EFFECTS OF PROTEIN-ENERGY MALNUTRITION ON THE INFLAMMATORY RESPONSE TO GLOBAL BRAIN ISCHEMIA

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon

By

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ABSTRACT

The overarching aim of the thesis research was to investigate mechanisms altered by protein-energy malnutrition (PEM), a common stroke co-morbidity factor that could affect the extent of brain damage and recovery following stroke. To model stroke, the rat 2-vessel occlusion model of global brain ischemia was employed. To characterize the effects of PEM, three states of malnutrition were assessed: PEM co-existing with brain ischemia (Study 1), effects of PEM independent of brain ischemia (Study 2), and PEM developing after brain ischemia (Study 3).

The first hypothesis tested was *co-existing PEM triggers an exacerbated glial response to global brain ischemia*. The failure to achieve a consistent model of global ischemia prevented us from drawing conclusions on whether co-existing PEM exacerbates reactive gliosis. Nonetheless, this study demonstrated that mean temperature and temperature fluctuation are increased within the first 24hr of exposure to a low protein diet.

The second hypothesis tested was *PEM causes sustained changes in core temperature that are associated with an inflammatory response.* Exposure to a low protein diet caused an immediate small and transient increase in mean temperature and a larger sustained increase in temperature amplitude. As malnutrition evolved, mean temperature declined. PEM stimulated an acute-phase response, characterized by an increase in the positive acute-phase protein, alpha-2-macroglobulin (A2M), and a decrease in the negative acute-phase protein, albumin. This response appeared to be aberrant, since the positive acute-phase protein, alpha-1-acid glycoprotein (AGP), was decreased with PEM.

The final hypothesis tested was *PEM developing after global brain ischemia exacerbates systemic and hippocampal inflammation, which is associated with diminished neuroplasticity*. The effects of PEM on the acute-phase response are persistent following brain ischemia, as demonstrated by decreased serum albumin and increased serum A2M. A decrease in the positive acute-phase protein, haptoglobin, strengthened the evidence that PEM triggers an atypical reaction. The strong glial response elicited by global ischemia was unaltered by PEM. However, PEM influenced hippocampal neuroplasticity mechanisms, as GAP-43 and synaptophysin were significantly lower at d21.

In summary, it has been demonstrated that PEM affects core temperature, the systemic acute-phase reaction and the neuroplasticity response to global brain ischemia.

ACKNOWLEDGEMENTS

This project would not have been possible without the support of many people. I would especially like to thank my supervisor, Dr. Phyllis Paterson, for her advice, guidance and support. I also thank the members of my graduate committee, Chair Dr. Brian Bandy, Dr. Adil Nazarali, Dr. Baljit Singh, Dr. Fred Colbourne and to my external examiner, Dr. Ursula Tuor, for their guidance and suggestions.

I would like to acknowledge financial support from the University of Saskatchewan (Graduate Scholarship) and the Natural Sciences and Engineering Research Council (NSERC Postgraduate Scholarship M). Research funding was provided by grants from the Heart and Stroke Foundation of Saskatchewan, Canadian Institutes of Health Research (CIHR), College of Pharmacy and Nutrition Internal College Trust Fund, Isabel Irwin Trust Fund, and University of Saskatchewan Tri-Council Bridge Funding.

Special thanks to my colleagues and friends who have supported and encouraged me to pursue this degree; Mark Hackett, Erin Prosser-Loose, Angela Cooper, Sonia Cyrenne, LaRhonda Sobchishin, and Barbara McEwen.

I owe my deepest gratitude to my parents, Elaine and Curt, and my sister Tara, for always offering support and love.

This thesis is dedicated to my loving parents, Curt and Elaine Smith, and to my sister, Tara Caswell.

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LIST OF ABBREVIATIONS & TERMINOLOGY

2-VO	2-vessel occlusion
4-VO	4-vessel occlusion
A2M	Alpha-2-macroglobulin
Acrophase	Time at which the daily rhythm peaks
AGP	Alpha-1-acid glycoprotein
AIN-93G	American Institute of Nutrition 1993 Growth Diet
AIN-93M	American Institute of Nutrition 1993 Maintenance Diet
Amplitude	Half the range of daily oscillation
ANOVA	Analysis of variance
APH-D	Acrophase difference
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
Co-existing PEM	Protein-energy malnutrition that is present at the time of brain ischemia
CON-7d	Fed control diet for 7 days
CON-28d	Fed control diet for 28 days
CON diet	Control diet (18% protein)
CON-ISC	Control diet ischemia surgery
CON-Sham	Control diet sham surgery
Ct	Cycle threshold
DAPI	4’6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ED-1	Antibody that recognizes CD68 antigen
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Fluctuation	Daily range of oscillation
FOOD trial	Feed or Ordinary Food trial

