the choice trial, the animal is free to choose either arm, but will only receive a reward if the arm opposite to the one entered in the forced trial is chosen. In the win-stay strategy the animal has to choose the same arm in the choice trial as in the forced trial in order to receive reward. Imposing a delay between trials increases the difficulty of the test (Corbett and Nurse. 1998). The win-stay strategy is more difficult to learn since it requires revisiting of the arm visited in the forced trial, which is opposite of the gerbil's normal foraging behaviour (Babcock and Graham-Goodwin. 1997). This test has been found to detect impairments in working memory, as indicated by decreased choice accuracy and increased time to reach criterion, following global ischemia (Babcock and Graham-Goodwin. 1997, Corbett and Nurse.

1998, Farrell et al. 2001). A potential disadvantage of this test in ischemia studies involving nutrition is the use of a food reward, which may confound results.

Other behavioural tests have also been used to test working and reference memory following global ischemia in the gerbil including the Morris water maze and the radial arm maze. These tests have indicated that ischemia does induce deficits in both reference and working memory, some of which can be recovered over time (Corbett and Nurse. 1998).

2.3.2.3.3 Regulation of Brain Temperature

As with all brain ischemia models, an important consideration includes regulation of brain temperature since hypothermia provides complete protection against the ischemic insult, (Nurse and Corbett. 1994), and hyperthermia can exacerbate damage (Minamisawa et al. 1990). When intraischemic brain temperature was maintained at 30ºC, histological appearance of CA1 neurons as well as field potentials from these cells, were indistinguishable from those of sham animals (Nurse and Corbett. 1994). Hypothermia during ischemia also resulted in sham-like performance in the open field (Nurse and Corbett. 1994). Post-ischemic brain temperature regulation is also important, as hypothermia during this period has been found to provide persistent neuroprotection in terms of CA1 neuronal survival, up to six months following ischemia (Colbourne and Corbett. 1995). In a rat model of global ischemia, when body and brain temperatures were held at 39ºC before, during, and after ischemia, damage was increased in the CA1 and subiculum regions of the hippocampus. Hyperthermia also caused ischemia-induced damage to brain structures that were not affected when temperature was normothermic (37ºC), including the caudoputanum and substantia nigra pars reticulata (Minamisawa et al. 1990).

2.3.2.3.4 Changes in Brain Vasculature Leading to Increased Variability

Unfortunately, during the course of this thesis work, the advantages of the gerbil BCCAO model began to come into question. Skepticism regarding the apparent absence of posterior communicating arteries in these animals has gradually been developing since the early 1990's. Recent experiments in our laboratory (Bobyn et al. 2005, Ji et al. 2008) and others (Laidley et al. 2005, Seal et al. 2005) have been showing an alarming increase in variability of hippocampal damage caused by the surgery.

A recent publication has clearly documented the problem. Laidley et al. (2005) compared the vasculature of gerbils from Charles River Canada (our supplier) with those from High Oak Farms (ON). Approximately 61% of animals from Charles River Canada 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 cell loss as well as attenuated behavioral deficits in the open field after undergoing BCCAO as compared to those with no posterior communicating arteries (Laidley et al. 2005). The presence of posterior communicating arteries (2.6% with bilateral and 13.2% with unilateral) was also found in gerbils from High Oak Farms, as was variable CA1 cell death and behavioural deficits after BCCAO. This suggests that the High Oaks Farms supply is also changing (Laidley et al. 2005). Similar results have been reported in gerbils from other North American suppliers as well (Seal et al. 2005). Gerbils with incomplete forebrain ischemia can be screened out of an experiment on the basis of either insufficient reduction in blood flow by laser Doppler flowmetry or lack of hyperactivity during the first day after surgery (Laidley et al. 2005). However, the problem occurs in such a large proportion of commercially available gerbils that most researchers are adopting other models of global ischemia.

2.3.2.4 The Rat 2-Vessel Occlusion Model

The rat 2-vessel occlusion (2-VO) model is more surgically invasive than the BCCAO model in the gerbil, as well as more complicated. However, it produces consistent and reliable damage, similar to that of the gerbil global ischemia model (Clark et al. 2007). Since the rat has a complete Circle of Willis and therefore collateral blood flow, the model combines temporary occlusion of the common carotid arteries (8-10min) with systemic hypotension in order to produce forebrain ischemia (Smith et al. 1984b). Monitoring of physiological variables such as blood gases and glucose concentration during the ischemic period is possible due to the larger size of the rat and is considered to be important information to obtain (Small and Buchan. 2000). Depending on the size of the rat, blood is withdrawn from either the jugular vein or the tail artery in order to induce hypotension. Tail artery cannulation is included in order to monitor blood pressure as well as to take blood samples prior to and following surgery in order to monitor blood gases and glucose.

Endpoints in the 2-VO model are similar to those established for the gerbil BCCAO model. The rat 2-VO model causes selective damage to the CA1 region of the hippocampus, in a spatial and temporal manner similar to that which occurs in the gerbil model. Deficits in behavioural tests of memory such as the water maze, the radial arm maze, and the T-maze post-ischemia are also evident (Corbett and Nurse. 1998), although the behavioural response in this model is just now being carefully characterized (Arvanitidis et al. 2009, Langdon et al. 2007).

2.4 Mechanisms Relevant to Recovery Following Cerebral Ischemia

2.4.1 Protective Mechanisms

While mechanisms involved in brain damage are extensive and can result in severe injury, there are endogenous, neuroprotective mechanisms that are also triggered during ischemia (reviewed in Lee et al. 2000). Acutely, neurotransmission of the inhibitory transmitter GABA (gamma-aminobutyric acid) is activated, which acts to reduce the excitability of circuits. Also, NMDA receptor function is downregulated, and extracellular Ca^{2+} and Na^{+} is reduced leading to decreased influx of these ions across membranes (Lee et al. 2000). Other protective responses are more ongoing and function to decrease the vulnerability of the brain to further damage. These responses include the concept of "ischemic tolerance", which involves the occurrence of the same mechanisms that cause damage, such as NMDA receptor activation and Ca^{2+} mediated pathways, but at sub-lethal levels. It is thought that when these mechanisms occur and the cell is able to survive, a tolerance to further damage can result (Lee et al. 2000). Eventually, pre-synaptic release of neurotransmitters is altered, leading to increased GABA release, and decreased glutamate release. Also involved in the protective response is upregulation of various cellular defenses such as heat-shock proteins, free-radical scavengers, calcium buffers, and growth factors, including neurotrophins (Lee et al. 2000).

2.4.2 Recovery Mechanisms

Until recently, it was believed that once the brain was injured, no regeneration or replacement of neurons could occur (Lee and van Donkelaar. 1995). Despite this thinking, survivors of stroke typically show some degree of functional recovery (Murphy and Corbett. 2009). In the first few days following a stroke, necrotic tissue is resorbed, edema subsides, and circulation to the salvageable tissue in the penumbra is re-established allowing recovery of

function of some nearby neurons (Lee and van Donkelaar. 1995). Diaschisis, a state of depressed function in remote regions that are interconnected with the ischemic core, is resolved, again allowing recovery of some neuronal function (Teasell et al. 2005). Late recovery, occurring weeks to months after ischemia, is due to events occurring in the brain, which fall under the term neuroplasticity (Lee and van Donkelaar. 1995, Teasell et al. 2005). Interestingly, plasticity mechanisms occurring during recovery from stroke are similar to those occurring in the developing brain and during experience-related changes such as learning (Murphy and Corbett. 2009). Plasticity refers to reorganization of the brain so that functions previously performed by now damaged regions, can be taken over by other intact regions (reviewed in Lee and van Donkelaar. 1995, Murphy and Corbett. 2009, Teasell et al. 2005). Anatomical mechanisms of neuroplasticity include unmasking of latent synapses, neuronal sprouting resulting in new synapse formation, and circuitry redundancy where a parallel pathway takes over the function of a damaged one (Lee and van Donkelaar. 1995, Murphy and Corbett. 2009). Chemical changes, involving neurotransmitters, neurotrophins, growth factors, hormones, and other molecules also occur in response to brain injury, contributing to neuroplastic events (Johansson. 2000, Murphy and Corbett. 2009). The local activities of neurotrophins specifically, have been found to promote neuronal survival, growth, and synaptic remodeling after ischemia, leading to enhanced recovery (Johansson. 2000). Neurogenesis, or birth of new neurons, is another mechanism now believed to be involved in recovery from ischemia, and may also require neurotrophic activities (Johansson. 2000).

2.5 BDNF and trkB

2.5.1 Overview of Neurotrophins

Neurotrophins are a group of secreted peptides that share a high degree of amino acid homology and protein structure. They are thought to be involved in various functions within the nervous system including cell survival, proliferation, differentiation, death, and plasticity (reviewed in Arevalo and Wu. 2006). Nerve Growth Factor (NGF) was the first neurotrophin to be discovered and described followed by Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5). Each neurotrophin binds a specific, high-affinity receptor through which it signals. TrkA (tropomyosin-related kinase A) is the receptor for NGF, BDNF binds trkB, NT-3 binds trkC, but can also bind trkA and B when there

is a short insert present in the extracellular domain of the receptor, and NT-4/5 binds trkB (reviewed in Arevalo and Wu. 2006). Neurotrophins form homodimers and bind to their specific trk receptor, causing dimerization of the receptor, and phosphorylation. Adaptor proteins and enzymes are recruited to the intracellular domain of the receptor, leading to the activation of cellular signaling pathways, triggering various transcription factors. This can then lead to activation of genes promoting pathways of survival and of apoptosis (Arevalo and Wu. 2006).

All neurotrophins can also bind to the low-affinity receptor, p75. This receptor has an intracellular death domain, but no intracellular tyrosine kinase domain. It is therefore believed to signal via constitutively associated proteins. Binding of this receptor can also cause ceramide production, which acts as a second messenger, activating various transcription factors, leading to either apoptosis or survival (Arevalo and Wu. 2006).

2.5.2 BDNF

BDNF plays an important role as a neuronal survival factor, both during development and after injury in the adult central nervous system (CNS). BDNF is unique among the neurotrophins as its release from secretory vesicles is activity-dependent and is therefore regulated and not constitutive (reviewed in Bolton et al. 2000). As well, BDNF can undergo both retrograde and anterograde transport and can have both autocrine and paracrine actions (reviewed in Nawa et al. 1997). In these ways BDNF has several characteristics of a neurotransmitter, which the other neurotrophins do not possess. One of the most interesting and unique characteristics of BDNF is its involvement in neuronal plasticity (Bolton et al. 2000, Nawa et al. 1997). It is able to modulate synthesis and release of various neurotransmitters, alter receptor sensitivity, and induce LTP (long-term potentiation), a form of synaptic plasticity involved in learning and memory (Nawa et al. 1997). BDNF can also alter morphological characteristics of synapses such as size, shape, and number (Nawa et al. 1997). It is due to these actions that BDNF is required for various fundamental functions, including spatial learning and memory (Mizuno et al. 2003, Mu et al. 1999). One study found that deprivation of endogenous BDNF by antibody delivery in rats resulted in impairments in water maze tasks, including increased escape latency, and poorer performance in the spatial probe test (Mu et al. 1999). Another study found that treatment with anti-sense BDNF oligonucleotide impaired acquisition, maintenance, and recall of spatial memory in the radial arm maze (Mizuno et al. 2003).

BDNF is also involved in the enhancement of neurogenesis. In the hippocampus, neurogenesis occurs throughout adult life in the dentate gyrus, specifically in the granule cell layer. A study by Lowenstein and Arsenault (1996) showed that exogenous BDNF increases survival and differentiation of cultured dentate granule cells, and also suggests that endogenous BDNF and trkB influence the growth and development of the cells in vivo as well. Scharfman et al. (2005) found that infusion of BDNF to the dentate gyrus causes increased neurogenesis of dentate granule cells, but also ectopic granule cell development. BDNF has been shown to promote neurogenesis in various other intact brain regions as well (Pencea et al. 2001). However, its role in insult-induced neurogenesis is more controversial (reviewed in Kokaia and Lindvall. 2003).

Prior to cleavage and its secretion as a mature ligand of trkB, BDNF is produced in its initial form, proBDNF. This pro-isoform has physiological significance as a ligand, and therefore a potential role in plasticity. Yang et al. (2009) found that proBDNF is produced in central neurons and can be secreted in this form, without being cleaved into the mature form. They further demonstrated that proBDNF expression increases in the hippocampus as development occurs in concert with the p75 receptor, through which it signals (rather than trkB as in the mature form), and that it is more abundant in juvenile mice than is the mature form, although expression is maintained in the adult. Spatial and temporal expression of proBDNF and p75 in this experiment suggest the involvement of proBDNF/p75 signalling during axonal extension, dendritic pruning, and synaptic maturation, implicating it in plasticity-related events (Yang et al. 2009). Further evidence for the involvement of proBDNF in plasticity is shown by the finding that proBDNF signals through p75 to facilitate hippocampal long-term depression (LTD) (Woo et al. 2005). Since mature BDNF is known to be involved in hippocampal long-term potentiation (LTP), the authors suggest that proBDNF and BDNF work in a bidirectional fashion in order to regulate synaptic plasticity (Woo et al. 2005). Signalling of proBDNF through p75 has also been shown to induce apoptosis in vitro (Fan et al. 2008, Koshimizu et al. 2010, Teng et al. 2005.); however no studies have been reported on the expression and actions of proBDNF in ischemia.

It is important to mention that BDNF signalling through trkB can be influenced by other proteins such as the leukocyte common antigen-related (LAR) protein. LAR is a member of the protein tyrosine phosphatase (PTP) family, members of which are involved in regulating activation of protein tyrosine kinase receptors (PTK), including trkB (Yang et al. 2006). LAR is

expressed in several neuronal populations that also express trk receptors and up- or down-regulates neurotrophic actions, depending on the context (Dunah et al. 2005, Tisi et al. 2000, Yang et al. 2006). Yang et al. (2006) demonstrated using embryonic hippocampal neuron cultures that LAR interacts with trkB and this is increased upon exposure to BDNF, whereas activation of trkB by BDNF was decreased in LAR-deficient cells. Several molecules downstream of BDNF were also found to respond in the same manner (Yang et al. 2006). These findings suggest that the interaction of LAR with the trkB receptor can promote BDNF signaling and therefore may play a role in plasticity-related mechanisms. The expression and role of LAR has not yet been examined in cerebral ischemia.

2.5.3 BDNF and trkB After Global Ischemia

Transcription of the BDNF gene is upregulated in response to ischemia, triggered by excess glutamate release and increasing intracellular Ca^{2+} levels (Tapia-Arancibia et al. 2004). In an effort to salvage neuronal circuitry, the translation of BDNF mRNA into protein is believed to promote neuronal survival, dendritic sprouting, and synaptic remodeling, leading to enhanced recovery (Tapia-Arancibia et al. 2004). Several studies have suggested that BDNF and its receptor trkB are involved in neuronal survival post-ischemia (Ferrer et al. 1998a, Ferrer et al. 1998b, Kiprianova et al. 1999a, Larsson et al. 1999, Lee et al. 2002, Tsukahara et al. 1994). Expressed another way, it is believed that brain areas lacking BDNF production may be more vulnerable to neuronal death post-ischemia. The dentate gyrus region of the hippocampus is able to maintain its levels of BDNF protein post-ischemia, which may in part explain the relative resistance of this region to ischemic-induced cell death (Kokaia et al. 1996). However, the high vulnerability of the CA1 region of the hippocampus to ischemic damage may be in part due to the relatively inability of this region to maintain its BDNF protein levels post-ischemia (Kokaia et al. 1996). For example, Lee et al. (2002) found, using in-situ hybridization, that while mRNA of BDNF was increased in both the CA1 and dentate gyrus regions of rat hippocampus in response to ischemia, protein levels, as indicated by immunohistochemistry, were significantly decreased in the CA1 region for up to 7d. Protein levels of BDNF also decreased in the dentate gyrus 1d post-ischemia, but this was transient. As expected, only the CA1 region showed significant neuronal damage and death at 7d post-ischemia (Lee et al. 2002). A separate study by Kokaia et al. (1996), found that following global ischemia in the rat, mRNA levels of BDNF

were increased in the dentate gyrus and unchanged in the CA1 region. Protein levels, as determined by a two-site enzyme immunoassay, in the dentate gyrus were also increased, but were decreased in the CA1 (Kokaia et al. 1996). Since ischemia is known to cause impaired protein synthesis (Martin de la Vega et al. 2001), it is possible that the translation of BDNF mRNA is interrupted, causing the mismatch of mRNA and protein levels, especially in the CA1 region. Protein levels of trkB in the CA1 however, do not seem to be affected by ischemia-induced interruption of translation. In fact, it is believed that the resistance of trkB neurons to ischemia-induced cell death is specifically how BDNF is able to render neuroprotection. Ferrer et al. (1998b) found that while there was a reduction of BDNF immunoreactive CA1 neurons in the gerbil hippocampus post-ischemia, trkB immunoreactive neurons survived the ischemic episode. Of note is that, of the BDNF immunoreactive neurons that did survive, 95% of them co-localized trkB. This co-localization only occurred in about 5% of CA1 neurons in sham animals, suggesting an autocrine loop in these surviving neurons induced by ischemia (Ferrer et al. 1998b).

Further support for the role of BDNF and trkB in survival of neurons post-ischemia comes from studies administering exogenous BDNF. In one study, grafting of BDNF-transfected fibroblasts into gerbil hippocampus was shown to cause upregulation of trkB in the CA1 and granule layer regions of the hippocampus, and was associated with the prevention of CA1 neuronal death post-ischemia (Ferrer et al. 1998a). Another study found that administration of BDNF intracerebroventricularly following global ischemia in the rat prevented neuronal death in the CA1 region of the hippocampus, and also inhibited post-ischemic activation of various glial cells (Kiprianova et al. 1999a).

Additional evidence for the neuroprotective effect of BDNF and trkB post-ischemia comes from studies in which endogenous BDNF levels are altered. Using intraventricular infusion of a trkB-Fc fusion protein in order to decrease endogenous BDNF activity, Larsson et al. (1999) found that endogenous BDNF was neuroprotective against damage induced by global ischemia in the hilar and dentate gyrus regions of the rat hippocampus and regions in the striatum. Using an asphyxia model of global ischemia, D'Cruz et al. (2002) found that the CA1 neuronal sparing effect of post-ischemic hypothermia correlated with increased levels of BDNF and trkB in the hippocampus as determined by immunoblots. Another study, using the calcineurin antagonist cyclosporin A, which is known to ameliorate CA1 neuronal death after

global ischemia, found that the mechanism of protection involves the induction of BDNF and trkB through activation of pCREB (phosphorylated cAMP response element binding protein) (Miyata et al. 2001).

While several studies have found that BDNF has an important role in plasticity and cognitive function, few studies have attempted to correlate this with outcome post-ischemia. Kiprianova et al. (1999b) found that administration of BDNF post-ischemia rescued induction of LTP, which could not be induced in non-BDNF treated ischemic animals. In this same study, BDNF administration increased both working and reference memory to the level of sham animals as indicated by improved performance in a hole-board test (Kiprianova et al. 1999b). Gobbo and O'Mara (2004) found that enriched environment increased BDNF protein levels and improved cognitive performance in the direct swim path task of the water maze post-ischemia, but did not correlate the two changes. Using a neonatal model of hypoxia-ischemia, Almli et al. (2000) found that administration of BDNF resulted in protection against post-ischemic spatial memory impairments as measured in the water maze.

Several studies have found that neurogenesis is induced by ischemia, likely by similar mechanisms as take place during development, involving various transcription factors, signaling molecules, and growth factors, including the neurotrophins (Kee et al. 2001, Nakatomi et al. 2002, Schmidt and Reymann. 2002). Treatment with various growth factors has been found to enhance neurogenesis post-ischemia and lead to the integration of new neurons into functional circuits (Nakatomi et al. 2002). However, the role of BDNF in post-ischemic neurogenesis has been controversial. Nomura et al. (2005) found that infusion of human mesenchymal stem cells expressing the BDNF gene reduced infarct volume and lead to improved functional recovery post-ischemia, to an extent greater than that of infusion of the stem cells alone. However, Larsson et al. (2002) found that intrahippocampal infusion of BDNF inhibited the formation of newly generated dentate granule cells following global ischemia in rats. Another study found that intraventricular infusion of trkB-Fc fusion protein, thereby decreasing BDNF, caused an increase in neurogenesis and neuronal differentiation in the dentate gyrus (Gustafsson et al. 2003). It is thought that long-term delivery of BDNF to the hippocampus may counteract neuronal differentiation but not proliferation or survival of new cells post-ischemia (Kokaia and Lindvall. 2003).

2.6 Synaptic Plasticity

Neurons communicate with each other and other cells via chemicals called neurotransmitters, which are transmitted across a junction between the two cells known as a synapse. In general, a synapse is comprised of the meeting of a process from the pre-synaptic cell, the axon, and a process from the post-synaptic cell, the dendrite. Neurotransmitters are typically synthesized in the pre-synaptic cell, released, and attached to receptors on the post-synaptic cell. Release of neurotransmitters occurs via a process called synaptic vesicle exocytosis (reviewed in Jahn and Südhof. 1994). This process involves the expulsion of neurotransmitters from vesicles, which are associated with the terminals of the pre-synapse (axon). The synaptic vesicle is then recycled within the pre-synapse for further transmitter release. A variety of proteins are present at the pre-synaptic site and are involved in the regulation of neurotransmitter release (reviewed in Jahn and Südhof. 1994).

Synaptic plasticity is an activity-dependent process that results in the sculpting of synaptic connections during development, as well as in the adult brain. It involves changes at the molecular level as well as larger structural changes such as the formation of new synapses. It is through these processes that memories are formed and stored, and that learning occurs (Gogolla et al. 2007). The hippocampus is an important area of the brain for the formation of memories, and as such, synaptic plasticity is an essential physiological event in this region. Since synaptic plasticity is an activity-dependent process, injury, including ischemia, can induce changes at both the pre- and post-synapse, resulting in altered synaptic functions (Briones et al. 2004, Dinocourt et al. 2006, Kovalenko et al. 2006, Ruan et al. 2006).

2.6.1 Axonal Plasticity

2.6.1.1 Overview

Axonal plasticity includes molecular changes involving alterations to neurotransmission, as well as larger structural changes, such as axonal regeneration and sprouting. These forms of axonal plasticity are not mutually exclusive and both can occur in physiological as well as pathological conditions (Gogolla et al. 2007, Leenders and Sheng. 2005).

Synaptic vesicle exocytosis is the process by which the release of neurotransmitters occurs across the synapse. This process can be divided into three stages (reviewed in Jahn and Südhof. 1994). The first step involves synaptic vesicles docking to an electron dense region of

the axon terminal, called the active zone. The second step occurs when an action potential causes Ca^{2+} influx into the pre-synaptic terminal, resulting in a priming reaction that renders the vesicles ready for release. Lastly, the Ca^{2+} influx causes vesicular membrane fusion to the membrane of the axon terminal, the neurotransmitter is released across the synapse, and the vesicle is then internalized and recycled back into the axon terminal (Jahn and Südhof. 1994). Several molecules are involved in the regulation of vesicle exocytosis, and therefore have roles in pre-synaptic plasticity (Jahn and Südhof. 1994, Leenders and Sheng. 2005).

Synaptic strength at the pre-synapse can be altered by the activation of intracellular second messengers, which regulate several proteins involved in vesicular release (Leenders and Sheng. 2005). The influx of Ca^{2+} that occurs with an action potential causes activation of calcium-binding proteins including several Ca^{2+}-dependent kinases. A variety of these enzymes are expressed in the pre-synapse and have been implicated in vesicle release and recycling. The kinase Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is highly expressed in the hippocampus, both at pre- and post-synaptic sites. Recent studies have found that pre-synaptic activation of this kinase is required for synaptic plasticity to occur in hippocampal neurons. By inhibiting this kinase at the pre-synapse, it was found that the number of pre-synaptic active sites induced by glutamate, was significantly reduced (Ninan and Arancio. 2004). The authors believe that glutamate activation of pre-synaptic CaMKII is necessary for the conversion of existing but silent synapses, to functional ones (Ninan and Arancio. 2004). Protein kinase C (PKC) and protein kinase A (PKA) are other enzymes believed to be involved in pre-synaptic plasticity, via phosphorylation of various proteins involved in vesicular release (Leenders and Sheng. 2005).

Larger scale pre-synaptic plasticity involves the growth of axonal branches and formation of new synapses. While the dynamics of axonal branching are fairly well understood during development, mechanisms occurring in the adult system are less understood (Gogolla et al. 2007). It has been found that learning a new skill as well as deafferentation of a target can cause axons to form side branches, a process which is enhanced by growth factors and neurotrophins. It is believed that other factors, specific to cell type, are necessary for the process to occur; however these factors have yet to be identified (Gogolla et al. 2007). Plasticity on a slightly smaller scale involves changes to axonal boutons. The term bouton refers to swollen areas on the axonal terminal, which are typically the sites where synapses are formed. There are different types of boutons, which display variable ranges and extents of plasticity (reviewed in Gogolla et

al. 2007); however the mechanism behind plasticity-induced changes of the boutons is not well known. It has been found that structural remodeling of axons may underlie mechanisms of learning and memory, and that new experiences can cause the formation of new synapses (Gogolla et al. 2007).

Injury in the adult brain can result in axonal plasticity, both at the molecular level, as well as at the ultrastructural level. Since one of the hallmarks of ischemic injury is increased intracellular Ca^{2+}, it follows that changes to pre-synaptic function, as well as structure, can occur (Briones et al. 2004). Using the rat 4-vessel occlusion model of global ischemia, Kovalenko et al. (2006) found changes in the number and spatial arrangement of synaptic vesicles at various time points post-injury. At 15min reperfusion following the insult, synaptic vesicles were arranged more distantly from the active zone of the axon terminal, compared to those in sham-operated animals. The vesicles were also less clustered. However, there was no change in number of synaptic vesicles. By 1d post-ischemia the number of synaptic vesicles decreased by 20% compared to sham, and by 44% by 7d. The authors believe that these changes represent membrane turnover due to the increased activity occurring at the synapse with ischemia. Synaptic vesicles exist in either reserve pools at the axon terminal, or in pools that are actively cycling and participating in neurotransmission. It is suggested in this study that the active pools were depleted and rearranged following ischemia. Impaired recycling of vesicles may also have contributed to the decreased number of vesicles seen at later time points following ischemia. The authors suggest that ischemia may have caused alterations to pre-synaptic proteins involved in the regulation of synaptic function, causing changes to number and arrangement of vesicles, and ultimately leading to synaptic dysfunction (Kovalenko et al. 2006).

Injury can also cause ultrastructural changes of the pre-synaptic component. Sprouting of hippocampal axons has been seen in the mossy fibres of the dentate granule cells 1-2wk after non-penetrating traumatic brain injury (Dinocourt et al. 2006). Axonal sprouting of CA1 and CA3 pyramidal cells has also been observed after seizure induction (Dinocourt et al. 2006). In these studies, new axons were found to be glutamatergic, forming functional, excitatory synapses (Dinocourt et al. 2006). Another study found increased synaptogenesis in the CA1 region of the hippocampus following global ischemia (Briones et al. 2004). This increase in the number of synapses per neuron was significant in the posterior region of the CA1, but not the anterior region. Authors believe that there may have been a less robust response in the anterior region,

due to the greater cell death that occurs here post-ischemia. The response in the posterior region might indicate a more rapid sprouting mechanism following the axonal degeneration due to the higher number of surviving cells (Briones et al. 2004).

During the process of exocytosis, synaptic vesicle activity is regulated, and can be altered by various synaptic proteins. For example, the activity-induced influx of intracellular Ca^{2+}, in both physiological and pathological conditions, causes activation of CaMKII, which phosphorylates synapsin-1, causing alterations to availability of vesicles for release (Leenders and Sheng. 2005). As well, growth-associated protein-43 (GAP-43) is a protein kinase C (PKC) substrate at the pre-synapse (Powell. 2006), and interacts with pre-synaptic vesicle release machinery, modulating neurotransmission. GAP-43 is important in axonal plasticity, and plays a role in the adult brain following injury, including ischemia.

2.6.1.2 GAP-43

The protein growth-associated protein-43 (GAP-43) is highly expressed in axonal growth cones during development, which is believed to be the first step in the process of neuronal branching (Denny. 2006). While this protein is involved in plasticity and neuronal growth and synapse formation during development, its expression is maintained in the mature CNS and is influenced by the expression of neurotrophic factors, including BDNF (Schmidt-Kastner et al. 1997, Tetzlaff et al. 1994). In the adult brain, GAP-43 has been implicated in modulation of neurotransmitter release, vesicular endocytosis and recycling at the pre-synapse (reviewed in Denny. 2006, Powell. 2006). It is believed to be a substrate of pre-synaptic protein kinase C (PKC) and interacts directly with components of vesicular exocytosis machinery (Powell. 2006). GAP-43 is also thought to be involved in memory formation and learning both in the developing brain, and in adulthood (Denny. 2006). Following injury, increased GAP-43 expression is believed to be due to the attempted regeneration of axons and reestablishment of synaptic connections (Dinocourt et al. 2006).

Ischemia alters the expression of GAP-43, at later stages of recovery, when surviving neurons are reorganizing axonal connections due to death of target cells (Schmidt-Kastner et al. 1997). However, some studies have found early changes occurring post-ischemia at the levels of both mRNA and protein. Tagaya et al. (1995) found that GAP-43 mRNA was increased in gerbil hippocampal granule cells beginning at 3hr and persisting through 7d post-ischemia, suggesting

that GAP-43 may be involved in axonal plasticity beginning soon after ischemia, but also indicating the occurrence of a possible pathological mechanism. Schmidt-Kastner et al. (1997) found using in-situ hybridization and immunohistochemistry that GAP-43 mRNA increased early post-ischemia in the granule and hilar cells of the dentate gyrus, and that protein was increased at 1d post-ischemia in the cytoplasm of hilar and CA3 neurons, which decreased from then on. Results of these two studies suggest an increase of GAP-43 in the dentate hilar region. It is thought that this axonal damage, not normally seen in global ischemia models, may have occurred due to the location and axonal arrangement of the hilar cells, rendering them vulnerable to edema (Schmidt-Kastner et al. 1997). As well, these cells are in close proximity to large amounts of trophic factors, including BDNF, which may lead to plastic responses (Schmidt-Kastner et al. 1997). Schmidt-Kastner et al. (1997) note that in their study, the increase of GAP-43 seen in hilar cells was minor. However the greater increases seen in the study by Tagaya et al. (1995) study may be pathological, possibly leading to post-ischemic seizures, to which gerbils are more vulnerable than rats (Schmidt-Kastner et al. 1997). Another study found an association between head injury, trkB and GAP-43 expression and post-traumatic epilepsy (Dinocourt et al. 2006). Using a hippocampal slice model, axonal transections were made between the CA3 and CA1 regions, resulting in deafferenation of the CA1 pyramidal neurons. GAP-43 expression was induced in sprouting CA3 pyramidal cell axons and this was preceded by increased BDNF and trkB protein in the CA3 area. The increased immunoreactivity of GAP-43 was impaired in hippocampal slices from trkB knock-down mice, which express reduced levels of the trkB gene. These results suggest that axonal sprouting following injury is induced by trkB signaling, causing up-regulation of GAP-43 (Dinocourt et al. 2006). This is likely a healing response, but the authors believe that excessive sprouting may alter the normal ratio of excitatory to inhibitory synapses, resulting in hyperexcitability, and possibly, post-traumatic epilepsy (Dinocourt et al. 2006). These studies suggest a role for GAP-43 in post-ischemic axonal sprouting and the formation of new synapses. However, it seems that excessive GAP-43 expression due to injury may result in abnormal connections, promoting hyperexcitability, and possibly seizure activity.

2.6.2 Dendritic Plasticity

2.6.2.1 Overview

Transmission of excitatory neurotransmitters from axon to dendrite terminates mainly on tiny protrusions on the post-synaptic membrane, known as dendritic spines (Luscher et al. 2000). These spines are characterized by the presence of an electron-dense area on their membranes, known as the post-synaptic density (PSD). The post-synaptic density is situated across from the pre-synaptic active zone and is the region where synaptic transmission occurs. Changes to the post-synaptic fraction of excitatory synapses, particularly in the hippocampus, are thought to underlie mechanisms of learning and memory (Luscher et al. 2000). The plasticity of the dendritic spines themselves has been demonstrated, as rapid changes to their structure and shape as well as changes in their biochemical composition, such as receptor type, have been observed (Luscher et al. 2000). These changes are believed to be activity-dependent, involving increases in intracellular Ca^{2+} levels; however, the degree to which intracellular Ca^{2+} increases is important in determining the extent of changes occurring to the dendritic spines. Small increases in intracellular Ca^{2+}, induced by minimal synaptic activity, are required for spine maintenance. Larger, yet brief increases cause formation of new spines and growth. Very large and sustained increases lead to retraction of dendritic spines (reviewed in Luscher et al. 2000). Activity is also believed to cause re-distribution of the AMPA type glutamate receptors on post-synaptic spines via exo- and endocytosis. A relationship between changes in dendritic spine morphology and redistribution of AMPA receptors is believed to exist. Increased activity and intracellular Ca^{2+} has been found to cause increased proportions of synapses with perforations within the post-synaptic density of dendritic spines, a process which is believed to be initiated by increased AMPA receptors in this region (Luscher et al. 2000). Activity has also been found to increase production of de novo dendritic spines. The mechanisms behind these morphological and biochemical changes induced by activity and increased intracellular Ca^{2+} is believed to be due to enhancement of actin-dependent dynamics thereby causing shape changes to the spines, as well as exo- and endocytosis leading to changes in receptor expression (Luscher et al. 2000).

While dendritic plasticity is known to occur in physiological conditions, such as memory and learning, it also occurs in pathological conditions, including ischemia (Ruan et al. 2006). Not only do the dendritic spines undergo changes in these conditions, the dendrites themselves also change. One study found that following global ischemia in rats, increased CA1 dendritic

disorientation occurred, meaning many apical dendrites branched into the region of basal dendrites, and vice-versa (Ruan et al. 2006). As well apical dendrite length was found to increase in length at 24hr following ischemia. This increased length is believed to be mainly due to sprouting of new dendrites rather than extension of existing ones. This alteration to dendrites directly alters the dendritic spines, and therefore synaptic transmission. The authors believe that this increase in dendritic branching 24hr post-ischemia may be pathological and contribute to excitotoxicity and cell death in the CA1 region of the hippocampus (Ruan et al. 2006). The underlying mechanisms of the ischemia-induced dendritic plasticity is believed to involve increased activation of CA3 pyramidal neurons which send their projections (Schaffer collaterals) to the CA1 pyramidal cells, causing activation of glutamate receptors and leading to dendritic branching. The authors suggest that neurotrophic factors may also have a role in dendritic plasticity following ischemia. BDNF in the dentate gyrus and CA3 regions following ischemia may then be anterogradely transported to the CA1 region, promoting branching of these dendrites. In conclusion, the authors of this study believe that the dendritic changes seen are a consequence of pathological events serving to increase the receptive field of the CA1 neurons, leading to increased excitotoxicity and promoting cell death (Ruan et al. 2006).

Several families of proteins are involved in the morphological and biochemical changes occurring during non-pathologic, as well as injury-induced, dendritic plasticity. Structural proteins, specifically microtubule-associated protein-2 (MAP-2), are involved in stabilizing dendritic spines and have roles in recovery of synaptic transmission by restructuring of damaged dendrites, post-ischemia (Briones et al. 2006).

2.6.2.2 MAP-2

MAP-2 belongs to a family of structural proteins, which also includes Tau and MAP-1 (Dehmelt and Halpain. 2005). Each protein in the family consists of a carboxyl terminus with microtubule-binding repeats, and various isoforms exist for each one. The forms of MAP-1 include MAP-1A and MAP-1B. MAP-2 forms include MAP-2a, MAP-2b, and MAP-2c. The MAP-2 isoforms are mainly expressed in neurons and oligodendrocytes. MAP-2c is the shortest isoform and is mostly expressed during development after which it is downregulated. MAP-2b is expressed during development as well as in adulthood, while MAP-2a begins being expressed as MAP-2c expression decreases (Dehmelt and Halpain. 2005). MAP-2 becomes segregated into

dendrites during development and is restricted to both cell bodies and dendrites in adulthood (Dehmelt and Halpain. 2005). The MAP family of proteins binds to microtubules as well as F-actin. MAP-2, specifically, is involved in stabilizing dendritic structural microtubules and restructuring of dendrites (Al-Bassam et al. 2002). Dendritic microtubules are very dynamic and go through phases of growth, shortening, and pausing. MAP-2 binds along the length of the microtubules and stabilizes them by controlling these changes (Dehmelt and Halpain. 2005). It responds to the activity-dependent induction of neurotrophins and aids in the formation of dendrites as a result (Vaillant et al. 2002).

MAP-2 is susceptible to degradation by mechanisms that occur in global ischemia, such as activation of calpains by increased intracellular Ca^{2+} (Briones et al. 2006). However, its induction in adjacent regions is believed to be a protective neuroplastic response to the injury (Briones et al. 2006, Li et al. 1998). Using a rat model of focal ischemia, Li et al. (1998) found that MAP-2 immunoreactivity decreased in the core region of damage in the cortex. Neurons that were still intact in this region expressed MAP-2 at 6hr post-ischemia, and a beaded pattern was seen in the dendrites of this region suggesting disruption and damage. MAP-2 immunoreactivity increased at later time points in the penumbra, the region bordering the ischemic core, suggesting regrowth and restructuring of dendrites in this region. The authors suggest that based on these results, MAP-2 can be used as an early marker of ischemic damage as well as later neural plasticity (Li et al. 1998). Results of another study suggest that post-ischemic expression of MAP-2 may have a role in functional outcome. Briones et al. (2006) found using the rat 4-vessel occlusion model of global ischemia that rats placed in complex environmental housing as opposed to simple social housing performed better in a water maze task post-ischemia. These animals were also found to have increased MAP-2 immunoreactivity in the CA3 region of the hippocampus compared to simple social housed rats. The authors speculated that the increased MAP-2 expression is a result of increased dendritic processes in the complex environment housed animals, therefore resulting in the enhanced functional outcome (Briones et al. 2006).

2.7 Protein-Energy Malnutrition

2.7.1 Definition

Protein-energy malnutrition is a nutritional state that results from inadequate dietary intake of protein, energy, and micronutrients needed to fuel the body (Becker. 1983, Torun.

2006). The relative deficiencies of these components, duration and cause of the deficiencies, age of the individual, and the presence of disease, all combine to determine the clinical manifestation of PEM. Primary PEM results from inadequate food intake, while secondary PEM is caused by the presence of other disease or treatment, such as pharmaceutical agents, leading to decreased food intake, loss of ability to absorb nutrients, and/or increased requirements (reviewed in Torun. 2006).

PEM is prevalent in developing countries and is associated with child mortality, and impaired growth (Pelletier et al. 1995). In these cases, PEM can be characterized as marasmus or kwashiorkor. Marasmus refers to a state of chronic, severe PEM in which there is a predominant deficit in energy, resulting in emaciation with no edema (Grover and Ee. 2009, Torun. 2006). Marasmus is thought to represent an adaptation to malnutrition and is often tolerated in adults (Emery. 2005). In kwashiorkor, there is a more rapid progression of the malnutrition, involving predominantly protein deficiency with some degree of energy deficit, and edema (Grover and Ee. 2009). Unlike marasmus, kwashiorkor is though to represent a failure to adapt to malnutrition (Emery. 2005). Marasmic kwashiorkor results from a chronic energy deficit, and either chronic or acute protein deficit and is characterized by both edema and emaciation (Grover and Ee. 2009, Torun. 2006).

In industrialized countries, individuals affected by PEM often include children in lower socioeconomic groups, solitary elderly individuals, and adults with substance abuse problems (reviewed in Torun. 2006). Individuals affected by disease states such as renal failure, liver disease and other stress states such as surgery, infection and trauma can also be affected by PEM (Chapman. 2006, Corish and Kennedy. 2000, Hoffer. 2001). PEM in these populations is often considered mild compared to those cases in children of underdeveloped countries, but is more difficult to diagnose and catagorize, and can still have devastating consequences (Torun. 2006).

2.7.2 PEM in the Elderly Population of Industrialized Countries

2.7.2.1 Overview

There is currently a dramatic increase occurring in the proportion of elderly individuals making up the Canadian population. It is estimated that 6.7 million Canadians will be age 65+ by 2021 (Institute of Aging. 2007). This group is at high risk for impairment of nutritional status. Healthy aging is associated with decreased hunger, intake of smaller meals, less snacking, and

more rapid satiety (Chapman. 2006, Morley. 1997, Wurtman et al. 1988). Elderly individuals also tend to eat a less varied diet. Daily energy intake decreases by about 30% between the ages of 20 and 80 (Wurtman et al. 1988). When decrease in food intake is greater than energy expenditure, body weight is lost. Body composition also changes with normal aging. Specifically skeletal muscle is lost while fat is increased (Chapman. 2006).

Protein-energy malnutrition is common in several elderly sub-populations in developed countries. Up to 15% of independent living elderly are affected by PEM and this increases to between 23-62% for the hospitalized, and up to 85% for those residing in nursing homes (Mion et al. 1994). Elderly individuals affected by PEM experience several adverse effects including impaired muscle and immune functions, decreased bone mass, and declining cognitive function (Visvanathan and Chapman. 2009). Several studies have found that the presence of PEM is a predictor of mortality in elderly populations, no matter where they reside at the time (Campbell et al. 1990, Cederholm et al. 1995, Morley and Silver 1995).

2.7.2.2 Causes

Several factors contribute to the development of PEM in the elderly populations of industrialized countries. Homeostatic mechanisms that are in place in younger individuals in order to restore food intake following periods of under-eating, do not seem to be present to the same extent in the elderly (Visvanathan and Chapman. 2009, Roberts et al. 1994). Aging also causes impairment of the sense of smell and taste, which is believed to contribute to the lack of interest in food exhibited by this population (Doty et al., 1994, Rolls and McDermott. 1991). Poor dentition in these individuals can lead to problems with chewing, biting, and swallowing, and has been found to result in poor protein intake (Sahyoun et al. 1988). An increase in the production of satiety-inducing cytokines, such as TNF-α, also can occur in elderly individuals who are also exposed to a high degree of stress, such as infection (Yeh and Schuster. 1999). Hormonal changes that occur with aging can lower physical activity, decrease appetite and food intake, and increase satiation (as reviewed in Visvanathan and Chapman. 2009). Several medical factors can also play a role in the development of PEM in this population, including infection, dysphagia, gastrointestinal symptoms, malabsorption syndromes, and the intake of multiple medications (reviewed in Chapman. 2006).

Social factors can also contribute to the development of PEM in the elderly populations. Many elderly live below the poverty line and therefore may be unable to afford to eat a nutritionally adequate diet (Chapman. 2006). These individuals are also more likely to live by themselves and therefore experience social isolation and loneliness, factors that have been associated with decreased appetite and energy intake (Walker and Beauchene. 1991). Elderly people living by themselves may also be unable to grocery shop, prepare food, and cook for themselves, again leading to decreased food intake. Psychological factors such as depression, dementia, alcoholism, and bereavement can also be a factor in the development of PEM in the elderly population (reviewed in Chapman. 2006).

2.7.2.3 Physiological and Pathological Effects

Physiologically, several initial adaptations will occur in response to protein-energy malnutrition (reviewed in Torun. 2006). Decreased energy expenditure occurs in individuals affected by PEM as an adaptation to the decreased energy intake. This is followed by mobilization of fat stores leading to weight loss (Torun and Viteri. 1981). As the energy deficit increases, subcutaneous fat decreases and protein catabolism occurs leading to muscle wasting and further weight loss, resulting in an emaciated appearance. Decreased protein intake results in reduced protein synthesis, contributing to skeletal muscle protein breakdown so that liver protein synthesis can be maintained. Protein deficiency causes an increase in the proportion of amino acids used and recycled for protein synthesis and a proportional reduction in amino acid catabolism. This results in decreased urea synthesis and nitrogen excretion (Tomkins et al. 1983). If PEM progresses to the point of depletion of non-essential tissue proteins, visceral proteins will be broken down and death may occur unless intervention is started (Torun. 2006).

Serum albumin synthesis decreases initially, but after a few days of PEM its half-life increases. In order to maintain circulating levels of this protein, serum albumin is moved from the extravascular to the intravascular pool. However, when PEM becomes prolonged and severe, serum albumin concentration decreases, along with other serum proteins (Gibson. 2005, Torun. 2006).

As summarized in Torun et al. (2006), hormonal adaptations also occur in PEM, contributing to the maintenance of energy homeostasis along with increased glycolysis, lipolysis, amino acid mobilization, breakdown of muscle protein, and decreased storage of glycogen, fat

and proteins. Decreased food intake leads to decreased glucose and free amino acids, resulting in decreased insulin secretion (Misra et al. 1980) and increased glucagon (Robinson and Seakins. 1982) and epinephrine (Parra et al. 1975). Low plasma amino acid concentration leads to increased release of human growth hormone (Soliman et al. 1986) and decreased somatomedin (insulin-like growth factors) activity (Smith et al. 1981). Reduced urea synthesis occurs (Iyengar and Narasinga Rao. 1982) due to the increase of growth hormone and epinephrine, contributing to amino acid recycling, rather than catabolism (Torun. 2006). Levels of thyroid hormones are decreased (Parra et al. 1975), which causes conservation of energy via decreasing thermogenesis and oxygen consumption (Torun. 2006).

An adaptive decrease in hemoglobin concentration and red cell mass also occurs in severe PEM (Viteri et al. 1968). Oxygen demands are decreased by reduced body mass and physical activity. However, this reduction is considered an adaptation rather than anemia, which will not develop unless iron, folate and vitamin B12 are no longer available (Torun. 2006).