GAP-43	Growth-associated protein-43
GFAP	Glial Fibrillary Acidic Protein
Hct	Hematocrit
H&E	Hematoxylin and eosin
Iba-1	Ionized calcium-binding adapter molecule-1
ICAM-1	Intracellular adhesion molecule-1
IDV	Integrated density value
IκB	Inhibitory component kappa B
IKK	Inhibitory component kappa B kinase
IL	Interleukin
ISC	Global forebrain ischemia
Low Protein Diet	Protein-deficient diet (2% protein)
LPS	Lipopolysaccharide
LSD	Least significant difference
LTP	Long term potentiation
MABP	Mean arterial blood pressure
MAP-2	Microtubule-associated protein 2
Mint1	Munc-18-interacting protein 1
mRNA	Messenger ribonucleic acid
N_20	Nitrous oxide
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NMDAR	N-methyl-D-aspartate receptor
NT-3	Neurotrophin-3
O_2	Oxygen
OCT	Optimal cutting temperature
OX-42	Antibody that recognizes CD11b antigen
PBS	Phosphate buffered saline
pCO_2	Partial pressure carbon dioxide
PCR	Polymerase chain reaction
PEM	Protein-energy malnutrition

PEM-7d	Fed protein-deficient diet for 7 days
PEM-28d	Fed protein-deficient diet for 28 days
PEM-ISC	Protein-energy malnutrition – ischemia surgery
PEM-Sham	Protein-energy malnutrition – sham surgery
pO_2	Partial pressure oxygen
Post-ischemic PEM	Protein-energy malnutrition that develops after brain ischemia
PSD-95	Postsynaptic density-95
REM	Rapid eye movement
RNA	Ribonucleic acid
Robustness	Strength of the rhythm
ROS	Reactive oxygen species
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
Sham	Sham surgery
SNAP-25	Synaptosomal-associated protein of 25 kDa
STAIR	Stroke Treatment Academic Industry Roundtable
TGF	Transforming growth factor
TNF	Tumor necrosis factor
t-PA	Tissue plasminogen activator
trkB	Tropomyosin-related kinase B
VLDL	Very-low-density lipoproteins

CHAPTER 1:
INTRODUCTION

1.1 Rationale

Stroke is the third leading cause of death in Canada, with approximately 300,000 Canadians living with the detrimental effects of stroke (Heart and Stroke Foundation, 2011). Effective treatments for stroke are scarce, resulting in only 10% of victims completely recovering from the insult. A stroke occurs every ten minutes in Canada, costing the economy $3.6 billion a year in medical expenses (Heart and Stroke Foundation, 2011). A stroke is defined as an interruption of blood supply to any area of the brain, which can be the result of either an ischemic or hemorrhagic episode. Ischemic strokes are four times more common, and arise when a vessel supplying blood to the brain is occluded. Occlusion can occur when a blood clot forms in an artery that is already very narrow, resulting in a thrombotic stroke, or the clot may originate in a distal location in the body and travel to the brain, causing an embolic stroke.

The pathogenesis of ischemic stroke is intricate and involves various complex mechanisms. Reduction in glucose and oxygen supply to the brain can result in a range of unfavorable outcomes, including energy failure, glutamate excitotoxicity, disrupted calcium homeostasis, and increased generation of reactive oxygen species (ROS). In addition, a robust inflammatory response is triggered immediately following brain ischemia and can be a long-lasting event, which profoundly affects the extent of brain injury and recovery (Xia et al., 2010). Activated microglia and macroglia are the predominant responders to ischemia injury, and in some incidences they are the sole source of inflammatory cells. Although there is extensive cell death following ischemia, the brain is plastic and can actively undergo remodeling. Neuroplasticity refers to the structural and functional changes that occur to surviving neurons, including axonal and dendritic sprouting, with an attempt to return synaptic activity to pre-stroke levels (Kriz & Lalancette-Hébert, 2009; Murphy & Corbett, 2009).

Animal models of brain ischemia are necessary to elucidate the cellular processes involved in the pathogenesis of human stroke. Models of ischemic brain injury are classified based on the location of occlusion (focal or global) and the extent of blood blockage (transient or permanent). Whereas reduction of blood flow to a specific brain region is termed focal ischemia, global ischemia is the result of deprivation of blood supply to the entire brain (Traystman, 2003). A global ischemia model, while being a mimic of ischemic brain injury due to cardiac arrest, has advantages of high reproducibility and induces many of the same pathophysiological mechanisms of focal stroke (Traystman, 2003). Thus, a global ischemia model was used in this thesis to investigate pathophysiological and biochemical mechanisms relevant to human stroke.

Despite years of animal research focusing on the pathophysiology of ischemic brain injury, little work has been translated into effective treatments in human stroke victims (Hackam & Redelmeier, 2006). Tissue plasminogen activator (t-PA), which restores blood flow by dissolving blood clots, is currently the only validated and approved stroke therapy (Fisher et al., 2009). The majority of pre-clinical experiments have focused on pharmacological agents that could decrease stroke-induced brain damage by targeting a single mechanism within the complex secondary cellular cascade of events that results in neuronal death. The focus on a single mechanism at least partially explains their failure in clinical trials. In response to the plethora of failed clinical trials, the Stroke Treatment Academic Industry Roundtable (STAIR) committee has recommended that stroke researchers broaden the scope of pre-clinical experiments (Albers et al., 2011). These recommendations include developing neuroprotective drugs that target multiple pathways, assessing the role of rehabilitation on recovery, and studying the effects of co-morbidity factors on stroke outcome (Albers et al., 2011; Fisher et al., 2009). Therefore, my laboratory is focused on elucidating the influences of pre- and post-ischemic nutritional status, as a way to improve stroke outcome with targeted nutritional treatments. Protein-energy malnutrition (PEM), a type of malnutrition commonly seen among elderly in North America, is characterized by deficits in both protein and energy status. At least 16% of stroke patients are protein-energy malnourished at the time of admission to hospital (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008), with prevalence rates reaching as high as 49% on admission to a rehabilitation setting (Finestone et al., 1995; Poels et al., 2006). Clinical studies have repeatedly demonstrated a strong correlation between nutritional status and outcome following stroke, suggesting the PEM may be an important co-morbidity factor. More specifically, protein-energy

malnourished stroke patients are more likely to experience post-stroke complications, as well as have longer hospital stays, increased functional dependency and decreased chances of survival (Axelsson et al., 1988; Davalos et al., 1996; FOOD Trial Collaboration, 2003; Gariballa et al., 1998; Olsen et al., 2008; Pandian et al., 2011). However, as a result of various methodological limitations, clinical studies have yet to show substantive proof of causation. In addition, the notion that PEM may exert direct effects on ischemic neuronal death pathways and/or brain remodeling is not very prevalent in the clinical literature. For these reasons, the use of animal models is crucial to further our knowledge of how PEM affects stroke outcome on both a cellular and functional level.

With the use of a gerbil model of global brain ischemia, my laboratory demonstrated that co-existing PEM (PEM that is present at the time of brain ischemia) worsens functional outcome (Bobyn et al., 2005). In addition, the data suggested, but did not prove, that co-existing PEM increased the neuroinflammatory response to brain ischemia (Bobyn et al., 2005; Ji et al., 2008). Therefore, the central focus of this thesis has been to investigate the inflammatory response to either co-existing or post-ischemic PEM.

The study described in Chapter 3 of this thesis was the first experiment performed in my laboratory using the rat 2-vessel occlusion (2-VO) model of global brain ischemia. Therefore, a primary objective was to investigate whether the 2-VO rat model would replicate previous findings of enhanced reactive gliosis in response to global ischemia in protein-energy malnourished gerbils (Bobyn et al., 2005). Hippocampal protein expression of ED-1, OX-42 and glial fibrillary acidic protein (GFAP) were examined by immunohistochemistry to assess the effects of co-existing PEM on the glial response to global brain ischemia in the rat. Since temperature regulation can directly alter the glial response following global ischemia (Ceulemans et al., 2010; Silasi & Colbourne, 2011), the effect of PEM on pre- and post-ischemic core temperature was also examined.