Physiological changes will continue to occur as PEM progresses and will eventually be harmful as opposed to adaptational. Central vascular circulation becomes more important than peripheral which can fail in severe PEM (Viart. 1977, Heymsfield et al. 1978). Renal filtration may also be reduced (Mahakur et al. 1983). T-lymphocytes are decreased in several regions including the thymus gland, spleen, and lymph nodes and impairment of the complement system also occurs in severe PEM, contributing to the susceptibility of malnourished individuals to infection and other complications (Chandra. 1991, Keusch. 1990). Potassium levels decrease and intracellular sodium increases which may contribute to reduced muscular strength and increased fatigue in these patients (Nichols et al. 1972). Intestinal absorption is impaired leading to diarrhea which may further aggravate the malabsorption causing further malnutrition. Malnutrition can also lead to impaired cognitive functions via decreased brain growth, neurotransmitter production, and nerve myelination, However, these effects have been seen mostly in developing children (Kar et al. 2008, Torun. 2006).

In summary, deficits to the cardiovascular, renal, immune, gastrointestinal, and nervous systems can all occur and will render the malnourished individual more susceptible to injury and illness as compared to a well-nourished person (Torun. 2006).

2.7.2.4 Diagnosis and Categorization

The categorization of marasmus or kwashiorkor generally refers to cases of severe undernutrition in children of developing countries. Characterization of PEM in elderly populations of developed countries is more difficult. PEM in this population may be the result of inadequate dietary intake, increased metabolic demands, or increased nutrient losses (Corish and Kennedy. 2000). All three of these conditions are typically seen in hospitalized elderly. Severe protein-energy malnutrition in this population causes muscle wasting and loss of subcutaneous tissue, similar to marasmus in starving children (Hill. 1992). Hypoalbuminemia and edema can also occur, resembling kwashiorkor (Hill. 1992). In hospitalized patients, 'marasmic kwashiorkor' is the most common categorization of PEM, expressed as muscle wasting, fat loss, and hypoalbuminemia (Corish and Kennedy. 2000, Hill. 1992).

A diagnosis of PEM in hospitalized elderly is based on screening protocols which may utilize a single parameter, or more desirably, multiparameter indices (reviewed in Gibson. 2005). Single index screens most often utilize anthropometric, biochemical, or functional indicators. Anthropometric measurements include height, weight, body mass index (BMI), triceps skinfold, and arm circumference (Edington et al. 1996, McWhirter and Pennington. 1994). These types of assessment are simple, cheap, and noninvasive. A BMI of $<20 kg/m^2$ has been used as an indicator of undernutrition. However, for many patients with weight loss or other clinical problems, this measure alone is not adequate. Biochemical measurements include serum albumin concentration, total iron binding capacity, serum transferrin, white blood cell count, urinary creatinine and urea nitrogen, all of which decrease with suboptimal protein status. The problem with measures such as serum albumin and transferrin is the long half-lives which translates into a slow response time to nutritional depletion. As well, these measurements can be affected by other non-nutritional disease states, as well as by age and medications (Taren and Schler. 1990), limiting their sensitivity and specificity. Functional indices include tests of muscle weakness, poor wound healing, impaired thermoregulation, depression, irritability, and fatigue. The value of using a single index screening protocol is unknown, but since these indices can often be non-specific when used alone, a multiparameter protocol may be more adequate (Gibson. 2005).

Multiparameter indicators of PEM include the prognostic nutritional index, the nutrition risk index, subjective global assessment, and the mini-nutritional assessment (reviewed in Gibson. 2005). The mini-nutritional assessment is the only indicator designed for use specifically

in elderly patients (Guigoz et al. 1996). Originally, various indices were grouped into four components: anthropometric assessment, general assessment (including lifestyle, medication and mobility), dietary assessment, and subjective assessment of health and nutrition. Scores from each component were totaled and used to classify the patient as normal, at risk for malnutrition, or undernourished (Vellas et al. 1999). The most recent version of the mini-nutritional assessment utilizes a two-step approach in which screening for potential malnutrition is done in the first step, followed by a more detailed nutritional assessment in the second step if the patient fails the initial screen (Rubenstein et al. 2001). Patients are then classified as either at risk of malnutrition or malnourished (Rubenstein et al. 2001).

Once a diagnosis is made, PEM can be further classified in terms of course or duration and severity, usually based on anthropological measurements (reviewed in Torun. 2006). A formal classification based on stunting and wasting is utilized in children suffering from PEM, but classification for adults and elderly in North America is less well developed. Usually, severity of PEM in adults is determined using anthropometric measurements, specifically BMI. For adults, a BMI of ≥18.5 is considered normal, 17.0-18.4 indicates mild PEM, 16.0-16.9 indicates moderate PEM, and <16.0 indicates severe PEM (Torun. 2006). Dietary history is also useful for assessing the relative contributions of protein and energy deficits in mild and moderate PEM, while clinical and biochemical data are used to confirm severe PEM (Torun. 2006).

Jensen et al. (2010) have recently highlighted the need to include inflammatory markers in the diagnosis of PEM for adult hospitalized individuals in developed countries. They suggest an approach that integrates the recognition of a continuum of inflammation from "starvation-related malnutrition", when there is chronic starvation without inflammation, "chronic disease-related malnutrition", when inflammation is chronic mild to moderate, and "acute disease or injury-related malnutrition", when inflammation is acute and severe (Jensen et al. 2010). It is important to emphasize that this approach is aimed at hospitalized individuals with underlying illness, and therefore the elderly population developing PEM prior to hospitalization who are the focus of this thesis, may not fit into this characterization. It is likely this population is mixed one, with differing degrees of malnutrition and some individuals affected by other illnesses and medications. These non-hospitalized individuals may also have some degree of underlying inflammation due to sarcopenia, which is defined as muscle loss that occurs with aging (Schaap et al. 2006). Jensen et al. (2009) proposes that individuals with sarcopenia may represent part of

the inflammation continuum overlapping with cachexia, where there is mild to moderate inflammation, and failure to thrive, which for the elderly population has been described as functional and/or cognitive decline (Jensen et al. 2009, Sarkisian and Lachs. 1996). Since serum albumin is often included as an indicator of PEM, and this protein is more sensitive to inflammatory states than it is nutritional status (Kaysen et al. 2001) the measurement of additional inflammatory markers such as C-reactive protein, may aid in further refinement of the PEM diagnosis and clarify whether underlying inflammation is also occurring (Jensen et al. 2009).

2.7.3 Animal Models of PEM

Unfortunately, there are currently no established standards of nutritional status for modeling protein-energy malnutrition in animals. Factors such as diet formulation and type of animal used for the model need to be carefully considered in order to mimic the important features of the nutritional paradigm being studied (Woodward. 1998).

Currently, investigators using animals to model PEM rely on several parameters to assess status. These include decreased body weight, food intake, serum albumin concentration, liver glutathione, and increased liver lipid. Commonly, either a paradigm of reduced feeding of a nutritionally-complete diet, or one of ad libitum feeding of a low-protein diet are used in rodent models of PEM (Woodward. 1998). Our laboratory has previously used a gerbil model of PEM in which animals fed a 2% protein diet voluntarily decrease their food intake resulting in a mixed protein-energy malnutrition. A study done in our laboratory using this model found that feeding the low protein diet for 4 weeks caused a 17% decrease in body weight, 15% decrease in food intake, 49% decrease in liver glutathione, and a 66% increase in liver lipids as compared to animals fed a control diet (12% protein) (Bobyn et al. 2005).

The decrease in food intake that occurs with the low protein feeding utilized in our model is essential for establishing PEM. However the reasons for this phenomenon are largely unknown. It has been suggested that an imbalance in the ratio of non-essential to essential amino acids may cause decreased food intake; specifically as this ratio increases, appetite may decrease (de Angelis et al. 1978). Results of another study suggest that the hypothalamus region of the brain likely plays a role in recognition of nutrient deficiencies, including protein malnutrition,

and that this may be coupled to changes in taste preference, thereby affecting food intake (Torii et al. 1998).

While serum albumin concentration may not be an ideal indicator of protein malnutrition in humans due to its non-specificity, it is a good marker in laboratory animals in which animal health, environment and other factors can be controlled. Since serum albumin has a long half-life, a reduction in its concentration is thought to occur as PEM progresses and protein synthesis therefore decreases (Gibson. 2005).

The cause of the increased liver lipid seen in our model is unknown, but has been hypothesized to be due to excess carbohydrate, or from decreased synthesis of the protein required for triglyceride transport out of the liver (Flores et al. 1970, Truswell et al. 1969). Liver gluthathione concentration decreases in this model of PEM, due to the decreased intake of sulphur amino acids (cysteine and methionine) necessary to synthesize glutathione (Dubick et al. 1985, Taylor et al. 1996).

The model of PEM utilized in our laboratory represents a moderate form of PEM. Decreased food intake and liver glutathione concentration have been shown in other rodent models of moderate protein deficiency (Eisenstein and Harper. 1991, Hum et al. 1992, Taylor et al. 1992). The lack of decreased voluntary food intake seen in models of mild protein deficiency (Bauman et al. 1988, Zhang et al. 2002), as well as the symptoms that develop in more severe PEM models (edema, diarrhea, sparse hair, staggering gait) (Flores et al. 1970, Leme-Brasil et al. 1980) also distinguish our model from these.

An acknowledged limitation of the studies of PEM in this thesis is the young age of the animals. This is in contrast to the problem of PEM in the human stroke patient, which predominantly affects the elderly. A feature of this problem, which is much more evident in the rat (Chapter 7) than in the gerbil (Chapter 5), is that rodents maintain a rapid growth rate long beyond adolescence.

2.7.4 PEM and Cerebral Ischemia

2.7.4.1 Overview

A number of studies over more than two decades have found that a proportion of elderly acute stroke patients are affected by protein-energy malnutrition upon admission to hospital, as assessed by mainly anthropometric and biochemical indices (Axelsson et al. 1988, Davalos et al.

1996, Davis et al. 2004, Martineau et al. 2005, Yoo et al. 2008). Davalos et al. (1996) used triceps skin-fold thickness, mid-arm muscle circumference, and serum albumin concentration to assess nutritional status of acute stroke patients upon admission to hospital and weekly during hospital stay. It was found that 16.3% of patients were affected by PEM upon admission and this increased to 26.4% after one week in the hospital. Malnutrition was associated with increased mortality, worse outcome, and longer duration of stay in the hospital (Davalos et al. 1996). Another study found that 16% of acute stroke patients were affected by PEM upon admission to hospital, which increased to 23% at time of discharge (Axelsson et al. 1988). Nutritional status was assessed using body weight, triceps skin-fold thickness, upper arm muscle circumference, plasma albumin and prealbumin, and transferrin (Axelsson et al. 1988). Using the multiparameter indicator subjective global assessment, within 24hr and 48hr of admission to hospital, Davis et al. (2004) and Martineau et al. (2005) found that 16.2% and 19.2%, respectively, of acute stroke patients were affected by PEM. In the Davis et al. (2004) study, malnourished patients were more likely to die or have a poor outcome, although this association lost significance when adjusted for age, stroke severity, and pre-morbid dependence. Low serum albumin concentration upon admission was associated with mortality one month following stroke (Davis et al. 2004). Martineau et al. (2005) found patients affected by PEM to have a longer hospital stay as well as increased incidence of dysphagia and post-stroke complications.

Nutritional status of a higher proportion of these acute stroke patients is adversely affected after stroke during hospital stay (Axelsson et al. 1988, Brynningsen et al. 2007, Davalos et al. 1996, Yoo et al. 2008). Yoo et al. (2008) found using weight loss, serum albumin concentration, serum transferrin concentration, and serum prealbumin concentration that 12.2% of acute stroke patients were affected by PEM upon admission which was in turn found to be predictive of undernutrition occurring 1wk later (19.8%) (Yoo et al. 2008). Bynningsen et al. (2007) found 35% of stroke patients to be affected by PEM 1wk following stroke based on several anthropometric and biochemical indices. The incidence of PEM in stroke patients continues to rise (35-49%) upon admission to rehabilitation units (Finestone et al. 1995, Poels et al. 2006).

It has been suggested that this worsening of PEM during hospital stay can have a negative effect on stroke outcome (Finestone et al. 1996). A study by Finestone et al. (1996) suggests that stroke patients with malnutrition prior to entering a rehabilitation unit will be more

functionally dependent than those who are adequately nourished upon entry. This dependency may result in an increased length of stay and decreased functional improvement rates. The authors suggest that malnutrition is the one variable significantly related to functional improvement rate that could possibly be reversed. The patients used in this study were restricted to those representing a relatively young and less severely impaired population of stroke victims. This may limit the usefulness of these results for the general population. As well, possible factors negatively affecting functional improvement, such as other medical complications, were not accounted for (Finestone et al. 1996).

A large epidemiological study examined the association of BMI and survival up to 5yr following stroke, in 21,884 hospitalized stroke patients (Olsen et al. 2008). BMI was calculated for all patients and stroke severity, computed tomography, and cardiovascular risk factors were evaluated. Results indicate that mortality following stroke is inversely related to BMI. Mortality was highest in the underweight group (BMI <18.5), followed by the normal weight group (BMI 18.5-24.9), severely obese (BMI ≥35), obese (BMI 30.0-34.9), and overweight group (BMI 25.0-29.9). These results suggest that malnutrition may increase risk of mortality within 5yr following stroke (Olsen et al. 2008).

Unlike the majority of studies that have been correlative in nature, a major study that had the potential to establish causality between protein-energy malnutrition and stroke outcome was the FOOD (Feed or Ordinary Diet) Collaboration Trial (Dennis et al. 2005a, Dennis et al. 2005b). This consisted of a family of 3 multicentre controlled trials with the same randomization, data collection, and follow-up procedures. The initial preliminary data from a prospective cohort consisting of the first 3012 patients randomized demonstrated that compromised baseline nutritional status of acute stroke patients was associated with decreased chance of survival and increased functional dependency six months later (FOOD Collaboration Trial. 2003). However, the completed interventions of the FOOD Trials failed in one of their goals, which was to test whether a causal relationship exists between nutritional status and stroke outcome (Prosser-Loose and Paterson. 2006). Since nutritional assessments were not standardized across centres, misclassification of patients into baseline nutritional categories may have occurred. Also, patients were assigned to one of several nutritional interventions during the six month period between baseline nutritional assessment and final follow-up, and the effect of these on nutritional

status and functional outcome were not monitored or considered in this analysis (FOOD Collaboration Trial. 2003, Prosser-Loose and Paterson. 2006).

Results of a study done in our laboratory indicated that PEM impairs functional outcome as measured in the open field in an animal model of global ischemia, suggesting a causal relationship between PEM and stroke outcome (Bobyn et al. 2005). The open field is a test of spatial learning and ability to adapt in a novel environment. It requires use of the hippocampus, which is selectively damaged in global ischemia (Colbourne and Corbett. 1995). Results indicate that PEM caused impaired performance in the open field post-ischemia, as measured by total distance traveled. While ischemic gerbils fed control diet habituated by 7d post-ischemia, PEM-ischemic gerbils were still impaired at 10d post-ischemia. A subset of these animals also displayed marked thigmotaxis in the field, which may indicate increased anxiety (Bobyn et al. 2005).

2.7.4.2 Effects of PEM on Mechanisms of Damage in Cerebral Ischemia

2.7.4.2.1 Glucose and Glucocorticoids

It is well documented that increased glucocorticoid concentration present at the time of cerebral ischemia has a detrimental effect on brain damage and the presence of hyperglycemia contributes to this increase (Lin et al. 1998, Pulsinelli et al. 1982). Cortisol is the predominant glucocorticoid in humans in contrast to corticosterone in the rat. Via different mechanisms, hypoglycemia has also been shown to aggravate ischemia-induced injury (Voll and Auer. 1988, Zhu and Auer. 2004). Increased glucocorticoid concentration often forms part of the physiological response to protein-energy malnutrition; however, in this case, it is not as a result of hyperglycemia. PEM can result in hypoglycemia and a re-organization of metabolism, which has been suggested as a mechanism for conserving energy and promoting glucose homeostasis (Pugliese. 1990). Human studies suggest that the occurrence of increased cortisol and hypoglycemia in the protein-energy malnourished state may depend on the severity of the nutritional deficiency.

2.7.4.2.1.1 Effects of Glucose and Glucocorticoids on Ischemic Damage

The issue of hyperglycemia contributing to ischemia-induced damage has been coined “the glucose paradox of cerebral ischemia”. The paradox lies in the fact that while energy failure

is a hallmark of the ischemic cascade, pre-ischemic hyperglycemia has been shown to worsen damage. Myers and Yamaguchi (1977) were the first to demonstrate this with the finding that infusion of glucose just prior to cardiac arrest in monkeys resulted in myoclonic seizures, opisthotonic spasms, decerebrate posturing, and widespread neuronal necrosis throughout the cortex and basal ganglia (Myers and Yamaguchi. 1977). Similar findings have since been demonstrated in rodent models of ischemia. Pulsinelli et al. (1982) found that hyperglycemia induced prior to global ischemia caused by 4-VO in the rat produced severe injury as compared to those receiving saline injection or glucose during or following ischemia. Neurological status and neuronal and glial death throughout the forebrain was significantly worsened in the pre-ischemic hyperglycemic group (Pulsinelli et al. 1982). Another study found significantly increased damage to the CA1 neurons in rats made hyperglycemic prior to the ischemic insult induced by 2-VO plus hypotension, as compared to normoglycemic rats. Hyperglycemic rats showed extensive damage to the striatum, neocortex, thalamus, and cortex, which was either absent or minimal in normoglycemic rats. Leukocyte activation on the vascular endothelium as well as heightened microglial activation was also seen in the hyperglycemic animals (Lin et al. 1998). A recent study measured blood glucose in 447 patients presenting with acute ischemic stroke to the emergency department. Results indicate that patients classified as hyperglycemic, without history of diabetes, had increased stroke severity and functional impairment compared to those classified as normoglycemic (Stead et al. 2009). Interestingly, these patients also had a poorer prognosis than patients classified as hyperglycemic with a history of diabetes. The authors suggest that patients with a previous diagnosis may have been somewhat protected by medications or perhaps effects of pre-conditioning (Stead et al. 2009).

There are several schools of thought on how hyperglycemia might contribute to ischemia-induced brain damage. The lactic acidosis hypothesis suggests that elevated blood glucose during ischemia causes increased lactate production leading to acidosis and increased damage to neurons (Kalimo et al. 1981). Kalimo et al. (1981) pre-treated rats with either a glucose infusion or saline prior to induction of global ischemia via the 2-VO model. Results indicate that structural alterations were exacerbated in glucose infused rats leading to irreversible cellular damage. These findings were attributed to an increase in tissue lactate concentration due to the glucose infusion, causing increased acidosis (Kalimo et al. 1981). Another study compared lactate levels and brain damage in fasted normoglycemic, acutely hyperglycemic, and

chronically hyperglycemic rats following cardiac arrest and resuscitation. Rats were made hyperglycemic by intraperitoneal injection of dextrose. Some of the animals were euthanized following 5min of reperfusion, and lactate levels and intracellular pH were measured. Both hyperglycemic groups showed a significant increase in brain cortical lactate levels; however, only the acutely hyperglycemic animals showed a significant increase in intracellular acidosis as compared to normoglycemic rats. In the rats that were allowed to recover, all of the chronically hyperglycemic animals died within 4d following injury. The acutely hyperglycemic rats showed significantly greater damage to the CA1 neurons as compared to the normoglycemic animals at 4d following the ischemic insult (Hoxworth et al. 1999). While these findings suggest a role for hyperglycemia and lactate production in ischemia-induced damage, they also suggest that factors other than pH contribute to outcome.

Skepticism surrounding the lactic acidosis hypothesis began to increase with the findings that the glucose paradox of cerebral ischemia could not be reproduced in vitro where no harmful effects of glucose or lactate were seen. In fact, some studies, both in vivo and in vitro, have observed a protective effect of glucose when given prior to ischemia (Ginsberg et al. 1987, Schurr et al. 1987). Scherr et al. (1987) used rat hippocampal slices that were perfused with either low glucose media (5mM) or high glucose media (10mM), and exposed them to a hypoxic insult of 10, 15, or 20min duration. Rate of recovery of synaptic function was significantly higher in the 10mM glucose slices after all durations of hypoxia. Slices were treated with lactate in order to determine if the lack of damage seen was due to limited lactic acid accumulation in the media; however this had no effect (Schurr et al. 1987). In a separate study, the same group used rat hippocampal slices exposed to hypoxic conditions and determined the effect of different concentrations of lactic acid on recovery rate of synaptic function. Lactic acid concentrations were 1, 2, 5, 10, and 20mM given 30min before and during hypoxia, which lasted 10, 12, or 15min. No significant differences in synaptic function were found among groups with the exception of 20mM lactic acid and 10min hypoxia in which a decrease in function was found. Also significant was an increase in recovery rate in the group receiving 10mM lactic acid and 12min hypoxia (Schurr et al. 1988). Interestingly, an in vivo study showed that when hyperglycemia was induced 15 to 60min prior to cardiac arrest followed by resuscitation, neuronal damage was worsened, whereas if hyperglycemia was induced 120 to 240min prior, neuronal damage was reduced as compared to normoglycemic conditions. It was also shown in

this study that glucose administration induced an increase in plasma corticosterone concentration 15-30min after glucose administration and a decrease to baseline 120min following glucose administration. From these findings, another possible explanation for the glucose paradox was developed (Schurr et al. 2001).

The corticosterone hypothesis postulates that hyperglycemia causes an increase in corticosterone resulting in an exacerbation of neuronal damage if the brain ischemia occurs while corticosterone levels are increased. If this increase in corticosterone is blocked (ie. with the drug metyrapone), elevated glucose levels are actually neuroprotective. This explains the consistent finding that glucose is neuroprotective in in vitro systems if an elevation in corticosterone concentration following ischemia-hypoxia is absent (Schurr et al. 2001). Further evidence in favor of the corticosterone hypothesis comes from studies finding a direct correlation between glucocorticoid concentration and neuronal injury. Payne et al. (2003) examined the effect of cardiac arrest followed by resuscitation on several experimental groups treated with glucose or corticosterone at different times prior to insult. Neuronal damage measured 7d after injury and the incidence of seizure correlated with plasma corticosterone concentration, and not glucose concentration, at the onset of ischemia in fasted rats (Payne et al. 2003). In the Schurr et al. (2001) experiment described above, when hyperglycemic rats (treated with glucose 15min prior to ischemia) were pre-treated with metyrapone, an inhibitor of corticosterone synthesis, CA1 neuronal damage was significantly attenuated compared to normoglycemic rats. In another study, gerbils administered glucose 15min prior to bilateral common carotid artery occlusion were also treated with a glucocortocoid receptor agonist (mifepristone) and displayed significantly less CA1 damage than that in hyperglycemic gerbils not receiving the agonist, and equal damage to that of normoglycemic gerbils (Antonawich et al. 1999).

While increased blood glucose concentration occurring as a result of increased glucocorticoid secretion is a well-described physiological response (Clore and Thurby-Hay. 2009), the mechanism by which hyperglycemia might increase glucocorticoid concentration is unknown. Several studies in humans with poorly controlled or uncontrolled diabetes, as well as in animal models of diabetes, have shown that these subjects have increased activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased circulating glucocorticoid concentrations (reviewed in Stranahan et al. 2008). It has been suggested that this is a result of either hyperglycemia and/or hypoinsulinemia since insulin therapy has been shown to restore

HPA activity (Chan et al. 2001). There is little information regarding how glucose levels can affect basal functioning of the HPA axis as well as its response to stress (Chan et al. 2005). The hypothalamus is known to consist of several glucose-sensing regions, suggesting that glucose levels may indeed play a role in regulation of the axis. However, a recent study by Chan et al. (2005) found that hypoinsulinemia is likely responsible for HPA dysregulation in an animal model of diabetes since correction of blood glucose levels in these animals, without alteration of insulin levels, was unable to normalize any HPA parameters. Hyperglycemic rats were, however, found to have impairment of the HPA axis in response to insulin-induced hypoglycemia (Chan et al. 2005). While these findings may not be directly relevant to this thesis since diabetes is not being studied, they do suggest that the mechanisms by which hyperglycemia increases glucocorticoid concentration may not be direct and are likely complicated. However, as reviewed above, a relationship does seem to exist between pre-ischemic hyperglycemia, increased glucocorticoids at the time of ischemia, and increased ischemia-induced damage.

While strong evidence exists that hyperglycemia worsens outcome from ischemia, hypoglycemia may also be detrimental. By itself, severe hypoglycemia can cause brain damage by actively killing neurons (reviewed in Auer. 2004). Neuronal necrosis from hypoglycemia only occurs when it is severe enough to cause a flattened electroenecephalogram. At this point, there is energy failure and aspartate is released into the extracellular space, overwhelming essential amino acid receptors on dendrites. Cell membranes eventually rupture and necrosis occurs (Auer. 2004).

In the context of ischemia, the degree of glycemic state seems to be important in determining whether additional brain damage will occur. Zhu and Auer (2004) found, using the MCAO model of focal ischemia, that insulin treatment was only beneficial when blood glucose levels were 6-7mM. In cases where blood glucose was very low at 2-3mM, necrosis in the hippocampus and neocortex occurred, in addition to cortical infarction caused by the ischemic-insult (Zhu and Auer. 2004). Voll and Auer (1988) found using the 2-VO model of global ischemia, that while high-dose administration of insulin, causing blood glucose levels of <3mM, resulted in decreased cellular necrosis to the hippocampus, striatum and cortex, it greatly increased seizure-related deaths, overshadowing the histological improvement (Voll and Auer. 1988).

In summary, both hyper- and hypoglycemia can increase ischemia-induced brain damage and there seems to be a narrow range of blood glucose levels in which the brain can be protected from injury. Hyperglycemia may exert damaging effects via increased glucocorticoid concentration. PEM has the potential to influence circulating concentrations of both glucose and glucocorticoids.

2.7.4.2.1.2 Effects of PEM on Glucocorticoids

The increase in glucocorticoids as a result of PEM is due to a restructuring of metabolic pathways resulting in a preference for fat as an energy source in order to promote glucose homeostasis (Pugliese. 1990). This endocrine response includes decreased leptin concentration, which leads to increased cortisol concentration in humans (Pugliese. 1990).

Animal studies have shown less variation than human studies and it has been found that, in general, glucocorticoid levels are high in animals with protein-deficiency or PEM. Fagundes et al. (2009) fed lactating dams a control diet (23% protein) or an isocaloric protein-restricted diet (8% protein). Following weaning, pups were placed on control diet. Pups from protein-restricted dams had decreased body weight and adiposity beginning at 90d of age. Serum corticosterone was measured using a radioimmunoassay specific for corticosterone, at six months of age at which time it was found that serum corticosterone concentration was significantly increased in pups from protein malnourished dams (Fagundes et al. 2009). Another study examined the effect of malnutrition on serum corticosterone concentration in two strains of weanling mice after 3, 6, and 14d on diet (Monk et al. 2006). Diet groups consisted of a control group fed a complete, protein sufficient diet (18% protein) *ad libitum*, an energy deficient group fed protein sufficient diet in restricted amounts (mimicking marasmus), and a protein deficient group fed an isocaloric, protein deficient diet (0.6% protein) *ad libitum* (mimicking kwashiorkor). Food intake and body weight were recorded and indicate malnutrition was achieved in both groups. Serum corticosterone levels were examined using both a double antibody radioimmunoassay and a competitive binding enzyme immunoassay. An exsanguination method was utilized that avoided pre-anaesthetic stress, which is known to falsely elevate corticosterone levels (Monk et al. 2006, Shipp and Woodward 1998). Findings indicate that both malnutrition protocols caused an elevation of blood corticosterone concentration at all time points, in both strains of mice and using both assays (Monk et al. 2006).

While the Monk et al. (2006) study did indeed induce PEM in their animals, the Fagundes et al. (2009) study likely induced protein-deficiency in their animals since food intake was not altered. Results of both studies do suggest an increase in glucocorticoids caused by the nutritional deficiency.

While several studies of malnourished children have found significantly increased cortisol levels compared to well-nourished counterparts (Kilic et al. 2004, Soliman et al. 2000), the effect seems to vary depending on severity of PEM and a number of other factors. In a study of malnourished children, Jaya Rao et al. (1968) found that fasting plasma cortisol was significantly elevated in children with kwashiorkor and those with marasmus compared to normal, well-nourished children. Interestingly, those with marasmus showed significantly increased levels as compared to those with kwashiorkor. In the same study, Synacthen, a synthetic corticotrophin, was used as an indicator of the reserve function of the adrenal gland via measurement of cortisol released. While neither malnourished group showed a significant difference compared to the well-nourished group in magnitude of change in cortisol levels, the absolute level in the marasmus group was much higher than that of the other two groups (Jaya Rao et al. 1968). A study by Smith et al. (1981) found that both free and total cortisol was significantly increased in children with both marasmus and kwashiorkor as compared to controls, and was significantly higher in those with marasmus as compared to those with kwashiorkor (Smith et al. 1981). However, another study found an increased response to corticotropin stimulation in children with kwashiorkor as compared to marasmus (Van Der Westhuysen et al. 1975) and yet another found children with kwashiorkor had a total cortisol level equal to those with marasmus (Olusi et al. 1977). It has been suggested that the differences in cortisol between malnourished groups may be due to differences in cortisol binding proteins (Olusi et al. 1977) and other factors such as differing degrees and etiologies of malnutrition and the presence of complicating factors, such as infection (Becker. 1983).

2.7.4.2.1.3 Effects of PEM on Glucose

Unlike the mechanisms occurring with brain ischemia, the increase in glucocorticoid concentration with PEM does not occur via hyperglycemia. As part of the physiological response to PEM, blood glucose levels are instead decreased (Torun. 2006). While decreased food intake leads to decreased intake of glucose, glucose absorption is also slowed in severe PEM. When the

supply for tissue and cell energy can no longer be maintained in patients with severe PEM, hypoglycemia results as does hypothermia and acidosis. Impaired thermoregulatory mechanisms occur with PEM and can contribute to hypoglycemia (Torun. 2006).

The presence of hypoglycemia as a result of PEM or protein-deficiency has been a consistent finding in animal studies; however, findings in children with PEM are more variable. In the Fagundes et al. (2009) study, described above, at 180d of age fasted offspring from protein-restricted dams were found to have a significantly lower (17%) blood glucose level as compared to pups from dams fed control diet (Fagundes et al. 2009). Gamallo et al. (1989) found pups of protein-deprived dams, fed a diet with 8% casein from conception to lactation had significantly decreased blood glucose concentration from 10d of age to euthanasia (20d); this was in comparison to pups from dams on the isocaloric control diet (23% casein) (Gamallo et al. 1989). Another study found that the presence of protein-deficiency in post-weaning rats had lasting effects on blood glucose concentration despite nutritional rehabilitation. Fasted rats were fed 6% protein diet from weaning to 55d of age at which time they were placed on control diet (23% protein) until 90d of age when blood glucose was measured. These animals had lower blood glucose as compared to those fed the isocaloric control diet from weaning (Miñana-Solis and Escobar. 2008). It is important to note that in this study, while diets were isocaloric, the protein deficient diet was purified while the control diet was rat chow. The different composition of these diets may have resulted in differences in glycemic indices, micronutrient makeup, and types of fat. Das et al. (2004) proposed that the presence of protein malnutrition in weanling rats caused permanent damage to intestinal mucosal cells of the small intestine, resulting in hypoglycemia. In this study, rats fed a protein-deficient diet (3% protein) had 42% decreased glucose absorption after 15d on the diet as compared to rats on control diet (18% protein) (Das et al. 2004). Since measurement of food intake was lacking in some of these animal studies, it is unclear whether these animals were protein-deprived, or protein and energy deprived. Nevertheless, the occurrence of hypoglycemia with protein-restriction or PEM seems to be a consistent finding.

However, results from studies in humans are less clear. Misra et al. (1980) found that while children with kwashiorkor and marasmus both had lower fasting blood glucose levels compared to healthy children**,** this was only significant in children with marasmus. However, differences between groups in response to a glucose tolerance test were found with more than

half of the children with kwashiorkor displaying a diabetic type response, while no abnormal response was found in any of the children with marasmus (Misra et al. 1980). Another study found that fasting blood glucose was significantly decreased in children with all types of PEM as compared to controls. However, differences between groups in terms of occurrence of hypoglycemia were reported, with 33% of children with marasmus, 14% of those with kwashiorkor, and 14% of those with marasmic kwashiorkor falling into this category (Das et al. 1998). Several studies have found low blood glucose in children with marasmus as well as those with kwashiorkor (Das et al. 1998, Hadden. 1967) whereas blood glucose levels comparable to that in healthy children have been reported in children with kwashiorkor (Misra et al. 1980). Another study of children with PEM found no significant difference in serum glucose concentration as compared to healthy children (Mishra et al. 2009). While differences in categorization of human patients into those with mixed PEM, marasmus, or kwashiorkor may account for some of the discrepancies reported, overall, the literature suggests that hypoglycemia occurs as a result of PEM. However, the degree to which this occurs likely differs depending on severity of the nutritional deficiency.

In summary, hyperglycemia present at the time of brain ischemia leads to increased glucocorticoid concentration and results in increased brain damage. PEM, through an endocrine response, also causes increased glucocorticoid concentration and therefore has the potential to worsen ischemic damage via this mechanism. Hypoglycemia, often a characteristic of PEM, also has the potential to increase brain damage caused by ischemia.

2.7.4.2.2 Oxidative Stress and Inflammation

There are a number of other mechanisms by which PEM might contribute to the cascade of events responsible for brain damage in the acute period following stroke. One hypothesis has been that PEM causes decreased cysteine availability, which is the cellular limiting amino acid for synthesis of the important antioxidant, reduced glutathione (GSH) (Paterson et al. 2001). While decreased production of lung and liver GSH occurs with PEM, the response of the brain is less clear (Taylor et al. 1992). In the gerbil model of global ischemia used in our laboratory, ischemia decreased hippocampal GSH, while PEM had no additional effect (Bobyn et al. 2005). However, these findings are a result of assessment at only one time point post-ischemia and are

confounded by variability in the global ischemia model. Therefore, the association between PEM and GSH concentration in the ischemic brain needs to be further examined.

Another study completed in our laboratory has elucidated another mechanism by which PEM may worsen brain damage. Marked reactive gliosis found in a subset of gerbils exposed to global ischemia and PEM (Bobyn et al. 2005) may indicate an increased inflammatory response, possibly due to increased activation of the transcription factor NFκB (nuclear factor kappa B) (Ji et al. 2008). This transcription factor is known to have many roles in inflammatory and immune responses, as well as control of cell division and apoptosis (Mattson and Camandola. 2001). In its inactive form, NFκB is present in the cytosol and is made up of three subunits, typically p65, p50, and IκB (inhibitory subunit). Activating signals include TNF-α (tumor necrosis factor), NGF (nerve growth factor), glutamate, increased intracellular Ca^{2+}, and reactive oxygen species (ROS). These molecules cause activation of IκB kinase, which causes degradation of IκBα. The p65/p50 dimer then translocates to the nucleus where it binds to consensus κB sequences in the enhancer region of κB-responsive genes, such as those promoting the production of pro-inflammatory cytokines and reactive oxygen species (Mattson and Camandola. 2001).

Previous work in our laboratory has found that ischemia and PEM result in increased oxidative stress, which is a known activator of NFκB (Bobyn et al. 2005). Our laboratory has recently discovered that PEM does in fact cause an increase in the activation of this transcription factor in the acute period post-ischemia (Ji et al. 2008). This finding extends to the CNS previous findings that protein malnutrition increases activation of NFκB as well as downstream genes, Il-1β and TNF-α, in liver of mice injected with the endotoxin lipopolysaccharide (LPS) (Li et al. 2002).

2.7.4.2.3 Thermoregulation

Since hypothermia has been shown to be protective against ischemia-induced damage (as reviewed in Section 2.3.2.3.3 of this thesis), it is important to briefly discuss the effect PEM may have on temperature. There is no evidence that PEM can cause hyperthermia and therefore would not contribute to ischemic damage in this way. Rather, the literature strongly points towards a deficit in thermoregulatory functions, often resulting in hypothermia. Therefore, the worsening of ischemic damage by PEM would be expected to occur by some other mechanisms, in spite of the potential protective effect offered by accompanying hypothermia. Impairments in

thermoregulation have been found in protein and protein-energy malnutrition in both human and animal studies. Devi et al. (1980) found that 26% of children with varying degrees of PEM experienced hypothermia at some point during their hospital stay, most often during the warm seasons. Anthropometric measures were not different between these children and those that did not experience hypothermia suggesting that loss of thermal insulation was not a factor (Devi and Parija. 1980). Brooke et al. (1972) measured body temperature of malnourished Jamaican children upon admission to hospital and during recovery via a high Calorie feed. No hypothermia, which was defined as ≤35.1°C, was reported among these children. Body temperature was found to be significantly lower, though not hypothermic, upon admission as well as more variable as compared to the recovery period. A decrease in body temperature was observed during sleep in the malnourished children, which decreased in magnitude during recovery. Rectal temperatures in a separate group of 5 children, all hypothermic, were measured just 2hr following administration of the high Calorie diet. Interestingly, temperature was found to increase to 36.6°C without the use of any external heating aids (Brooke. 1972).

The results of a study in which offspring of protein-deprived rats were examined at 20d of age showed deficits in thermoregulation (Conradi et al. 1988). These rats had brain and rectal temperatures similar to those of animals born to dams on control diet (14% protein) when measured directly following removal from the nest or if they were kept in an environment where temperature was 32.5°C. Following removal from the nest, both brain and rectal temperatures fell quickly in the protein-deprived rats. In rats allowed to recover in the warmer environment (32.5°C), brain and rectal temperatures of protein malnourished rats significantly increased above those kept at room temperature, but did not reach the level of controls (Conradi et al. 1988). While the method of brain temperature measurement was quite crude, involving decapitation and insertion of a thermocouple probe into the central forebrain, results do indicate an effect of protein deprivation on thermoregulation. Balmagiya et al. (1983) fed rats a protein-deprived diet (6% casein) or a control diet (25% casein) for 6wk. Core (rectal) and shell (paws and tail) temperatures were measured once a week. Rectal temperature was found to increase in the protein deficient group significantly following 2, 3, and 4wk on the diet and returned to the level of control animals by 5wk. Shell temperature was significantly lower than controls after 1wk on diet and remained decreased, but not significantly, for the remainder of the study. When a cold challenge (18-19°C) was administered after 6wk, protein-deprived rats showed a larger

drop in body temperature, as compared to controls, and took longer to restore to normal following the challenge (Balmagiya and Rozovski. 1983). The authors suggest that constriction of blood vessels and a decrease in skin blood flow explains the difference between core and shell temperature regulation in these protein-deprived animals (Balmagiya and Rozovski. 1983). However, a study that has employed telemetry to allow chronic monitoring of core temperature has shown that the effect of protein malnutrition on body temperature can vary with light conditions when rats are tested at an advanced age (550d) (Durn et al. 2008). In summary, these studies strongly suggest that protein and protein-energy malnutrition have effects on thermoregulatory function. However no investigation has been carried out on how this altered thermoregulatory capacity in protein-energy malnutrition would respond to ischemia, and therefore this is an important area to examine.

2.7.4.3 Effects on Mechanisms of Recovery from Cerebral Ischemia

While PEM has been found to impair spatial learning following global ischemia (Bobyn et al. 2005), the mechanisms behind this impairment have not been determined. Since neurotrophins have been implicated in learning and memory, it is possible that an alteration to their levels and/or activities may affect functional outcome post-ischemia. This is particularly important for the hippocampus, which is especially vulnerable to protein malnutrition and also heavily reliant on neurotrophins (Mesquita et al. 2002). Results from one study suggest that protein malnutrition in adult rats results in decreased numbers of hippocampal neurons expressing BDNF and trkB as well as decreased mRNA levels in surviving neurons (Mesquita et al. 2002). However, in this study, diets were not matched for nutrients other than protein and were therefore likely of different energy and micronutrient density as well. Thus, these findings may not be due specifically to protein deficiency (Mesquita et al. 2002). In any case, the idea that altered BDNF levels, or other molecules involved in plasticity mechanisms during the recovery period, due to PEM may contribute to the impaired functional outcome observed following global ischemia, has been completely unexplored.

To date, most research examining the effect of PEM on cerebral ischemia has been focused on how pre-exisiting PEM can exacerbate damage by escalating the ischemic cascade. Unfortunately, potential links between PEM and plasticity mechanisms following ischemia have remained entirely unexplored. This is an important area to research since the incidence of PEM

has been found to increase with time spent in hospital (Davalos et al. 1996). A higher proportion of stroke victims (20-35%) are affected by PEM following stroke (Axelsson et al. 1988, Brynningsen et al. 2007, Yoo et al. 2008) as compared to those affected prior to stroke (16%) (Axelsson et al. 1988, Davalos et al. 1996, Davis et al. 2004, Gariballa and Sinclair. 1998, Martineau et al. 2005, Yoo et al. 2008). If PEM does have an effect on plasticity mechanisms occurring immediately following stroke, the potential exists for PEM to interfere with the benefits of rehabilitation during recovery. A high proportion of patients (35-49%) have poor nutritional status at this stage (Finestone et al. 1995, Poels et al. 2006). The importance of examining the effect of nutritional status during recovery and rehabilitation following stroke is emphasized by the finding that current clinical studies in this area have been judged by a Canadian Stroke Network Consensus Conference group to be of a lower caliber than that of other stroke-related rehabilitation studies (Bayley et al. 2008).

CHAPTER 3

EXPERIMENTAL PROGRESSION OF THESIS

Although all aspects of this thesis relate to the major goal of understanding the mechanisms by which pre-existing PEM can adversely affect outcome from global ischemia, three relatively distinct research questions were investigated. A major consideration was the need to keep examining the experimental conditions underlying the various animal models. Our laboratory has previously shown that protein-energy malnutrition (PEM) worsens functional outcome following global forebrain ischemia in the gerbil as shown by an inability to habituate in the open field behavioural test (Bobyn et al. 2005). In order to further characterize functional outcome following ischemia, I wanted to use the behavioural test the T-maze as an indicator of working memory (Corbett and Nurse. 1998). However, it was important to first clarify the effect of PEM on this task before combining it with ischemia since previous experiments have found protein malnutrition can independently affect behaviour (Almeida et al. 1996, Fukuda et al. 2002, Hernandes and Almeida. 2003, Lukoyanov and Andrade. 2000). My first experiment, described in Chapter 4, investigated whether PEM independently impaired working memory in the T-maze and if the associated food reward would affect our dietary model of PEM in the gerbil. Based on food intake, body weight, and serum albumin concentration it was concluded that the sunflower seed reward required for the behavioural test did not interfere with the model of PEM. In order to assess the effect of PEM on working memory, two different criteria for the T-maze were examined. Contrary to the hypothesis, it was found that the more stringent criterion detected a significant improvement in performance in the PEM group as compared to the control (CON) group. It was theorized that this was indicative of an increased motivation for the food reward in these malnourished animals rather than an improvement in hippocampal function. It was believed that this motivation for the food reward had the potential to cloud the effects of ischemia and therefore the T-maze was not utilized in subsequent experiments.

After choosing to abandon behavioural testing for the second experiment, it was decided to focus on mechanisms that may contribute to the functional detriment found in the study previously performed in our laboratory (Bobyn et al. 2005). This previous study found that the functional deficit was not explained by increased CA1 neuronal death; therefore, other possible explanations include deficits that may be related to recovery mechanisms. The second experiment examined the effect of PEM on molecules involved in plasticity-related recovery mechanisms following global ischemia, specifically brain-derived neurotrophic factor (BDNF), its receptor tropomyosin-related kinase B (trkB), and growth-associated protein-43 (GAP-43). For this experiment, the bilateral common carotid artery occlusion (BCCAO) model of global ischemia in the gerbil was utilized. This model had been used in several experiments in our laboratory (Bobyn et al. 2005, Ji et al. 2008) and had been well characterized histologically and behaviourally (Nurse and Corbett. 1994). The gerbil historically lacked posterior communicating arteries in the brain vasculature forming an incomplete Circle of Willis and because of this, transient occlusion of the common carotid arteries produced consistent damage to the CA1 region of the hippocampus (Traystman. 2003). As reviewed in Chapter 2, section 2.3.2.3.4, of this thesis, recent experiments have shown increased variability in CA1 hippocampal damage in the BCCAO gerbil model of global ischemia. Therefore, gerbils with incomplete forebrain ischemia were screened out of the study if they did not display hyperactivity during the first day following surgery. The latter is characteristic of severe forebrain ischemia (Corbett and Nurse. 1998).

Experiment 2 (described in Chapter 5 of this thesis) describes male, Mongolian gerbils randomized to either control (12.5% protein) or PEM diet (2% protein) for 4wk prior to 5min bilateral common carotid artery occlusion or sham surgery. Brains were processed at 1, 3 and 7d post-surgery for in-situ hybridization or immunofluorescence to detect mRNA and protein expression, respectively, of BDNF, trkB and GAP-43. Results indicated that ischemia increased mRNA expression of BDNF and trkB at all time points in the CA1 region. Ischemia also increased trkB protein expression at 3 and 7d in the CA1 pyramidal neurons and this was further increased by PEM at 7d especially in the fibres surrounding the CA1 region. GAP-43 expression was increased at 3 and 7d post-ischemia in the CA1 region and this was intensified by PEM at 3d and extended to the CA3 and dentate gyrus regions. We have proposed that these findings

suggest an increased stress-response and/or hyperexcitability in the hippocampus of PEM ischemic animals.

While the screening procedure utilized to indirectly determine the success of ischemia surgery is effective (McEwen and Paterson. 2009), it does result in a large number of unusable animals. Therefore it was decided to abandon the gerbil model for the third experiment, as many researchers in the stroke field have done, and adopt the rat 2-vessel occlusion with hypotension model (2-VO) of global ischemia. This model of global ischemia was chosen since it also produces consistent and reliable damage to the CA1 region, similar to that historically seen in the gerbil BCCAO (Clark et al. 2007, Small and Buchan. 2000).

Prior to development of the 2-VO model, a fellow student in our laboratory tested an extension of the dietary paradigm we had previously developed for PEM in the 11-12wk old male gerbil in the 10wk old male Sprague-Dawley rat. It was the intent to use the 2-VO model in the same age of rodent as was used in previous gerbil BCCAO studies. Rats were fed a diet containing 2% protein for 2 weeks. Based on 11%, 17%, 12% reductions in food intake, body weight, serum albumin and a 125% increase in liver lipid, it was decided to use the 2% protein diet to induce PEM in the 10wk old rat.

With the dietary paradigm in place, the next step was to develop the surgical model for our laboratory. However, this model is more surgically invasive and complicated, and therefore learning and practicing of several new surgical techniques was required. Several pilot studies were then performed before consistent bilateral hippocampal CA1 injury was demonstrated. The development of the 2-VO model is described in Chapter 6 of this thesis. Due to the complexity of the surgery, another Ph.D. student in our laboratory, Shari Smith, and I worked as co-surgeons. Since the rat has a complete Circle of Willis, induction of systemic hypotension via jugular blood withdrawal along with transient occlusion of the common carotid arteries is necessary to produce forebrain ischemia (Clark et al. 2007, Langdon et al. 2008, Smith et al. 1984b). Tail artery cannulation is also necessary in order to monitor blood pressure and obtain arterial blood samples for blood gas analysis. Once the surgical procedures were perfected and other variables controlled such as appropriate age of rat and length of occlusion time, experiment 3 (Chapter 7) was begun using this model of global ischemia. An important observation from the pilot studies that guided us in Chapter 7 was the finding that the 2-VO surgical procedure was only successful when performed on small, relatively younger rats (~300g).

The data collected from this experiment with the rat 2-VO model has been separated into two manuscripts, one of which is described in Chapter 7. The other data have been collected for the thesis of Shari Smith, and I will be second author on the accompanying manuscript. The primary objectives of this experiment were to determine whether PEM influenced CA1 neuronal death in this global ischemia model. The dendritic marker MAP-2 was investigated as another marker of injury. Post-ischemic corticosterone concentration was also examined as an indicator of the stress response. As this was the first study in the laboratory utilizing the rat 2-VO model, a secondary objective was to determine whether pre-existing PEM affected several physiological parameters that are essential to control in order to achieve consistent forebrain ischemia in the 2-VO model. A final objective was to investigate if the dietary protocol chosen induced PEM in the Sprague-Dawley rat similar to what had been observed in the gerbil.

In order to address these questions, male Sprague-Dawley rats were acclimatized on control diet (18% protein) before implanting bioelectric temperature sensors in the peritoneal cavity to examine core temperature throughout the experiment. Selected results of this portion of the experiment are presented in Appendix A and discussed in Chapter 7. One week later, animals were randomized to control diet or PEM (2% protein) for 7-8d prior to 2-VO surgery. Animals were then exposed to global ischemia or sham surgery. Arterial blood samples were taken to monitor blood gases (pH, pCO_2, pO_2), hematocrit and blood glucose prior to and following the ischemic period. Animals recovered for 7d following surgery, at which time blood was collected for serum albumin and corticosterone analysis and brains were processed for viable hippocampal CA1 neuron counts.

Based on food intake, body weight, and serum albumin analysis, PEM was successfully induced in the Sprague-Dawley rat. Histological results indicate that PEM did not exacerbate the decrease in CA1 neuronal cell counts and MAP-2 protein expression caused by exposure to global ischemia. Serum corticosterone was not altered by either independent variable. However, these questions may not have been addressed accurately since inducing PEM prior to global ischemia appears to have interfered with some of the physiological parameters that are key determinants of consistent hippocampal injury in the 2-VO model. PEM affected pre- and post-ischemic acid-base balance as well as fasting glucose concentration. Intra-ischemic blood pressure was significantly higher in PEM sham animals as compared to CON sham, suggesting that post-ischemic blood pressure in the PEM ischemic group may have been higher than CON

ischemics. It is possible an increase in blood pressure in these animals may have provided some protection against the ischemic insult. As detailed in Appendix A, thermoregulation was altered in PEM animals both prior to and following surgery. It is proposed that the alterations in these physiological variables are responsible for the trend towards enhanced CA1 survival observed in the malnourished group. This study suggests that the clinical problem of pre-existing PEM cannot be accurately studied in the 2-VO model.

CHAPTER 4

CAN A REWARD-BASED BEHAVIOURAL TEST BE USED TO INVESTIGATE THE EFFECT OF PROTEIN-ENERGY MALNUTRITION ON HIPPOCAMPAL FUNCTION?

This chapter has been published as follows:
Prosser-Loose EJ, Saucier DM, Paterson PG. Can a reward-based behavioural test be used to investigate the effect of protein-energy malnutrition on hippocampal function? Nutritional Neuroscience 2007;10:145-150.

4.1 Abstract

Our laboratory is investigating the effects of protein-energy malnutrition (PEM) on cognitive outcome following global ischemia. Here, we investigated whether PEM independently impairs working memory in the T-maze and if the associated food reward reverses PEM. Gerbils were fed 12.5% (control diet) or 2% protein. A loss of body weight (20.1%) in the 2% protein group and decreased food intake and serum albumin concentration compared to controls (17.5% and 18.2%, respectively) indicated that PEM was achieved. Based on T-maze criterion frequently used in ischemia studies, no difference was observed in the mean (±SEM) number of trials required (control, 5.2 ± 0.7; PEM, 4.9 ± 0.4; $p = 0.758$) or the number of animals reaching criterion (control, 10/12; PEM,12/12; $p = 0.140$). Using more stringent criterion, PEM animals required fewer trials (control 7.3 ± 0.7; PEM 5.4 ± 0.4; $p = 0.035$), and more reached criterion (control 8/12; PEM 12/12; $p = 0.028$). PEM may increase motivation to obtain a food reward.

4.2 Introduction

The elderly population is at highest risk for stroke, and their prognosis may be worsened by the presence of a nutritional deficiency. Approximately 16% of elderly stroke patients have protein-energy malnutrition (PEM) upon admission to hospital, and this condition can worsen during hospital stay (Axelsson et al. 1988, Davalos et al. 1996, Davis et al. 2004). Using a model of global ischemia in the gerbil, in which the CA1 region of the hippocampus is particularly susceptible to damage (Corbett and Nurse. 1998), our laboratory has demonstrated that functional outcome as measured in the open field is impaired by PEM (Bobyn et al. 2005). The T-maze is another behavioural test that reliably detects impairments in working memory post-ischemia but requires a nutritive reward (reviewed in Corbett and Nurse. 1998). Before utilizing this test for studies of global ischemia, we wanted to first establish if PEM affects performance of the adult gerbil in the T-maze prior to induction of ischemia.

Prenatal and early postnatal malnutrition has well-established detrimental effects on the developing hippocampus with effects in adult life that are not reversed when malnutrition is corrected later. This is best described in one particular series of studies. Although the dietary paradigm has been described as protein deficiency (Almeida et al. 1996, Blatt et al. 1994, Bronzino et al. 1997, Cintra et al. 1997a, Cintra et al. 1997b, Diaz-Cintra et al. 1994, Fukuda et al. 2002, Hernandes and Almeida. 2003, Lister et al. 2005, Tonkiss and Galler. 1990, Tonkiss et al. 1990), the level of casein fed in these studies (6%) often results in a voluntary reduction in food intake (Kanarek et al. 1986, Langley and Jackson. 1994, Zeman et al. 1986). Therefore the deficits described more likely represent the effects of a mixed protein-energy deficiency. Unfortunately, food intake was not reported**,** and biochemical indices such as serum albumin and liver lipid were not measured to clarify nutritional status. Regardless, these studies reported structural and biochemical alterations in the CA1 and CA3 regions of the hippocampus of the adult rat as a result of prenatal protein or protein-energy deficiency (Blatt et al. 1994, Bronzino et al. 1997, Cintra et al. 1997a, Cintra et al. 1997b, Diaz-Cintra et al. 1994, Lister et al. 2005, Morgane et al. 2002). Perinatal malnutrition was also reported to impair hippocampal-dependent functions, including significant impairment in the water maze (Fukuda et al. 2002). Deficits in inhibitory avoidance associated with both pre- and postnatal protein-energy malnutrition are also believed to result from alterations to the hippocampus (Almeida et al. 1996, Hernandes and Almeida. 2003). Prenatal protein-energy malnutrition causes behavioural inflexibility during

tests that require the animal to change its response strategy (Tonkiss and Galler. 1990). Possible explanations include dysfunction of the prefrontal cortex, to which the CA1 pyramidal cells project, or an imbalance in the ratio of interneurons to pyramidal cells, leading to reduced output from the hippocampus (Lister et al. 2005, Strupp and Levitsky. 1995).

More relevant to our studies is the influence that changes in protein-energy status occurring during adulthood has on behaviour. A growing body of evidence has suggested that protein deprivation during adulthood can also cause important structural and biochemical alterations in the hippocampus, including a reduction in the number of pyramidal neurons, synapses and arborizations in the CA1 and CA3 regions (Andrade et al. 1996a, Andrade et al. 1996b). Decreased expression of neurotrophic factors as well as a reduction in the number of cholinergic and GABAergic neurons has also been reported in the hippocampus (Andrade and Paula-Barbosa. 1996, Mesquita et al. 2002). The behavioural consequences have been less studied although in adult rats fed 8% casein diet for eight months, performance in the open field and the water maze indicated significant impairments of hippocampal-dependent functions such as emotionality, habituation and spatial learning (Lukoyanov and Andrade. 2000).

While these findings from a nutritional insult imposed during adulthood are exciting, there are some major flaws in these studies. The nutritional model used in this series of studies (8% casein) was intended to induce protein deficiency, yet weight gain was unaltered and no biochemical evidence of protein deficiency was provided. Although food intake was reported to be unchanged (Andrade et al. 1996a, Andrade et al. 1996b, Andrade and Paula-Barbosa. 1996, Lukoyanov and Andrade. 2000, Mesquita et al. 2002), it is difficult to interpret these data because the low protein diet was of entirely different composition than that of the control diet and thus likely of different energy and micronutrient density. Further, since diets were not matched for nutrients other than protein, the structural and behavioural deficits cannot be specifically attributed to protein status.

Despite the limitations, the studies cited do suggest that the model of adult gerbil protein-energy malnutrition used in our laboratory might impair working memory measured in the T-maze. Our nutritional paradigm of PEM is characterized by decreased food intake, weight loss, decreased liver glutathione, increased liver lipid (Bobyn et al. 2005), and a decline in serum albumin (Harmon et al. 2006). The severity of protein-energy deficiency and age and species of rodent differ between our model and the previous studies reviewed, making it difficult to

extrapolate previous results to our model. This is further hindered by the experimental flaws discussed. Therefore, our first objective was to investigate whether working memory in the T-maze is impaired by a model of protein-energy malnutrition induced by feeding a diet containing 2% protein to the adult gerbil. Since the T-maze requires a nutritive reward, a second objective was to examine whether the use of sunflower seeds would interfere with the induction of PEM.

4.3 Materials and Methods

4.3.1 Animals and Diets

Twenty-four male Mongolian gerbils (Charles River Canada, Saint-Constant, QC), 11-12wk of age, were acclimatized for 5d during which time they were fed standard laboratory rodent chow. Animals (60.8–72.2g) were then randomized to receive either control diet (CON group, 12.5% protein, n = 12), or protein deficient diet (PEM group, 2% protein, n = 12) for 28d prior to behavioural testing. Diets were obtained from Dyets, Inc (Bethlehem, PA) and were based on the AIN-93M diet (Reeves et al. 1993) with τ-butylhydroquinone omitted as previously described (Bobyn et al. 2005). Gerbils fed the 2% protein diet voluntarily reduce food intake, resulting in a mixed protein-energy malnutrition (PEM) (Bobyn et al. 2005). Gerbils remained on diet throughout the behavioural testing period, resulting in a total of 57d spent on diets. Gerbils were caged in groups of four at 22°C with a 12hr light/dark cycle, and allowed free access to food and water. Body weight was recorded weekly and food intake per cage daily. All animal procedures were in compliance with the guidelines of the Canadian Council on Animal Care.

4.3.2 Behavioural Testing

Gerbils underwent behavioural testing in the T-maze in order to assess working memory as previously described (Farrell et al. 2001). The structure measured 47cm (stem) x 30cm (arms) x 10cm (width). Environmental cues (e.g. experimenter, shelving, lighting) were kept constant during testing. Since previous pilot study data suggested that sucrose tablets would be an acceptable reward for use in the T-maze, habituation was begun using this reward at 28d. When it was quickly discovered that this reward was not sufficiently motivating, gerbils were placed back in their home cages for an additional 14d during which time there was no behavioural testing. Gerbils continued on the same diets.

On d42, testing resumed in the T-maze with a reward of sunflower seed portions, which contained on average 0.01g protein/seed and 0.33 kcal/seed. Habituation was carried out for 5d, during which the animal was placed in the maze and allowed to explore for three 5min sessions per day. During this period, sunflower seed portions were initially distributed throughout the maze, and then gradually localized to the ends of the arms by d5. After d5, gerbils underwent 10 pairs of win-shift trials/day for 10d, during which they received sunflower seed portions as a reward. In the first trial, the animal was allowed access to one arm of the maze where a reward was received (win trial). In the second trial, both arms of the maze were accessible, but a reward was only received if the animal entered the opposite arm (shift trial). The maze was cleaned with 70% ethanol after each trial. The criterion for learning the strategy was set at an average of 9/10 trials correct over three consecutive days to mimic criteria commonly used for global ischemia studies (Farrell et al. 2001). Results based on a more stringent criterion of a minimum of 9/10 trials correct on each of three consecutive days were also analyzed.

4.3.3 Serum Albumin Analysis

At the end of behavioural testing, animals were humanely killed by an overdose of isoflurane, and blood was collected for serum albumin analysis by the bromcresol green method (Doumas et al. 1971). One control animal was omitted from the serum albumin analysis due to small serum sample size.

4.3.4 Statistical Analysis

Statistical analysis was carried out using SPSS v13.0 for Windows. Body weight, food intake, serum albumin and the number of trials to criterion in the T-maze were analyzed by unpaired t-test. The number of animals reaching criterion in the T-maze was analyzed by Chi-square analysis. Differences were considered significant at $p < 0.05$.

4.4 Results

4.4.1 Indices of PEM

Mean (±SEM) initial body weight was not significantly different between the two experimental groups, being 64.9 ± 1.1g in the control group and 65.2 ± 0.7g in the PEM group ($p = 0.813$). The pattern of weight change is shown in Figure 4.1. There was a significant difference

between groups in body weight change over 57d ($p < 0.001$). Control animals increased body weight by 12.2%, while PEM animals lost 20.1% of their initial body weight by d57. The two groups differed in body weight by d7.

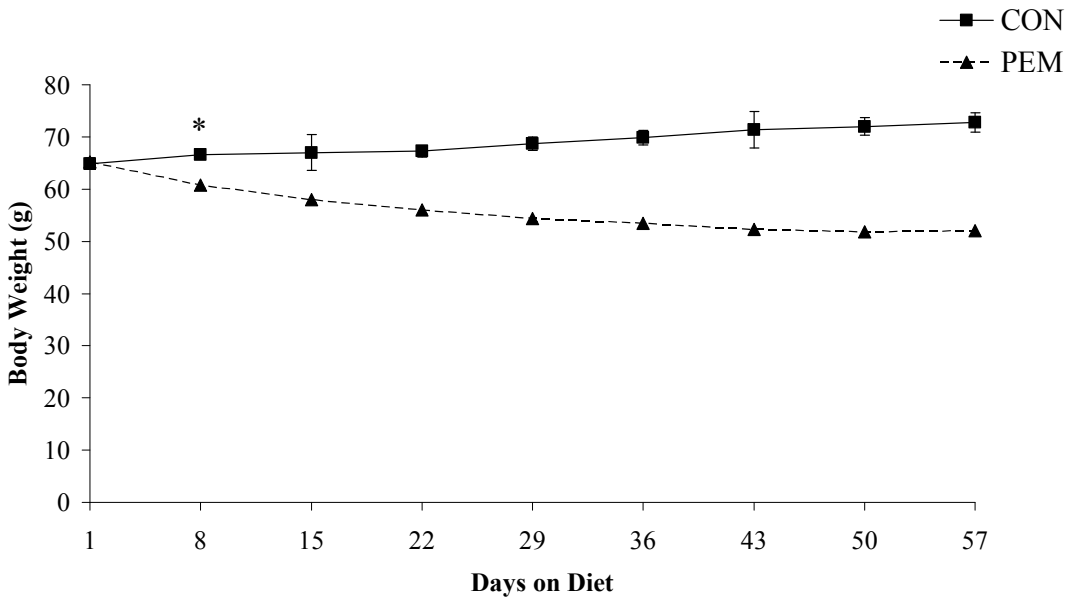

Figure 4.1. Pattern of mean (±SEM) body weight of control gerbils (squares) and PEM gerbils (triangles) over 57d. n = 12. *Indicates the point at which a significant difference in body weight was first detected between control and PEM gerbils, and this was sustained throughout the study.

Total food intake was decreased significantly in the PEM group to 17.5% less than that of the control group ($p = 0.025$) (Table 4.1). Mean (±SEM) serum albumin concentration of the PEM group declined significantly to 18.2% lower than that of the control group ($p < 0.001$) (Table 4.1).

Table 4.1. Food intake and serum albumin concentration in control and PEM gerbils

	Total Food Intake/57d (g)*	Serum Albumin Concentration (g/L)[†]
Control	837.3 ± 13.4	46.7 ± 0.1
PEM	690.4 ± 39.8[‡]	38.2 ± 0.1[¶]

Values expressed as mean (±SEM). *Food intake is based on cage data [n = 3 (4 animals/cage)]. [†]n = 11 (Control) and n = 12 (PEM). [‡]p = 0.025 as compared to controls, based on unpaired t-test. [¶]p < 0.001 as compared to controls, based on unpaired t-test.

4.4.2 Behavioural Testing

There was no significant difference between diet groups on performance in the T-maze when criterion was set at an average of 9/10 correct trials over three consecutive days (Table 4.2) ($p = 0.758$). There was also no significant effect of diet on the number of animals reaching this criterion by 10 days [$X^2(n = 24, df = 1) = 2.182, p = 0.140$]. However, when criterion was raised to a minimum of 9/10 correct trials on each of three consecutive days, the PEM group required significantly fewer trials to reach criterion ($p = 0.035$), and more PEM animals reached criterion as compared to controls [$X^2(n = 24, df = 1) = 4.800, p = 0.028$].

Table 4.2. Acquisition of the T-maze win-shift trials by control and PEM gerbils

	Criterion = average 9/10 correct		Criterion = minimum 9/10 correct	
	# Trials to Criterion	# Animals to Criterion	# Trials to Criterion	# Animals to Criterion
Control	5.2 ± 0.7	10/12	7.3 ± 0.7	8/12
PEM	4.9 ± 0.4	12/12	5.4 ± 0.4*	12/12†

Trials to criterion expressed as mean (±SEM); n = 12. *p = 0.035 as compared to controls based on unpaired t-test. †Significantly different than controls based on chi-square analysis [$X^2(n = 24, df=1) = 4.800, p = 0.028$].

4.5 Discussion

The first issue addressed in this study was whether a sunflower seed reward used for the T-maze would elevate intake of protein and energy sufficiently to interfere with the induction of protein-energy malnutrition. Sucrose tablets were initially tested as a reward, but, unlike sunflower seeds, they did not provide sufficient motivation for the gerbils to explore the T-maze. Based on nutrient analysis of the sunflower seeds and the highest reward consumption, it is estimated that the maximum extra protein consumed was approximately 0.03g/day. Therefore, animals in the protein-energy malnourished group ingested a maximum of 0.10g of protein/day, which is below the estimated requirement for maintenance of 0.20g protein/day. This estimate

was extrapolated from rat data (National Research Council. 1995), since protein requirements for maintenance in the gerbil are not known.

In agreement with these calculations, the results demonstrate that the food reward associated with the T-maze did not reverse protein-energy malnutrition achieved by feeding a 2% protein diet for six weeks. Body weight and food intake remained significantly decreased in the PEM animals throughout the experimental period to an extent consistent with results from a previous 4wk study in our laboratory in which no food reward was present (Bobyn et al. 2005). Serum albumin concentration was also significantly lower in the PEM animals at the end of the testing period, confirming that PEM was induced and was not interfered with by the sunflower seed reward. The decrease in serum albumin concentration of 18% is consistent with our previous data obtained in the absence of a food reward (Harmon et al. 2006).

Using criterion typically employed in studies of global ischemia, our results suggest that working memory tested in the T-maze was not impaired by 6wk of protein-energy malnutrition in the gerbil. Although findings from a study by Tonkiss and Galler (1990) cannot be directly extrapolated to our own due to the difference in age at which deficiency was imposed, their results do support our findings. They investigated the effect of prenatal protein-energy malnutrition on working memory in adulthood and reported no effect on performance on win-shift trials in the T-maze. Our results are in contrast to those of some other studies in which protein deficiency or protein-energy malnutrition were found to have a negative effect on hippocampal function measured by the elevated T-maze and the water maze. This is not surprising given that the studies differed from ours in basal diet composition, age of rodent, and length of feeding period (Almeida et al. 1996, Fukuda et al. 2002, Hernandes and Almeida. 2003, Lukoyanov and Andrade. 2000, Tonkiss and Galler. 1990, Tonkiss et al. 1990, Strupp and Levitsky. 1995). This would have resulted in varying degrees of severity of protein-energy malnutrition, which was often not characterized by measuring indices of protein-energy status.

When criterion in the T-maze was set more stringently, protein-energy malnourished animals required significantly fewer trials to reach criterion, and more of these animals reached criterion. We hypothesize that these results reflect increased motivation to obtain the food reward. This appears to be a novel finding as we are not aware of a previous report of PEM exerting such an effect in animals unless they were food-deprived to motivate them to perform in the T-maze. We avoided this food deprivation by using a preferred food for the gerbil as reward.

Increased motivation to obtain a food reward in an operant task requiring pressing of a lever was reported in adult rats that had been protein-energy deprived during prenatal life (Tonkiss and Galler. 1990). However, a major difference in study design was that the animals were food-deprived and thus presumably hungry when they performed the task. In a second study by Tonkiss et al. (1990) using a food rewarded operant task, prenatally malnourished rats also performed better when a non-nutritive reward (saccharin solution) was used, but not to the same extent as when the nutritive reward was offered.

It is possible that the increased motivation detected with the most sensitive criterion would cloud interpretation of future studies assessing how protein-energy malnutrition influences global ischemia-induced changes in T-maze performance. However, criterion adopted for evaluating performance in the T-maze for studies of global ischemia are typically less stringent (Babcock and Graham-Goodwin. 1997, Colbourne and Corbett. 1995, Farrell et al. 2001). In rat models of global ischemia, this potential confounder can also be avoided by using behavioral tests such as the water maze that are sensitive to ischemic injury but do not require the use of a food reward. Unfortunately, the water maze is not ethologically well suited for the gerbil because of the stress generated when a desert rodent is required to swim to solve a task (Corbett and Nurse. 1998).

The results of this study should be of interest to others studying the effects of nutritional status on stroke outcome using rodent models of global ischemia. Use of the gerbil model of global ischemia is likely to decline in future given two recent reports of changes in cerebral vasculature, accompanied by loss of morphological and behavioral consistency, in a high proportion of gerbils from North American suppliers (Laidley et al. 2005, Seal et al. 2005). As investigators turn to other models of global ischemia such as two- and four-vessel occlusion in the rat, we predict that the effect of protein-energy malnutrition on cognitive outcome will be similar to what we have previously reported in the gerbil (Bobyn et al. 2005), but this is yet to be studied. Similarly, any confounding influences of nutritional intervention on the behavioral tests used to assess functional outcome in these models of stroke would need to be assessed.

CHAPTER 5

DOES PROTEIN-ENERGY MALNUTRITION ALTER EXPRESSION OF MARKERS OF HIPPOCAMPAL PLASTICITY FOLLOWING GLOBAL ISCHEMIA IN THE GERBIL?

5.1 Abstract

Our laboratory has previously demonstrated that protein-energy malnutrition (PEM) impairs habituation in the open field following global ischemia. The present study examined the hypothesis that PEM exerts some of its deleterious effects on functional outcome by altering the post-ischemic expression of the plasticity-associated genes brain-derived neurotrophic factor (BDNF), its receptor tropomyosin-related kinase B (trkB), and growth-associated protein-43 (GAP-43). Male, Mongolian gerbils (11-12wk) were randomized to either control diet (12.5% protein) or PEM (2% protein) for 4wk, at which time animals underwent 5min bilateral common carotid artery occlusion or sham surgery. Tympanic temperature was maintained at 36.5 ± 0.5°C during surgery, and animals were screened for incomplete ischemia after surgery. Brains were removed at 1, 3 and 7d post-surgery and processed for in-situ hybridization or immunofluorescence to detect mRNA and protein expression, respectively. Ischemia increased mRNA expression of BDNF and trkB at all time points in hippocampal CA1 neurons. Increased trkB protein levels were evident at 3 and 7d, and this was augmented by PEM at 7d, particularly in the stratum radiatum and stratum oriens of the CA1 regions. Post-ischemic GAP-43 protein expression was increased at 3 and 7d in the CA1 region. This elevated GAP-43 expression was intensified by PEM at 3d and extended beyond CA1 to the CA3 and hilar regions. These findings, which were independent of an increase in CA1 neuron loss, suggest that PEM increases the stress response and/or hyperexcitability in the hippocampus after global ischemia. Nutritional care, although a frequently disregarded component of therapy, appears to have robust effects on mechanisms important to recovery after brain ischemia.

5.2 Introduction

Stroke is the leading cause of disability worldwide, leaving a large proportion of survivors with moderate to severe impairment (Heart and Stroke Foundation. 2008). Other than the thrombolytic agent, tissue-plasminogen activator (t-PA), which breaks up the blood clot and allows reperfusion, there is limited treatment for stroke (Besancon et al. 2008, Ginsberg. 2009). Drug therapies aimed at lowering the vulnerability of brain tissue after stroke by targeting individual mechanisms of the acute ischemic cascade, such as glutamate-induced excitotoxicity, increased intracellular Ca^{2+}, and elevated production of reactive oxygen species and inflammatory mediators, have been extensively tested as neuroprotective strategies (Lee et al. 1999). Despite their success in animal studies, they have not been efficacious in clinical trials (Cheng et al. 2004, Dirnagl et al. 1999). Therefore, novel methods of neuroprotection with a focus on combination therapy are needed (Cheng et al. 2004).

Optimal nutritional care should receive more attention as part of this therapy, since impaired nutritional status before and after a stroke appears to exacerbate brain damage and worsen recovery. Estimates made more than a decade ago identified at least 16% of elderly acute stroke patients as already protein-energy malnourished upon admission to hospital (Axelsson et al. 1988, Davalos et al. 1996, Gariballa and Sinclair. 1998), and more recent studies suggest that the magnitude of the problem has not decreased (Davis et al. 2004, Martineau et al. 2005, Yoo et al. 2008). Nutritional status worsens during post-stroke hospital stay, with estimates of protein-energy malnutrition (PEM) affecting 20-35% of patients at 1wk (Axelsson et al. 1988, Brynningsen et al. 2007, Yoo et al. 2008) and 35-49% on admission to a rehabilitation setting (Finestone et al. 1995, Poels et al. 2006). Despite methodological criticisms of these studies and a recognition that weaknesses in choice of nutritional assessment tools have hampered our understanding of the relationship between nutritional status and stroke (Foley et al. 2009), the evidence suggests that poor nutritional status contributes to an adverse stroke outcome (Davalos et al. 1996, Davis et al. 2004, Finestone et al. 1996, FOOD Trial Collaboration. 2003, Martineau et al. 2005). That malnutrition can aggravate brain injury is also supported by the results of a recent large epidemiological study that surveyed body mass index in 21,884 patients at admission for stroke and found subsequent 5yr mortality to be highest in the underweight group (Olsen et al. 2008).

We have employed a rodent model of global ischemia to study the effects of pre-existing PEM (Bobyn et al. 2005, Ji et al. 2008). Although global ischemia models mimic cardiac arrest and not focal stroke, the similar pathophysiological mechanisms and more consistent injury (Small and Buchan. 2000, Traystman. 2003) make the model desirable for confirming the link between nutritional status and outcome after brain ischemia. We have demonstrated that PEM impairs functional outcome measured by inability to habituate in the open field (Bobyn et al. 2005). Elevated oxidative stress (Bobyn et al. 2005) and increased activation of the transcription factor, nuclear factor kappa B (NFκB) (Ji et al. 2008), may contribute to this functional deficit by amplifying the ischemic cascade. We hypothesize that PEM continuing uncorrected after stroke will exert further deleterious effects by altering recovery mechanisms.

Stroke survivors can show some degree of functional recovery as a result of neural plasticity that includes the actions of neurotrophic factors (Lee and van Donkelaar. 1995, Teasell et al. 2005). Brain-derived neurotrophic factor (BDNF) is upregulated as a consequence of glutamate release and calcium influx after brain ischemia (Tapia-Arancibia et al. 2004). BDNF gene expression in the hippocampus increases after global ischemia, but this is region-specific, and the temporal and spatial profile for BDNF mRNA varies considerably among reports (Kokaia and Lindvall. 2003, Lee et al. 2002, Takeda et al. 1993, Tsukahara et al. 1998, Yang et al. 2002). BDNF protein expression can increase in CA3 and dentate gyrus regions, depending on the length of reperfusion studied (Kokaia et al. 1996, Lee et al. 2002, Lee et al. 2008, Miyata et al. 2001, Yamasaki et al. 1998). Many investigators have described a decrease in BDNF protein levels in the highly vulnerable CA1 region after global ischemia (Ferrer et al. 1998b, Kokaia et al. 1996, Lee et al. 2002, Lee et al. 2008, Miyata et al. 2001, Yamasaki et al. 1998), although others report a transient increase in the first few hours (Miyata et al. 2001). In general, some degree of disparity between hippocampal BDNF mRNA and protein has been described after global ischemia (Kokaia et al. 1996, Lee et al. 2002, Miyata et al. 2001).

BDNF and activation of its receptor, tropomyosin-related kinase B (trkB), promote neuronal survival following brain ischemia (Ferrer et al. 1998a, Ferrer et al. 1998b, Kiprianova et al. 1999a, Larsson et al. 1999, Lee et al. 2002, Tsukahara et al. 1994). Post-ischemic hypothermia enhances total hippocampal BDNF and trkB protein levels after cardiac arrest (D'Cruz et al. 2002), suggesting their involvement in the striking benefits accrued from this treatment after both focal and global ischemia (Ginsberg. 2008, Krieger and Yenari. 2004, Nagel

et al. 2008). It has been suggested that CA1 neurons that can survive forebrain ischemia do so by maintaining the autocrine signaling of BDNF whereas this function is lost in those neurons undergoing delayed cell death (Ferrer et al. 1997, Yamasaki et al. 1998). Exogenous administration of BDNF into the brain upregulates trkB (Ferrer et al. 1998a), and postischemic administration of BDNF, either intracerebroventricularly (Kiprianova et al. 1999a, Kiprianova et al. 1999b) or by viral vector-mediated gene delivery (Shirakura et al. 2004) decreased hippocampal CA1 neuronal death following global ischemia. In addition, because of their established functions in neural plasticity mechanisms triggered by injury, endogenous BDNF and trkB are proposed to have central roles in recovery from brain ischemia by promoting dendritic sprouting and repair of synaptic connections (Connor and Dragunow. 1998, Komitova et al. 2006, Lindvall et al. 1994, Schinder and Poo. 2000, Schallert et al. 2000, Tapia-Arancibia et al. 2004). Exogenous BDNF improves hippocampal long-term potentiation and spatial memory after forebrain ischemia (Kiprianova et al. 1999b). Although other data linking BDNF to recovery after global ischemia are based on correlation, a recent study has provided direct evidence that BDNF is essential for rehabilitation-induced motor relearning after focal ischemia (Ploughman et al. 2009).

PEM may exert some of its deleterious effects following brain ischemia by impairing BDNF activity. This could be particularly important for the hippocampus, which is heavily reliant on neurotrophins (Connor and Dragunow. 1998, Mufson et al. 1999) and potentially vulnerable to protein malnutrition (Andrade et al. 1996a, Andrade et al. 1996b, Andrade and Paula-Barbosa. 1996). Feeding a low protein diet has been reported to decrease the number of dentate gyrus granule neurons immunoreactive for BDNF or trkB and the neuronal mRNA levels of both molecules (Mesquita et al. 2002). Although this study provided evidence that BDNF activity can be altered by a change in diet, the specific role of protein deficiency is uncertain. Since no biochemical evidence of protein deficiency was obtained and the control and low protein diets were not matched for other nutrients, BDNF activity may also have been influenced by differences in energy and micronutrient density. Nonetheless, the results are intriguing.

Growth-associated protein-43 (GAP-43) is associated with plasticity and neuronal growth and synapse formation during development, and its expression is maintained in the mature central nervous system and influenced by BDNF (Schmidt-Kastner et al. 1997, Tetzlaff et al. 1994). Although increased GAP-43 following injury is believed to be part of an attempt to

regenerate axons and reestablish synaptic connections, and trkB appears important for its induction (Dinocourt et al. 2006), this has received little attention in global ischemia. GAP-43 increases in distinct hippocampal regions following global ischemia, but the mRNA pattern varies in the only two studies conducted (Schmidt-Kastner et al. 1997, Tagaya et al. 1995); only one study has described changes in protein (Schmidt-Kastner et al. 1997). Whether the changes are indicative of beneficial synaptic re-organization or pathophysiology related to increased excitability is yet unclear (Schmidt-Kastner et al. 1997, Tagaya et al. 1995).

We therefore hypothesized that PEM would impair BDNF and trkB expression in the hippocampus induced by global ischemia and result in a decreased GAP-43 response. Hippocampal mRNA and protein expression of these molecules were examined in the well-characterized model of global ischemia induced by bilateral carotid artery occlusion in the gerbil.

5.3 Material and Methods

5.3.1 Animals and Diets

Male Mongolian gerbils (11-12wk) (Charles River Canada, QC, Canada) were randomly assigned to one of two diet groups, a protein adequate diet (Control, 12.5% protein) or a protein-deficient diet (PEM, 2% protein) for 4wk. Both diets are based on the AIN-93M rodent diet (Reeves et al. 1993) but do not contain τ-butylhydroquinone (Ji et al. 2008). The protein deficient diet causes a voluntary decrease in caloric intake, thereby causing a deficit in both protein and energy intake (Bobyn et al. 2005, Ji et al. 2008). That this feeding paradigm induces PEM has been previously established on the basis of decreased food intake, body weight, liver reduced-glutathione and serum albumin, and increased liver lipid concentration (Bobyn et al. 2005, Harmon et al. 2006).

Animals were caged in groups of two to four in shoebox cages with bedding and free access to food and water. Housing facilities were maintained at 22°C and had a 12hr light/dark cycle. Body weight was recorded weekly and food intake daily. All animal care and procedures followed the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply.

5.3.2 Bilateral Common Carotid Artery Occlusion Procedure

After 4wk on the assigned diet, gerbils underwent either sham or global ischemia surgery. Animals in the ischemia group were anaesthetized with ~4% isoflurane and 1L/min oxygen for induction. Gerbils were then placed on a homeothermic blanket and anaesthetic was set at ~2.5% for maintenance. Both common carotid arteries were isolated through a ventral midline incision and occluded for 5min with microaneurysm clips. Occlusion and reperfusion were visually verified. During ischemia, brain temperature was approximated via a tympanic probe and maintained at 36.5 ± 0.5˚C by wrapping a water-heated blanket around the gerbil's head. The incision was closed and 2% Xylocaine (lidocaine hydrochloride) applied. Gerbils were then placed in individual cages under a heat lamp for 2hr and monitored; once mobile, gerbils could choose whether they remained under the heat lamp. The same methods were applied to animals undergoing sham surgery, except that isolated carotid arteries were not occluded. Therefore the four experimental groups generated were: Control diet-Sham surgery (CON-S), Control diet-Ischemia (CON-I), PEM-Sham surgery (PEM-S), and PEM-Ischemia (PEM-I), each with n = 6 for each timepoint (1, 3, and 7d post-surgery). Following surgery, each gerbil continued on the same diet as assigned during the pre-surgical period.

5.3.3 Screening for Complete Ischemia

Due to the increased incidence of complete or partial Circle of Willis in the North American gerbil supply, forebrain ischemia will be incomplete in some animals following the standard methodology of 5min bilateral common carotid artery occlusion (Laidley et al. 2005, Seal et al. 2005). To avoid this problem and to maintain consistent hippocampal CA1 damage in the current study, we excluded any gerbils that did not demonstrate the characteristic persistent hyperactivity that is related to ischemic severity in this global ischemia model (Corbett and Nurse. 1998). Since this hyperactivity normally develops 2-3hr after the ischemic insult and lasts for up to 2d (Corbett et al. 1997), gerbil activity was monitored for 20hr, beginning 2hr after surgery, using an Opto-M3 Activity Meter (Columbus Instruments, Columbus, OH, USA). To be included in the experiment, the mean activity level of a gerbil exposed to ischemia had to meet the following criteria: 1) greater than 3 standard deviations above our laboratory mean activity level for sham animals, and 2) continuously high for 20hr. The rationale for the first criterion is that, assuming a normal distribution, the mean ±3 standard deviations should encompass greater

than 99% of the sham population. Previous activity data from gerbils exposed to varying ischemic damage support this criterion (Corbett et al. 1997). The rationale for the second criterion is based on data demonstrating that gerbils with incomplete forebrain ischemia can have high initial activity that subsequently decreases (Corbett et al. 1997). A previous study from our laboratory demonstrated that 27/27 (100%) gerbils exposed to bilateral carotid artery occlusion and selected on the basis of these inclusion criteria had extensive CA1 neuron loss confirmed by histology (McEwen and Paterson. 2009). We have also previously demonstrated that a combination of pre- and post-ischemic PEM does not confound the screening tool, as it neither independently affects the hyperactivity ($p = 0.897$), nor interacts with the ischemic effect ($p = 0.782$) (P.G. Paterson, unpublished observations).

5.3.4 Tissue Preparation

At 1, 3, and 7d after surgery, deeply anesthetized animals were euthanized by trans-cardial perfusion with heparinized saline followed by 4% paraformaldehyde. The head was removed and fixed overnight in 4% paraformaldehyde. The brain was then removed and fixed overnight in 4% paraformaldehyde and placed in 20% sucrose solution for cryoprotection. Subsequently, brains were placed in OCT (Optimal Cutting Temperature) compound for approximately 30min, after which they were frozen using dry ice-cooled isopentane in a mold containing the OCT compound. Samples were then stored at -80°C until sectioning. Tissue was sectioned through the rostral hippocampus at 10μm thickness with a cryostat and thaw-mounted onto slides (VWR Superfrost Plus). Four sections, one representing each experimental group**,** were placed on each slide so that all experimental groupings were processed under identical conditions. This allowed for accurate assessment of relative changes in hybridization or fluorescence signal among experimental groups on the same slide and avoids any bias due to slide to slide variabilities in signal intensities.

5.3.5 BDNF, trkB, and GAP-43 In-Situ Hybridization

In-situ hybridization was carried out on tissue using 48 base pair oligonucleotide probes (University Core DNA Services, Calgary, AB, Canada) complementary to and selective for trkB (full length) bases 1361–1408 (Middlemas et al. 1991) (Genbank accession number - M55291.1), GAP-43 complementary to bases 70-117 (Karns et al. 1987) (Genbank accession number –

M16228), rat BDNF (1) complementary to bases 213–260 (Maisonpierre et al. 1991) (GenBank accession number - M61175) or rat BDNF (2) complementary to bases 626–673 (Leibrock et al. 1989) (GenBank accession number - X16713). Probes were checked against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure that no greater than 75% homology was found to sequences other than the cognate. Each oligonucleotide probe (80ng) was labelled at the 3' end with α-[^{35}S]dATP using terminal deoxynucleotidyltransferase (Amersham, Canada) in a buffer containing 10mM $CoCl_2$, 1mM dithiothreitol, 300mM Tris base and 1.4M potassium cacodylate (pH 7.2), purified using the QIAquick Nucleotide Removal Kit (Qiagen) and dithiothreitol added to a final concentration of 10nM. Labelled probe was stored at 4°C until use. The specific activities used ranged from 2.0 to 5.2 × 10^6 cpm/ng oligonucleotide.

Prior to hybridization, slides were air dried, fixed in 4% paraformaldehyde, and washed in 1X PBS. Sections were then treated with proteinase K (20µg/ml) containing 1ml 1M Tris-HCl (pH 7.6), 2ml 0.5 M EDTA, 200µl proteinase K stock (20mg/ml) and 188µl ddH_20, rinsed in 1X PBS, and post-fixed in 4% paraformaldehyde. Slides were then rinsed and dehydrated in ascending alcohols.

Hybridization was carried out according to published procedures (Karchewski et al., 2002). Briefly, the sections were hybridized at 43°C for 14-18hr in a buffer containing 50% formamide (Sigma Aldrich, Canada), 4X SSC (1X SSC – 0.15M NaCl, 0.015M sodium citrate, 1X Denhart's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02M phosphate buffer (pH 7.0), 10% dextran sulphate, 500µg/ml heat-denatured sheared salmon sperm DNA, 200mM dithiothreitol and 10^7cpm/ml of probe. After hybridization, the slides were washed for 4 X 15min in 1X SSC at 55°C, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al. (2002) and exposed for 7 to 10d before developing in D-19 (Kodak, Rochester, NY, USA). Slides were stained with toluidine blue for viewing under brightfield conditions. Photographs were taken at 400x and 1000x magnification.

We have previously confirmed the specificity of hybridization signal for the BDNF and trkB probes as described in Karchewski et al. (2002). That is, sections were hybridized with labeled probe, labeled probe with a 1000-fold excess of cold probe, or labeled probe with a 1000-fold excess of another, dissimilar cold probe of the same length and similar guanine-cytosine content, in order to ensure specificity of the labeled probe.

All experimental groups were mounted on the same slide to ensure that slide to slide variability in hybridization signal would not bias the relative changes between experimental groups to be quantified. Three samples from each experimental group (representing 3 different animals per group) were used for quantification, which was performed in a blinded fashion. Photographs of the CA1 region taken at 200x magnification were used for analysis. To assure that grains could be readily detected over tissue Nissl stain, the images were first adjusted in Photoshop so that silver grains were distinct. The threshold function was then applied in order to obtain a black and white image consisting of grains only, with no cellular structure. These images were then analyzed using spot densitometry (AlphaEaseFC Imaging Software, Alpha Innotech). Five boxes (pixel area of 30,765 each) were placed at approximately equal intervals along the CA1 pyramidal layer. An integrated density value (IDV=sum of pixel values in the region of the interest) for each box was obtained and then the 5 values averaged. Values from the left and right hemispheres were then averaged. Average values from the CON-I, PEM-S, and PEM-I groups were then normalized to the respective CON-S value on the same slide.

5.3.6 Hippocampal CA1 Neuron Counts

Sections that had been prepared for in-situ hybridization were stained with toluidine blue. With observer blinded to treatment, the number of viable-looking CA1 neurons that had a distinct nucleus and an intact cellular membrane were counted bilaterally at 400x magnification in a section representing the rostral hippocampus from each of three different animals in each experimental group. Using a 200μm square (10x10) microscope grid, cells were counted in medial, middle, and lateral sectors of the CA1 region in one section ranging from -1.5 to -1.9mm bregma (Loskota. 1974).

5.3.7 BDNF, trkB, and GAP-43 Immunofluorescence

Slides were brought to room temperature and washed in 0.1M PBS, pH 7.4, for 1hr. Prior to incubation with primary antibodies for BDNF or trkB, citrate antigen retrieval was performed. Briefly, slides were placed in 0.01M citrate buffer (10% 0.1M sodium citrate buffer in ddH_2O, pH 6) at 50°C and then warmed to 90°C over 45min. Slides were then allowed to cool for 20min. All slides were then incubated with blocking solution containing 5% normal goat serum in primary diluent (0.1% Triton X-100 in 0.1M PBS) for 1hr at room temperature. Primary

antibodies were diluted with 2% normal goat serum in primary diluent to the following concentrations: rabbit anti-BDNF (N-20, sc-546, Santa Cruz Biotech, Inc) at 1:500, rabbit anti-trkB (794, sc-12, Santa Cruz Biotech, Inc) at 1:200, and mouse anti-GAP-43 (9-1E12 Ascites, kind gift of Dr. David J. Schreyer) at 1:5000. After 24hr incubation at 4°C in diluted primary antibody, slides were washed for 30min in 0.1M PBS. Slides labeled with BDNF and trkB primary antibodies were then incubated in the secondary fluorescent antibody, goat-anti-rabbit Alexa Fluor 594 (IgG, A11012, Molecular Probes, Invitrogen), diluted to a concentration of 1:400 in 0.1M PBS. Slides labeled with the primary antibody GAP-43 were incubated in the secondary fluorescent antibody goat-anti-mouse Cy3 (AffiniPure IgG (H+L), Jackson Immuno) and diluted to a concentration of 1:200 in 0.1M PBS. After 1hr incubation in the dark at room temperature, slides were washed for 30min in 0.1M PBS, and then coverslipped using ProLong Gold Antifade Reagent with DAPI in order to visualize cell nuclei (P36931, Molecular Probes, Invitrogen). Control sections were processed in the same manner, but no primary antibody was applied. Photographs were taken at 40x, 200x, and 1000x magnification.

Sections from animals in each experimental group were mounted on the same slide to ensure that slide to slide variability in fluorescence signal would not bias the relative changes between experimental groups to be quantified. Three to 4 samples from each experimental group (representing different animals per group) were used for quantification, which was performed in a blinded fashion. Photographs of all hippocampal regions of interest of sections mounted on the same slide were taken at 200x magnification under identical conditions. BDNF, trkB, and GAP-43 signals in the CA1 region were analyzed separately for the CA1 pyramidal cell layer, stratum oriens, and stratum radiatum. In addition, the GAP-43 signal only was analyzed in the CA3 and dentate gyrus regions. The hippocampal subregions can be viewed in Figure 2.2 of the thesis. In Photoshop, all images were changed to gray scale and then analyzed using spot densitometry (AlphaEaseFC Imaging Software, Alpha Innotech). For CA1 pyramidal layer analysis, 5 boxes (pixel area of 28,405 each) were placed at approximately equal intervals along the cell layer. For stratum oriens and stratum radiatum analysis, 3 boxes (pixel area of 48,125 each) in each layer were analyzed. In the CA3 region, 3 boxes (pixel area of 33,441 each) were placed along the CA3 neuron layer. In the dentate gyrus, 4 boxes (pixel area of 24,255 each) were placed along the granule cell layer and 3 boxes (2 with pixel area of 93,611 each, and 1 with pixel area of 84,847) were placed within the hilar region. An integrated density value (IDV=sum of pixel

values in the region of interest) was obtained for each box, and then these values were averaged for the region of interest. Values from the left and right hemispheres were averaged. Average values for the CON-I, PEM-S, and PEM-I groups were normalized by expressing them as a ratio to the respective CON-S value on the same slide.