PEM may exert some of its effects on outcome after brain ischemia by altering key physiological determinants of ischemic brain injury. Changes in body temperature during the acute ischemic period can radically alter the cascade of cellular events. Post-ischemic hypothermia can markedly reduce neuronal damage (MacLellan et al., 2009; van der Worp et al., 2007), while hyperthermia can aggravate injury (Wang et al., 2009). Since changes in core temperature in the face of PEM had been identified in the first study, the study described in

Chapter 4 assessed the duration and severity of temperature changes that can occur with prolonged PEM. A second goal was to examine whether PEM is an independent stimulus of inflammation. Previously it has been demonstrated that activation of the major inflammatory transcription factor, nuclear factor kappa B (NFκB), is increased in the hippocampus of non-operated malnourished gerbils (Ji et al., 2008). Given that pro-inflammatory mediators are predominantly regulated at the level of transcription by NFκB, these results suggest that PEM can independently induce hippocampal inflammation. This was assessed in the second study of this thesis by examining the mRNA expression of pro-inflammatory genes regulated by the NFκB cascade in the hippocampus of malnourished rats. Lastly, there is compelling evidence that PEM can be an independent stimulus of systemic inflammation (Ling et al., 2004; Lyoumi et al., 1998). Therefore, serum concentrations of acute-phase proteins were assessed to determine if the PEM model used in this research elicits a systemic inflammatory response.

Given that compromised nutritional status often develops in patients within the weeks following stroke, the focus of the final study of this thesis was to assess the effects of PEM when it develops after brain ischemia (post-ischemic PEM). In addition to being clinically significant, the results from this study are also novel, since the mechanistic effects of post-ischemic PEM have never been studied. A primary objective was to investigate the influence of post-ischemic PEM on systemic and neuroinflammation following global brain ischemia. Serum concentrations of various acute-phase proteins were measured to assess systemic inflammation, while hippocampal inflammation was examined by staining for GFAP (astrocytic marker) and ionized calcium-binding adapter molecule 1 (Iba-1; activated microglia marker). Given that the inflammatory response after brain ischemia can affect recovery processes (Banati, 2002; Katsuki et al., 1990; Kriz & Lalancette-Hébert, 2009), an additional goal was to assess the effects of post-ischemic PEM on neuroplasticity markers. Immuno-staining of hippocampal plasticity-associated proteins, including growth-associated protein-43 (GAP-43) and synaptophysin, provided insight on the effects of post-ischemic PEM on neuronal plasticity following global brain ischemia.

1.2 Hypotheses & Objectives

Study 1 (Chapter 3):

Hypothesis: Co-existing PEM triggers an exacerbated glial response to global brain ischemia.

Objectives:

1. Investigate if the astrocytic (GFAP) and microglial (ED-1 and OX-42) responses after global brain ischemia are altered by co-existing PEM.
2. Determine if PEM affects core temperature during the pre- and post-ischemic periods.

Study 2 (Chapter 4):

Hypothesis: PEM causes sustained changes in core temperature that are associated with an inflammatory response.

Objectives:

1. Investigate whether acute and chronic PEM affect NFκB mediated signaling in the hippocampus.
2. Determine if our model of PEM is an independent stimulus of an acute-phase response, as reflected by serum albumin, alpha-2-macroglobulin (A2M), and alpha-1-acid glycoprotein (AGP) concentrations.
3. Determine whether PEM-induced alterations to core temperature are sustained long term.
4. Examine if the immediate increase in temperature previously reported in rats fed a low protein diet is caused by a temporary stress-induced increase in activity.

Study 3 (Chapter 5):

Hypothesis: PEM developing after global brain ischemia exacerbates systemic and hippocampal inflammation, which in turn is associated with a diminished neuroplastic response to hippocampal injury.

Objectives:

1. Examine whether post-ischemic PEM exacerbates hippocampal inflammation following global brain ischemia, as reflected by increased astrocytic (GFAP) and microglial (Iba-1) responses.
2. Determine if the ability of PEM to induce an acute-phase response differs following global brain ischemia.
3. Investigate whether PEM following global brain ischemia alters the expression of plasticity-associated proteins (GAP-43 and synaptophysin).

CHAPTER 2:
REVIEW OF THE LITERATURE

2.1 Introduction

Stroke, defined as an interruption of blood supply to any area of the brain, is the third leading cause of death in Canada (Heart and Stroke Foundation, 2011). There are over 50,000 strokes each year in Canada, with only 10% of victims completely recovering from the insult. Approximately 75% of stroke victims suffer from some form of disability including paralysis, learning impairments, aphasia, memory loss, vision problems, dysphagia, and depression (Heart and Stroke Foundation, 2011). Ischemia, which accounts for 80% of stroke cases, occurs when there is disruption of blood flow to the brain due to a clot.