5.3.8 Statistical Analysis

Presurgical body weight and food intake were analyzed using an unpaired t-test. Postsurgical body weight and CA1 neuron counts were analyzed by 2-factor ANOVA.

5.4 Results

5.4.1 Impact of Diet and Ischemia on Food Intake and Body Weight

The pattern of body weight change prior to surgery is shown in Figure 5.1. Mean (±SEM) initial body weight was not significantly different between the two diet groups ($p > 0.05$). There was a significant difference in body weight between groups starting after 1wk on diet and continuing throughout the 4wk pre-surgical period ($p = .020$ at 1 wk; $p < 0.001$ for each of the following weeks). By 4wk, CON animals had increased body weight by 5.3%, while PEM animals had decreased body weight by 11.7%, resulting in a difference in body weight of 14.4%. As shown in Table 5.1, weekly food intake was not significantly different between groups until the fourth week, at which time food intake was 9% lower in the PEM gerbils ($p < 0.001$). Analysis of post-surgery body weight on both d3 and 7 showed a statistically significant decrease with PEM ($p = .001$), but there was no independent effect of ischemia nor an interaction between the diet and ischemia. Mean (±SEM) final body weight (g) of the four experimental groups on d3 post-surgery were as follows: CON-S = 60.7 ± 2.8, CON-I = 62.5 ± 2.1, PEM-S = 55.7 ± 2.0, PEM-I = 51.3 ± 1.7 (n = 6) (diet effect $p = 0.001$, surgery effect $p = 0.551$, diet and surgery interaction $p=0.171$). Mean (±SEM) final body weight of the four experimental groups on d7 post-surgery were as follows: CON-S = 69.6 ± 3.3, CON-I = 65.5 ± 2.2, PEM-S = 54.3 ± 0.9, PEM-I = 57.5 ± 2.0 (n = 6) (diet effect $p = 0.001$, surgery effect $p = 0.848$, diet and surgery interaction $p = 0.120$).

Following surgery, sample size (on a cage basis) was insufficient to analyze food intake data statistically. After the acute surgical period (by 3d), the pattern suggested food intake suppressed both by PEM and ischemia relative to the CON-Sham group. For example, mean

(±SEM) food intake (g) on d6 was as follows: CON-S = 4.2 ± 0.2, CON-I = 2.5 ± 0.6, PEM-S = 3.3 ± 0.2, PEM-I = 2.8 ± 0.2.

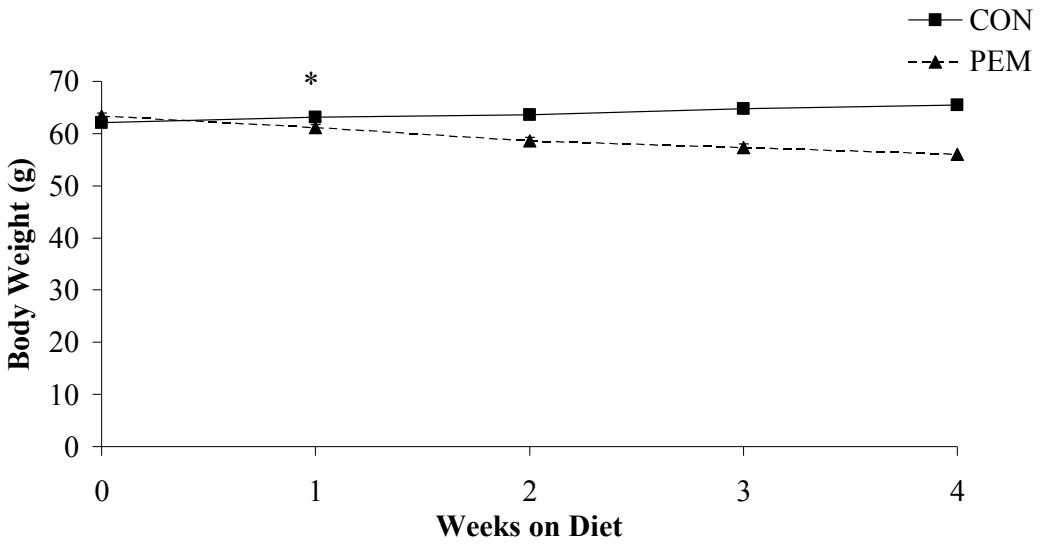

Figure 5.1. Mean (±SEM) body weight (g) of control (squares) and PEM gerbils (triangles) over the 4wk feeding period prior to surgery, n = 36. *Indicates the point at which a significant difference in body weight was first detected by unpaired t-test ($p = 0.02$); this continued throughout the pre-surgery feeding period ($p < 0.001$).

Table 5.1. Effect of experimental diet on food intake of gerbils

	Week 1	Week 2	Week 3	Week 4
CON	28.6 ± 0.7	24.4 ± 0.7	24.4 ± 0.7	24.5 ± 0.6
PEM	27.9 ± 0.6	25.1 ± 0.6	23.8 ± 0.5	22.3 ± 0.5*

Values expressed as mean (±SEM). Food intake is based on cage data [n = 16 (2-4animals/cage)]. *$p = 0.005$ as compared to controls, based on unpaired t-test.

5.4.2 Hippocampal CA1 Neuron Counts

Hippocampal CA1 cell counts are shown in Table 5.2. No effect of ischemia or diet was found at 1d. Exposure to global ischemia independently decreased total CA1 neuronal cell counts at 3d and 7d ($p \leq 0.001$). PEM did not exacerbate CA1 neuronal loss nor was there an independent effect of PEM.

Table 5.2. Effect of global ischemia and PEM on hippocampal CA1 neuron counts

Time Point Post-Ischemia	CON-S	CON-I	PEM-S	PEM-I
1d	325.0 ± 13.5	327.7 ± 11.0	345.0 ± 15.7	319.0 ± 13.5
3d*	304.7 ± 4.3	45.3 ± 6.7	333.0 ± 24.8	52.3 ± 13.4
7d*	299.7 ± 16.9	43.0 ± 11.3	299.7 ± 20.3	36.3 ± 9.4

Values expressed as mean (±SEM). *Indicates a significant independent effect of ischemia based on 2-factor ANOVA ($p \leq 0.001$). n = 3 for each group.

5.4.3 Sample Size for Analysis of In-Situ Hybridization and Immunofluorescence

BDNF, trkB, and GAP-43 expression were examined to determine whether PEM altered the neuroplastic response following global ischemia. All tissue processed for qualitative analysis (in-situ hybridization or immunofluorescence) showed consistent results regarding the relative changes observed for the markers BDNF, trkB, and GAP-43 based on an analysis of n = 3-6 animals per experimental group. Slides with poor signal to noise ratios were excluded from the analysis. Quantification of alterations in hybridization or fluorescence signal was then performed in a blinded fashion for 3-4 animals/experimental group as described in the methods section.

5.4.4 BDNF

Representative bright field photographs of BDNF mRNA expression in the CA1 region at 3d post-surgery as detected by in-situ hybridization are shown in Figure 5.2 and results of quantitative analysis are shown in Table 5.3. An independent effect of ischemia was evident as an increase in BDNF mRNA expression in the CA1 pyramidal layer of the hippocampus in both ischemic groups (Table 5.3). There was no apparent independent effect of diet or any interaction between diet and ischemia on BDNF mRNA in the CA1 region. This pattern was already evident on d1 post-ischemia before the neurons appeared shrunken. By d7, expression was decreasing, at which time there were many fewer surviving neurons. No specific mRNA expression was evident in any other region of the hippocampus in any group at any timepoint.

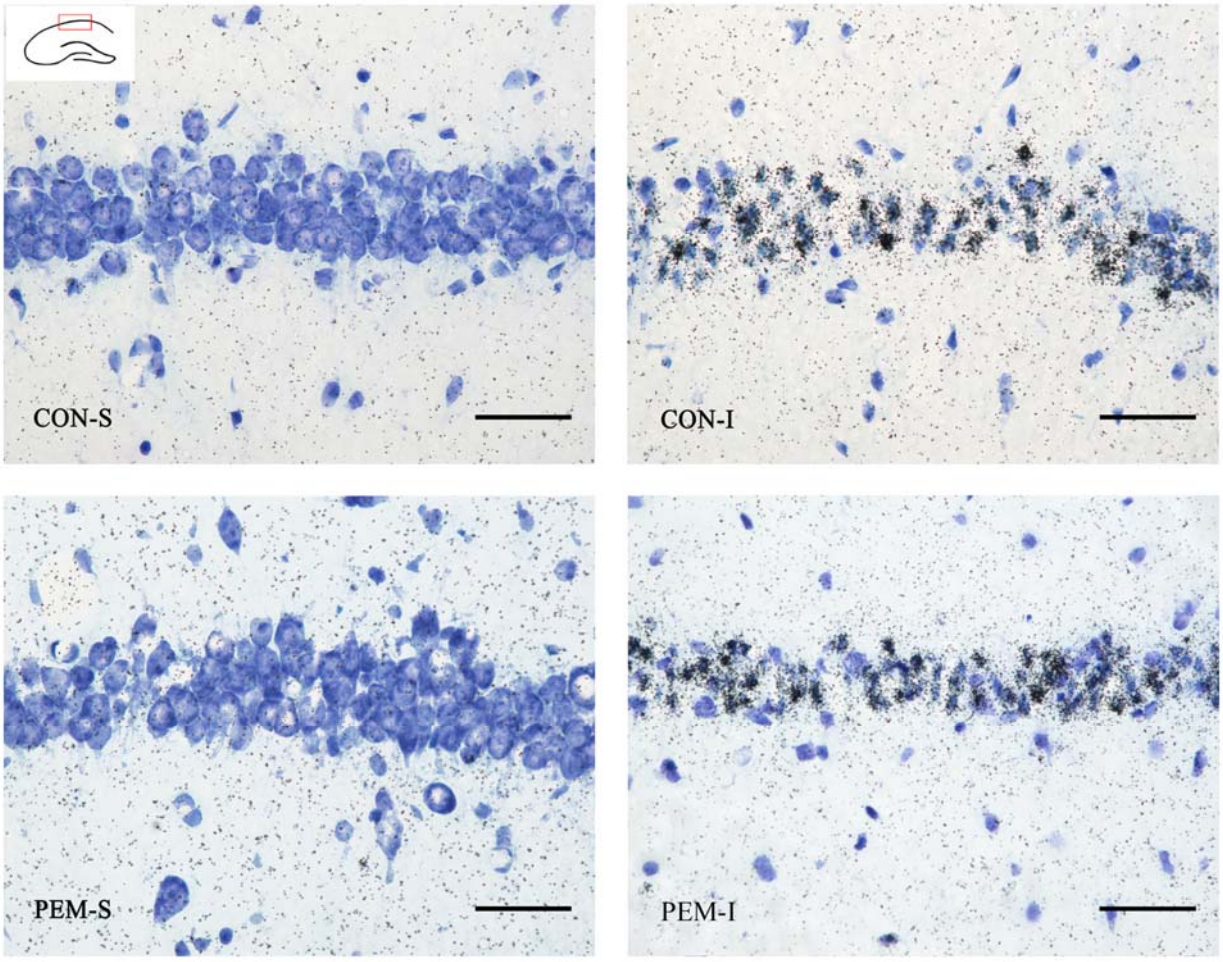

Figure 5.2. Representative bright field photographs of BDNF mRNA expression as detected by in-situ hybridization in the CA1 region of the hippocampus at 3d post-surgery. Protein-energy malnutrition does not alter the global ischemia-induced increase in CA1 expression of BDNF mRNA. Cell bodies are stained with toluidine blue, and BDNF mRNA hybridization signal is identified by the presence of black silver grains over tissue for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 50 μm.

Table 5.3. Effect of ischemia and PEM on hippocampal CA1 BDNF and trkB mRNA expression

	Time Point Post-Ischemia	CON-I	PEM-S	PEM-I
BDNF	1d	3.6 ± 0.6	1.2 ± 0.2	4.2 ± 1.5
	3d	8.5 ± 2.0	1.5 ± 0.1	9.8 ± 3.1
	7d	7.7 ± 4.5	1.9 ± 1.3	7.6 ± 4.4
trkB	1d	3.9 ± 0.5	1.2 ± 0.2	3.8 ± 0.7
	3d	10.2 ± 2.6	0.8 ± 0.2	9.3 ± 4.2
	7d	3.4 ± 2.0	0.4 ± 0.1	2.1 ± 0.9

Values are expressed as mean (±SEM) integrated density value normalized to the respective CON-S value on the same slide. n = 3 animals analyzed per group.

Figure 5.3 shows BDNF protein expression in the hippocampal formation in a representative CON-S animal at 3d and indicates the location of the stratum oriens and stratum radiatum layers. In both sham groups, BDNF protein expression was mainly confined to the mossy fibre axons and the polymorphic layer of the dentate gyrus (Figure 5.3) with less expression in the CA1 pyramidal region at all times after surgery; this is illustrated for d3 in Figure 5.3 and 5.4. The characteristic CA1 neuronal death that occurs around 3d post-ischemia in this model was evident as a loss of the well-defined CA1 pyramidal cell layer and is illustrated in Table 5.2. Note the increase in DAPI-stained cell bodies scattered throughout the CA1 region by 3d in both ischemic groups, which contrasts sharply with the orderly CA1 pyramidal neuron layer observed in both sham groups (Figure 5.4). No change in BDNF protein expression was evident at any timepoint as a result of ischemia or PEM in the CA1 pyramidal cell layer (shown for d3 in Figure 5.4), stratum oriens or stratum radiatum (Figure 5.5) or any other region of the hippocampus. Note the large variability in signal intensity evident in Figure 5.5 even after normalization of all data to that from the CON-S gerbil on the same slide. High magnification pictures of BDNF mRNA and protein expression in the CA1 pyramidal neurons of a CON-I animal are shown in Figure 5.6 to illustrate the disparity between mRNA and protein expression after global ischemia.

5.4.5 trkB

Global ischemia resulted in increased expression of trkB mRNA in the hippocampal CA1 pyramidal neuron layer at 1, 3, and 7d, with a pattern similar to that observed for BDNF mRNA

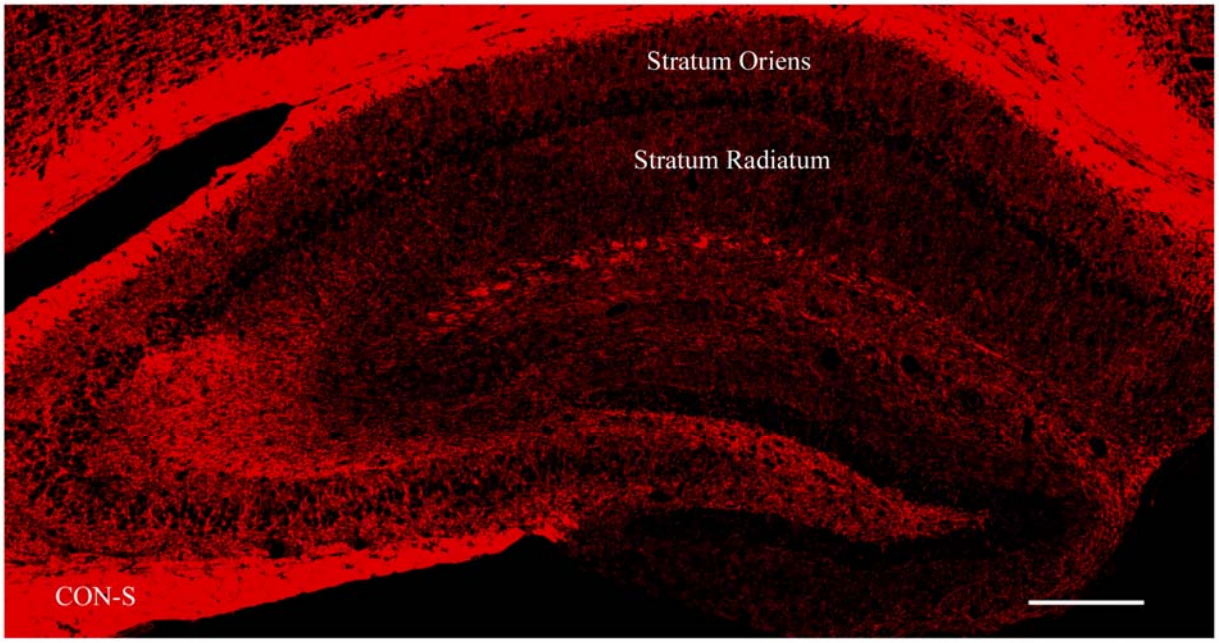

Figure 5.3. A representative photograph of BDNF immunoreactivity in the hippocampal formation of a control sham gerbil at 3d post-surgery. The red stain is BDNF-LI. A. Scale bar = 500 μm.

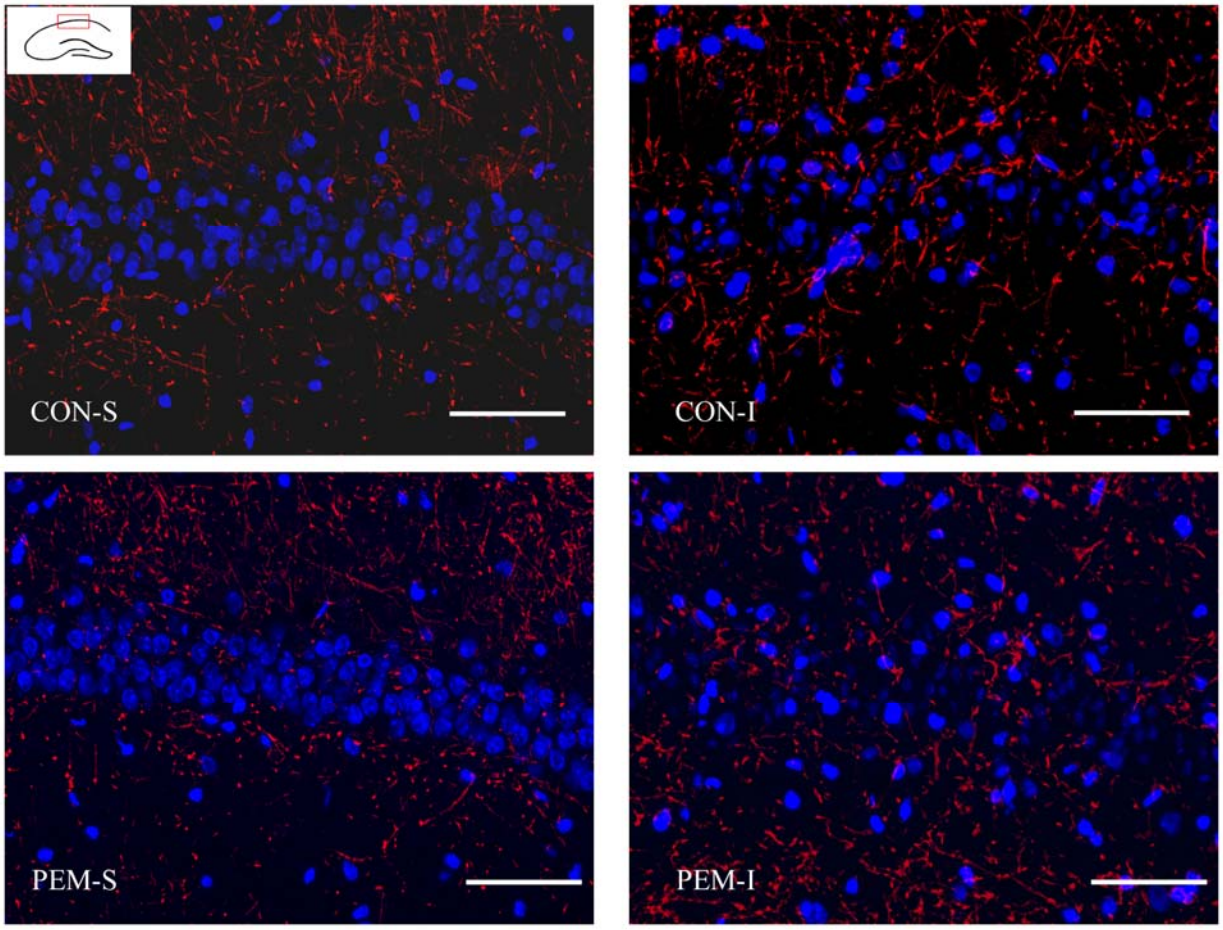

Figure 5.4. Representative photographs of BDNF immunoreactivity in the CA1 region of the hippocampus at 3d post-surgery. Blue staining is DAPI representing cell nuclei, and red staining is BDNF-LI for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 100 μm.

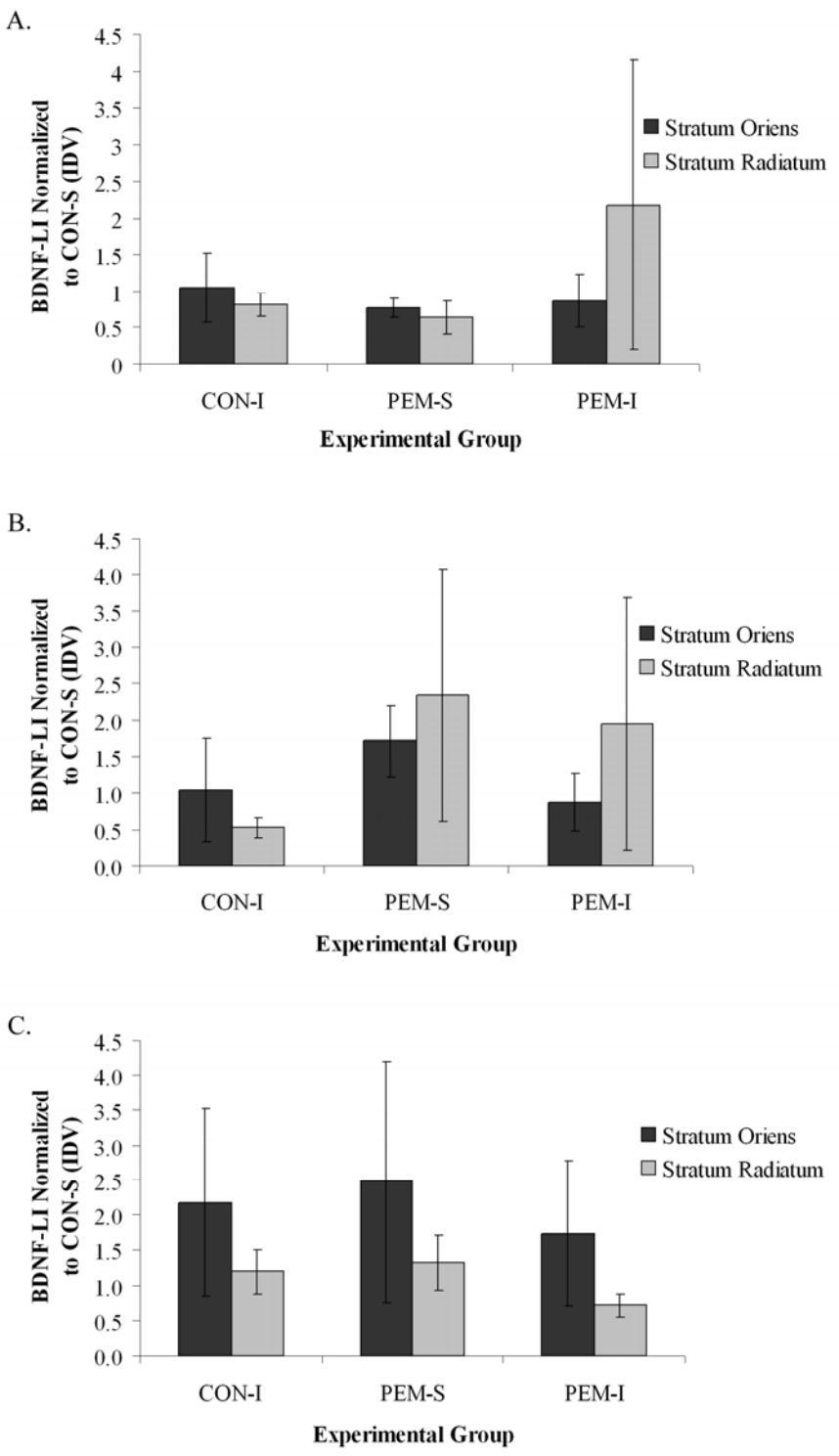

Figure 5.5. Relative changes in fluorescence signal for BDNF protein in CA1 stratum oriens and stratum radiatum expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 1d (A), 3d (B), and 7d (C) following surgery. n = 3 for 1d time point and n = 4 for 3 and 7d time points per group.

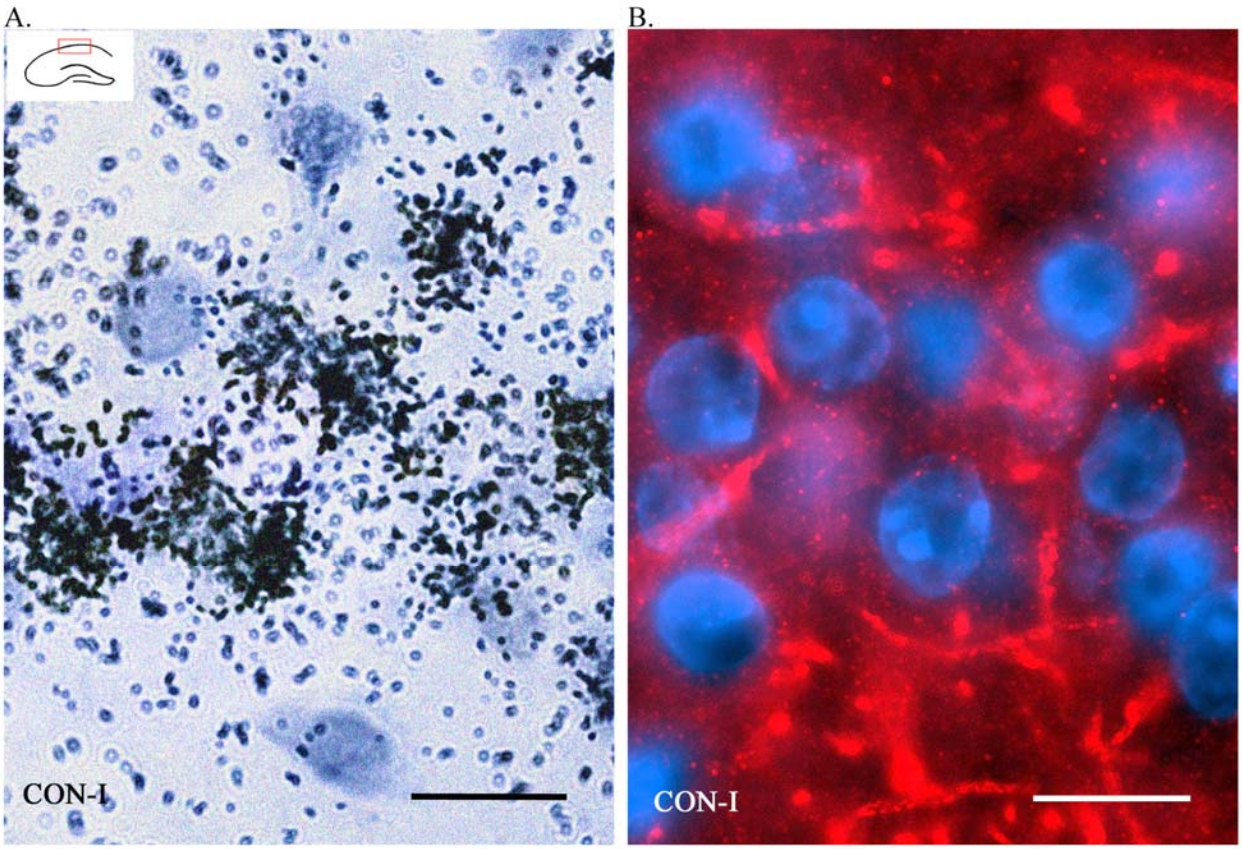

Figure 5.6. Representative high magnification photographs highlighting the disparity between hippocampal CA1 expression of BDNF mRNA (A) and protein (B) at 3d post-ischemia in a control diet-ischemic gerbil. A. Cell bodies are stained with toluidine blue and BDNF mRNA hybridization signal is identified by the presence of black silver grains in the CA1 region. Scale bar = 20µm. B. Blue staining is DAPI representing cell nuclei, and the red stain is BDNF-LI. Scale bar = 20 µm.

(Table 5.3). Representative bright field photographs of trkB mRNA at 3d post-surgery are shown in Figure 5.7. There was no apparent independent effect of diet and no interaction evident between diet and ischemia on trkB mRNA expression in the CA1 region. There were also no apparent differences among experimental groups in any other region of the hippocampus at any time point examined.

Ischemia increased trkB protein expression in the CA1 pyramidal layer in both diet groups by 3d (Figure 5.8 and 5.9) which was still present at 7d, but to a lesser degree (Figure 5.8 and 5.9). By 7d, the increased trkB in the CA1 pyramidal cell layer appeared to be further elevated when the gerbils were also protein-energy malnourished (Figure 5.9), but this more subtle observation was somewhat obscured by the dramatic increase in protein expression in the stratum radiatum and stratum oriens in what are hypothesized to be CA1 neuronal fibres in the PEM-I group (Figure 5.8 and 5.10). There were no apparent differences in trkB protein among groups in any other region of the hippocampus.

5.4.6 GAP-43

At 1d following global ischemia, no difference in GAP-43 mRNA expression was apparent among experimental groups in any part of the hippocampus. There was no analysis of GAP-43 hybridization signal at 3 and 7d due to a poor signal to noise ratio.

Ischemia increased GAP-43 protein expression within the CA1 neurons as demonstrated by increased GAP-43-like immunoreactivity in both the CON-I and PEM-I groups evident by 3d and continuing, although diminishing, to 7d (Figure 5.11 and 5.12). By 3d, PEM exacerbated this increase in GAP-43, not only in the CA1 neurons (Figure 5.11 and 5.12) but also in CA3 and hilar (Figure 5.13 and 5.14) regions as compared to CON-I gerbils. By 7d after global ischemia, this expression had decreased in all regions, the exception being that GAP-43 expression in the CA1 region was still higher in the PEM-I animals (Figure 5.11 and 5.12). PEM also increased the extent of GAP-43 protein induced by ischemia in the stratum oriens and stratum radiatum surrounding the CA1 pyramidal layer as compared to that observed in the CON-I group at 3d (Figure 5.11 and 5.15).

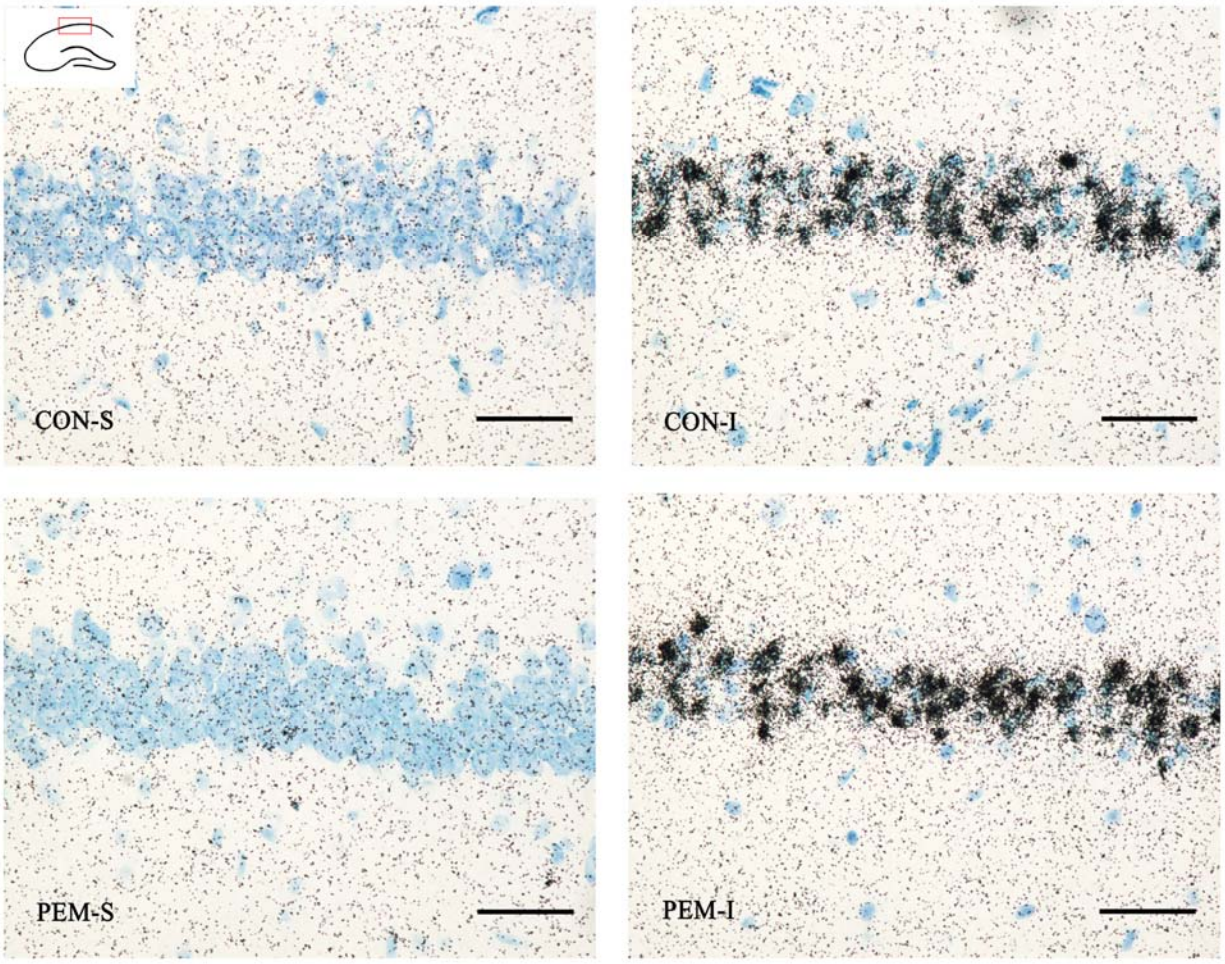

Figure 5.7. Representative bright field photographs of trkB mRNA expression as detected by in-situ hybridization in the CA1 region of the hippocampus at 3d post-surgery. Protein-energy malnutrition does not alter the global ischemia-induced increase in CA1 expression of trkB mRNA. Cell bodies are stained with toluidine blue, and trkB mRNA hybridization signal is identified by the presence of black silver grains over tissue for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 50 μm.

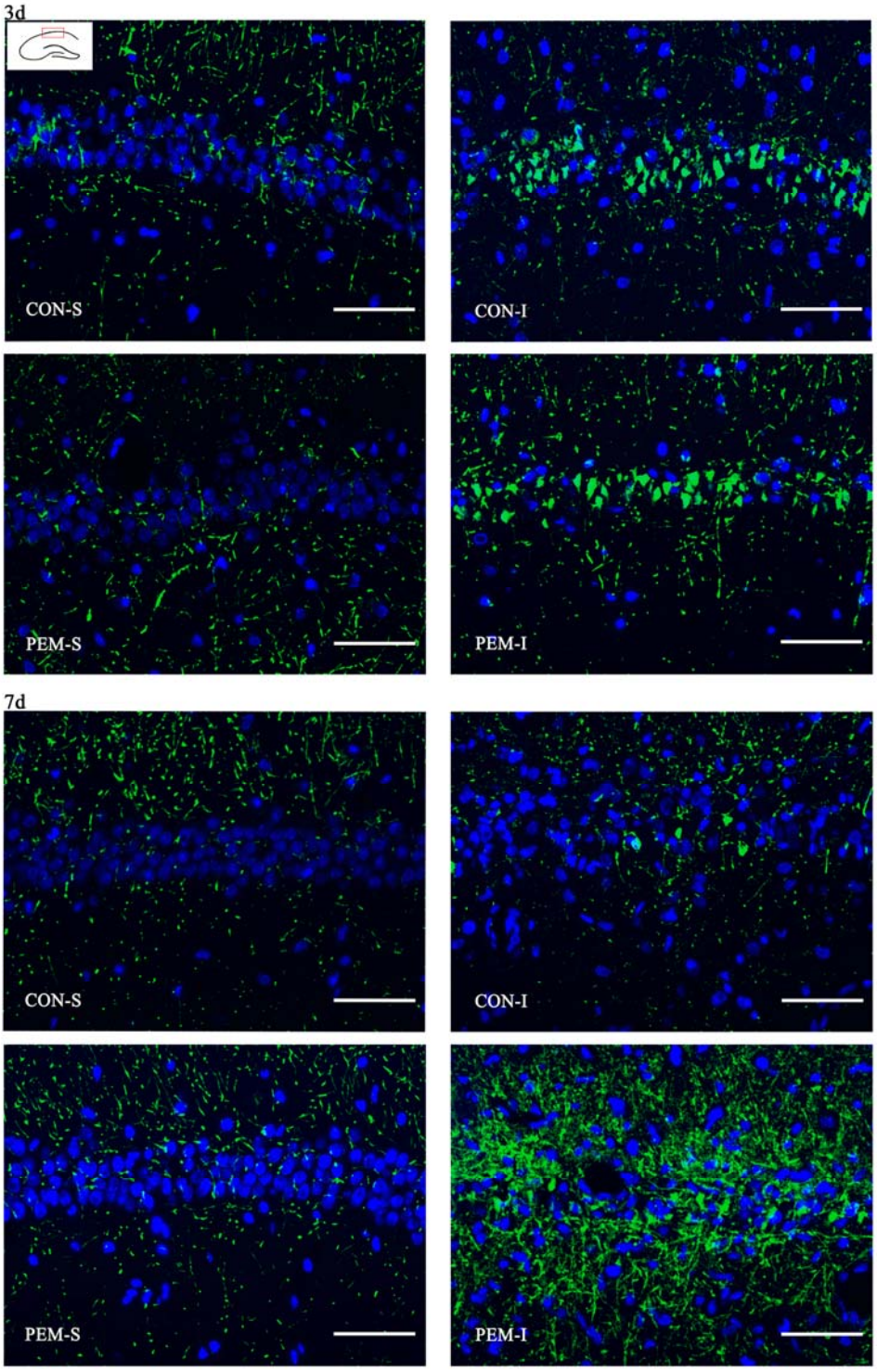

Figure 5.8. Representative photographs of trkB immunoreactivity in the CA1 region of the hippocampus at 3 and 7d post-surgery. Protein-energy malnutrition magnifies the hippocampal CA1 trkB protein response to global ischemia. Blue staining is DAPI representing cell nuclei, and green staining is trkB-LI for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 100 μm.

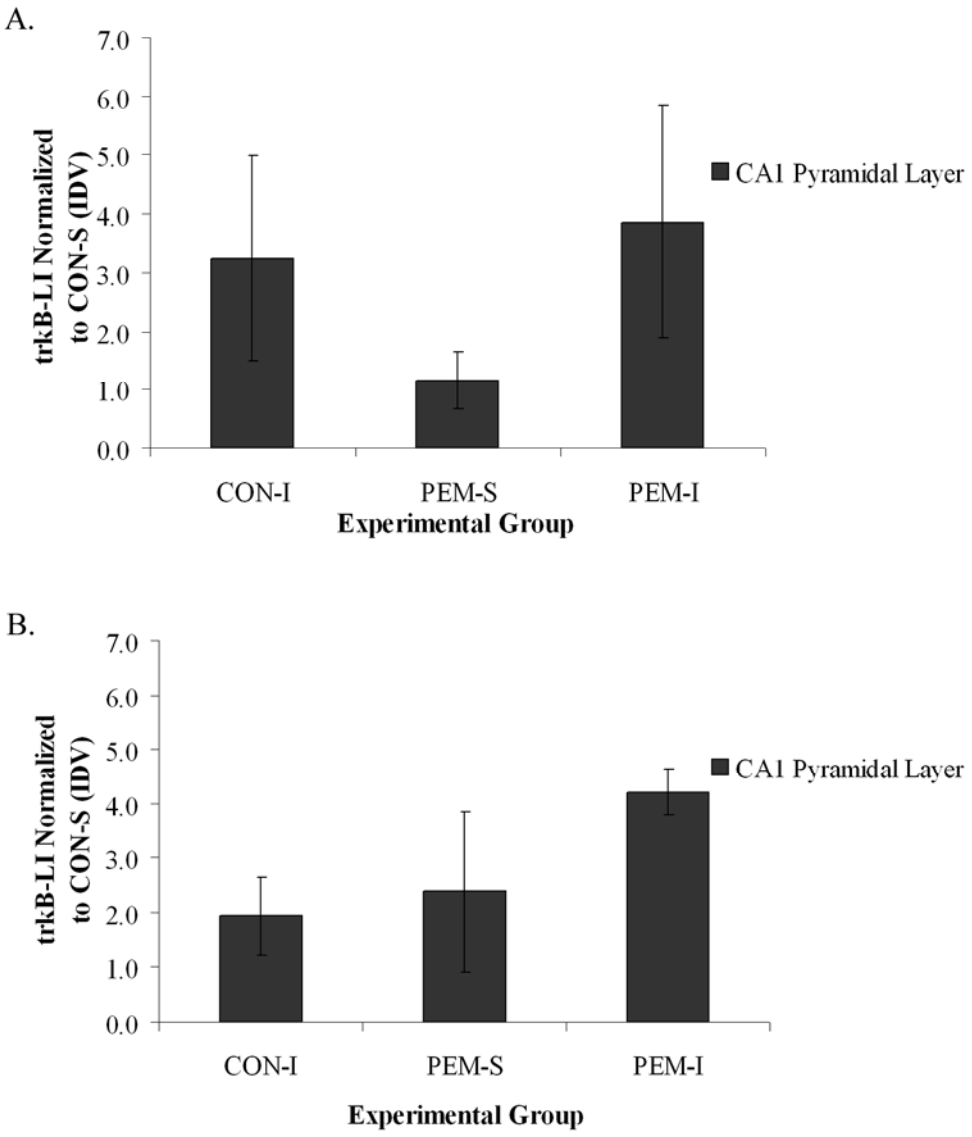

Figure 5.9. Relative changes in fluorescence signal for trkB protein in the CA1 pyramidal layer expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 3d (A), and 7d (B) following surgery. n = 4 animals per group.

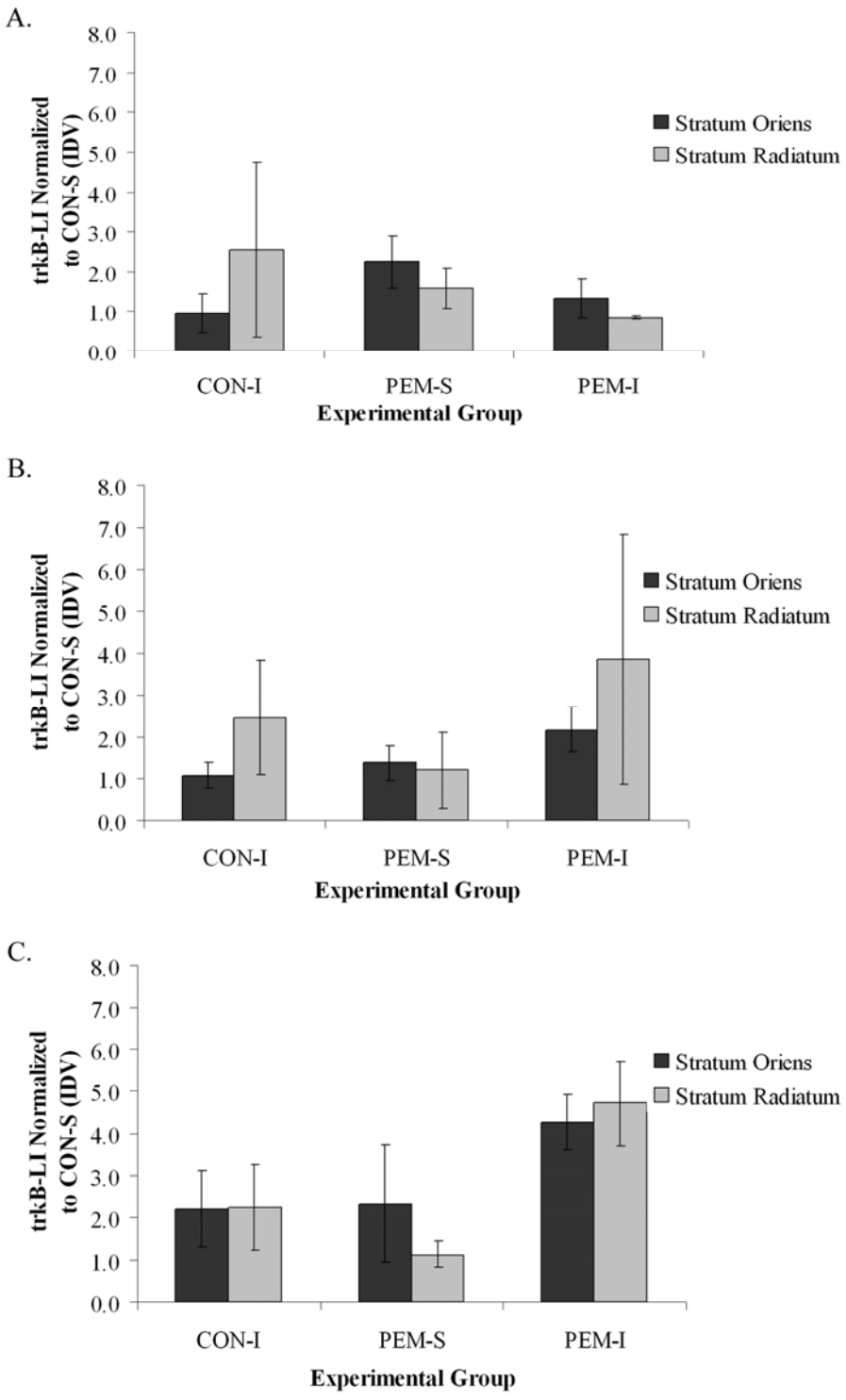

Figure 5.10. Relative changes in fluorescence signal for trkB protein in CA1 stratum oriens and stratum radiatum expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 1d (A), 3d (B), and 7d (C) following surgery. n = 3 animals for 1d time point and n = 4 animals for 3 and 7d time points per group.

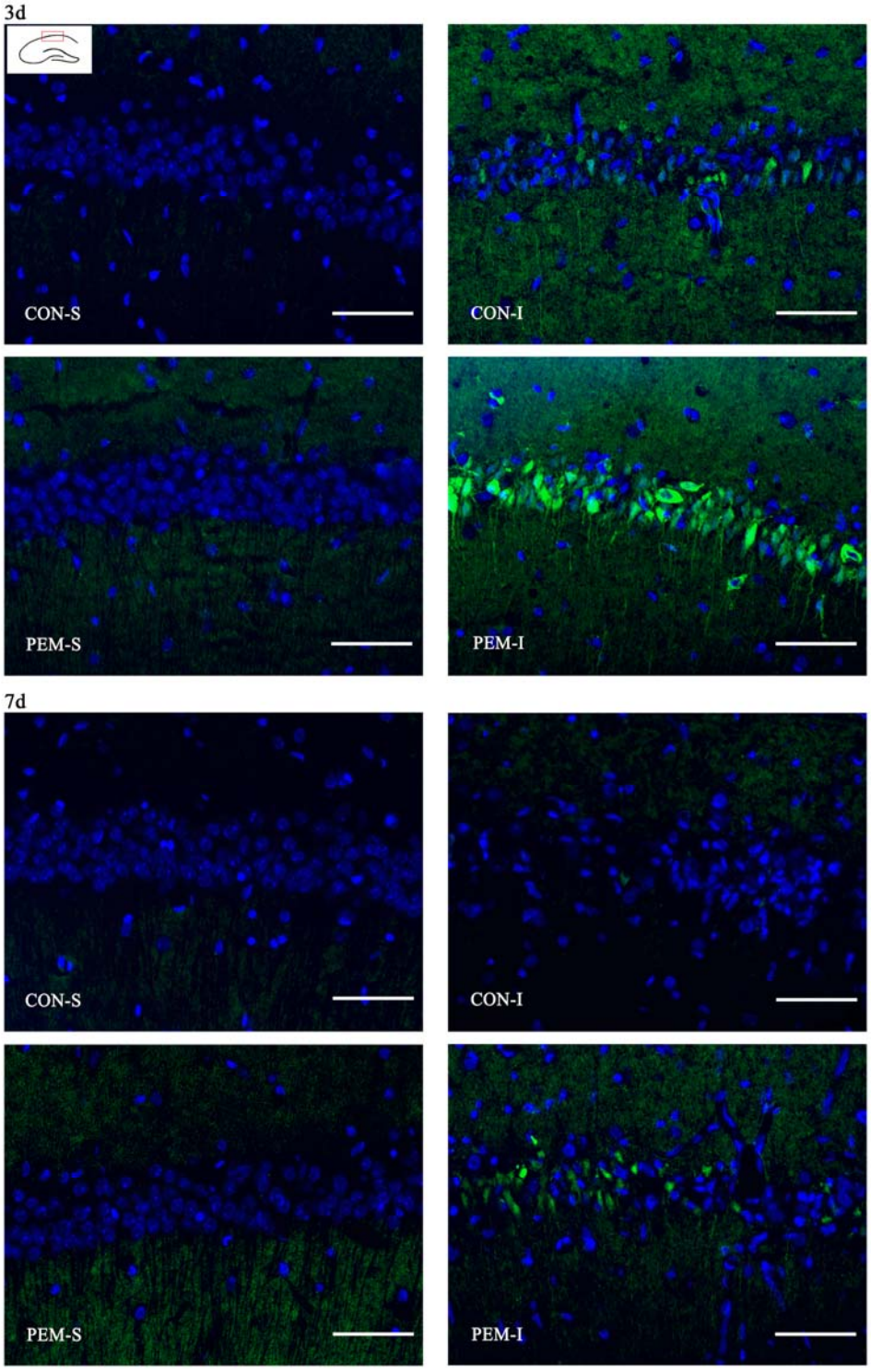

Figure 5.11. Representative photographs of GAP-43 immunoreactivity in the CA1 region of the hippocampus at 3 and 7d post-surgery. Protein-energy malnutrition exacerbates the increase in hippocampal CA1 GAP-43 protein after global ischemia. Blue staining is DAPI representing cell nuclei, and the green stain is GAP-43-LI for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 100 μm.

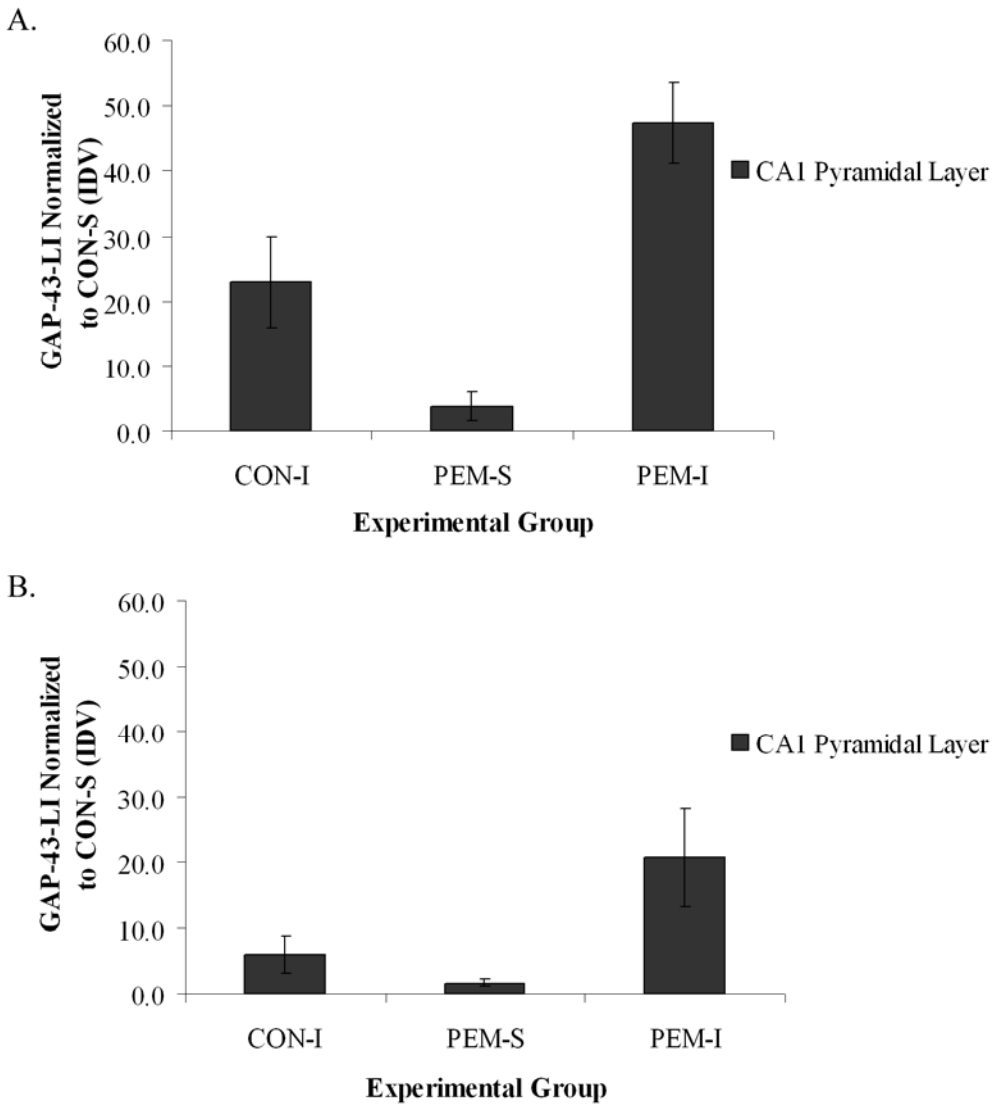

Figure 5.12. Relative changes in fluorescence signal for GAP-43 protein in the CA1 pyramidal layer expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 3d (A) and 7d (B) following surgery. n = 4 animals per group.

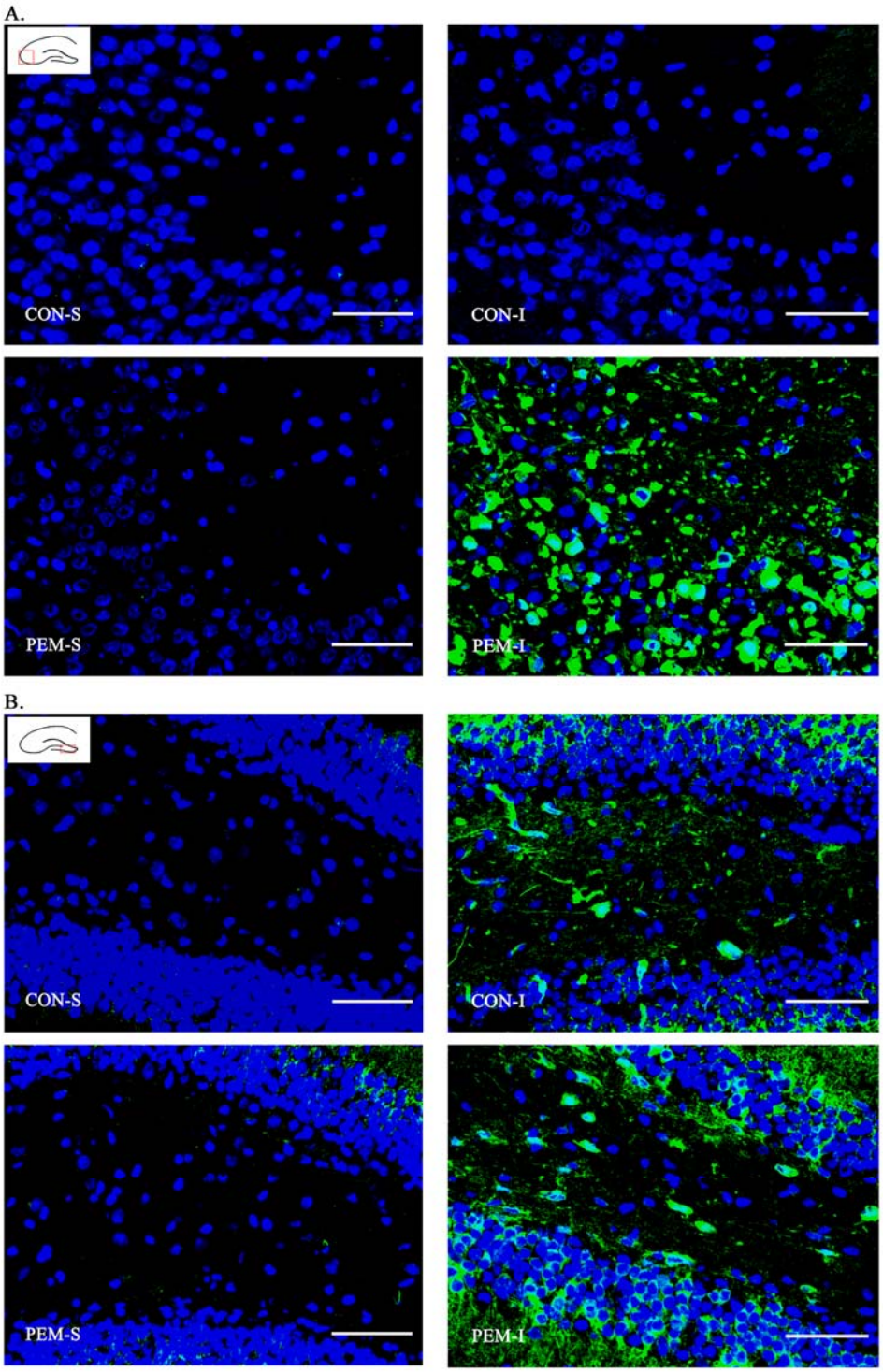

Figure 5.13. Representative photographs of GAP-43 immunoreactivity in the CA3 (A) and dentate gyrus (B) regions of the hippocampus at 3d post-surgery. Protein-energy malnutrition also magnifies the GAP-43 protein response to global ischemia in CA3 and dentate gyrus regions. Blue staining is DAPI representing cell nuclei, and the green stain is GAP-43-LI for control diet-sham (CON-S), control diet-ischemic (CON-I), PEM-sham (PEM-S), and PEM-ischemic (PEM-I) animals. Scale bar = 100 µm.

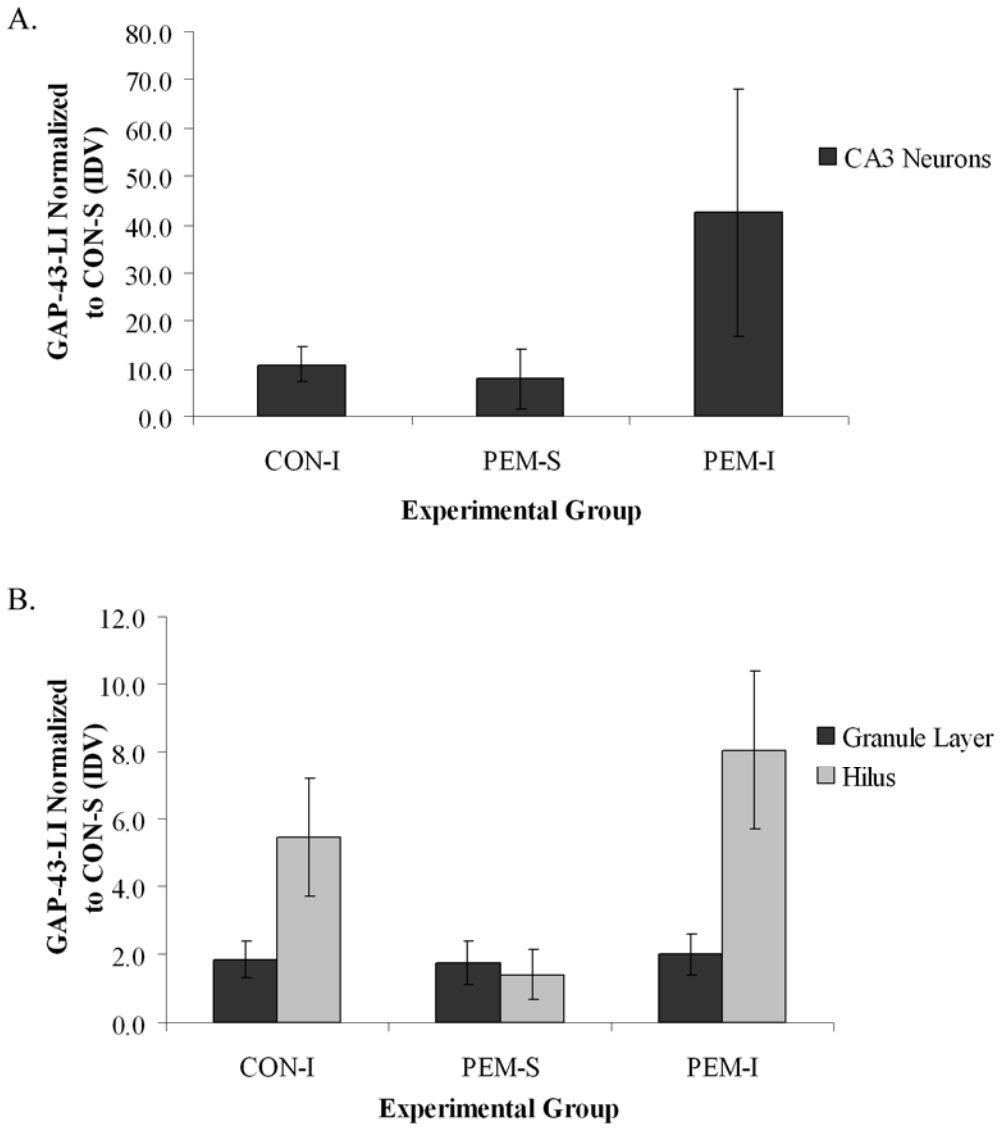

Figure 5.14. Relative changes in fluorescence signal for GAP-43 protein in the CA3 pyramidal neurons (A) and dentate gyrus regions (B) expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 3d following surgery. n = 4 animals per group.

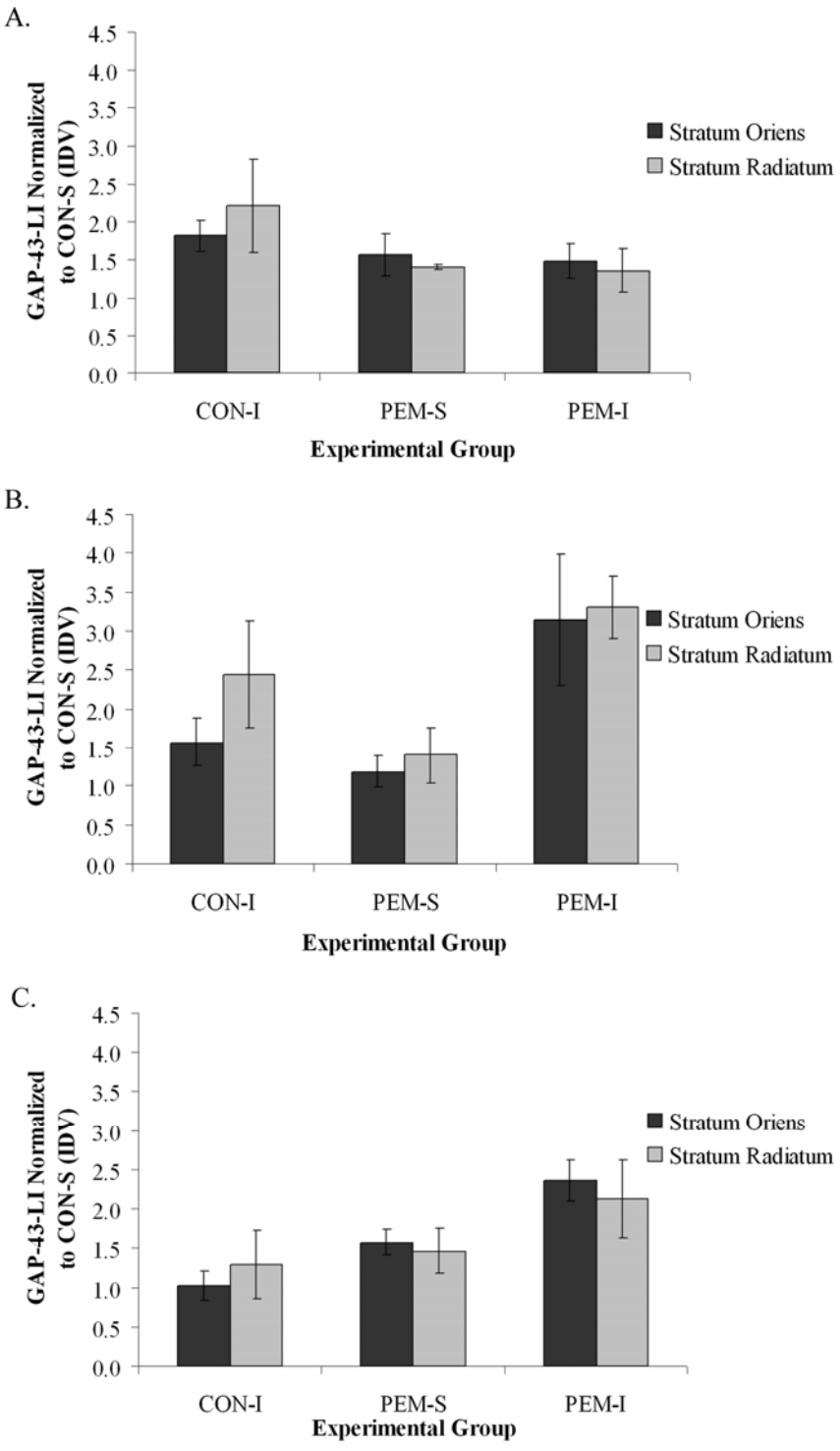

Figure 5.15. Relative changes in fluorescence signal for GAP-43 protein in CA1 stratum oriens and stratum radiatum expressed as integrated density value (IDV) (±SEM) for CON-I, PEM-S, and PEM-I groups normalized to the respective CON-S value on the same slide. Results are shown for 1d (A), 3d (B), and 7d (C) following surgery. n = 3 animals for 1d time point and n = 4 animals for 3 and 7d time points per group.

5.5 Discussion

This study investigated if pre-existing PEM alters plasticity-associated gene expression associated with recovery mechanisms after global ischemia. The low protein diet utilized has previously been shown to cause PEM on the basis of a voluntary reduction of food intake (15%), decreases in body weight (17%), liver reduced-glutathione (49%), and serum albumin (18%), and an increase in liver lipid concentration (66%) (Bobyn et al. 2005, Harmon et al. 2006). The decrease in food intake caused by the low protein diet was slower to arise in the current study, with intake 9% lower than in gerbils fed control diet by the fourth week, just prior to inducing ischemia. However, the decline in body weight occurred after only 1 week on experimental diet, reaching 14% after 4 weeks. Thus, PEM was induced, but to a milder degree than previously observed. Food intake was also temporarily decreased by exposure to ischemia. Although changes in food intake and weight loss caused by both brain ischemia and the treatment under investigation could influence outcome in rodent surgical models of stroke, collection of such data is often neglected.

In-situ hybridization and immunofluorescence were employed to study the effect of PEM on global ischemia-induced changes in BDNF, trkB and GAP-43 mRNA and protein expression in the hippocampus. We used a sample of Mongolian gerbils screened by hyperactivity testing to avoid the extreme inconsistencies in hippocampal CA1 injury that can occur in this species after bilateral carotid artery occlusion (Laidley et al. 2005). The alterations in trkB and GAP-43 protein expression observed in protein-energy malnourished gerbils occurred without exacerbation of hippocampal CA1 death as assessed by routine histology at 3 and 7d. Although based on a small sample size, this result is in agreement with our previous conclusion from a large sample which demonstrated altered functional outcome without an accompanying increase in cell death (Bobyn et al. 2005). The absence of CA1 cell loss in any group at d1 and extensive loss of CA1 neurons in both ischemic groups by d3 is typical of bilateral carotid artery occlusion in the gerbil since CA1 neuron death is not evident in this model until 2-3d post-ischemia (Kirino. 1982).

Contrary to our hypothesis, PEM did not diminish the post-ischemic increase in BDNF mRNA observed in the CA1 region at 1, 3, and 7d. The increase in BDNF mRNA was not mirrored at the protein level at any sampling time. This mismatch has been reported previously after global ischemia, although the pattern and extent of BDNF expression varies among studies

(Kokaia et al. 1996, Lee et al. 2002, Yang et al. 2002). The latter likely reflects differences in insult severity due to differences in regulation of important determinants of injury such as intra- and post-ischemic brain temperature as well as variation in sampling times and the extent to which the postischemic environment stimulates BDNF production.

The discord between BDNF mRNA and protein expression after brain ischemia has been attributed to translational arrest caused by dysfunctional protein synthesis, release, or transport (Lee et al. 2002, Martin de la Vega et al. 2001). The CA1 region of the hippocampus is the most vulnerable to irreversible translational arrest after global ischemia and reperfusion (Kayali et al. 2005). A relative inability to maintain BDNF protein levels, relative to regions such as the dentate gyrus, has been suggested to contribute to the high susceptibility of the hippocampal CA1 region to ischemic damage (Kokaia et al. 1996). However, a lack of overlap between BDNF mRNA and protein has also been observed in uninjured hippocampus and in other brain regions (reviewed in Tapia-Arancibia et al. 2004), suggesting that there are other contributing mechanisms. Because of the capacity for both anterograde and retrograde transport of BDNF (Nawa et al. 1997), increased BDNF protein synthesized in CA1 neurons in response to ischemia could be rapidly transported retrogradely to the mossy fibres and dentate gyrus. Our data provide no evidence to support the latter explanation though, since post-ischemic BDNF protein levels did not increase in these regions above that observed in sham animals. The entorhinal cortex is the destination for anterograde transport from the CA1 and although we did not observe differences here, a more detailed examination could be done in the future.

It is possible that a small rise in BDNF in the CA1 region occurring within the first few hours was missed. Studying the influence of PEM under conditions that stimulate more BDNF synthesis after global ischemia, such as environmental enrichment (Gobbo and O'Mara. 2004), might yield quite different results. These factors may also account for some of the disagreement among the few studies of global ischemia.

The increased mRNA expression of trkB in the CA1 region observed at 1, 3, and 7d following ischemia was not affected by PEM. Others have also reported this increase (Majda et al. 2001). Merlio et al. (1993) found the increase restricted to the dentate gyrus but may have missed changes in CA1 neurons by examining only early timepoints (2-24hr). Increased trkB protein in the CA1 pyramidal neuron layer was observed in our study at 3d after ischemia, and this was declining by 7d. Since extensive CA1 neuronal death occurs between 1 and 3d after

global ischemia (Kirino. 1982), increased trkB would provide a mechanism by which more BDNF could be sequestered for neuroprotection. The timeframe of trkB protein expression in the current study also differs from previous reports Ferrer et al. (1997) observed no change in trkB at similar times after bilateral carotid artery occlusion in the gerbil while Miyata et al. (Miyata et al. 2001) reported an increase only from 1 to 12hr after global ischemia in the rat. Neither of these studies controlled intra-ischemic brain temperature, and thus, our more prolonged pattern of expression likely reflects a more severe and consistent insult.

Although the number of CA1 neuronal bodies expressing trkB protein after global ischemia appeared slightly increased by PEM, the most striking feature was the heightened trkB protein response to ischemia at 7d in what we hypothesize are CA1 dendrites in the stratum oriens and stratum radiatum. However, without counterstaining, the possibility cannot be excluded that this increased expression is on astroglial processes. At 3 and 7d, when there is extensive loss of CA1 neurons, Figure 5.8 shows an increase in DAPI-stained cell bodies scattered throughout the CA1 region in both ischemic groups. Although the cells were not identified, the smaller cell body, the scattered distribution, and the timing (coincident with expected reactive gliosis and the death of a large percentage of CA1 neurons) all strongly point to these being glial cells. While the truncated form of trkB is generally predominant in astrocytes (Rose et al. 2003), which would not have been recognized by the antibody used in this study, full-length trkB receptors have been demonstrated on reactive astrocytes in the cerebral cortex in response to chronic injury simulated by a nitrocellulose filter implant (McKeon et al. 1997). In contrast, other studies have reported exclusively expression of truncated trkB receptors by glial cells after brain injury and most notably, the expression of full-length trkB by astrocytes has not been observed in *in vivo* models of either global (Ferrer et al. 1998a) or focal (Ferrer et al. 2001) ischemia. This variation in astrocytic response has been suggested to relate to the type or region of brain injury (McKeon et al. 1997).

Translational regulation of trkB, such as has been observed in the current study as a result of PEM, has previously been reported in the dentate gyrus following kainic acid injection in the mouse (Inoue et al. 1998). The enhanced trkB response could indicate that PEM is magnifying the stress response to brain ischemia, since trkB is increased by stressors (Tapia-Arancibia et al. 2004) such as kainic acid administration (Goutan et al. 1998), experimental brain injury (Hicks et al. 1998), repeated immobilization stress (Nibuya et al. 1999), behavioral tasks (Croll et al.

1998), and long-term potentiation (Dragunow et al. 1997). This receptor can also be induced by increased glucocorticoid concentration or a glucocorticoid receptor agonist (Jeanneteau et al. 2008). An increase in serum glucocorticoids is characteristic of PEM (Monk et al. 2006), although the response does fluctuate with severity and acuteness of onset of the malnutrition (Pugliese. 1990). Although cortisol concentration was not measured in the current study, we were previously unable to detect an elevation by PEM at 6hr after global ischemia in the gerbil (Ji et al. 2008). However, since we had observed higher than normal physiological cortisol concentrations, preanaesthesia or surgical stress-induced inflation of basal cortisol levels may have masked an independent effect of PEM, as has been previously shown (Monk et al. 2006, Shipp and Woodward. 1998). Glucocorticoid response will be important to monitor in future studies, as higher levels of free urinary cortisol have been reported in protein-energy malnourished patients during the first week after stroke (Davalos et al. 1996).

While parallel data on GAP-43 mRNA were not obtained, the increase in GAP-43 protein in the CA1 region after global ischemia was magnified by PEM. Combined with the striking increase in GAP-43 in the CA3 and hilar regions, this suggests that PEM caused a more extensive and prolonged response to ischemic injury. Although an increase in GAP-43 after injury can represent a protective neuroplastic response, it has also been suggested as an indicator of pathophysiology underlying the formation of post-ischemic seizure activity (Schmidt-Kastner et al. 1997, Tagaya et al. 1995). Late-onset epilepsy associated with brain ischemia is believed to result from long-term hyperexcitability in the hippocampus. The latter has been associated with a reduction in GABAergic interneurons and synaptic transmission and an increase in glutamatergic terminals and transmission in the CA3 region several months following global ischemia (Epsztein et al. 2006). Enhanced GAP-43 and trkB expression have been associated with detrimental hyperexcitability. In a hippocampal slice model of injury in which excessive sprouting causes hyperexcitability (McKinney et al. 1997), elevated GAP-43 protein in sprouting CA3 pyramidal cell axons was preceded by increased BDNF and trkB protein (Dinocourt et al. 2006). An impaired response in slices from trkB knock-down mice suggests that this axonal sprouting is induced by trkB signaling and subsequent to upregulation of GAP-43 (Dinocourt et al. 2006). While likely a repair response, excessive sprouting may alter the normal ratio of excitatory to inhibitory synapses, resulting in over-excitability (Diaz-Cintra et al. 2007). Although the protein-energy malnourished gerbils in our study showed no seizures, they were

observed only up to 7d after global ischemia. The enhanced hippocampal expression of trkB and GAP-43 may be indicative of developing hyperexcitability.

Alterations in both inhibitory and excitatory circuits have been reported with protein malnutrition imposed during hippocampal development in numerous studies, including the recent ones of Diaz-Cintra et al. (2007) and Chang et al. (2003); the findings vary depending on developmental stage of exposure to malnutrition. That nutritional status during adulthood can also independently affect functioning of hippocampal circuitry is suggested by one report of low protein feeding in the rat causing decreases in cholinergic innervation of the hippocampus and hippocampal cholinergic and GABAergic neurons (Andrade and Paula-Barbosa. 1996). The specific influence of nutrition remains to be established, however, since control and low protein diets were frequently not matched for other nutrients in the developmental studies, and the adult study did not provide convincing evidence of protein deficiency. Future research should utilize well-characterized nutritional paradigms to investigate whether PEM affects the ratio of excitatory to inhibitory synapses and electrophysiological recordings following global ischemia. These mechanisms could contribute to the impaired habituation in the open field previously reported in protein-energy malnourished gerbils following global ischemia (Bobyn et al. 2005). As shown here and in a previous study (Bobyn et al. 2005), this functional impairment is not explained by a greater loss of CA1 neurons detectable by a basic histological analysis. This suggests that there are functional abnormalities in the remaining viable CA1 neuron population or in other hippocampal subregions. The current data suggest that PEM could induce abnormalities in structure, function, and plasticity of hippocampal fibres.

The robust GAP-43 response observed in the protein-energy malnourished gerbils might also reflect an enhanced inflammatory response to global ischemia. While this is supported by our previous findings of PEM increasing hippocampal NFκB activation after global ischemia (Ji et al. 2008), it is yet to be confirmed if there is also enhanced expression of the pro-inflammatory target genes that are regulated by NFκB and important in stroke pathophysiology. GAP-43 can be induced by inflammation in the central nervous system caused by lipopolysaccharide application to the motor cortex (Hossain-Ibrahim et al. 2006) or experimental autoimmune encephalomyelitis (Kerschensteiner et al. 2004), but a relationship between GAP-43 and inflammation after brain ischemia has not been explored.

There are limitations to the methodology used for quantification of the in-situ hybridization and immunofluorescence signals observed in this study. Sample size and inter-animal differences likely contributed to the large degree of variability noted in some cases. Given the large changes in trkB and GAP-43 protein expression observed by immunohistochemical analysis, undertaking more animal experiments to generate hippocampal tissue for quantification by western blot analysis may have strengthened the conclusions for the protein data. This approach is not a simple undertaking, however, since this study involved a long period of pre-surgical feeding, followed by sham or ischemia surgery and multiple post-surgical sampling points. A strength of the conclusions that have been drawn, however, is that they are based on quantitative densitometric analyses that were generally in agreement with what was observed by qualitative assessment of the slides.

In summary, our results demonstrate that PEM intensifies the expression of trkB and GAP-43 protein in the hippocampus following global ischemia. While there are likely multiple contributing mechanisms, these findings may be indicative of both an increased stress response and hyperexcitability in the hippocampal circuitry. Thus, nutritional care appears to be an important but often ignored component of therapy after brain ischemia that may have powerful effects on mechanisms key for optimal recovery. The influence of nutritional status on hippocampal hyperexcitability and the potential for the development of post-stroke epilepsy is an interesting question that also warrants investigation.

CHAPTER 6

DEVELOPMENT OF THE RAT 2-VESSEL OCCLUSION MODEL OF GLOBAL ISCHEMIA

6.1 Rationale

Previous experiments in our laboratory utilized the gerbil model of global ischemia induced by bilateral common carotid artery occlusion (BCCAO). Since this model was no longer reliable as discussed in Chapter 2, section 2.3.2.3.4 of this thesis, an alternate model of global ischemia, the 2-vessel occlusion model with hypotension (2-VO) in the Sprague-Dawley rat, needed to be adopted. Another Ph.D. student in our laboratory (Shari Smith) and I were responsible for developing and running this model. Due to the complexity of the model, we worked as co-surgeons. The methodology is based on that of Smith et al. (1984b) as modified by Dr. Fred Colbourne's research group at the University of Alberta (Arvanitidis et al. 2009). To learn the procedures involved in this model, we visited the laboratory of Dr. Fred Colbourne at the University of Alberta. New techniques required for this model included anaesthetic set-up using isoflurane in a N_2O and O_2 mixture, artery and vein cannulations, blood pressure regulation, blood gas analysis, and skull temperature regulation. Learning of the surgical technique for implantation into the peritoneal cavity of small, sterilized bio-electrical sensor transmitters encapsulated in biocompatible silicone (*SubCue*™ dataloggers) was also required. These dataloggers were utilized for chronic core temperature monitoring during both the pre- and post-ischemic periods (results to be reported in the thesis of Shari Smith). Once the basic methodology was transferred to our laboratory, the focus moved to the most important determinants of consistent hippocampal injury. These factors needed to be controlled and adjusted as needed for the laboratory equipment used.

Brain temperature is an essential variable to measure and control in models of brain ischemia since hypothermia has been found to protect against ischemia-induced damage (Colbourne and Corbett. 1995, Nurse and Corbett. 1994), while hyperthermia worsens damage

(Minamisawa et al. 1990). Both intra- and post-ischemic hypothermia have been shown to provide neuroprotection after global ischemia in terms of CA1 neuronal survival (Colbourne and Corbett. 1995, MacLellan et al. 2009, Nurse and Corbett. 1994). Hyperthermia increases CA1 and subiculum damage and causes damage in other brain regions not normally affected by global ischemia (Minamisawa et al. 1990). Both skull and tympanic temperature have been measured as the basis for estimating and controlling brain temperature during the ischemic period. Since temperature changes following ischemia can also alter the extent of brain damage, monitoring post-ischemic temperature is ideal. For this purpose, implanted telemetry probes or bioelectric sensors allow for repeated readings to be taken and avoid the stress of rectal temperature monitoring (MacLellan et al. 2009).

Length of carotid artery occlusion time was important to consider since brain damage is affected by this variable. Smith et al. (1984a) used a range of 2-10min occlusion times in the 2-VO with hypotension model of global ischemia and assessed brain damage at 7d following ischemia. Mild damage was found in the CA1, hilar, and subiculum regions after 2min occlusion, and this was more consistent after 4min. Neocortex showed damage after 4min, while the CA3 region showed cell death following 6min occlusion. Damage was found in the caudoputamun region after 8-10min of occlusion (Smith et al. 1984a). Variability in brain damage due to different occlusion times may be further complicated by other factors including brain temperature (which was not controlled nor monitored in the Smith et al. (1984a) study), rat strain (Iwasaki et al. 1995) and size/age of rat (Sutherland et al. 1996). A study by Clark et al. (2007) compared 8, 9, and 10min carotid occlusion times in the rat 2-VO model in combination with blood pressure at 35-45mmHg with brain temperature maintained at approximately 37°C. All three occlusion times resulted in significant hippocampal CA1 neuronal loss, but there was no difference among groups (Clark et al. 2007).

The 2-VO model also requires the monitoring and control of several important physiological variables that if out of the desired range, have the potential to significantly reduce the consistency of hippocampal CA1 neuronal death. In order to monitor acid-base balance, arterial blood pH, pO_2 and pCO_2 can be measured in the 2-VO model. Arterial samples have been shown to detect changes in ventilation rapidly (Andrews et al. 1994) and therefore these measurements can be used to detect potential respiratory acidosis. In cases in which a ventilator is not used to assist breathing while the animal is under anesthesia, this is especially important

and allows the investigator to take appropriate actions such as adjusting anaesthetic. While ischemia has been shown to cause a decrease in brain tissue pH, acidosis itself increases brain damage following stroke (Huang and McNamara. 2004). Acidosis is thought to promote free radical formation and activate pH-dependent endonucleases (Anderson et al. 1999) as well as lead to increased intracellular Ca^{2+} via activation of acid sensing ion channels (ASIC), contributing to neuronal death (Huang and McNamara. 2004). In focal stroke, acidosis has been found to lead penumbral tissue into infarction, thereby increasing injury (de Courten-Myers et al. 1992). The goal with pO_2 in animal stroke models is to achieve saturation (>100%) to ensure adequate oxygenation while avoiding excess, which can generate more free radicals and oxidative stress than would normally be present under brain ischemia conditions. Hematocrit is an important parameter to measure as an indicator of the oxygen-carrying capacity of the blood (Kiyohara et al. 1985). While low hematocrit may indicate inadequate oxygenation of tissues, high hematocrit can also be detrimental during brain ischemia. A high hematocrit level is indicative of increased blood viscosity and therefore decreased cerebral blood flow. This decreased blood flow supersedes the increased oxygen content of the blood and actually results in less oxygen being delivered to the brain (Kiyohara et al. 1985). With less blood flow comes less oxygen transport, contributing to brain damage.

Blood glucose concentration is important to monitor and control as hyperglycemia has been shown to exacerbate brain injury if present at the time of induction of global ischemia. Hyperglycemia causes decreased cerebral blood flow and increases lactic acid and mitochondrial dysfunction**,** leading to brain acidosis (de Courten-Myers et al. 1992). High glucose levels also cause increased extracellular glutamate leading to increased inflammation and oxidative stress (de Courten-Myers et al. 1992). Fasting has been found to minimize, though not entirely eliminate, variability in blood glucose concentration between animals (Ginsberg and Busto. 1989).

After considering these variables and learning the surgical techniques required for the 2-VO model, it was necessary to first undertake pilot studies in order to ensure the injury to the CA1 region of the hippocampus was consistent and at the desired level. The expected timeframe of injury from the 2-VO model is similar to that seen in the gerbil, that is, delayed CA1 neuronal death beginning ~3d post-ischemia with the majority of it complete by 7d (Small and Buchan. 2000). Neuronal death was examined at 7 or 16d post-ischemia in these pilot studies, allowing

sufficient time for CA1 neuronal death to develop. A secondary objective in the pilot studies was to investigate the extent to which food intake and growth were impaired by global ischemia and to determine how many days were required for these parameters to normalize.

6.2 Pilot Study 1

6.2.1 Materials and Methods

6.2.1.1 Animals and Surgical Procedure

Male Sprague-Dawley rats (11-18wk) were housed individually and allowed *ad libitum* access to rat chow and water. Prior to surgery, animals were fasted for 12-18hr in order to bring blood glucose values within a consistent range. Animals weighed 412-605g at the time of surgery. Rats in the global ischemia group (n = 10) were anaesthetized using 4% isoflurane for induction and ~2.5% for maintenance in 60% N_2O and 40% O_2. A thermocouple probe was inserted subcutaneously and placed against the skull, and temperature was maintained at 37.0 ± 0.2°C via a heat lamp with a 250W infrared bulb. The heat lamp was automated to turn on and off in response to signals from a temperature controller (OMEGA CN9500), which received feedback from the thermocouple probe. A ventral midline incision was made in the neck region for isolation of both common carotid arteries. A separate incision was then made through which the jugular vein was isolated and cannulated. A catheter attached to a blood pressure transducer and monitor was inserted into the tail artery and a pre-ischemic blood sample (100µl) was taken for measurement of blood gases, hematocrit and glucose. Target ranges for these parameters were as follows: pH 7.25-7.45, pCO_2 35-45mmHg, pO_2 125-135mmHg, glucose 4-8mmol/L (webpage of Dr. Fred Colbourne). Normal hematocrit values for the rat range between 35-57% (Sharp. 1998), the large range reflecting differences in strain and age. Hematocrit values for 15wk old male Sprague-Dawley rats have been reported to be ~40% (Probst et al. 2006). Blood was extracted via a syringe attached to the jugular catheter in order to decrease blood pressure to 35mmHg. At this point, microaneurysm clips (S&T Vascular Clamps 3V, Fine Science Tools) were used to occlude the carotid arteries for 8min. The goal was to maintain blood pressure at 37-43mmHg throughout the 8min ischemic period by withdrawing or re-infusing blood. Blood pressure and skull temperature were manually recorded each minute. After 8min, the clips were removed and reperfusion was visually verified. Blood was slowly re-infused into the jugular vein to restore normal blood pressure. A final post-ischemic arterial blood sample was taken to

determine blood gas, hematocrit, and glucose values. Vessels were then tied off, and incisions sutured. Marcaine (Bupivacaine) (2mg/kg) was applied at all three incision sites. Sham surgeries (n=3) were identical to global ischemia surgeries except hypotension was not induced and carotid arteries were not occluded. Animals were carefully monitored following surgery for signs of pain or seizures. Following surgery, animals were caged individually. Body weight and food intake were recorded daily until 7d following surgery**,** at which time animals were euthanized.

6.2.1.2 Hippocampal CA1 Histology

At 7d following surgery, rats were deeply anaesthetized with 5% isoflurane (100% O_2) and humanely killed via transcardial perfusion with heparinized saline followed by 10% phosphate-buffered formalin. Heads were removed and kept in formalin at 4°C overnight at which time brains were removed and stored in formalin at 4°C until processing. Paraffin-embedded brains were sectioned at 6µm and stained with hematoxylin and eosin (H&E). Brain sections approximately at coordinate -3.8mm relative to bregma, were examined at 400x magnification. Hippocampal CA1 cell counts were not performed on these sections as it was apparent that very little to no damage had occurred and hippocampal CA1 viability was similar to that of sham animals.

6.2.2 Results

6.2.2.1 Food Intake and Body Weight

A summary of post-ischemic food intake and body weight results beginning at surgery day (d0) and ending on perfusion day (d7) is shown in Table 6.1. A high degree of variability was present for both variables between animals and between days. Body weight on d0 was greater in sham animals and thus the two groups were not matched at baseline. Although body weight did decrease slightly in the 2d following surgery, in general, there were not large decreases in either food intake or body weight caused by 2-VO surgery in this study.

6.2.2.2 Physiological Variables

Table 6.2 shows mean (±SEM) pre- and post-ischemic physiological variables as well as the range. Since skull temperature and blood pressure were recorded each minute during the 8min occlusion period, the range shown includes both the between rat and between minute range

Table 6.1. Post-ischemic food intake and body weight for pilot study 1

Day	Food Intake		Body Weight	
	Sham (n=3)	Ischemic (n=10)	Sham (n=3)	Ischemic (n=10)
0	-	-	583.5 ± 14.3	514.5 ± 21.4
1	26.0 ± 0.6	24.8 ± 2.5	590.1 ± 14.0	512.0 ± 19.6
2	24.7 ± 1.9	20.5 ± 3.2	586.3 ± 12.0	510.6 ± 18.6
3	36.0 ± 9.0	26.6 ± 2.3	588.6 ± 13.9	513.0 ± 18.0
4	20.0 ± 8.2	26.8 ± 2.2	592.5 ± 9.2	516.1 ± 17.7
5	26.6 ± 0.9	29.7 ± 2.2	597.2 ± 9.4	522.2 ± 16.6
6	31.6 ± 4.9	26.1 ± 1.2	612.1 ± 15.3	528.3 ± 15.4
7	26.1 ± 1.3	28.3 ± 1.9	605.3 ± 11.2	532.6 ± 15.5

Values are expressed as mean (±SEM).

Table 6.2. Pre and post-ischemic physiological values for pilot study 1

	Global Ischemia (n=10)		Sham (n=3)	
	Pre-Ischemia	Post- Ischemia	Pre-Ischemia	Post-Ischemia
pH†	7.41 ± 0.01 (7.38-7.49)	7.28 ± 0.04 (7.05-7.53)	7.41 ± 0.04 (7.38-7.48)	7.37 ± 0.04 (7.32-7.43)
pCO_2 (mmHg)†	42 ± 1 (36-48)	54 ± 6 (23-94)	37 ± 3 (32-44)	46 ± 3 (42-52)
pO_2 (mmHg)†	154 ± 11 (91-216)	145 ± 9 (101-181)	181 ± 19 (144-206)	168 ± 13 (143-183)
Hematocrit (%)†	48 ± 1 (43-52)	41 ± 1 (37-46)	44 ± 2 (40-48)	51 ± 5 (44-61)
Blood Glucose† (mmol/L)	11.7 ± 0.6 (8.5-14.9)	9.8 ± 0.8 (6.3-14.9)	11.1 ± 0.7 (10.3-12.5)	12.0 ± 0.4 (11.1-12.4)
Skull Temperature (°C)	36.9 ± 0.03 (36.5-37.2)		37.0 ± 0.06 (36.7-37.2)	
Blood Pressure (mmHg)	40 ± 0.3 (35-48)		89 ± 4 (78-99)	

Values are mean (±SEM) (range). †Measured on arterial blood.

of values. In the pre-ischemic measurements, the mean values for pH and pCO_2 for both experimental groups were within the desired range, whereas the average pO_2 value was higher than the desired range for this ischemic model (125-135mmHg). However, values for some individual rats were out of target range. This included 3/10 rats (2 ischemic and 1 sham) for pH (all had higher than desired values) and pCO_2 (1↓ and 2↑) and 12/13 (9 ischemic and all 3 shams) for pO_2 (2↓ and 10↑). All individual values for both experimental treatments fell within the expected range for hematocrit, whereas the group means and all individual values were higher than the desired range for blood glucose.