Despite years of animal research focused on developing effective stroke treatments, t-PA is the only validated and approved stroke therapy included in the Canadian Best Practice Recommendations for Stroke Care (Lindsay et al., 2010). However, since the therapeutic time window is short and many patients do not meet the criteria to receive the treatment, less than 2% of Canadian stroke victims receive this type of intervention (Yip & Demaerschalk, 2007). Due to the ongoing challenges of developing effective treatments for stroke victims, my laboratory has taken a different approach to research that is focused on enhancing the outcome of stroke. This promising area of research investigates the implications of stroke co-morbidity factors, such as compromised nutritional status, with the intent to highlight novel targeted treatments. A significant proportion of stroke patients are protein-energy malnourished at the time of admission to hospital (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008), with this subset of malnourished patients drastically rising by the time of admission to a rehabilitation unit (Finestone et al., 1995; Poels et al., 2006). Using an experimental model of brain ischemia, studies from my laboratory have demonstrated that co-existing PEM impairs functional outcome

(Bobyn et al., 2005) and alters mechanisms underlying both neuronal death and neuroplasticity (Bobyn et al., 2005; Ji et al., 2008; Prosser-Loose et al., 2010). The research described in this thesis examined whether the inflammatory and neuroplasticity responses to global brain ischemia are altered by either co-existing PEM or compromised protein-energy status that arises after brain ischemia. Ultimately, research in this area may aid in the design of targeted nutritional interventions that will prevent or reverse PEM and enhance stroke outcome.

2.2 Pathophysiology of Ischemic Stroke

2.2.1 Overview

The pathogenesis of ischemic stroke is intricate and involves various complex mechanisms. These processes that lead to brain injury act at different times and can develop over several hours or even days following brain ischemia. The severity of each mechanism is dependent on the extent of cessation of cerebral blood flow and where in the brain it is occurring. Although reperfusion is necessary to restore blood flow, it also induces secondary injury caused by an influx in inflammatory cells, ROS, excitatory amino acids and intracellular calcium (Tang et al., 2007).

Blood flow interruption results in the depletion of energy supply, which is the primary event that leads to cell necrosis (Astrup et al., 1977). Various pathways are disrupted in response to energy depletion, resulting in unfavorable outcomes that include glutamate excitotoxicity, calcium overload, and oxidative stress. Rapid depletion in adenosine triphosphate (ATP) that occurs at the time of ischemia has detrimental effects on a neuron by interfering with energy-dependent ionic pumps, such as the sodium-potassium ATPase pump (Hansen, 1985). This critical active transporter is responsible for maintaining a high cellular concentration of potassium and low cellular levels of sodium. During ischemia this system is disrupted, resulting in the accumulation of sodium ions within a cell. The influx of cations causes membrane depolarization, which if significant enough, will result in the generation of an action potential. Glutamate, an excitatory amino acid found at high concentrations in normal neurons, is released by the pre-synaptic neuron during an action potential and rapidly binds to its ionotropic receptor on the post-synaptic neuron. Binding of glutamate opens voltage-gated calcium channels on the post-synaptic terminal, thereby facilitating calcium entry into the cell (Tang et al., 2007). Ultimately, over-firing of neurons during the post-ischemic period can cause intracellular calcium concentrations to rise by 1000-fold (Tang et al., 2007).

Given that calcium is a regulator of several enzyme systems, excess levels of calcium can result in over-activation of various pathways leading to detrimental outcomes. For example, calcium activation of proteases, such as calpain 1, leads to degradation of vital structural proteins, and calcium-activated endonucleases cause DNA cleavage (Choi, 1990). Production of toxic free radicals, such as nitric oxide, is also an unfavorable consequence of calcium influx and is the result of activation of nitric oxide synthase (Iadecola, 1997). Oxidative stress is another detrimental outcome of brain ischemia. At the time of reperfusion, oxygenated blood is supplied to deprived brain regions resulting in the generation of ROS. The brain has minimal antioxidant capacity, making this biological system very susceptible to oxidative stress (Chan, 2001). Therefore, extensive generation of ROS following ischemia overpowers the antioxidant defense system, and in turn the imbalance in the production and manifestation of ROS generates oxidative stress. Consequently, ROS can trigger the degradation of proteins, lipids and DNA, causing irreparable damage (Chan, 2001). Ultimately, marked levels of glutamate, calcium, and ROS following brain ischemia can cause significant cellular stress and lead to cell death.