In the post-ischemic measurements, average pH was within range for both experimental groups. Average pCO_2 and pO_2 values were higher than the desired range for both groups. Values for several individual animals were out of target range. This included 5/10 ischemic animals for pH (1↑ and 4↓), 8 animals (7 ischemic and 1 sham) for pCO_2 (8↑ and 1↓), and 10 animals (8 ischemic and 2 sham) for pO_2 (7↑ and 3↓). Average and individual post-ischemic hematocrit values were all within range with the exception of one sham animal (↑). Eight of the 10 ischemic animals had higher than desired blood glucose values as did all three shams and both average values were higher than desired. Average skull temperature over the 8min occlusion period was lower than the target range (36.8-37.2°C) for both ischemic and sham groups. Four individual ischemic animals and 1 sham had lower than the target temperature range for 3-5min during the occlusion period. Average blood pressure in the ischemic group fell within the desired range (37-43mmHg); however, 7 animals fell out of range for 1min (5↓ and 1↑) and 1 had a higher than desired blood pressure for 2min.

6.2.2.3 Hippocampal CA1 Neuron Death

Based on the morphology observed on H&E staining, 7 of the 10 animals exposed to the global ischemia protocol appeared to have no hippocampal CA1 damage; the CA1 neurons appeared similar to those of sham animals. The other 3 rats had unilateral damage confined to hippocampal CA1 neuron death in either the right or left hemisphere. Photographs representative of each of these subcategories are included below as part of the results of the third pilot study.

6.2.3 Conclusions

There was a high degree of variability in food intake and body weight between animals and between days following surgery. Although ischemic animals decreased in body weight in the first 2d, which would be expected following an ischemic insult, this decrease was minimal. The mismatch of body weight at baseline between sham and ischemic groups was likely due to the small sample size in the sham group and the large range of weights of animals used in this study. Food intake was slightly decreased on d2 in both groups, suggesting an effect of surgery in general; however this decrease was greater in the ischemic group.

The 2-VO protocol used to induce forebrain ischemia did not result in the desired extensive hippocampal CA1 neuron death. Several factors were considered to explain these findings. One hypothesis was that the microaneurysm clips may have not been placed correctly on the arteries or that they did not exert enough pressure to completely occlude the arteries. Magnifying loops and a larger size of microaneurysm clip were planned for future studies in order to ensure complete occlusion of the carotid arteries. Time was also spent re-calibrating equipment, including the temperature feedback system and the blood pressure transducer, in order to ensure that these critical instruments were controlling for the narrow ranges required; no problems were identified with the equipment. Since outliers for skull temperature and blood pressure were evident during the 8min occlusion period, these parameters needed to be improved upon; inconsistency in these crucial variables may have contributed to the lack of hippocampal damage. As well, the decision was made to change to the more accurate estimate of brain temperature, tympanic temperature (Sutherland et al. 1992), instead of skull temperature. The out of range individual values for pCO_2 (generally↑) and pH (generally↓), indicative of respiratory acidosis, may be due to the long period of time animals were under anaesthesia (average 2hr for ischemic rats and 1.5hr for shams). Since pO_2 values from the first pilot study were higher than desired, it was concluded that anesthesia should be adjusted to isoflurane in 70% N_2O and 30% O_2 for the second pilot study. As it is another important determinant of injury from global ischemia, an increase in occlusion time was also planned for the next pilot study.

The high blood glucose values for almost all animals in this study, while not considered hyperglycemic for the rat and therefore not a key factor in determining outcome from ischemia, was another issue that needed to be dealt with. Animals in pilot study 1 had been fasted for an average of 16hr and therefore it was believed that an increase in fasting period was not required.

Rather, it was decided to change the bedding in the cage at the beginning of the fasting period in order to avoid very small pieces of food pellet or pellet dust, that while not visible, might be present and potentially interfering with the fast.

It was also decided to group house the animals in the next pilot study in order to mimic the planned experiment.

6.3 Pilot Study 2

6.3.1 Materials and Methods

6.3.1.1 Animals and Surgical Procedure

Eight male Sprague-Dawley rats (11-12wk) were used for this study. Animals were housed in pairs and allowed *ad libitum* access to rat chow and water. The 2-VO surgical procedure followed the same protocol as discussed in pilot study 1 with the following modifications: 1) carotid artery occlusion time was increased to 10min, 2) tympanic temperature replaced skull temperature as an indicator of brain temperature, 3) for anaesthesia, isoflurane in 60% N_2O and 40% O_2 was adjusted to 70% N_2O and 30% O_2**,** 4) larger microaneurysm clips were used (S&T Vascular Clamps HD-S, Fine Science Tools). These animals weighed 372-418g at the time of surgery, which is a smaller range than used in the first pilot study. This unintentional change reflects the shorter period of time these animals spent in the animal facility due to the greater availability of the surgical room. Six animals underwent ischemia surgery and two underwent sham surgery. Post-surgery, animals were housed individually for 1d to allow wound recovery, and then housed in pairs until euthanasia.

6.3.1.2 Hippocampal CA1 Histology

A survival time point of 16d was chosen for this second pilot study in order to test the survival time planned for the upcoming experimental study and mimic a study (Langdon et al. 2007) in which the short- and long-term glial response had been established in the 2-VO model. This allowed us to add the secondary objective to this study of testing some glial immunohistochemical protocols important to my co-worker's next study. Rats were deeply anaesthetized 16d following surgery with 5% isoflurane and transcardially perfused with 4% paraformaldehyde**.** Processing of the brains was modified from the first pilot study to be optimal for the accompanying immunohistochemistry. Heads were kept overnight in 4%

paraformaldehyde at 4°C at which time brains were removed and then kept in 4% paraformaldehyde at 4°C overnight. Brains were then transferred to 20% sucrose at 4°C until saturated. Brains were frozen in OCT (optimal cutting temperature) compound using dry ice-cooled isopentane and kept at -20°C until sectioning. Rostral hippocampal sections were taken at approximately the same coordinate as indicated in pilot study 1. These sections were 14μm thick and stained with cresyl violet (in place of hematoxylin and eosin used in the previous study) to obtain good staining results on frozen sections (Langdon et al. 2007). CA1 cell counts were not performed as it was again apparent that little to no damage had occurred.

6.3.2 Results

6.3.2.1 Food Intake and Body Weight

A summary of post-ischemic food intake and body weight results beginning at surgery day (d0) and ending on perfusion day (d16) is shown in Table 6.3. On average, ischemic animals showed a decrease in body weight on d1 and seemed to recover weight gain by d2. Some variability was present between animals as well as between days as some individual rats took longer to recover, while some did not decrease body weight at all. The recovery of weight gain was not to the same degree as that of shams. By d16, it was evident that on average, ischemic animals had a slower growth rate (18% gain as compared to baseline) as compared to sham animals (27% gain as compared to baseline). Average food intake was reduced on d1 in ischemic animals as compared to shams, but also recovered by d2; only one ischemic animal did not show a decrease in food intake.

6.3.2.2 Physiological Variables

Table 6.4 shows mean (±SEM) pre and post-ischemic physiological variables, as well as the range for individual animals. The range for tympanic temperature and blood pressure includes both the between rat and between minute range of values. Pre-ischemic average values were within the desired range for pH for the ischemic group and pCO_2 for both experimental groups. Mean pO_2 values were higher than desired in both groups. Several individual values were out of target range including 4 animals (3 ischemic and 1 sham) for pH (all↑), 3 ischemic animals for pCO_2 (2↓ and 1↑), and all animals with the exception of 1 sham for pO_2 (all↑). All

individual values fell within the desired range for hematocrit, while mean values and all but 2 individual values (1 ischemic and 1 sham) were higher than desired for glucose.

Table 6.3. Post-ischemic food intake and body weight for pilot study 2

Day	Food Intake		Body Weight	
	Sham (n=2)	Ischemic (n=6)	Sham (n=2)	Ischemic (n=6)
0	-	-	410.2 ± 1.3	395.0 ± 6.2
1	30.7 ± 0.5	18.6 ± 3.5	434.2 ± 6.3	387.1 ± 7.5
2	30.0	29.3 ± 1.2	436.5 ± 3.6	397.7 ± 7.0
3	27.4	28.3 ± 3.4	441.8 ± 5.5	401.2 ± 7.5
4	29.1	27.9 ± 1.4	448.2 ± 4.7	406.4 ± 7.3
5	32.8	31.4 ± 1.3	-	413.3 ± 6.2
6	27.6	28.2 ± 2.8	463.9 ± 3.2	411.0 ± 7.2
7	26.1	24.1 ± 3.4	467.0 ± 0.9	422.8 ± 6.9
8	33.7	28.3 ± 3.9	479.2 ± 2.8	425.6 ±6.8
9	24.0	27.0 ± 3.9	482.7 ± 2.6	435.7 ± 7.1
10	28.0	28.9 ± 1.0	486.6 ± 4.5	440.3 ± 7.3
11	29.6	31.1 ± 1.7	488.9 ± 8.1	437.8 ± 7.2
12	33.6	27.9 ± 1.1	483.9 ± 8.5	447.5 ± 7.3
13	26.7	32.7 ± 1.6	500.2 ± 9.6	449.2 ± 6.5
14	28.3	25.6 ± 2.8	504.7 ± 7.2	456.2 ± 7.0
15	30.4	24.5 ± 3.7	513.8 ± 8.2	461.5 ± 7.9
16	29.5	30.2 ± 3.3	519.8 ± 7.0	466.8 ± 8.0

Values are expressed as mean (±SEM). Food intake is based on individual data on d1 and cage data beginning at d2. The 2 sham animals were housed together and therefore there is no SEM beginning at d2.

Post-ischemic average pH and pCO_2 values were within the desired range for both experimental groups. The mean pO_2 value was higher than desired in the ischemic group and within range for the sham group. Individual values that fell out of range included 1 animal from

each group for pH (ischemic animal was ↑, sham animal was ↑), 2 animals in the ischemic group for pCO_2 (both ↑), and all but 1 ischemic animal (5 ischemics and 2 shams) for pO_2 (all ↑ with the exception of 1 sham). The sham group consisted of 1 rat with a higher than desired value and 1 rat with a lower than desired value, which caused the mean value to fall within range. All individual values fell within the desired range for hematocrit. All but 2 animals (1 ischemic and 1 sham) had higher than desired glucose values.

Table 6.4. Pre and post-ischemic physiological values for pilot study 2

	Global Ischemia (n=6)		Sham (n=2)	
	Pre-Ischemia	Post-Ischemia	Pre-Ischemia	Post-Ischemia
pH†	7.45 ± 0.01 (7.39-7.49)	7.36 ± 0.03 (7.24-7.45)	7.46 ± 0.04 (7.42-7.5)	7.43 ± 0.06 (7.37-7.48)
pCO_2 (mmHg)†	36 ± 2 (30-46)	44 ± 3 (37-58)	38 ± 4 (34-42)	40 ± 6 (34-45)
pO_2 (mmHg)†	162 ± 7 (138-178)	149 ± 6 (128-166)	147 ± 19 (128-165)	127 ± 10 (117-136)
Hematocrit (%)†	45 ± 1 (41-47)	41 ± 1 (38-45)	47 ± 1 (46-47)	47 ± 2 (45-49)
Blood Glucose (mmol/L)†	9.4 ± 0.7 (7.3-12.5)	9.7 ± 1 (6.3-13.6)	8.8 ± 0.9 (7.9-9.6)	8.1 ± 1 (7.1-9.0)
Tympanic Temperature (°C)	37.0 ± 0.02 (36.7-37.2)		37.1 ± 0.00 (37.0-37.3)	
Blood Pressure (mmHg)	41 ± 0.2 (35-49)		78 ± 2 (74-82)	

Values are expressed as mean (±SEM) (range). †Measured on arterial blood.

Tympanic temperature regulation was very tight in this pilot study with only 1 animal from each group falling out of range (36.8-37.2°C) for 1min. Six ischemic animals fell out of blood pressure range (37-43mmHg) for 1min.

6.3.2.3 Hippocampal CA1 Neuron Death

Histological results indicated that the changes made in pilot study 2 helped to increase the number of animals with CA1 hippocampal damage. Three (50%) of the 6 rats that underwent 2-

VO surgery had severe unilateral damage, one rat had minimal unilateral damage, and two appeared to have no damage with an appearance similar to shams.

6.3.3 Conclusions

Variability in both food intake and body weight was less than that seen in pilot study 1 due to the smaller range of initial body weight used in this study. While some degree of variability was still present in this study, on average, both variables were decreased by ischemia on d1 and seemed to recover by d2. However, the sustained slower growth rate in the ischemic groups may be related to the more severe hippocampal injury than that which was obtained in pilot study 1.

The results of pilot study 2 showed improvement over those obtained in the first pilot study; however the degree of CA1 hippocampal damage was still not consistent nor severe enough. In order to determine which factors should be changed for the third pilot study, we conducted further literature review and further compared the existing protocol with details from the publications on the 2-VO model out of the laboratories of Dr. Fred Colbourne (University of Alberta) (Arvanitidis et al. 2009) and Dr. Dale Corbett (Memorial University) laboratories (Langdon et al. 2007). Both laboratories have recently adopted this model and are obtaining quite consistent results (~80% CA1 neuron death in 80% of animals [Dr. Fred Colbourne, unpublished communication]).

The most significant difference between our protocol and theirs was the size of rat utilized. These groups have had success with the 2-VO model of global ischemia when using smaller rats weighing approximately 300g. A study in Dr. Fred Colbourne's laboratory in which investigators attempted to perform the 2-VO model on obese rats (600-1000g) had to be aborted due to unsuccessful CA1 damage (unpublished data). In fact, aged rats would be ideal for our experiments, given the research objectives; since this is difficult with global ischemia models our laboratory had originally chosen adolescent aged rats (10-12wk, ~400g). However, since this no longer appeared to be a feasible goal, it was concluded that smaller rats (~300g) would have to be employed.

Since pO_2 values were somewhat high, it was decided to decrease oxygen flow just slightly below 30% when indicated by pre-ischemic blood gas readings for the next pilot study. Post-ischemic pH and pCO_2 were greatly improved upon from pilot study 1, perhaps due to the

shorter surgery time (average 100min for ischemics, 70min for shams). A goal of reducing this time even more was set for pilot study 3. Although the range of blood glucose levels were slightly improved from pilot study 1, most animals still had high values indicating that a longer fasting period may be required.

It was also decided to decrease the target blood pressure range further to 35-40mmHg (from 37-43mmHg) and increase the target tympanic temperature to 37.5 ± 0.2°C (from 37.0 ± 0.2°C) with the goal of producing more severe damage to the CA1 hippocampal region. Carotid artery occlusion times of both 8 and 10min would be included in the third pilot study, since it was possible that a 10min occlusion would produce too severe damage in the presence of the additional changes. Another suggestion from the Colbourne laboratory was that the length of time under anaesthesia may have been contributing to the lack of brain damage and so as mentioned previously, it was our goal to decrease this time with further surgical refinements and practice.

6.4 Pilot Study 3

6.4.1 Materials and Methods

6.4.1.1 Animals and Surgical Procedure

Male Sprague-Dawley rats (7wk), housed in groups of 2-3, were acclimatized on rat chow for 3d and then placed on the control diet planned for use in future studies in order to more closely mimic planned experimental procedures (details of the diet are shown in Table 7.1 in Chapter 7 of this thesis). Surgeries were performed on rats weighing 273-339g. Rats were anaesthetized with isoflurane (70% N_2O and 30% O_2) and then underwent ischemia surgery (n=6 for each of 8min and 10min occlusion time groups) or sham surgery (n=2). A nonsurgical control group (n=2) was also included in order to ensure the hippocampal morphology of the sham animals was not being affected by the surgical procedure. The 2-VO procedure followed the protocol previously described with the incorporation of the following changes: 1) smaller rats weighing ~300g were utilized, 2) the goal for blood pressure maintenance was 35-40mmHg during the occlusion period, 3) the goal for tympanic temperature maintenance was 37.5 ± 0.2°C, and 4) oxygen flow was slightly decreased to below 30% when indicated by excessively high pre-surgery pO_2 readings. Arterial blood samples (100µL) were taken several times prior to the induction of ischemia to ensure the pO_2 values fell within the desired range of 125-135mmHg.

Following surgery, animals continued on the control diet and were individually housed for the first 24-48hr. Rats were then group housed with their original cage mates and then euthanized 7d later.

6.4.1.2 Hippocampal CA1 Histology

A survival time point of 7d was chosen in this study since the majority of CA1 neuronal death occurs by this time. The processing of brains was identical to that performed in pilot study 2. Cresyl violet staining was again performed on 14μm thick sections. CA1 cell counts were performed on hippocampal sections at approximately -3.8mm from bregma. Using a 200μm square (10x10) microscope grid, viable cells with a distinct nucleus and an intact cellular membrane were counted in middle, medial, and lateral sectors (sector length 0.2mm) of the CA1 region.

6.4.2 Results

6.4.2.1 Food Intake and Body Weight

A summary of post-ischemic food intake and body weight results beginning at surgery day (d0) and ending on perfusion day (d7) is shown in Table 6.5. Both ischemic groups decreased food intake following ischemia, which on average seemed to recover by d5 in both ischemic groups as compared to the sham group. Some variability was present between animals as well as between days since some animals did not decrease food intake while some took longer to recover. Average body weight was also decreased in both ischemic groups and appeared to recover to baseline by d2 in the 8min occlusion group and d3 in the 10min group. By d7 sham animals had gained 21% of their body weight as compared to baseline, whereas the 8min ischemic group gained 15% and the 10min group gained 13%, indicating a slower growth rate following true ischemia. Again, some variability in body weight was present between animals as well as between days as some individual rats took longer to recover, while some did not decrease body weight at all.

Table 6.5. Post-ischemic food intake and body weight for pilot study 3

Day	Food Intake			Body Weight		
	Sham (n=2)	Ischemic (8min) (n=6)	Ischemic (10min) (n=6)	Sham (n=2)	Ischemic (8min) (n=6)	Ischemic (10min) (n=6)
0	-	-	-	286.6 ± 5.7	315.7 ± 8.6	298.0 ± 7.6
1	27.8 ± 0.5	12.8 ± 2.4	5.6 ± 1.2	303.3 ± 2.2	308.1 ± 11.7	276.7 ± 8.4
2	28.6	21.5 ± 2.6	14.4 ± 3.7	309.2 ± 5.7	320.3 ± 12.7	286.6 ± 9.5
3	28.9	20.0 ± 2.8	19.8 ± 3.5	316.7 ± 5.2	330.5 ± 12.1	291.7 ± 11.2
4	25.3	21.2 ± 2.2	20.5 ± 4.8	332.2 ± 6.2	323.8 ± 22.8	303.0 ± 11.2
5	24.2	24.2 ± 1.7	28.9 ± 0.7	336.8 ± 8.5	344.9 ± 17.8	317.8 ± 9.6
6	23.2	24.8 ± 1.6	25.9 ± 0.6	342.6 ± 8.4	354.4 ± 11.3	329.1 ± 8.8
7	21.8	25.2 ± 0.9	27.6 ± 2.1	347.4 ± 10.4	363.0 ± 11.1	338.6 ± 8.8

Values are expressed as mean (±SEM). Food intake is based on individual data on d1 and cage data beginning at d2. The 2 sham animals were housed together therefore there is no SEM beginning at d2.

6.4.2.2 Physiological Variables

Table 6.6 shows average pre and post-ischemic physiological variables, as well as the range. The range for tympanic temperature and blood pressure includes both the between rat and between minute range of values. In the pre-ischemic measurements, the mean values for pH fell within range for both ischemic groups and the sham group while the mean value for pCO_2 fell out of range for the 8min occlusion group only. Mean pO_2 values fell out of range for all three experimental groups. Only one individual value fell out of range for pH (↑), coming from the 10min occlusion group. Four animals (3 from the 8min occlusion group, 1 from the 10min occlusion group) fell out of the desired range for PCO_2 (all ↓). Eight animals (5 from the 8min occlusion group, 1 from the 10min occlusion group, and both shams) fell out of range for pO_2 (all↑). All individual and mean values fell within the expected range for hematocrit, as did mean values for glucose. Only 2 individual values (both from the 10min occlusion group) fell out of the desired range for glucose (both↑).

Table 6.6. Pre and post-ischemic physiological values for pilot study 3

	Global Ischemia 8min Occlusion (n=6)		Global Ischemia 10min Occlusion (n=6)		Sham (n=2)	
	Pre-Ischemia	Post-Ischemia	Pre-Ischemia	Post-Ischemia	Pre-Ischemia	Post-Ischemia
pH†	7.41 ± 0.02 (7.39-7.45)	7.35 ± 0.02 (7.28-7.39)	7.42 ± 0.02 (7.36-7.47)	7.32 ± 0.02 (7.25-7.36)	7.41 ± 0.01 (7.40-7.42)	7.40 ± 0.01 (7.39-7.40)
pCO_2 (mmHg)†	34 ± 2 (28-43)	39 ± 2 (33-44)	37 ± 2 (31-44)	41 ± 3 (34-52)	39 ± 0 (39)	38 ± 1 (37-39)
pO_2 (mmHg)†	142 ± 3 (131-153)	136 ± 5 (117-151)	141 ± 1 (134-143)	136 ± 10 (108-180)	146 ± 8 (139-154)	139 ± 8 (131-147)
Hematocrit (%)†	43 ± 2 (39-52)	43 ± 2 (38-50)	45 ± 2 (41-52)	41 ± 1 (39-42)	44 ± 3 (41-46)	44 ± 2 (37-39)
Blood Glucose (mmol/L)†	6.2 ± 0.3 (5.0-7.1)	6.1 ± 0.3 (5.3-7.0)	7.2 ± 0.6 (5.8-9.2)	7.0 ± 0.7 (5.4-9.1)	6.4 ± 0.1 (6.3-6.5)	6.5 ± 0.4 (6.1-6.9)
Tympanic Temperature (°C)	37.4 ± 0.03 (37.1-37.5)		37.4 ± 0.02 (37.1-37.7)		37.5 ± 0.01 (37.4-37.6)	
Blood Pressure (mmHg)	38.0 ± 0.2 (35-43)		38 ± 0.3 (33-44)		81 ± 4 (75-88)	

Values are expressed as mean (±SEM) (range). †Measured on arterial blood.

In the post-ischemic measurements, average values fell within range for pH and pCO_2 for all groups. Mean pO_2 values were higher than desired for all groups. All individual values for pH fell within the desired range. Only 3 animals (1 from the 8min occlusion group (↓), 2 from the 10min occlusion group (1↓ and 1↑) fell out of range for pCO_2. Nine individual values (4 from the 8min occlusion group, 3 from the 10min occlusion group, and both shams) fell out of range for pO_2 (all ↑, with the exception of one value from each ischemic group, which were ↓). All individual and mean values were within range for hematocrit as were mean values for glucose. Only 2 animals fell out of range for glucose (both↑).

Tympanic temperature was increased to 37.5 ± 0.2°C for this pilot study and 4 ischemic animals from the 8min occlusion group and 2 from the 10min group fell out of range for 1-3min (all↓). Both sham animals remained within the desired range. Only 1 ischemic animal from the 8min group fell out of range for blood pressure and this was for only 1min (↑). All of the ischemic animals from the 10min group fell out of range for 1-3min during the occlusion period (all ↑ with the exception of 2 animals which were ↓ for 1min).

6.4.2.3 Hippocampal CA1 Neuron Death

Figure 6.1 shows examples of animals with bilateral damage (A), unilateral damage (B), and no damage (C), relative to results from a sham animal (D). Of the 6 rats in the 8min occlusion group, 4 showed bilateral damage, 1 showed unilateral damage, and 1 showed no damage. The average % damage in the animals with bilateral damage was 74.3%. Of the six rats in the 10min occlusion group, 5 showed bilateral damage, and 1 showed unilateral damage. The average % damage in the animals with bilateral damage was 73.2%. Table 6.7 shows CA1 neuronal cell counts for each animal.

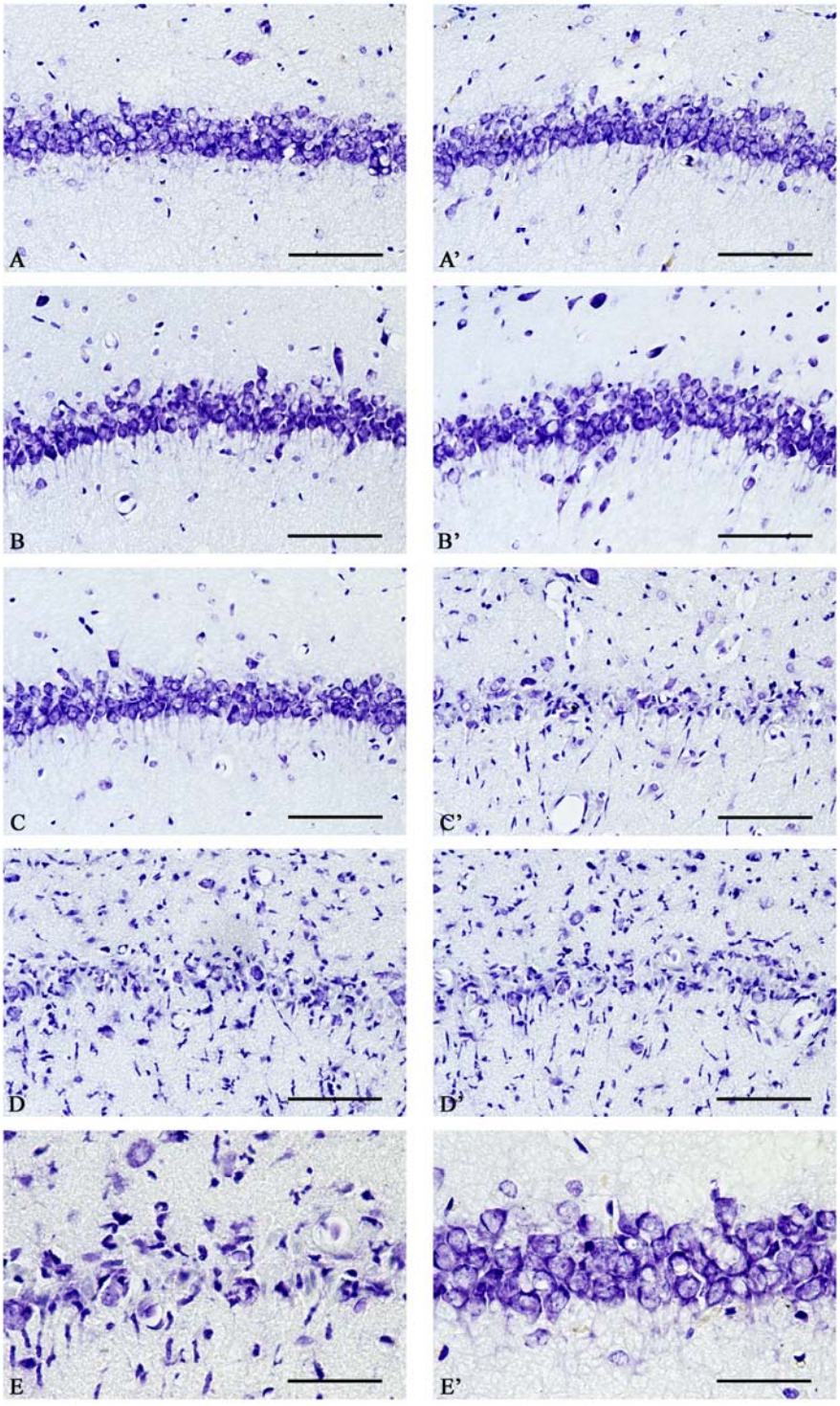

Figure 6.1. Representative photographs of left and right hippocampus from a sham animal (A, A'), an ischemic animal with no damage (B, B'), an ischemic animal with unilateral damage (C, C'), and an ischemic animal with bilateral damage (D, D'). E and E' show higher magnification pictures of a damaged CA1 pyramidal layer and one with no damage, respectively. For A-D, scale bar = 100μm. For E and E', scale bar = 50μm.

Table 6.7. Hippocampal CA1 neuron counts from individual rats

Experimental Group	Individual Rat #	CA1 cell counts (% damage)
Global Ischemia 8min occlusion (n=6)	3-1	106 (62.7%)
	4-0*	205 (27.8%)
	4-1	71 (75.0%)
	5-0¶	325 (0%)
	5-1	71 (75.0%)
	5-2	55 (80.6%)
Global Ischemia 10min occlusion (n=6)	1-0	76 (73.2%)
	1-1*	207 (27.1%)
	3-0	77 (72.9%)
	3-2	54 (81.0%)
	6-0	85 (70.1%)
	6-1	102 (64.1%)
Sham (n=2)	2-0	269
	2-1	299
Non-surgical (n=2)	7-0	326
	7-1	286

Values are expressed as the sum of left and right CA1 neuronal cell counts. *Indicates unilateral damage. ¶ Indicates no damage. % damage is based on total CA1 cells as compared to the average total CA1 cell count in sham animals.

6.4.3 Conclusions

Food intake and body weight were both decreased by 8 and 10min of ischemia in this study. It was evident that 10min of ischemia caused a greater and more sustained decrease in body weight, suggesting a more severe injury with the longer occlusion period.

Histological results indicate that the changes to the 2-VO procedure produced the outcome desired. The 2-VO model does not produce 100% consistency in hippocampal brain damage, even under ideal methodological conditions, and an average of 80% CA1 cell death in

80% of animals was expected based on discussions with our collaborator (Dr. Fred Colbourne, unpublished communication). The results from pilot study 3 indicated that this success rate was being approached, particularly in the 10min occlusion group. In fact, the slightly lower (72%) CA1 cell death achieved in 83% of these rats is advantageous for our studies to avoid a "ceiling effect" and thus be able to detect the effect of a condition that could exacerbate this degree of CA1 neuron loss (e.g. protein-energy malnutrition). It is acknowledged that this rate may also be a slight under- or overestimate due to the relatively small sample size. The changes made in the size of rat utilized, tympanic temperature, blood pressure and shortened surgery time were believed to be effective in producing the CA1 damage expected from this model. On the basis of pH and pCO_2 values, both pre- and post-ischemic respiratory status and acid-base balance were slightly improved over that observed in the second pilot study (and much improved over that demonstrated in the first pilot study), although there were still some values out of target range for individual rats. This improvement was likely primarily due to a decrease in the time required to complete surgical procedures and thus a shorter period of anaesthesia. Ischemic surgery time was reduced in this study to approximately 70min, as compared to approximately 120min in pilot study 1 and 100min in pilot study 2. With more precise control of the ratio of N_2O:O_2 run with the isoflurane anaesthetic, the pO_2 values were also less excessive in the two ischemic groups than had been observed in the second pilot study. However, many of the values were still above the desired range of 125-135mmHg. Only 2 ischemic animals fell out of range for glucose, indicating the change to the purified diet and/or the slight increase in fasting period (approximately 17hr as compared to 16hr in pilot study 1, and 15hr in pilot study 2) helped to achieve this.

Given the experimental designs of the three pilot studies, which were intended to minimize use of experimental animals, it was not known which of these variables, individually or in combination, contributed to the success of the third pilot study. Based on these results, it was decided that the 2-VO model was ready for use in my next experiment. Although the small sample size makes it difficult to draw a definite conclusion on whether the damage differed between an 8 and 10min occlusion, the 10min occlusion time was selected for the next experiment since one more animal had extensive damage in this group as compared to the 8min occlusion group. Due to the difficulty in reaching the target pO_2 range of 125-135mmHg and no clear evidence that a slightly higher range would be detrimental, 130-140mmHg (and relying on

the 70% N_2O: 30% O_2 mixture with anaesthetic) was set as the target range for the next experiment.

CHAPTER 7

CAN THE INFLUENCE OF PRE-EXISTING PROTEIN-ENERGY MALNUTRITION ON OUTCOME FROM BRAIN ISCHEMIA BE RELIABLY STUDIED WITH THE RAT 2-VESSEL OCCLUSION MODEL?

7.1 Abstract

Our laboratory has previously found that PEM affects mechanisms of damage and recovery as well as functional response to global ischemia induced by bilateral common carotid artery occlusion in the gerbil. The primary objectives of this experiment were to determine whether PEM influenced CA1 neuronal death, MAP-2 expression, and corticosterone concentration following global ischemia induced by 2-vessel occlusion (2-VO) with hypotension in the rat. Secondary objectives were to determine whether pre-existing PEM affected several physiological parameters, which are important for achieving consistent forebrain damage, and to ensure PEM was induced in the rat. Male Sprague-Dawley rats (30-32d) were implanted with small bioelectric sensors to monitor core temperature. Five to 6d later, rats were randomized to protein adequate diet (18%; CON) or protein deficient diet (2%; PEM) modified from the American Institute of Nutrition-93G diet. Seven to 8d later, rats underwent 10min of bilateral carotid artery occlusion with hypotension (35-40mmHg) or sham surgery. Tympanic temperature was maintained at 37.5 ± 0.2°C throughout the surgical period. Intra-ischemic blood pressure was significantly higher in PEM sham animals as compared to the CON sham group. Arterial blood samples were taken to monitor pH, pCO_2, pO_2, hematocrit and blood glucose prior to and following the ischemic period. Animals were perfused 7d later and blood was collected for serum albumin and corticosterone analysis. Brains were sectioned and either stained with cresyl violet for CA1 neuronal counts or processed for MAP-2 immunohistochemistry. Based on 41.4%, 30.6%, and 23.0% decreases in food intake, body weight, and serum albumin, respectively, PEM was successfully induced in the Sprague-Dawley rat. Histological results indicate that PEM did not augment the decrease in CA1 neuronal cell counts and MAP-2 protein

expression caused by exposure to global ischemia; however, a trend was present for neuroprotection in the PEM group. Serum corticosterone concentration was not altered by either independent variable. PEM animals showed a significantly lower core temperature within the 8hr following induction of surgical anaesthetic. PEM significantly increased pH, pO_2, and blood glucose, and decreased pCO_2 prior to carotid artery occlusion as well as increased pO_2 and decreased pCO_2 following ischemia. A positive correlation was detected between CA1 neuron counts and post-ischemic pH and hematocrit when all experimental groups were analyzed. A trend for a positive correlation was found between CA1 neuron counts and pre-ischemic pO_2 when ischemic animals only were analyzed. Since PEM interfered with several physiological parameters that are key determinants of consistent hippocampal injury in the 2-VO model, the surgical model of brain ischemia was confounded and therefore experimental objectives may not have been accurately addressed.

7.2 Introduction

Stroke is the leading cause of disability worldwide, leaving approximately 75% of survivors with some form of impairment (Heart and Stroke Foundation. 2008). Treatment for stroke is limited to tissue-plasminogen activator (t-PA), a thrombolytic agent that breaks up the blood clot and allows reperfusion (Besancon et al. 2008, Ginsberg. 2009). However this drug is only effective if given within 4.5hr following onset of stroke, and the risk for hemorrhagic transformation is present (Besancon et al. 2008, Ginsberg. 2009). Unfortunately, neuroprotective drugs developed to target individual mechanisms of the acute ischemic cascade have not been efficacious in clinical trials despite their success in animal studies (Cheng et al. 2004, Dirnagl et al. 1999). More focus on combination therapies is needed (Cheng et al. 2004), and optimal nutritional care should be included as part of this therapy.

Based on estimates made up to two decades ago, approximately 16% of elderly acute stroke patients are already protein-energy malnourished upon admission to hospital, and the problem has not improved based on recent estimates (Axelsson et al. 1988, Davalos et al. 1996, Davis et al. 2004, Gariballa and Sinclair. 1998, Martineau et al. 2005, Yoo et al. 2008). Physical and mental incapacities develop as a result of stroke, resulting in feeding problems causing nutritional status to worsen during post-stroke hospital stay, with 20-35% of patients being affected at 1wk (Axelsson et al. 1988, Brynningsen et al. 2007, Yoo et al. 2008) and 35-49% by

the time of admission to a rehabilitation setting (Finestone et al. 1995, Poels et al. 2006). Therefore, protein-energy malnutrition (PEM) could affect both mechanisms key to the acute ischemic cascade as well as those central to recovery and rehabilitation. The clinical evidence suggests that poor nutritional status adversely affects stroke outcome (Davis et al. 2004, Davalos et al. 1996, Finestone et al. 1996, FOOD Trial Collaboration. 2003, Martineau et al. 2005) although there are numerous criticisms of these studies including the variation in nutritional assessment tools used to diagnose PEM (Foley et al. 2009). Further support for this link has been provided by the epidemiological finding that 5yr mortality following stroke was highest in an underweight group based on body mass index surveyed in 21,884 patients at admission (Olsen et al. 2008).

In order to establish a causative link between PEM and outcome after brain ischemia, our group has been utilizing a gerbil model of global ischemia (Bobyn et al. 2005, Ji et al. 2008). While global ischemia models are more representative of cardiac arrest and not focal stroke, they generally result in more consistent injury and share many of the same pathophysiological mechanisms (Small and Buchan. 2000, Traystman. 2003). This renders the global ischemia model a useful tool for examining the relationship between nutritional status and outcome after brain ischemia.

Opportunities to alter outcome from brain ischemia exist in two broad mechanistic categories that include targeting of 1) the acute ischemic cascade, and 2) recovery mechanisms. Mechanisms responsible for brain cell death in the acute ischemic period include, but are not limited to, decreased glucose and oxygen supply to the brain, decreased ATP production, glutamate excitotoxicity, increased intracellular Ca^{2+}, increased reactive oxygen species generation and inflammation (Lee et al. 1999). Our laboratory has shown that PEM can elevate oxidative stress (Bobyn et al. 2005) and increase activation of nuclear factor kappa-B (NFκB), a transcription factor involved in the promotion of inflammation (Ji et al. 2008). These mechanisms could contribute to the functional impairment previously reported as an inability of protein-energy malnourished gerbils to habituate in the open field following global ischemia (Bobyn et al. 2005). Mechanisms of recovery involve anatomical and chemical changes that occur in order to protect and promote recovery, all collectively termed neuroplasticity (Lee and van Donkelaar. 1995, Teasell et al. 2005). These changes include unmasking of latent synapses, neuronal sprouting resulting in new synapse formation, and changes to neurotransmitters,

neurotrophins, growth factors and hormones (Johansson. 2000, Lee and van Donkelaar. 1995) (Johansson. 2000, Lee and van Donkelaar. 1995). A recent study from our group demonstrated that PEM can affect plasticity-related recovery mechanisms following global ischemia as well. As pre-existing PEM caused exacerbation of the receptor tropomyosin-related kinase B (trkB) and growth-associated protein-43 (GAP-43) protein expression in the hippocampus following global ischemia, we propose that PEM may be increasing hyperexcitability within hippocampal circuits and/or the stress response to global ischemia (Chapter 5).

We have recently discontinued use of the gerbil bilateral common carotid artery occlusion model of global ischemia due to increasing inconsistency explained by the development of posterior communicating arteries in the North American commercial gerbil supply (Laidley et al. 2005, Seal et al. 2005). Here we describe our first study of the influence of pre-existing PEM in the rat 2–vessel occlusion with hypotension (2-VO) model of global ischemia. This model, which combines temporary occlusion of the common carotid arteries with systemic hypotension to produce forebrain ischemia, is considerably more surgically invasive than the gerbil global ischemia model. Although it produces consistent and selective damage to the CA1 region of the hippocampus in a spatial and temporal manner typical of global ischemia models (Small and Buchan. 2000), its utility for studying nutritional influences on brain ischemia has not been established. While our previous study demonstrated that the heightened functional impairment caused by PEM was not accompanied by increased neuronal cell death at 10d following global ischemia in the gerbil (Bobyn et al. 2005), this finding needs to be confirmed in the rat 2-VO model. Therefore, a primary objective of the current study was to determine the effect of pre-existing PEM on short-term CA1 hippocampal neuronal death. The dendritic structural protein, microtubule-associated protein-2 (MAP-2), a marker of injury following global ischemia (Li et al. 1998), was also examined. Corticosterone concentration, which can be increased by PEM (Monk et al. 2006), was also measured at 7d after global ischemia as an indicator of the stress response and another potential mechanism by which PEM could worsen outcome.

Monitoring of physiological variables (pH, pCO_2, pO_2, hematocrit and glucose) that can alter the extent of ischemic cell death following brain ischemia is possible in the 2-VO model due to the larger size of the rat. Thus, a secondary objective was to investigate the influence of PEM on these variables. Blood glucose concentration was of particular interest since both hyper-

(Lin et al. 1998, Pulsinelli et al. 1982) and hypoglycemia (Zhu and Auer. 2004) have been shown to increase neuronal cell death following ischemia, and PEM can lower blood glucose concentration (Torun. 2006). Finally, data on food intake, weight gain, and serum albumin are provided to confirm that PEM was induced in the 30-32d old male, Sprague-Dawley rat by feeding a diet containing 2% protein.

7.3 Materials and Methods

7.3.1 Animals

Male Sprague-Dawley rats (30-32d old) (Charles River Canada, QC, Canada) were acclimatized on control diet (Table 7.1) for 5-6d. All animal care and procedures followed the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply.

7.3.2. *SubCue*™ Datalogger Implantation and Body Temperature Analysis

All surgical procedures were done using aseptic technique. After 5-6d of acclimatization, animals underwent surgery to implant small, sterilized bio-electrical sensor transmitters encapsulated in biocompatible silicone (*SubCue*™ Dataloggers) to gather information on pre and post-ischemic core temperature. Animals were anaesthetized (4% isoflurane for induction and ~2% for maintenance with 1L/min oxygen) and placed on a heated water blanket. An incision was made in the mid-abdominal region and the temperature sensor inserted into the peritoneal cavity. The incision was closed and Ketaprofen (5mg/kg) injected subcutaneously. The probe was programmed to take a reading every 30min and was active from initial implantation until perfusion.

7.3.3 Diet Assignment

At 5-7d following the abdominal surgery, animals were randomly assigned to one of two diet groups, a protein adequate diet (CON, 18% protein) or a protein-deficient diet (PEM, 2% protein). Both diets are based on the AIN-93G rodent diet (Reeves et al. 1993) but do not contain τ-butylhydroquinone (Table 7.1). Our laboratory has previously shown that feeding a 2% protein diet to the young adult male gerbil (Bobyn et al. 2005, Ji et al. 2008) and the 10wk old male

Sprague-Dawley rat (unpublished observations) causes a voluntary decrease in caloric intake, thereby causing protein-energy malnutrition **(**PEM).

Table 7.1. Composition of experimental diets*

Component	Adequate Protein[§] (CON) g/kg	Low Protein[§§] (PEM) g/kg
Vitamin Free Casein	200	22.4
L-Cysteine	3	0.29
Sucrose	100	100
Cornstarch	397.5	520.05
Dextrinized Cornstarch	132	174
Soybean Oil (without TBHQ)	70	70
Cellulose	50	50
Mineral Mix[£]	35	0
Mineral Mix [¶]	0	35
Calcium Phosphate, dibasic	0	12.4
Calcium Carbonate	0	3.36
Vitamin Mix[†]	10	10
Choline Bitartrate	2.25	2.25

*Diets were purchased from Dyets Inc. (Bethlehem, PA). [§]Control diet was formulated to contain 18% protein. [§§]The low protein diet used to induce PEM was formulated to contain 2% protein. [£]AIN-93G mineral mix (Reeves et al., 1993). [¶]AIN-93G modified mineral mix with calcium and phosphorus deleted, potassium citrate H_2O increased from 28 to 226.55 g/kg, sucrose increased from 209.806 to 618.256 g/kg mineral mix. [†]AIN-93G vitamin mix (Reeves et al., 1993).

Animals were caged in groups of 2-4 in shoebox cages with bedding and free access to food and water. Housing facilities were maintained at 22°C and had a 12hr light/dark cycle. Food intake was recorded daily with the exception of d7 since this included the pre-surgery fasting period. Body weight was recorded on the day animals were placed on diet (d0), surgery day (d7), for 2d following surgery (d8 and 9), and perfusion day (d14).

7.3.4 2-Vessel Occlusion to Induce Global Ischemia

After animals had been on assigned diet for 7-8d, they underwent the 2-VO procedure of bilateral common carotid artery occlusion combined with hypotension to induce global forebrain ischemia, modified, as described in Chapter 6, from the procedure of Arvanitidis et al. (2009). All rats were food deprived for 16-20hr prior to surgery in order to lower blood glucose levels into a consistent range (target range was 4-8mmol/L). Animals were anaesthetized (4% isoflurane for induction and ~2% for maintenance in 70%N_2O and 30% O_2) and placed on a heated water blanket. A ventral midline neck incision was made in order to isolate both common carotid arteries. A second incision was made to isolate and cannulate the right jugular vein for blood withdrawal and re-infusion to induce systemic hypotension. The tail artery was cannulated for monitoring of mean arterial blood pressure and measurement of blood gases. Initial arterial blood samples were taken to measure blood pH, pCO_2, pO_2, hematocrit, and glucose levels prior to inducing hypotension. Our target ranges for these parameters were as follows: pH 7.25-7.35, pCO_2 35-45mmHg, pO_2 130-140mmHg, glucose 4-8mmol/L. Once the blood pressure reached 35mmHg, the common carotid arteries were occluded for 10min using non-traumatic micro-aneurysm clips. Tympanic temperature, an indicator of brain temperature, was monitored and maintained throughout the ischemic period using an automatic feedback temperature controller (Omega, CN9500) connected to a heatlamp outfitted with a 250W infrared bulb; the goal was to maintain at 37.5 ± 0.2C°. Hypotension was achieved by maintaining arterial blood pressure at 35-40mmHg during the ischemic period. Carotid reperfusion was visually verified after removal of the clips and blood was re-infused into the jugular vein to restore normal blood pressure. After ~10min equilibration period another arterial blood sample was taken to determine post-ischemic blood gas values. All incisions were sutured and Marcaine (Bupivacaine) (2mg/kg) was injected subcutaneously at each incision site. A subcutaneous injection of 0.9% sterile saline (10mg/kg/hr) was given to each rat following surgery. Animals were carefully monitored following surgery for seizures or signs of pain or discomfort. The same protocol was applied for sham surgeries except that carotid arteries were not occluded and hypotension was not induced.

The four experimental groups generated by d7 were therefore: Control diet-Sham surgery (CON-S), Control diet-Ischemia (CON-I), PEM-Sham surgery (PEM-S), and PEM-Ischemia (PEM-I), each with n = 10. Food intake was monitored each day following surgery. Animals were housed singly for 2d following surgery, and body weight was obtained on each of these

days. Animals were then placed back with their original cage mates. Following surgery, each rat continued on the same diet as assigned during the pre-surgical period. A timeline of experimental events and experimental groups is presented in Figure 7.1.

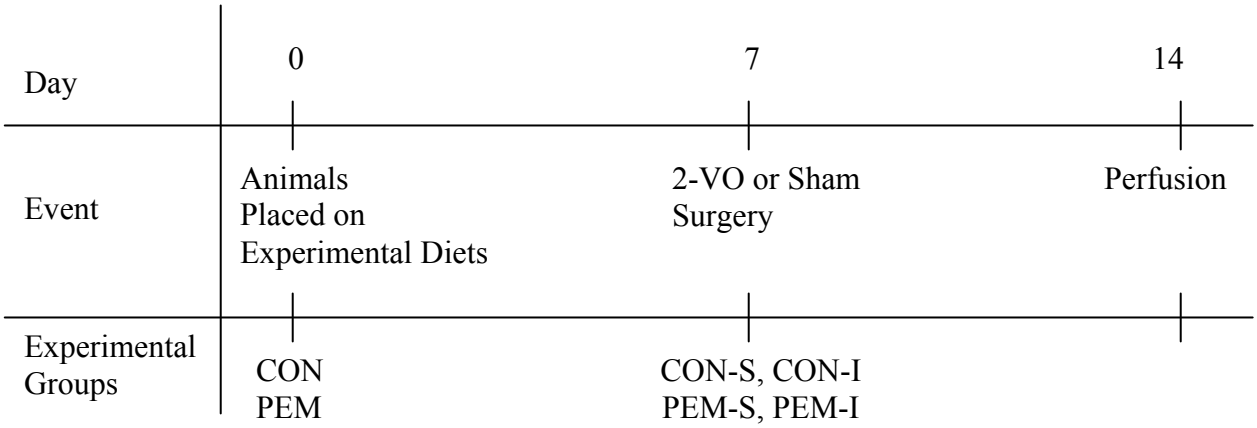

Figure 7.1 Timeline of experimental events and experimental groups present.

7.3.5 Blood Collection and Tissue Preparation

At 7d following surgery (d14 of the study), animals were placed under deep anaesthesia and blood was withdrawn from the left ventricle of the heart for determination of serum albumin and corticosterone concentration. Animals were then euthanized via trans-cardial perfusion with heparinized saline followed by 4% paraformaldehyde. Heads were placed overnight in 4% paraformaldehyde for fixation in order to avoid dark neuron artifact (Cammermeyer. 1962). The brain was then removed and fixed overnight in 4% paraformaldehyde followed by cryoprotection for 3-5d in 20% sucrose solution. Subsequently, brains were frozen using dry ice-cooled isopentane in a mould containing OCT (Optimal Cutting Temperature) compound. Samples were stored at -20°C until sectioning. Coronal sections were taken through the hippocampus at 14μm thickness with a cryostat and mounted onto gelatin-subbed slides. Four sections, one representing each experimental group**,** were placed on each slide so that all experimental groupings were processed under identical conditions. This allowed for accurate assessment of relative immunohistochemical changes among experimental groups.

7.3.6 Serum Albumin and Corticosterone Analysis

Blood samples were centrifuged at 1500 x g for 10min after clotting at room temperature. Serum was transferred to 500µl microcentrifuge tubes and stored at -80C° until analysis. Serum albumin analysis was performed using the bromocresol green method (Doumas et al. 1971). Briefly, 25µl of sample was added to 5.0ml bromocresol green solution (bromocresol green in a 0.075M succinate buffer). After 30min, serum albumin levels were analyzed spectrophotometrically at 628nm (Biochrim Ultrospec 3100 Pro). Serum albumin concentration of the sample relative to a series of protein standards was determined by linear regression.

Samples for corticosterone analysis were analyzed by Prairie Diagnostic Services, College of Veterinary Medicine, University of Saskatchewan. Samples were analyzed using ImmuChem double antibody corticosterone ^{125}I radioimmunoassay designed to measure total corticosterone (MP Biomedicals). Briefly, anti-corticosterone antibody was reacted with ^{125}I labeled corticosterone and differing concentrations of serum sample. Competition for the antibody occurred between the sample corticosterone and the labeled corticosterone and the resultant immune complexes were precipitated out and radioactivity measured. A standard curve (25-1000ng/mL) was made in which the concentration of serum corticosterone was inversely proportional to measured radioactivity.

7.3.7 Histology

Every tenth section through the hippocampus was stained with cresyl violet for CA1 cell counts. These sections were dehydrated through graded alcohols (70%, 95%, 100%) followed by rehydration into ddH_2O. Slides were placed in 0.25% cresyl violet for 10min followed by ddH_2O, 70% ethanol and 95% ethanol. Sections were differentiated in 0.25% glacial acetic acid in 100% ethanol and then cleared in 95% ethanol, 100% ethanol and xylene. Slides were coverslipped with cytoseal.

Viable cells in the CA1 region that had a distinct nucleus and an intact cellular membrane were counted. Neurons were counted bilaterally at 400x magnification in 3 sections representing the entire rostral-caudal axis of the hippocampus. Using a 200µm square (10X10) microscope grid, cells were counted in medial, middle, and lateral sectors of the CA1 region at -3.8 (Level A) and -4.8mm (Level B) relative to bregma and in a single middle sector at -6.3mm (Level C) from bregma.

7.3.8 Immunohistochemistry

Every fifth consecutive section (14μm) taken from the rostral hippocampus was used for MAP-2 immunohistochemistry. Following a wash in phosphate-buffered saline, sections underwent citrate antigen retrieval. Briefly, slides were placed in 0.01M citrate buffer (10% 0.1M sodium citrate in ddH_2O, pH 6) at 50°C and then warmed to 90°C over 45min. Slides were then allowed to cool for 30min. Sections were then incubated with blocking solution containing 5% normal goat serum in 0.1% Triton X-100 for 1hr at room temperature. Slides were incubated overnight at 4°C with primary antibody (MAP-2, 1:200) diluted in 2% normal goat serum in 0.1% Triton X-100. Slides were then washed in PBS and then incubated with goat anti-rabbit biotinylated secondary antibody (1:1000) in PBS for 1hr at room temperature. Slides were washed and quenched for endogenous peroxidases in 0.3% H_2O_2 in methanol for 20min followed by another wash in PBS. Sections were then treated with avidin biotin (Vectastain) for 1hr at room temperature followed by a wash in PBS and a 5min reaction with diaminobenzidine. Slides were washed under running dH_2O and cleared in increasing concentrations (70%, 95%, and 100%) of ethanol and xylene and then coverslipped with cytoseal. Control sections were processed in the same manner, but no primary antibody was applied.

One animal from each of the experimental groups was mounted on the same slide to ensure that slide to slide variability in signal would not bias the determination of relative changes between experimental groups to be quantified. Ten samples from each experimental group (representing different animals per group) were used for quantification, which was performed in a blinded fashion. Photographs of the CA1 pyramidal layer for all sections on the slide, including the stratum oriens and stratum radiatum were taken under identical conditions at 400x magnification. A box (pixel area of 367,845) was placed over a region containing the CA1 pyramidal layer, the stratum oriens and stratum radiatum and analyzed using spot densitometry (AlphaEaseFC Imaging Software, Alpha Innotech). An integrated density value (IDV=sum of pixel values in the region of interest) was obtained and then values from the left and right hemispheres were averaged. Average values for the CON-I, PEM-S, and PEM-I groups were normalized by expressing them as a ratio to the respective CON-S value on the same slide. MAP-2 expression was analyzed in 2 ways: 1) with all rats exposed to global ischemia included, and 2) with only those rats included that showed bilateral hippocampal injury in response to the 2-VO protocol (see rationale below).

7.3.9 Statistical Analysis

All data are presented as mean (±SEM). Pre-surgery body weight and food intake were analyzed using unpaired t-tests. Post-surgery body weight, food intake, CA1 cell counts, serum corticosterone concentration, and serum albumin were analyzed using 2-factor ANOVA, as were blood gases, blood glucose concentration, and hematocrit. When significant interactions were identified, post-hoc LSD (least significant difference) was performed. Because the extent to which the 2-VO procedure resulted in extensive bilateral hippocampal injury differed between the CON and PEM groups, CA1 cell counts were analyzed in 2 ways: 1) with all rats exposed to global ischemia included, and 2) with only those rats included that showed bilateral hippocampal injury in response to the 2-VO protocol.

To determine if hippocampal CA1 injury was correlated with a number of physiological parameters, the correlation between total CA1 cell counts and each of the following parameters was determined by Pearson's correlation coefficient: pre- and post-ischemic pH, pCO_2, pO_2, glucose, and hematocrit, and the lowest core temperature observed during the 8hr immediately following anaesthetic induction for 2-VO or sham surgery. These correlations were performed both with all experimental groups included and with only rats exposed to global ischemia included. The latter approach was used to increase sensitivity for detecting correlations among ischemic animals with varying degrees of hippocampal damage. Statistical significance for all analyses was set at $p \leq 0.05$.

7.4 Results

No rats were removed from the study due to inadequate control of intra-ischemic tympanic temperature or postsurgical seizures.

7.4.1 Indices of PEM

As shown in Figure 7.2, pre-surgery food intake was declining in the PEM group but this was not yet significant as compared to controls ($p > 0.05$), with the exception of d4 where a significant decrease in the PEM group was transiently detected ($p = 0.019$).

Figure 7.3 shows average daily post-surgery food intake, which is based on cage data (n = 2-4 rats/cage) with the exception of d8, 9 and 10, when individual food intake could be monitored due to individual rat housing. There was a significant interaction between diet and

ischemia at d8 ($p = 0.038$), and post hoc analysis demonstrated that ischemia caused a greater decrease in CON animals (64.0% as compared to CON-S) than in PEM animals (35.5% as compared to PEM-S). On d8, mean (±SEM) food intake (g) was as follows, with different superscripts designating differences among groups: CON-S = 26.4 ± 1.2^{a}, CON-I = 10.4 ± 2.4^{b}, PEM-S = 19.7 ± 1.2^{c}, PEM-I = 12.7 ± 3.0^{b}. An independent effect of ischemia in depressing food intake was present on d9 (41.6% decrease as compared to shams) ($p = 0.001$). PEM independently decreased food intake at 10d ($p = 0.005$) (24.9% less than CON groups); this effect remained significant for the rest of the study ($p \leq 0.001$).

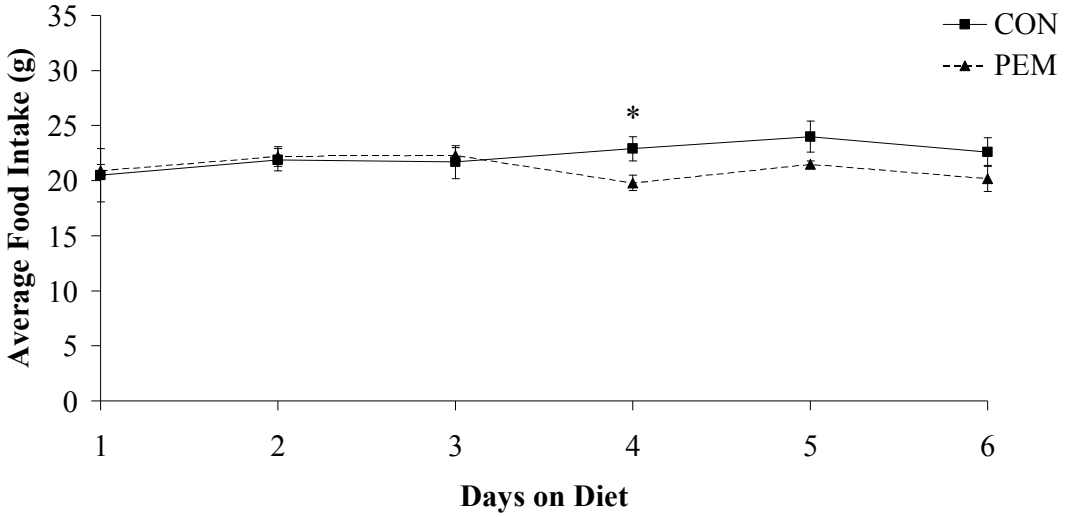

Figure 7.2. Pattern of average daily pre-surgery food intake. Data are shown as mean (±SEM). Food intake is based on cage data [n = 6 for CON, n = 8 for PEM (3-4 animals/cage)]. *Indicates a significant difference between groups as detected by unpaired t-test ($p < 0.05$).

Mean (±SEM) initial body weight (d0) was not significantly different between the two diet groups ($p > 0.05$) as shown under pre-surgery body weight in Table 7.2. There was weight loss in the PEM group as shown by a significantly lower body weight in PEM animals (16.9%) as compared to CON rats after 7d on diet (day of surgery) ($p < 0.001$).

Analysis of post-surgery body weight on d8 and 14 showed a statistically significant independent decrease with PEM (17.6 and 30.6%, respectively, as compared to CON groups) ($p < .001$) as shown in Table 7.2. Analysis of body weight at d9 demonstrated an interaction between diet and surgery ($p = 0.022$), indicating that ischemia caused a decrease in body weight in CON (12.5% as compared to CON-S) but not PEM rats.

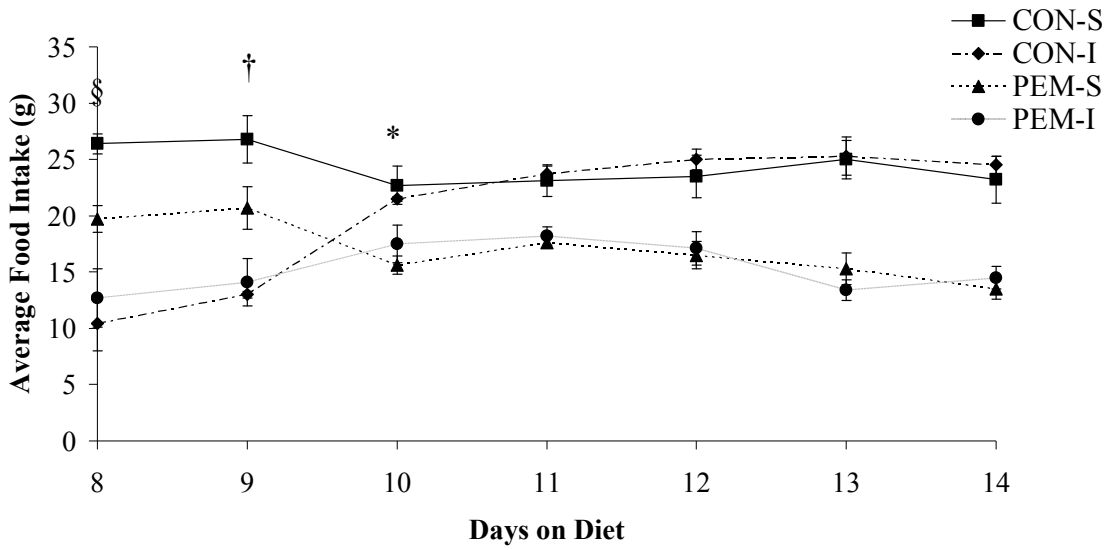

Figure 7.3. Pattern of average daily post-surgery food intake. Data are shown as mean (±SEM). Food intake is based on individual rat housing data for d8, 9, and 10. On the other days, food intake is based on cage data [n = 3 for CON-S and CON-I, n = 4 for PEM-S and PEM-I (2-4 animals/cage)]. [§]Diet and surgery interaction ($p = 0.038$); the decrease after global ischemia was greater in CON than in PEM animals by post-hoc LSD test. [†]Surgery effect as indicated by 2-way ANOVA ($p = 0.001$). *Diet effect as indicated by 2-way ANOVA ($p = 0.005$), which continued for the remainder of the study.

Table 7.2. Pre- and post-surgery body weight as influenced by PEM and global ischemia.

Pre-Surgery Body Weight (g)	CON		PEM	
Day 0	229.8 ± 5.2		234.7 ± 3.7	
Day 7	272.0 ± 6.2		226.0 ± 3.4*	
Post-Surgery Body Weight (g)	**CON-S**	**CON-I**	**PEM-S**	**PEM-I**
Day 8[£]	282.1 ± 11.9	262.3 ± 5.5	228.5 ± 5.6	219.9 ± 3.8
Day 9[¶]	308.3 ± 13.2[a]	269.8 ± 6.6[b]	231.0 ± 5.7[c]	226.8 ± 3.2[c]
Day 14[£]	336.7 ± 14.7	326.1 ± 6.6	230.8 ± 6.2	229.1 ± 3.0

Data are mean (±SEM). n = 10 for all groups on each day with the exception of n = 7 for CON-S group on d9. *Indicates a significant difference from CON groups by unpaired t-test ($p < 0.001$). [£]Indicates a significant effect of diet ($p < 0.001$) by 2-factor ANOVA. [¶]Indicates an interaction of diet and surgery with differences among groups detected by LSD posthoc test and denoted by different superscripts ($p = 0.022$).

7.4.2 Serum Albumin

Figure 7.4 shows results of serum albumin analysis. Diet had an independent effect as PEM animals had a significantly lower serum albumin concentration (23%) as compared to CON ($p < 0.001$). There was no effect of ischemia nor an interaction.

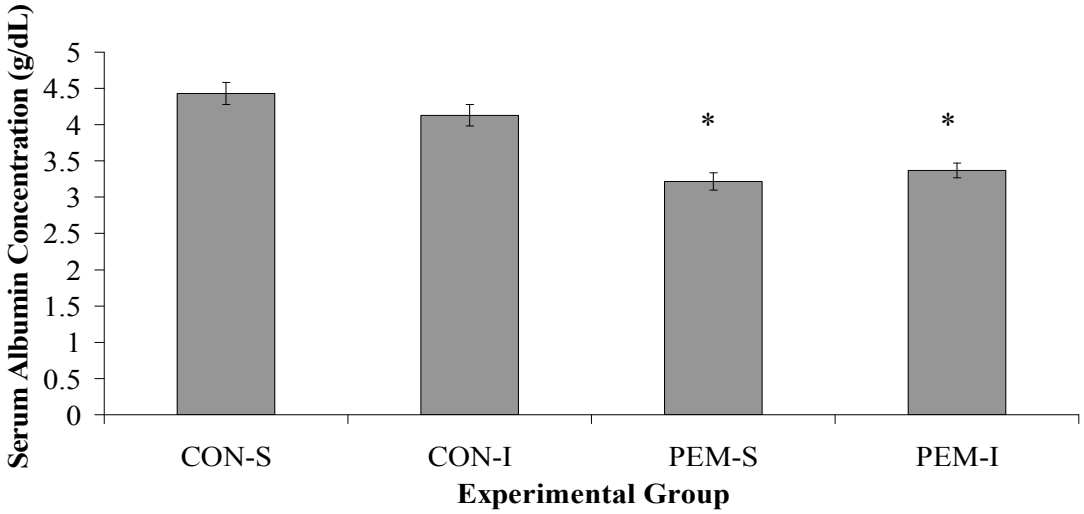

Figure 7.4. The low protein diet independently decreases serum albumin. Data are shown as mean (±SEM) serum albumin concentration (g/dL) at 14d. *Independent diet effect as indicated by 2-way ANOVA ($p < 0.001$).

7.4.3 Physiological Parameters

A summary of physiological results related to the 2-VO model is presented in Table 7.3. In the pre-ischemic blood sample, PEM independently increased arterial blood pH ($p = 0.001$), pO_2 ($p = 0.019$), and fasting blood glucose concentration ($p = 0.041$) and decreased pCO_2 ($p = 0.003$). Mean values that fell out of target range included pCO_2 in the CON-S and CON-I groups, and pO_2 in the PEM-S and PEM-I groups. Several individual rat values were out of range for these parameters. For pH, this included 2/10 (↓) CON-S and 2/10 (↓) CON-I animals. For pCO_2, 7/10 (↑) CON-S, 7/10 (↑) CON-I, and 4/10 (3↑, 1↓) PEM-S animals were out of range. For pO_2, this included 6/10 CON-S (3↑, 3↓), 4/10 CON-I (3↑, 1↓), 7/10 PEM-S (all ↑), and 7/10 PEM-I (all ↑). Individual values for hematocrit and glucose were all within the desired range.

In the post-ischemic blood sample, PEM significantly increased pO_2 ($p = 0.019$) and decreased pCO_2 ($p = 0.003$) while pH ($p = 0.003$) and hematocrit ($p < 0.001$) were independently decreased

by ischemia. There was a trend for increased blood glucose by PEM ($p = 0.084$). Mean values for, pCO_2 and pO_2 fell out of range in the CON-S group, as did pH and pCO_2 in the CON-I group, and pH, pCO_2 and pO_2 in the PEM-I group. Again, several individual values fell out of range. For pH, this included 4/10 CON-S (all ↓), 6/10 CON-I (all↓), and 6/10 PEM-I (all ↓). For pCO_2, 8/10 (7↑, 1↓) CON-S, 7/10 (all ↑) CON-I, 2/10 (1↑, 1↓) PEM-S, and 7/10 (all ↑) PEM-I animals were out of range. For pO_2, this included 6/10 (2↑, 4↓) CON-S, 6/10 (3↑, 3↓) CON-I, 8/10 (6↑, 2↓) PEM-S, and 9/10 (5↑, 4↓) PEM-I. All hematocrit values were within range. One animal from the CON-S, and 1 from the CON-I groups had a lower than desired glucose value, while 1 PEM-I animals had a higher than desired value.

Table 7.3. Physiological parameters measured during 2-VO surgery

		CON-S	CON-I	PEM-S	PEM-I
Pre-Ischemia Values	pH* 7.25-7.45	7.28 ± 0.03	7.31 ± 0.02	7.37 ± 0.01	7.36 ± 0.01
	pCO_2* 35-45mmHg	53.9 ± 5.0[¶]	47.5 ±1.9[¶]	41.5 ± 1.7	41.3 ± 1.0
	pO_2* 125-135mmHg	131.5 ± 3.4	132.5 ± 2.7	137.7 ± 1.3[¶]	138.8 ± 2.2[¶]
	Hematocrit (%)	40.4 ± 0.3	41.5 ± 1.1	44.2 ± 2.2	41.0 ± 1.1
	Blood Glucose* 4-8mmol/L	5.3 ± 0.3	5.4 ± 0.2	5.8 ± 0.3	6.2 ± 0.4
Post-Ischemic Values	pH[†] 7.25-7.45	7.34 ± 0.03	7.22 ± 0.02[¶]	7.35 ± 0.01	7.23 ± 0.03[¶]
	pCO_2* 35-45mmHg	57.2 ± 6.4[¶]	56.1 ± 3.0[¶]	41.7 ± 1.6	49.7 ± 2.1[¶]
	pO_2* 125-135mmHg	121.4 ± 4.0[¶]	126.5 ± 5.6	132.6 ± 3.8	140.9 ± 6.8[¶]
	Hematocrit (%)[†]	41.0 ± 0.5	36.4 ± 1.4	43.1 ± 0.9	36.2 ± 1.7
	Blood Glucose 4-8mmol/L	5.4 ± 0.3	5.3 ± 0.2	6.0 ± 0.3	6.1 ± 0.6
Intra-Ischemic Values	Tympanic Temperature °C	37.5 ± 0.01	37.4 ± 0.03	37.5 ± 0.03	37.5 ± 0.04
	Blood Pressure[§] mmHg	73.0 ± 1.9[a]	37.8 ± 0.2[b]	82.0 ± 2.8[c]	38.1 ± 0.2[b]

Data are expressed as mean (±SEM) with desired ranges of values indicated below each parameter heading. *Indicates a significant effect of diet based on 2-factor ANOVA ($p < 0.05$). [†]Indicates a significant effect of surgery ($p \leq 0.003$). [§]Indicates an interaction of diet and surgery with differences among groups detected by LSD posthoc test and denoted by different superscripts. [¶]Indicates parameters for which mean values have fallen out of range. n = 10.

Tympanic temperature and blood pressure were recorded each minute during the 10min carotid artery occlusion period. There was no significant difference in tympanic temperature among groups and only 8 individual animals (4/10 CON-I and 4/10 PEM-I) had values that fell slightly out of range transiently during the 10min period. All of these out of range individual values were lower than desired, the lowest reaching 36.9°C for one minute, with the exception of one high value reaching 37.8°C for one minute. The remaining animals fell between the desired 37.3-37.7°C for the entire 10min period, indicating good control via the heatlamp system. For mean arterial blood pressure, there was an interaction between diet and surgery ($p = 0.014$). Blood pressure was not significantly different between the 2 ischemic groups, in which blood pressure was regulated, but PEM significantly raised blood pressure (12.3%) in the sham group over that observed in sham rats fed control diet ($p = 0.010$). For the two ischemic groups thirteen individual values (8/10 CON-I, 5/10 PEM-I) fell out of the desired range for 1-3 consecutive minutes. The most extreme range in a single animal was 32-44mmHg; however, 8/13 out of range values fell into the narrower range of 35-43mmHg.

7.4.4 Serum Corticosterone Concentration

Figure 7.5 shows serum corticosterone concentrations at d14. No significant effects of ischemia, diet, nor interactions were found in serum corticosterone concentration.

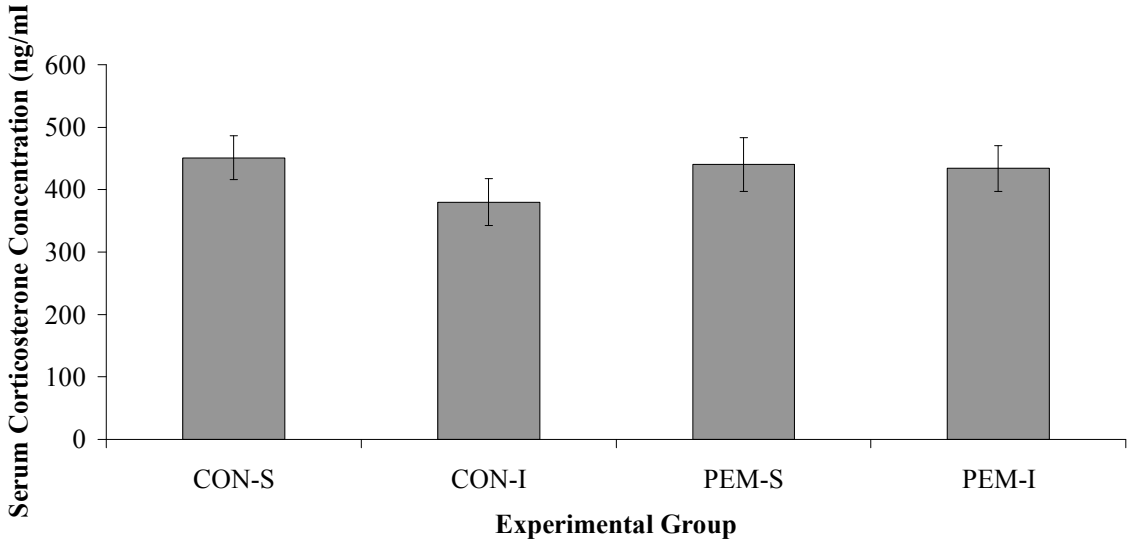

Figure 7.5. Impact of diet and surgery on serum corticosterone concentration. Data are shown as mean (±SEM) serum corticosterone concentration (ng/mL) at 14d. No effect of diet or surgery was detected by 2-way ANOVA ($p > 0.05$).

7.4.5 Hippocampal CA1 Neuron Counts

Representative photographs of cresyl violet stained hippocampal sections are shown in Figure 7.6 and CA1 cell counts are shown in Table 7.4. Figure 7.6 also highlights what appears to be reactive gliosis that is typical in global ischemia. These cells were not examined further in this study, but will be characterized in the thesis of Shari Smith. When statistical analysis was performed on all experimental animals, exposure to global ischemia independently decreased CA1 neuronal cell counts at all three levels and total counts ($p \leq 0.001$). PEM did not significantly exacerbate CA1 neuronal loss as shown by the interaction term ($p=0.195$) nor was there an independent effect of PEM ($p=0.080$).

However, PEM appeared to increase variability in CA1 neuron loss after the 2-VO surgery with a trend towards neuroprotection. Only 5/10 PEM-I rats demonstrated extensive bilateral CA1 damage. Either unilateral (1/10 rats) or no apparent damage (4/10) was evident in the other 50% of the PEM-I group. Hippocampal CA1 loss in this group therefore ranged from 0-92.1% as compared to the average of the 2 sham groups. In contrast, 7/10 of the CON-I group demonstrated extensive bilateral CA1 damage. The remaining 3/10 rats in the CON-I group showed unilateral damage, and cell death in this group therefore ranged from 25.2-91.4%.

Because of this finding, statistical analysis was performed again including only those rats in the two ischemic groups that showed bilateral hippocampal CA1 neuronal loss. An independent effect of ischemia was present ($p<0.001$), with no independent effect of PEM ($p=0.221$), nor an interaction between ischemia and PEM ($p=0.732$).

Correlation analyses between total CA1 cell counts and pre- and post-ischemic physiological parameters are summarized in Table 7.5. Significant positive correlations were detected between CA1 cell counts and post-ischemic pH ($r=0.384$, $p=0.014$) as well as hematocrit ($r=0.453$, $p=0.003$) only when all 4 experimental groups were included in the analysis. There was a trend for a positive correlation between total CA1 cell counts and pre-ischemic pO_2 when ischemic animals alone were analyzed ($r=0.440$, $p=0.052$).

The detailed analysis of the core temperature data collected by SubCue™ temperature sensors will form part of the thesis of Shari Smith, and this will not be presented here. However, key findings are presented in Appendix A. Figure A.1 shows mean temperature pattern for each group, pre- and post-surgery. In general, it can be observed that PEM increases the daily

fluctuation in core temperature (range between lowest and highest temperature over a 24hr diurnal cycle) within a short period of being placed on a low protein diet; this pattern gradually re-establishes following global ischemia (S. Smith, unpublished observations). Table A.1 shows the mean (±SEM) lowest core temperature observed within 8hr following surgical anaesthetic induction for each group. There was a significant independent effect of diet, as PEM animals had a more pronounced drop in core temperature as compared to those rats on CON diet (p=0.003). There was no independent effect of surgery (p=0.747), nor an interaction between diet and surgery (p=0.241).

Table 7.4. Effect of 2-VO surgery and PEM on hippocampal CA1 neuronal cell counts

	CON-S	CON-I		PEM-S	PEM-I	
	(n=10)	(n=10)†*	(n=7)§*	(n=10)	(n=10)†*$^{£}$	(n=5)§*
Level A	301.7 ± 9.7	78.8 ± 23.6	33.4 ± 3.6	313.1 ± 8.7	155.5 ± 49.2	37.4 ± 6.5
Level B	299.0 ± 9.8	109.7 ± 25.2	64.6 ± 11.4	319.3 ± 10.4	172.6 ± 34.0	88.6 ± 24.0
Level C	108.7 ± 5.1	63.1 ± 9.9	55.0 ± 13.0	102.2 ± 5.4	86.6 ± 12.6	71.0 ± 23.3
Total	709.4 ± 19.6	251.6 ± 54.3	153 ± 26.0	734.6 ± 21.2	414.7 ± 84.5	197 ± 52.0

Data are presented as mean (±SEM).†Includes all experimental rats.§Includes only rats with bilateral hippocampal CA1 injury.*Indicates a significant independent effect of surgery based on 2-factor ANOVA ($p \leq 0.001$). $^{£}$There was a trend for PEM to be neuroprotective ($p = 0.080$). Level A = -3.8mm from bregma, Level B = -4.8mm from bregma, Level C = -6.3mm from bregma.

Correlation analysis was performed between total CA1 cell counts and this measurement for: 1) all 4 experimental groups combined, 2) the PEM-I and CON-I groups combined, and 3) PEM-I and CON-I groups separately as shown in Table 7.6. A significant negative correlation was detected between CA1 counts and the drop in temperature occurring within 8hr of anaesthetic induction only when CON-I animals alone were included in the analysis ($r=-0.897$, $p<0.001$).

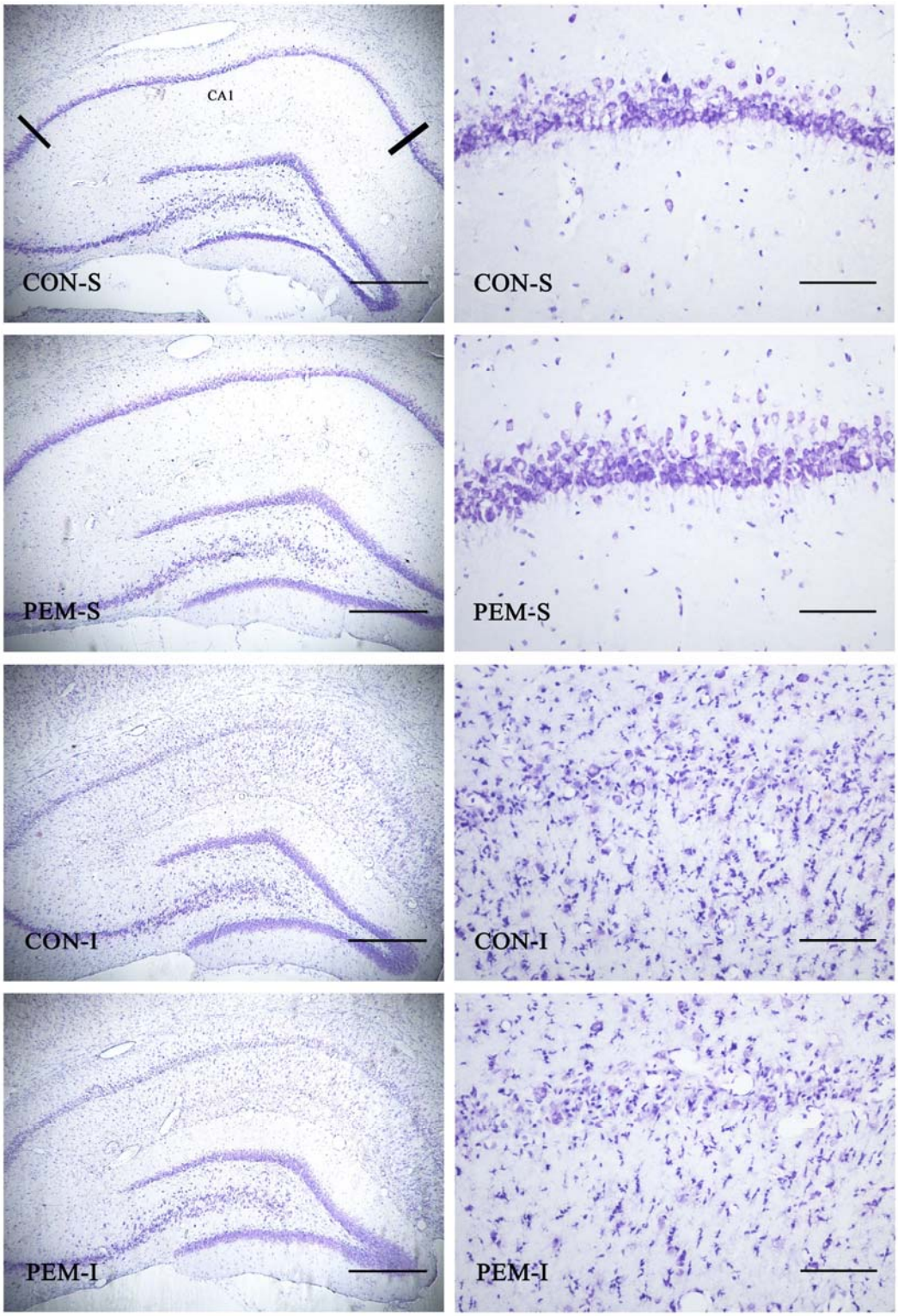

Figure 7.6. Representative photographs of cresyl violet staining in the hippocampal formation (left column; scale bar = 500μm) and the CA1 region (right column; scale bar = 100μm) observed in those rats demonstrating bilateral hippocampal injury at 7d post-surgery.

Table 7.5. Correlation of hippocampal CA1 total cell counts with physiological parameters

		All Groups (n=40)		CON-I + PEM-I (n=20)	
		r	p	r	p
Pre-Ischemia	pH	0.026	0.873	0.089	0.708
	pCO_2	0.043	0.793	-0.182	0.442
	pO_2	0.219	0.175	0.440	0.052
	Hematocrit	0.120	0.462	-0.61	0.798
	Blood Glucose	0.019	0.909	0.197	0.405
Post-Ischemia	pH	0.384	0.014*	0.081	0.733
	pCO_2	-0.162	0.319	-0.124	0.601
	pO_2	-0.198	0.220	-0.127	0.595
	Hematocrit	0.453	0.003*	-0.050	0.833
	Blood Glucose	0.218	0.183	0.405	0.085

*Indicates a significant correlation as detected by Pearson's Correlation.

Table 7.6. Correlation of hippocampal CA1 total cell counts with post-surgery core temperature

	All groups (n=40)		CON-I + PEM-I (n=20)		CON-I (n=10)		PEM-I (n=10)	
	r	p	r	p	r	p	r	p
Core Temperature °C†	-0.111	0.494	-0.352	0.127	-0.897	<0.001*	0.010	0.978

*Indicates a significant correlation as detected by Pearson's Correlation. †Lowest core temperature observed within 8hr following surgical anaesthetic induction.

7.4.6 Hippocampal CA1 MAP-2 Expression

Representative photographs of MAP-2 immunohistochemistry are shown in Figure 7.7. Mean (±SEM) integrated density values for the CA1 region for the three experimental groups expressed relative to the control (CON-S) section on the same slide were as follows: CON-I = 0.86 ± 0.06, PEM-S = 1.11 ± 0.04, PEM-I = 0.89 ± 0.06 (n = 10), indicating a decrease in MAP-2 expression caused by global ischemia. There was no apparent effect of PEM. When analysis was performed after excluding rats in the ischemic groups that did not show bilateral damage, integrated density values were as follows: CON-I = 0.77 ± 0.05, PEM-I = 0.73 ± 0.05. This again indicates a decrease in MAP-2 expression caused by global ischemia.

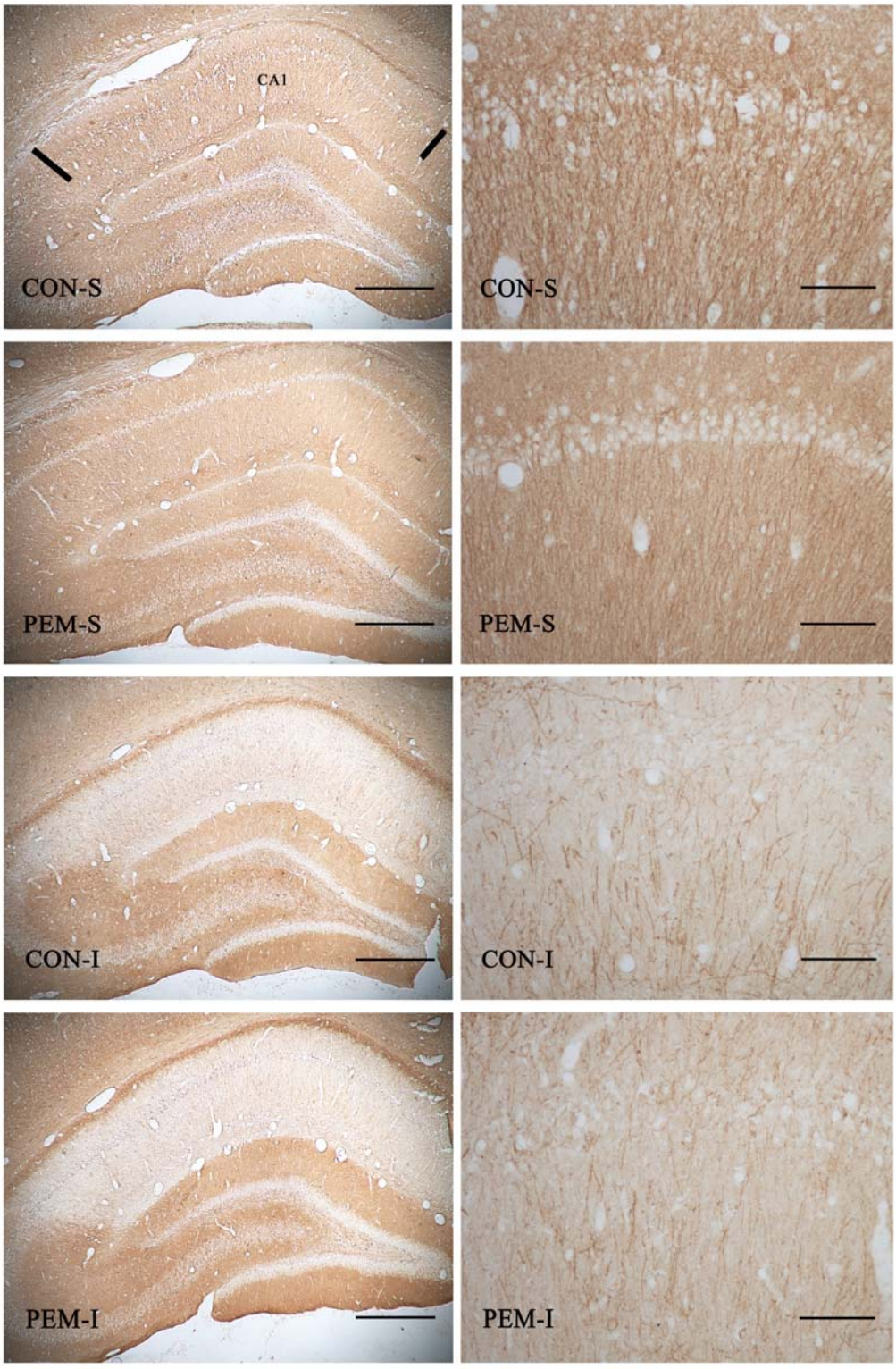

Figure 7.7. Representative photographs of MAP-2 immunoreactivity in the hippocampal formation (left column; scale bar = 500μm) and the CA1 region (right column; scale bar = 100μm) at 7d post-surgery. MAP-2 expression was decreased by global ischemia with no apparent effect of PEM.

7.5 Discussion

The results of this study confirm that PEM was induced in the Sprague-Dawley rat. By the end of the study, after feeding young (30-32d or ~4.5wk) rats a low protein (2%) diet for 14d, food intake, body weight, and serum albumin were depressed, relative to that of control diet–fed rats, by 41.4%, 30.6%, and 23.0%, respectively. However, the model of PEM has some very different features from that used previously in which young adult (11-12wk-old) gerbils were fed a protein-deficient diet (2% protein) for 35d and 38d (Bobyn et al. 2005, Chapter 5). In these gerbil studies, food intake and body weight in protein-energy malnourished gerbils were depressed by 15.0-16.5% and 17.3-21.6% respectively, as compared to control diet-fed animals (Chapter 5, Bobyn et al. 2005); serum albumin concentration decreased in this model by 17.8% after feeding the low protein diet for 31d (Harmon et al. 2006). The variation in the two models of PEM can be readily explained by species differences such as their growth patterns, age at time of placement on experimental diet, length of feeding period, and relative protein requirement.

A major contributor to the differences in the models is the steeper slope of the growth curve of the rat at the time of introduction of the 2% protein diet. To illustrate the dissimilarity in the growth patterns of the two species and the greater impact of reducing dietary protein content in a younger and more rapidly growing rodent, control diet-fed rats in the current study gained 18.4% and 44.2% of their initial body weight after 7 and 14d on the experimental diet, respectively, as compared to gerbils in previous studies from our laboratory that gained 1.6-2.6% (Prosser-Loose, EJ unpublished observation, Chapter 5) after 7d, 5.3-6.8% after 28d (Chapter 5, Bobyn et al. 2005), and 9% after both 35d and 38d (Chapter 5, Bobyn et al. 2005). These estimates of 44.2% after 14d for the rat and that of 9% after 35d and 38d for the gerbil are both slightly underestimated because of the additional transient growth-depressing effect of global ischemia. Rats fed the low protein diet in the current study lost only a small amount of weight (2.0% as compared to initial body weight), whereas protein-energy malnourished gerbils demonstrated greater weight loss (11.8% and 16.5% relative to baseline) (Bobyn et al. 2005, Chapter 5). The higher protein requirement and growth potential of the young rat was accounted for in diet formulation in that the control rats were fed a diet modified from the American Institute of Nutrition-93 Growth diet (AIN-93G) containing 18% protein whereas adult gerbils in previous studies were fed a diet based on the AIN-93 Maintenance (M) diet containing 12.5% protein (Reeves et al. 1993). To summarize, the model of PEM described in the current study in

the rat is characterized by mild weight loss in a rapidly growing young rat, whereas that in the previous studies resulted in greater weight loss in the adult gerbil in which growth rate is approaching a plateau. In both cases, animals appeared clinically healthy with the exception of stunting. Given the differences described, it is difficult to compare severity of the PEM, but the greater depression in serum albumin concentration suggests that protein synthesis has been inhibited more by the end of the current study.

A second important difference between the two models of PEM is the extent to which malnutrition had developed by the time of induction of brain ischemia. In the current study, although body weight was depressed in the low protein diet-fed group by 16.9% by the time of the ischemic insult and food intake was declining after 6d of experimental diet (10.6%), the latter did not reach statistical significance until 1d after induction of brain ischemia. In contrast, we previously described not only a 17.5% decrease in body weight in the gerbil by the day of exposure to global ischemia (and 28d of experimental diet) but also a clear voluntary suppression of food intake (14.8%) (Bobyn et al. 2005). This difference in response in food intake may indicate a milder PEM, although any conclusion would be strengthened by the addition of biochemical markers of protein status at this time. This was the first experiment using the 2-VO model in our laboratory and since there was uncertainty as to how well the animals would recover after this more invasive surgery, we were conservative in placing the animals on the protein deficient diet for only 1wk prior to surgery. In future studies, the length of time spent on the protein-deficient diet prior to brain ischemia could be extended. However, for reasons outlined below, studies of pre-existing PEM on outcome following global ischemia may not be possible.

A final difference to highlight is how the effects of the PEM model on food intake and body weight interrelate with that caused by exposure to brain ischemia. In the current study, the growth depressing effect of brain ischemia was transient and had resolved by 7d post-ischemia in parallel with the return to usual food intake in control diet-fed rats after 3d. In comparison, a small growth depressing effect of ischemia at 10d after exposure was reported in one previous study in the gerbil (Bobyn et al. 2005) whereas this was absent in a subsequent study (Chapter 5). We have also reported both prolonged suppression of food intake following global ischemia in the control diet-fed gerbil (Bobyn et al. 2005) as well as a transient decrease followed by an increase sustained for 21d (McEwen and Paterson. 2009), and we believe this difference relates

to single versus group housing after the ischemic insult. This point draws attention to the unavoidable disadvantage of having to use surgical models to study brain ischemia experimentally. This point is largely ignored by researchers who generally do not report food intake and body weight following experimental stroke. However, alterations in these variables could potentially either confound or help to explain results, particularly in the case in which a putative neuroprotectant affects food intake or weight gain.

Upon comparison of the two models of PEM used in this thesis, it seems that the gerbil model is a better representation of the clinical situation of interest. Although neither was conducted in an aged animal, as would be preferred for mimicking the elderly stroke patient, the growth pattern of the gerbil is closer to that of a human in that a plateau is approached at young adulthood. Unfortunately, as discussed in Chapter 6, the rat 2-VO model requires the use of young, rapidly growing rats (weighing ≤300g) to induce consistent hippocampal injury. This age restriction has disadvantages for modeling the adult stroke patient, and this is of particular concern for nutritional studies. Also, even if a rat of comparable age could be used for such studies, the slope of the growth curve is much steeper than that of the gerbil (Charles River website) and thus a poor mimic of the adult stroke patient. Of particular benefit to future studies of protein-energy malnutrition and brain ischemia would be to add markers of inflammation, such as C-reactive protein, that would help to further classify the type of protein-energy malnutrition. This is similar to what has been recommended for human studies (Jensen et al. 2010)

Given the differences in models of global ischemia and PEM from those employed previously (Bobyn et al. 2005), a major objective of the current study was to confirm whether PEM exacerbated short-term hippocampal CA1 neuron loss in the rat 2-VO model of global ischemia. As expected, based on a previous study using the gerbil model of global ischemia (Bobyn et al. 2005), PEM did not appear to worsen the hippocampal CA1 death. However, no definite conclusion can be drawn as the experiment has been confounded by other variables such that pre-existing PEM cannot be accurately studied in the 2-VO model. Some variability was expected with use of the 2-VO model and in the current study, bilateral hippocampal CA1 damage in the CON-I group occurred in 70% of rats and averaged 78.8% (range 68.1-91.4%) with 30% of rats demonstrating unilateral CA1 injury. This range of variability is close to though slightly above the 80% damage in 80% of animals expected from communications with our

collaborator (Dr. Fred Colbourne, unpublished communication) as well as the results of the final pilot study described in Chapter 6 of this thesis in which bilateral damage was present in 83.3% of animals and averaged 72.3% (range 64.1-81.0%) in the 10min occlusion group. However, the inconsistency in injury was substantially increased by the pre-existence of PEM, as shown by bilateral CA1 damage in only 50% of the group with the remaining rats demonstrating either no (40%) or unilateral (10%) injury. When these data were re-analyzed after excluding any rat exposed to global ischemia that did not sustain bilateral hippocampal injury, the trend for PEM to exert a neuroprotective influence was removed. However, this type of analysis is suboptimal as it can lead to biased or incomplete interpretation. For example, if the neuroprotective effect of PEM is, in fact, real, and not due to model deficiencies as proposed below, then removing these rats from analysis leads to incorrect conclusions. In addition, performing this type of analysis further compromises the results by reducing power due to the smaller sample size.

While these findings could be interpreted as suggestive that PEM truly offers neuroprotection, other experimental data obtained support the idea that pre-existing PEM has altered key physiological variables and invalidated the 2-VO model for studying the questions of interest. Control of such variables is key to producing consistent hippocampal injury with the 2-VO model so that experimental variables such as pre-existing nutritional status can be studied. Hypothermia is known to be neuroprotective when administered during or following global ischemia (Colbourne and Corbett. 1995, Nurse and Corbett. 1994). The data from this experiment demonstrate that brain temperature was well controlled in the normothermic range in all experimental groups during the intra-ischemic period. However, the drop in core temperature caused by PEM immediately following surgery, during the time when the rats were coming out of anaesthesia, may have played an important role in reducing hippocampal injury. While this hypothermia was considerably milder and shorter in duration than that required for permanent neuroprotection after global ischemia (Colbourne and Corbett. 1995, Colbourne et al. 1998, Colbourne et al. 1999), it certainly may have played a role in combination with changes in other key variables discussed below. However, the drop in post-surgical core temperature was not limited to those PEM-I gerbils with minimal hippocampal damage, and thus CA1 neuron counts were not correlated with this drop. This suggests that other physiological factors (as discussed below) in addition to transient post-ischemic hypothermia have contributed to the reduction in hippocampal injury. Given the transient and modest decline in core temperature caused by PEM,

any influence on CA1 neuron survival may be temporary, as has been shown for some putative neuroprotective drugs (as reviewed in Corbett and Nurse. 1998). This could only be determined by assessing long-term hippocampal CA1 survival. A smaller decline in core temperature was also evident in only 30% of CON-I animals, and this was more closely related to unilateral CA1 injury as supported by the significant negative correlation between post-ischemic core temperature and surviving CA1 neurons.

Alterations in a number of other key physiological variables may have further confounded interpretation of results in the protein-energy malnourished group exposed to 2-vessel occlusion and hypotension. It was important to determine whether PEM altered baseline acid-base balance and also to ensure that both sham and ischemic groups remained within the target physiological ranges for these parameters. Departure from these ranges could potentially interfere with the hippocampal damage induced by the 2-VO surgery or impair recovery from surgery. On first examination, rats on the protein-deficient diet appeared to have a better ventilatory response to anaesthetic based on significantly improved pH, pCO_2, and pO_2 values prior to ischemia or sham surgery. However, it is difficult to draw any definite conclusion on this point because this effect has been magnified, perhaps falsely, by the atypically low pH and high pCO_2 baseline values in the control diet-fed sham group. In contrast, mean values (±SEM) of control diet-fed animals in the three pilot studies described in Chapter 6 of this thesis were 7.41 ± 0.04, 7.46 ± 0.04, and 7.41 ± 0.01, respectively. In particular, in this study, two animals in the CON-S group had very low pH values of 7.16 and 7.11, in parallel with high pCO_2 values of 76 and 88, respectively, indicative of respiratory acidosis. Correlation analysis suggests that baseline pH and pCO_2 did not influence total CA1 cell counts. However, a trend for a positive correlation ($p=0.052$) between neuron counts and pO_2 was present on analysis of all rats exposed to global ischemia, suggesting that the higher baseline pO_2 values of the PEM group contributed to neuroprotection in this group.

If PEM truly improved respiratory response to isoflurane anaesthesia, there is no apparent explanation. This finding was surprising since the literature suggests a detrimental effect of malnutrition on respiratory function. Studies in rats have found changes in lung morphology and biochemistry suggesting emphysema-like characteristics (Sahebjami and MacGee. 1982, Sahebjami and Vassallo. 1979, Sahebjami and Wirman. 1981). Studies in otherwise healthy humans have found decreased basal oxygen consumption, decreased response of ventilation to

hypoxia, and decreased neural ventilatory drive, suggesting an effect of malnutrition on respiratory muscles as well as the central nervous system (Doekel et al. 1976, Weissman et al. 1983). It is important to note that these studies were not examining protein-energy malnutrition specifically. The studies by Sahebjami et al. (Sahebjami and MacGee. 1982, Sahebjami and Vassallo. 1979, Sahebjami and Wirman. 1981) fed adult rats one fifth of their daily food consumption until a loss of 40% of body weight was achieved. Doekel et al. (1976) fed healthy individuals a 500kcal/d carbohydrate diet with supplemental electrolytes in order to mimic the effect of intra-venous fluid infusion. Weissman et al. (1983) infused healthy individuals with 5% dextrose for 7d (400kcal/d) followed by 3.5% amino acids for 24hr in order to separate the effects of these two constituents of parenteral nutrition. The different nutritional paradigms examined in these studies likely explain the discrepancy between these findings and those of the current study. Also, unlike these studies, rats in the current study were exposed to anaesthetic for approximately 30min prior to the first blood gas measurement, which will have a major impact on ventilatory ability.

The influence of PEM on blood pH disappeared by the time of post-ischemic blood sampling, at which time the major finding was a significant number of cases of respiratory acidosis in both ischemic groups as shown by high pCO_2 and low pH values. The positive correlation detected between total CA1 cell counts and pH when all groups were included in analysis can be explained by this effect of 2-VO surgery (carotid artery occlusion and hypotension) to cause respiratory acidosis. The rise in pCO_2 appears to not completely account for the fall in pH, and thus there may also be an element of metabolic acidosis. That this significant correlation disappears when only rats exposed to global ischemia are included in analysis supports the conclusion that pH has not contributed to the variability in extent of hippocampal damage. An apparent independent improvement in ventilatory response by PEM, shown by higher pO_2 and lower pCO_2 values, was still evident following exposure to sham or 2-VO surgery, but again the significance is uncertain given the interpretation of baseline values in the control diet-fed group discussed above.

PEM did not alter hematocrit in either pre- or post-ischemic samples, and thus a change in oxygen-carrying capacity of the blood does not offer an explanation for the decreased hippocampal damage in the PEM ischemic group. This conclusion is supported by the lack of correlation between CA1 cell counts and pre-ischemic hematocrit values. The pre-ischemic

values for all groups fell within the normal range for the rat of 35-57%, with this large range reflecting differences in age, sex, and strain (Sharp. 1998). Similar to our values, hematocrit values for ~15wk old male Sprague-Dawley rats have been reported to be ~40% (Probst et al. 2006). The hematocrit measured on post-surgical blood samples was decreased in both ischemic groups although still within the normal physiological range. The positive correlation detected between total CA1 cell counts and hematocrit values on these samples with all groups included in analysis detected this relationship. However, this decrease in hematocrit likely occurred as a result of the surgical procedures, in particular because of hemodilution caused by the small amount of saline that was infused as blood was withdrawn and later re-infused to alter blood pressure.

Blood glucose concentration, since it was increased by PEM but still within the expected fasting range in the pre-ischemic blood samples, does not offer a clear contribution to the apparent reduction in hippocampal damage. This is supported by the lack of correlation between pre-ischemic glucose and total CA1 cell counts. However, fasting blood glucose is an incomplete measure of glucose regulation. These data, along with the trend towards a positive association between post-ischemic blood glucose and total surviving CA1 neurons ($p = 0.085$) may be indicative of altered glucose regulation in the protein-energy malnourished group in response to anaesthesia, surgery, and global ischemia that might have influenced ischemic outcome.

While a number of statistically significant findings have been discussed above, all of the differences are small and thus do not provide strong evidence for the apparent confounding of the model. It is likely that PEM has induced changes in other physiological factors that account for the findings. Blood pressure regulation is one factor to consider. The higher blood pressure readings in protein-energy malnourished sham rats during surgery suggest this as another possible physiological contributor to the apparent neuroprotection offered by PEM. Although blood pressure was strictly controlled during the 10min period of ischemia and was not different between control-fed and protein-energy malnourished rats, PEM may have elevated blood pressure in the post-ischemic period. Enhanced brain reperfusion as a result may have afforded some protection in the PEM-I animals since high blood pressure has the potential to save penumbral tissue after focal ischemia (Verro and Chow. 2009). This would be important to monitor in future studies. A limitation of this conclusion is that in the current study, both sham groups showed a low mean arterial blood pressure in comparison to what has been previously

reported for the Sprague-Dawley rat under isoflurane anaesthetic (96.7 ± 1.2mmHg) (MacLellan et al. 2006). While excess anaesthesia could contribute to lowering of blood pressure, we do not believe that this was an important factor since isoflurane was decreased to as low a level as possible based on toe pinch reflex, blood pressure readings and respiratory rate. Suboptimal location for placement of the tail artery catheter is a more likely explanation for the low and variable blood pressure readings. Positioning of the catheter is important since the tail has a pressure gradient in which a drop of 2mmHg occurs per centimetre of tail length (Fregly. 1963). Cannulation at the base of the tail is therefore ideal. In this experiment tail cannulations were generally performed ~1.3-2.5cm from the base of the tail in case re-cannulation was required due to artery breakage or unsuccessful cannulation. Now that additional surgical experience has been obtained, the cannula should be kept close to the base of the tail for future experiments and, most importantly, be consistently placed between animals.

These findings for such a broad range of physiological variables emphasize the challenge of studying clinically relevant co-morbid factors that can influence outcome after brain ischemia. Considering the confounding influences identified, we do not believe that the problem of pre-existing protein-energy malnutrition can be accurately studied in the 2-VO model of global ischemia. While blood gases could be maintained at the same levels as those of control-diet fed rats by using mechanical ventilation, as discussed above, it is suspected that other variables are more important. Thus, the findings are also inconclusive in addressing the second major hypothesis of the study that PEM would exacerbate the decrease in MAP-2 expression caused by global ischemia. The data showed that while ischemia significantly decreased the expression of MAP-2 expression in the CA1 pyramidal layer, stratum oriens and stratum radiatum of the rostral hippocampus, no effect of diet was observed; this conclusion was unaltered by excluding rats with unilateral or no hippocampal damage. The pattern of MAP-2 expression followed that of CA1 cell death such that animals with unilateral CA1 cell death also presented with unilateral dendritic damage. It is therefore evident that confounders of the 2-VO surgery in this study also had an effect on MAP-2 expression. A second constraint of the study was that the decrease in MAP-2 expression at 7d post-ischemia was so extensive that PEM may not have been able to cause a further detectable decrease. In future studies, it would be of value to examine MAP-2 expression at several time points and in other hippocampal regions after global ischemia. By examining earlier time points, differences in onset or pattern of dendritic damage may be found,

whereas examination at later time points may detect recovery of dendritic structure and plasticity. It would be interesting to examine treatments that promote dendritic recovery and plasticity post-ischemia, modeling a rehabilitation setting, such as environmental enrichment (Nithianantharajah and Hannan. 2006), and determine whether PEM impairs those mechanisms.

The confounding variables discussed above do not preclude future study of the influence of PEM that develops after brain ischemia, thereby avoiding complications with the 2-VO surgery caused by altered nutritional status. This is an equally, if not more important, clinically relevant question since nutritional status tends to worsen in stroke patients, affecting up to 26.4%, after 1wk in the hospital (Davalos et al. 1996). As demonstrated in Chapter 5, PEM can affect mechanisms relevant to post-ischemic recovery. Since molecules involved in plasticity mechanisms following ischemia (trkB and GAP-43) are affected by the presence of pre-existing PEM, malnutrition developing after brain ischemia may induce similar changes.

This study also identified other physiological influences of PEM that could exert important influences on clinical stroke outcome independent of artifacts related to the necessity of using surgical models to study stroke. Contrary to altered thermoregulation that occurs in response to anaesthesia and surgery, any changes in temperature regulation caused by malnutrition either before or after stroke could have a true effect on outcome. An increase in diurnal temperature fluctuation occurred rapidly in response to the low protein diet. This factor should be studied in both malnourished stroke patients and experimental stroke models to determine its influence on brain injury.

The possible implications of PEM confounding the 2-VO surgical model of brain ischemia by altering glucose regulation have been discussed above. However, similarly to changes in thermoregulation, if PEM causes chronic alterations in glucose homeostasis, which in turn can influence brain injury caused by ischemia (Lin et al. 1998, Pulsinelli et al. 1982, Voll and Auer. 1988, Zhu and Auer. 2004), this could be relevant to clinical stroke. Previous literature indicates that hypoglycemia commonly occurs with protein or protein-energy malnutrition (Das et al. 2004, Fagundes et al. 2009, Gamallo et al. 1989), and our data indicate that PEM can increase fasting blood glucose concentration under surgical conditions. Chronic monitoring of glucose fluctuations in both the fed and fasted state prior to and after clinical stroke (and without the confounding influence of surgery) would be essential to determine whether PEM can truly influence the extent of brain injury through this mechanism.

While PEM did not affect serum corticosterone concentration in the current study, this factor is an important one to re-examine as it is known to influence outcome from ischemia (Payne et al. 2003, Schurr et al. 2001) and glucocorticoid concentration is often elevated by PEM (Monk et al. 2006). Previous findings of enhanced trkB expression (outlined in Chapter 5) suggest that PEM might increase the stress response to global ischemia since this receptor can be induced by increased glucocorticoids (Jeanneteau et al. 2008). Rats in the current study had an un-physiologically high mean (±SEM) corticosterone concentrations of 426.3 ± 15.8 ng/ml, as compared to what has been reported in non-stressed male Sprague-Dawley rats (~275-300 ng/ml) (Shors et al. 1999). This suggests that pre-anaesthetic stress may have masked a smaller increase caused by PEM, as this problem has been demonstrated in previous studies in mice (Monk et al. 2006, Shipp and Woodward. 1998). In future studies, efforts to minimize pre-anaesthetic stress should be taken in order to avoid artificially increased values and thus increase sensitivity for detecting an effect of malnutrition.

The possibility that post-surgical alterations in blood pressure regulation have confused the findings of this study are discussed above. In addition, however, if PEM chronically raises blood pressure, this could also be a clinically relevant determinant of stroke outcome. Effects of protein deficiency and protein-energy deprivation on blood pressure have been previously reported (Almeida and Mandarim-de-Lacerda. 2005, Tonkiss et al. 1998). Using radiotelemetry, Tonkiss et al. (1998) found that pre-natally protein malnourished rats had a small, but significant, increase in diastolic blood pressure. Upon the presentation of an olfactory stress (ammonia), both systolic and diastolic pressures increased significantly in the protein malnourished rats (Tonkiss et al. 1998). This raises the possibility that PEM could exert a beneficial effect by enhancing brain reperfusion after ischemia. Conversely, higher blood pressure could have adverse effects later on following ischemia as hypertension following stroke may promote edema and increase the risk of hemorrhagic transformation (Britton and Carlsson. 1990). In future experimental studies of PEM and brain ischemia, chronic blood pressure should be monitored directly with the use of implantable radiotelemetry probes. These allow the collection of continuous blood pressure data without the confounding effect of anaesthesia (Huetteman and Bogie. 2009). Blood pressure is evidently an important variable to monitor following stroke in patients, in whom it would be of particular interest to examine whether pre-existing PEM causes hypertension.

Aside from the specific effects of PEM discussed above, the occurrence of respiratory acidosis in the current study indicates that further trouble-shooting needs to be carried out in the 2-VO model. While many animals had good acid-base balance, several fell out of range, even during baseline sampling. Respiratory acidosis was indicated when pCO_2 values increased above the desired range, with a corresponding decrease in pH values below the desired range. Animals were not artificially ventilated during the surgery and therefore the ability to prevent respiratory acidosis during anaesthesia was limited to avoiding excessive anaesthetic and minimizing surgery time. Given the extensive surgical practice obtained during the pilot studies discussed in Chapter 6, the reduction in surgical time that followed from this, and the excellent blood gas values obtained in the final pilot study, respiratory acidosis in the current study was not anticipated. In addition to signs of respiratory acidosis in post-ischemic blood samples, what was most unexpected was the high pCO_2 and low pH values observed in pre-surgical samples from some control diet-fed sham rats. There is no obvious explanation for the general deterioration in acid-base balance found in this study although it is worth noting that some problems with the anaesthetic machine and N_2O tank were encountered that may have contributed to some of the abnormal blood gas values.

The findings of this study highlight the difficulty in using surgical models to induce stroke. These experimental models introduce variables that are not true to the clinical situation and become especially challenging when studying co-morbid conditions, such as PEM. The inclusion of a sham surgical group is useful for controlling some, but not all, of these factors. A large confounder is the use of anaesthetic, which can interfere with several physiological parameters including acid-base balance and blood pressure. Changes in both of these variables are known to occur with stroke and the use of anaesthetic in the animal model limits the mimicking of the human situation and the ability to draw conclusions about these factors. Another limitation of the 2-VO model is that it negates variables that may potentially be present in human stroke. Brain temperature (via tympanic measurements) and blood pressure are controlled during the 2-VO surgery and are kept within specific ranges, again excluding these variables that may change during human stroke and in response to the experimental variables being studied. The need to fast rats prior to surgery is another example, since altered blood glucose often occurs in human stroke (reviewed in Wong and Read. 2008). In human studies,

blood glucose is affected by PEM (Torun. 2006) and therefore it would be ideal to test the true effect of this clinical situation in the animal model of global ischemia.

A limitation of this study, as with many experimental stroke studies, was the lack of monitoring of physiological variables post-ischemia. Several of these parameters are altered in the clinical stroke patient in the days and weeks following stroke and are associated with outcome. In our study, oxygenation in the 2-VO model was measured just following reperfusion but was not monitored further. This may be an important variable to monitor since hypoxia is often reported following human stroke and has been associated with co-morbidities and poor prognosis (reviewed in Wong and Read. 2008). Blood glucose was also not monitored during the post-ischemic period, and readings taken prior to and following occlusion are additionally affected by pre-ischemic fasting. In addition to the association between pre-stroke hyperglycemia and poor stroke outcome, post-stroke hyperglycemia is also thought to be detrimental. A large proportion of stroke patients show a rise in serum glucose following ischemic stroke and this seems to be related to increased stroke severity (reviewed in Wong and Read. 2008). Blood pressure has also been reported to increase following ischemic stroke; however, both low and high blood pressure have been associated with poor outcome, suggesting a U-shaped relationship. Cerebral autoregulation, which normally maintains cerebral blood flow in the face of altered systemic blood pressure, is thought to be affected post-stroke, likely affecting cerebral perfusion (reviewed in Wong and Read. 2008). Again, mean arterial blood pressure was monitored only during the occlusion period in our study, leaving post-ischemic effects unknown.

In summary, it has been demonstrated that PEM can be induced by feeding a diet containing 2% protein to the young male Sprague-Dawley rat. While there was no apparent influence of PEM on CA1 neuronal cell death or MAP-2 expression following global ischemia induced by 2-VO with hypotension, these conclusions are confounded by the effects of pre-existing PEM on numerous physiological variables that interfere with a consistent 2-VO model. Care should be taken if using this model to examine the effect of other pre-existing nutritional states on ischemic outcome, and physiological parameters should be closely monitored.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Evidence collected over the last two decades indicates that a proportion of acute stroke patients are already affected by protein-energy malnutrition upon admission to the hospital (Axelsson et al. 1988, Davalos et al. 1996, Davis et al. 2004, Gariballa and Sinclair. 1998, Martineau et al. 2005, Yoo et al. 2008). Since these patients have been shown to have a worsened functional recovery (Davalos et al. 1996, Finestone et al. 1996), this problem is an important one to research. Animal studies using rodent models of brain ischemia have found not only a detrimental effect of PEM on functional recovery (Bobyn et al. 2005), but also effects on several mechanisms of both damage and recovery (Bobyn et al. 2005, Ji et al. 2008, Chapter 5). The experiments described in this thesis examined cognitive effects of PEM, as well as mechanistic effects on how PEM influences outcome in rodent models of global ischemia. The overall goal of the research has been to study the effects of clinically relevant nutritional situations in experimental models that can link histological and functional outcome to the underlying mechanisms.

This thesis is centered around the study of a disease co-morbidity factor, PEM, on stroke outcome. Such variables are often not considered. In the first experiment, examination of whether PEM might confound one of the standard reliable tests of functional outcome used in experimental stroke was carried out. Previous research found protein-energy malnourished gerbils to be significantly impaired in terms of ability to habituate in the open field behavioural test following global ischemia (Bobyn et al. 2005). In order to further characterize the functional effects, the effect of PEM on performance in the T-maze was examined. The T-maze is a test of working memory, which refers to a short-term type of memory that requires the use of the hippocampus (Corbett and Nurse. 1998). The high sensitivity of the T-maze for detecting functional impairments after global ischemia has been well described (Corbett and Nurse. 1998); however the effect of PEM on performance in the T-maze in the adult rodent had not been

thoroughly studied. In order to avoid potential interpretational complications of combining PEM and ischemia, the effect of PEM alone was studied on performance in this food-baited test. The first finding was that the sunflower seed reward, shown to be a highly motivating reward for gerbils (Farrell et al. 2001), did not interfere with the induction of PEM produced by feeding a 2% protein diet for 6wk. Food intake, body weight and serum albumin analysis were decreased to a similar extent as reported in previous experiments using the same protein-deficient diet with no food reward present (Bobyn et al. 2005, Harmon et al. 2006). This clearly demonstrates that PEM can be successfully induced in the gerbil even with the use of a small food reward.

The second finding was that working memory as assessed in the T-maze was not impaired by 6wk of protein-energy malnutrition when analyzed using criterion typically employed in studies of global ischemia. This is supported by findings of a previous study in which prenatal PEM had no effect on performance in the T-maze (Tonkiss and Galler. 1990). However, several other studies found a negative effect on hippocampal function with protein deficiency or protein-energy malnutrition as measured in the T-maze and the water maze (Fukuda et al. 2002, Lukoyanov and Andrade. 2000, Tonkiss and Galler. 1990, Tonkiss et al. 1990). This contrast can likely be explained by differences in diet composition, length of feeding, and age of animal, which would have resulted in varying degrees of severity of protein-energy malnutrition. This problem is further complicated by the lack of measurement of biochemical indices of PEM in these studies. These discrepancies highlight the need to confirm the effects of specific types of PEM and other nutritional paradigms on cognitive performance in functional tests used in experimental stroke models.

An interesting finding in this experiment was that when criterion in the T-maze was set more stringently, protein-energy malnutrition altered performance but not in the direction that was hypothesized. Animals on the protein-deficient diet actually required significantly fewer trials to reach criterion, and more of these animals reached criterion, which would be suggestive of improved working memory. However, we hypothesize that these results reflect an increased motivation to obtain the food reward rather than an enhancement of hippocampal function. No other studies indicated such an effect in animals unless they had been fasted prior to T-maze testing (Tonkiss and Galler. 1990, Tonkiss et al. 1990), and therefore our finding seems to be a novel one.

Although evaluation criterion for performance in the T-maze typically used for assessing outcome following global ischemia in the gerbil typically consists of a less stringent requirement than that discussed above (Babcock and Graham-Goodwin. 1997, Colbourne and Corbett. 1995, Farrell et al. 2001), the possibility remains that the increased motivation detected by the more stringent criterion in nutritionally deprived animals would cloud interpretation of functional outcome from ischemia. However, since this effect was small, this theory would have to be confirmed by testing gerbils exposed to both PEM and global ischemia in the T-maze. Unfortunately the use of the water maze, a test of working memory that does not require a food reward, cannot be used in the gerbil due to the stress generated when a desert rodent is required to swim to solve a task (Corbett and Nurse. 1998).

Since the gerbil model of global ischemia has been abandoned by many investigators due to issues of consistency and reliability of the model (Laidley et al. 2005, Seal et al. 2005), there is increasing use of other models of global ischemia, and in particular the rat 2-vessel occlusion and the rat 4-vessel occlusion. It will be important to clarify any confounding influences of pre-existing nutritional status on the behavioral tests used to assess functional outcome in these models of stroke. The assessment of behavioural tests requiring food rewards for nutritional studies will likely present many challenges including differences between species. For example, while we, and others (Babcock and Graham-Goodwin. 1997, Colbourne and Corbett. 1995, Farrell et al. 2001) have shown that the gerbil will work for a specific type of food reward, the rat requires food deprivation prior to this type of behavioural testing in order to induce sufficient motivation (Langdon et al. 2007). It is impossible to predict the outcome without testing it, but it seems likely that the test results would be difficult to interpret if chronic PEM (that causes a voluntary decrease in food intake) were combined with acute food deprivation. The potential confounder of food motivation can be avoided in rat models of global ischemia by choosing behavioural tests, such as the water maze, that do not require a food reward.

Several paths can be taken from the work completed in this first experiment. The effect of PEM on working memory post-ischemia should be examined using a non-food baited test. The rat would be a more suitable species to use for this since a wider variety of tests could be used. The water maze is a test of working memory and the rat has been shown to be able to complete this task; however, it should be noted that this is not a very sensitive test for discerning effects in the 2-VO model of global ischemia (Arvanitidis et al. 2009, Langdon et al. 2007). If the T-maze

is to be used in conjunction with the 2-VO model, it has been suggested that the most difficult forms of the maze should be used and that other behavioural tests should be included (Langdon et al. 2007). Ideally, a combination of food reward-based and non-food reward based tests should be used. The latter might include object recognition tests (Gobbo and O'Mara. 2004, Plamondon et al. 2008), fear conditioning tests (Spencer et al. 2008), and assessment of retrograde amnesia (Travis et al. 2009). The behavioural consequences of global ischemia using the rat 2-VO model have been nicely documented in one experiment in which histological findings were also presented from the same group of animals, thereby confirming successful ischemia (Langdon et al. 2007). Long-term (270d post-ischemia) deficits were apparent in learning, working, and reference memory, which were measured by various behavioural tests including the T-maze, the radial arm maze, and the Morris water maze (Langdon et al. 2007). It will be interesting to see how PEM affects these findings.

In experiment 2, the focus turned to pursuing examination of the mechanisms that might be a factor in the functional deficits caused by PEM following global ischemia in a previous study (Bobyn et al. 2005). Since a large proportion of survivors of stroke do show some spontaneous recovery, the effect of PEM on recovery-related mechanisms was an important area to study. The neurotrophin BDNF and its receptor trkB have been implicated in promoting recovery following ischemia, although this has been shown mostly with the use of exogenous BDNF (Ferrer et al. 1998a, Kiprianova et al. 1999a). Since PEM had previously been shown to decrease endogenous BDNF expression in the hippocampus (Mesquita et al. 2002), it was believed that this could potentially worsen recovery from ischemia and might help explain the functional deficit that has been evident in these animals. GAP-43 is another recovery-related molecule that had been suggested to be beneficial following global ischemia (Schmidt-Kastner et al. 1997, Tagaya et al. 1995), although the evidence behind this was lacking. It was therefore decided to characterize the effect of PEM on mRNA and protein expression of BDNF, trkB, and GAP-43 in the hippocampus following global ischemia in the Mongolian gerbil.

The first finding from this study was that PEM induced by feeding 2% protein to the gerbil resulted in a milder degree of PEM than has previously been observed. In the current study, food intake decreased by 9% in PEM animals as compared to CON after 4wk on diet. Previous studies in our laboratory found food intake to decrease by 15% (Bobyn et al. 2005) and 14.5% (Prosser-Loose et al. 2007) at 4wk as compared to CON. In the current study, body

weight in PEM animals was decreased by 14% as compared to CON. This is also milder than values previously reported of 17% (Bobyn et al. 2005) and 21% (Prosser-Loose et al. 2007) at 4wk as compared to CON. It is difficult to explain what caused the milder form of PEM that arose in this study. It is possible that the housing of animals in varying numbers (2-4/cage) and the social effects this might have had, changed the extent to which the low protein diet caused a reduction in food intake and therefore affected body weight reduction.

Another major finding was that BDNF mRNA was increased in both ischemic groups at every time point but this was not mirrored at the protein level where no changes were observed with either surgery or diet. Previous studies observing the mismatch between BDNF mRNA and protein have attributed the issue to a problem in protein synthesis, release, or transport causing translational arrest (Lee et al. 2002, Martin de la Vega et al. 2001). Another possibility is that the BDNF protein was rapidly transported away from the CA1 region and therefore went undetected; however our findings show no evidence of a change in expression in destination regions. It is possible that we missed a small rise in the CA1 region occurring within the first few hours post-ischemia. In order to clarify this issue in future studies it would be of use to examine BDNF protein expression at earlier time points, for example, within the first few hours following ischemia, since BDNF has been shown to be an immediate early gene (Hughes et al. 1993). Detailed examination of the destination regions (CA3, entorhinal cortex) of BDNF transport would also be needed to confirm whether retrograde or anterograde transport is occurring. Further examination of the problem of translational arrest in global ischemia is also warranted since several other molecules may be affected by this (Kozak. 1991, Martin de la Vega et al. 2001). It would also be of interest to determine if proBDNF is released upon ischemic insult, and the effect PEM may have on it. This is a precursor form of BDNF that can be released from neurons, but that signals through the receptor p75 and not trkB (Yang et al. 2009). Since p75 signaling promotes cell death and interferes with synaptic transmission, it will be important to determine whether this form of the neurotrophin is expressed following ischemia and whether it is affected by nutritional status.

Another finding from this experiment was the increase in trkB mRNA and protein expression in the CA1 region after global ischemia suggesting an effort to sequester more BDNF for neuroprotection (Kirino. 1982). Especially striking was the amplified expression of trkB protein observed in the fibres surrounding the CA1 region in the PEM ischemic group, at 7d

post-ischemia. A possible explanation for this finding is that PEM caused an increased stress response to ischemia, thereby increasing trkB expression. Increased expression of the receptor has been shown in response to a number of different stressors (Croll et al. 1998, Dragunow et al. 1997, Goutan et al. 1998, Hicks et al. 1998, Nibuya et al. 1999, Tapia-Arancibia et al. 2004) and can be induced by increased glucocorticoid concentration (Jeanneteau et al. 2008). PEM causes increased serum glucocorticoid concentration as part of the endocrine response to the nutritional deficiency (Monk et al. 2006) and relevant to the current thesis, protein-energy malnourished patients have been reported to have higher levels of free urinary cortisol during the first week after stroke (Davalos et al. 1996). The main glucocorticoid in the gerbil is cortisol and a previous study in our laboratory found no change in its concentration by PEM 6hr following global ischemia (Ji et al. 2008). PEM also did not affect serum corticosterone concentration in rats as shown in experiment 3 of this thesis. However, since the glucocorticoid levels were higher than normal physiological values in both of these studies, it is likely that pre-surgical stress affected these animals. Future studies should address the problem of pre-anaesthetic stress in rodents in order to obtain accurate measurements of glucocorticoids.

Another interesting finding from this study was that ischemia increased GAP-43 expression in the CA1 region at 3 and 7d and this was magnified by PEM. Interestingly, at 7d this exacerbation of expression in PEM-I animals extended to the CA3 region and the dentate gyrus of the hippocampus. One hypothesis formed is that this exacerbated expression is reflective of a pathophysiological mechanism, potentially involved in post-ischemic seizure activity (Schmidt-Kastner et al. 1997, Tagaya et al. 1995). Interestingly, both GAP-43 and trkB have been implicated in hyperexcitability of hippocampal circuits (McKinney et al. 1997), a known cause of ischemia-associated epilepsy (Epsztein et al. 2006). Relevant to our studies, protein malnutrition has also been shown to alter hippocampal circuits, both during development (Chang et al. 2003, Diaz-Cintra et al. 2007) and in the adult (Andrade and Paula-Barbosa. 1996), although the nutritional paradigms in these studies were not well characterized. No seizure activity was evident in the animals in this study; however since they were observed only up to 7d after global ischemia, the increased hippocampal expression of trkB and GAP-43 may be indicative of developing hyperexcitability.

While my data and that of others from my laboratory (Bobyn et al. 2005, Harmon et al. 2006) suggest that PEM does not exacerbate CA1 neuronal death caused by global ischemia, the

results from my second experiment indicate that there may be abnormalities in structure, function, and plasticity of hippocampal fibres. Since these mechanisms may potentially contribute to the impaired functional recovery previously observed in PEM ischemic gerbils (Bobyn et al. 2005), it would be of value in future studies to specifically investigate whether the ratio of excitatory to inhibitory synapses is affected by PEM by use of electrophysiological recordings. CA1 field excitatory post-synaptic potentials (fEPSPs) could be recorded from the stratum radiatum region upon stimulation of the Schaffer collaterals. This would allow changes in membrane hyperexcitability to be detected in each experimental group. The potential association of nutritional status with the development of post-stroke epilepsy is another interesting question that should be investigated further by examining longer survival time points following ischemia.

Another possible explanation for the exacerbated GAP-43 expression observed in the PEM ischemic animals, is that PEM caused an enhanced inflammatory response. A previous study in our laboratory found that PEM caused increased activation of the transcription factor NFκB (Ji et al. 2008); however, the effect on pro-inflammatory target genes of NFκB is yet to be determined. While the relationship between GAP-43 and inflammation following global ischemia has not specifically been explored, central nervous system inflammation has been shown to induce GAP-43 expression (Hossain-Ibrahim et al. 2006, Kerschensteiner et al. 2004). Therefore future experiments should examine the effect of PEM and global ischemia on the relationship between the inflammatory reponse, including NFκB and its downstream pro-inflammatory molecules, and GAP-43 expression.

In summary, this experiment has shown that nutritional status prior to and following brain ischemia may have important effects on several mechanisms involved in recovery, such as the stress-response, hippocampal hyperexcitability, and inflammation. In the future, these potential mechanisms should be studied in combination with behavioural outcome since it is important to show that a change in mechanisms translates into a change in function.

The third experiment in this thesis represents the first study in our laboratory to use the rat 2-VO model of global ischemia to model and extend findings on the significance of pre-existing PEM as a co-morbid factor in determining stroke outcome. The first important finding was that PEM was successfully induced in the Sprague-Dawley rat based on food intake, body weight, and serum albumin concentration. However, the model of PEM in the rat described in

Chapter 7 was quite different from that induced in the gerbil in Chapters 4 and 5. Rats fed the protein-deficient diet were found to have a much different pattern of food intake and body weight, versus control rats, as compared to the same measurements in the gerbil. These differences are due to several factors including different lengths of feeding prior to ischemia, different ages of the animals, and a much steeper growth curve in the rat, even when compared at the same ages. The rats used in this study are not ideal for the PEM model since they are young (30-32d) and rapidly growing and therefore not representative of an elderly patient. Unfortunately, the use of adolescent rats is required for the 2-VO global ischemia model since results of the pilot studies described in Chapter 6 of this thesis suggests that consistent injury cannot be induced in older rats. However, it is important to note that several variables were changed during the course of these pilot studies, which may have also contributed to the findings.

A major finding of this experiment was that several factors important to control for a consistent 2-VO surgical model of brain ischemia were affected by PEM. As such, this represents a confounding influence important to highlight for researchers using surgical models of brain ischemia. PEM significantly affected respiratory status and acid-base balance prior to carotid artery occlusion. Core temperature was also affected by PEM both prior to and following global ischemia, and the most significant confounding influence in this experiment was likely the transient hypothermia observed in the immediate post-surgical period. In combination, these confounding variables interfered with adequately addressing the major objectives of determining the effect of PEM on CA1 neuronal death and dendritic damage.

In contrast, other factors affected by PEM are likely to be important to the clinical situation of PEM and stroke outcome. The increase in fasting blood glucose concentration caused by PEM is clinically relevant since increased blood glucose is detrimental to stroke outcome (de Courten-Myers et al. 1992). Future studies should monitor chronic glucose fluctuations in both the fed and fasted state in order to determine the full effect of PEM.

The increase in blood pressure caused by PEM in anaesthetized sham rats raises the possibility that PEM causes chronic elevations in blood pressure that should be considered in explaining its influence on outcome from brain ischemia. In future studies, blood pressure should be monitored chronically with the use of implantable radiotelemetry probes. These factors are

not only important to monitor in experimental models of stroke, but in clinical situations as well, where they are important indicators of outcome (Wong and Read. 2008).

While the potential confounders noted in this experiment suggest that the 2-VO model cannot be used to obtain reliable results on the effect of pre-existing PEM on brain ischemia, the model remains valid for the examination of PEM developing post-ischemia. This question may be even more clinically relevant since nutritional status has been found to worsen as length of stay in the hospital increases (Axelsson et al. 1988, Davalos et al. 1996). Based on the finding from the second experiment, that pre-ischemic pre-existing PEM affects recovery mechanisms from global ischemia, it is suggested that the potential for post-ischemic PEM to induce similar changes exists.

The two models of global ischemia utilized in this thesis were quite different. The rat 2-VO model allowed monitoring of many physiological variables during the surgery that was not possible in the gerbil BCCAO model. This monitoring allowed us to identify problems in surgical management, and we could then make changes and ensure our procedures were perfected. Since this was not possible in the gerbil experiment, it is unknown if some of these animals experienced problems such as acidosis, which may have altered hippocampal damage resulting from the ischemic-insult. The lack of physiological monitoring also may have led to a worsened recovery in some gerbils.

Based on findings with the rat 2-VO model, it is suggested that development of a focal ischemia model may be necessary at this stage in order to continue examining the effect of PEM on stroke outcome. While focal models are ideal since they are much more representative of a human stroke, global models have been described as being more consistent in resulting damage (Small and Buchan. 2000). The gerbil global ischemia model certainly was useful in terms of simplicity of the surgery and consistency of the damage. However, as described in Chapters 6 and 7 of this thesis, the 2-VO rat model of global ischemia is much more surgically invasive, shows acceptable but not excellent consistency, and is evidently problematic for studying PEM developing pre-ischemia. An additional advantage of using a focal ischemia model is that older rats can be used which would be more useful for mimicking PEM in the elderly stroke patient. Together, these factors all suggest that a focal model of ischemia should be used for future studies examining the effect of PEM on outcome from stroke.

It will also be of interest to determine the effect of PEM on treatment methods, such as hypothermia. Induction of hypothermia post-ischemia appears to currently be the most promising treatment in terms of dramatically reducing brain damage and improving behavioural outcome following ischemia (Krieger and Yenari. 2004, MacLellan et al. 2009). Results of experiment 3 of this thesis indicate that PEM induces a wider fluctuation of daily core temperature both prior to and following global ischemia and that it might also induce a degree of cooling. Aside from its potential to influence thermoregulation PEM also causes many effects that may be detrimental to hypothermic treatment. Hypothermia is proposed to exert some of its beneficial effects by decreasing the production of reactive oxygen and nitrogen species (Globus et al. 1995, Kumura et al. 1996), decreasing inflammation (Deng et al. 2003), and inhibiting activation of the transcription factor NFκB (Webster et al. 2009). It is very possible that PEM would interfere with some of the benefits of hypothermic treatment since it has been shown to increase oxidative stress (Bobyn et al. 2005), and increase activation of NFκB (Ji et al. 2008). This is an important area to examine since combinational therapies are becoming more of interest in the treatment of stroke, of which nutrition is sure to be a part of.

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APPENDIX A

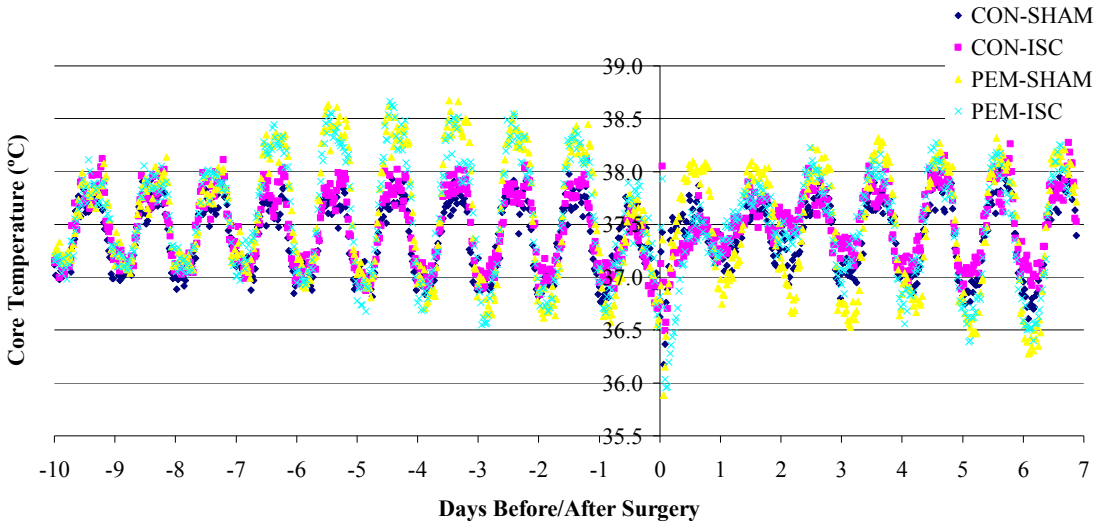

Figure A.1. Mean daily core temperature patterns for each experimental group. Values were collected in 30min intervals beginning at 10d prior to 2-VO surgery and ending at 7d following surgery (n = 10). Time 0 indicates time of anaesthetic induction for 2-VO surgery. PEM increases the daily fluctuation in core temperature (range between lowest and highest temperature over a 24hr diurnal cycle) shortly after being placed on a low protein diet. This increased fluctuation gradually re-establishes following global ischemia after a brief period of postsurgical hypothermia caused by PEM.

Table A.1. Lowest core temperature observed within 8hr following anaesthetic induction

	CON-S	CON-I	PEM-S	PEM-I
Core Temperature °C	35.9 ± 0.1	36.1 ± 0.1	35.6 ± 0.2*	35.3 ± 0.3*

Data are presented as mean (±SEM). *Indicates a significant independent effect of diet by 2-factor ANOVA (p = 0.003). n = 10 for each group.