Neuronal death following brain ischemia is primarily the result of necrosis or apoptosis. Necrosis is the premature death of cells, and is characterized by cellular swelling and rupturing of the plasma membrane. As a result, the cellular contents, including toxic products of cell death, are released into the surrounding tissue and trigger an inflammatory response. Apoptosis, or programmed cell death, is an energy-dependent pathway that involves chromatin condensation, nuclear blebbing, DNA fragmentation and cell shrinkage. Apoptosis is considerably less destructive to the surrounding tissue, given that dying cells are broken up into apoptotic bodies and quickly degraded by phagocytic cells (Colbourne & Auer, 2010). Given that necrosis and apoptosis each have unique morphological features, it is possible to distinguish the mode of cell death by examining for cellular swelling, chromatin condensation, and the presence of apoptotic bodies (Colbourne & Auer, 2010). Evidence from rodent models of brain ischemia suggests that neuronal death is mostly necrotic. The mode of cell death is to some extent dependent on the type of brain ischemia; however, most evidence suggests that the global brain ischemia model used in this thesis results in cells with necrotic morphology (Colbourne & Auer, 2010).

2.2.2 Inflammation

2.2.2.1 Neuroinflammation

The inflammatory response is another key constituent of this complex cascade of secondary mechanisms involved in brain injury. The orchestrated inflammatory response following an ischemic insult can play both beneficial and detrimental roles (Kriz & Lalancette-Hébert, 2009). The inflammatory response can trigger oxidative stress and cytokine production, ultimately damaging the brain. Conversely, inflammatory cells can be beneficial by removing cell debris, repairing tissue and preventing further neuronal damage. The exact role inflammation has on ischemic brain injury is heavily dependent upon the severity of inflammation and the time over which it occurs following insult. Numerous studies have identified specific inflammatory cytokines as either having neuroprotective or detrimental effects on the damaged brain. Whereas increased levels of transforming growth factor (TGF)-α and interleukin (IL)-1 can exacerbate neuronal injury (Allan & Rothwell, 2001; Patel et al., 2003), TGF-β and IL-10 can have neuroprotective effects on an ischemic brain (Allan & Rothwell, 2001; Froen et al., 2002; Grilli et al., 2000). Similarly, activated microglia can play differential roles following brain ischemia. Microglia are valuable in removing dead neurons, but can have damaging effects by releasing cytokines and ROS (Lai & Todd, 2006). Although there are advantageous outcomes of inflammation, most of the evidence suggests that the inflammatory response following brain ischemia is more detrimental than beneficial (Feuerstein, 2001).

Under normal conditions, the brain is an immune-privileged organ, implying that the blood-brain barrier prevents the infiltration of systemic inflammatory cells. Damage to the blood-brain barrier following an ischemic episode makes the diseased brain susceptible to systemic inflammation (Danton & Dietrich, 2003). Leukocytes migrate to the damaged brain and are involved in the local inflammatory response mediated by activated microglia. Inflammatory cells follow a chemokine concentration gradient to the damaged region and gain access into the tissue through interactions between adhesion molecules.

2.2.2.1.1 Neuroinflammatory Mediators

Secretion of cytokines by glial cells precedes the infiltration of inflammatory cells, and thus plays a major role in initiating and maintaining the inflammatory response following brain ischemia. Marked increases in cytokine concentrations, in both the brain and systemic circulation, are evident within hours following stroke (Ahmad & Graham, 2010). The best-

characterized cytokines that are up-regulated in response to ischemia include tumor necrosis factor (TNF) and members of the interleukin family (IL-1, IL-6, IL-8, IL-10, IL-12) (Ahmad & Graham, 2010). The two contradicting signals involved in the inflammatory response are the pro-inflammatory and anti-inflammatory pathways. Pro-inflammatory cytokines (e.g. TNF-α, IL-1) amplify the response, while anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-13) are involved in turning off an immune reaction (Planas et al., 2006). The majority of these cytokines have dual functions in the ischemic brain; therefore, simply inhibiting the expression does not provide neuroprotection. For example, one report has shown that inhibition of TNF-α in mice can reduce ischemic brain injury (Yang et al., 1998), while another mouse ischemic model illustrated that TNF-α is involved in ischemic tolerance and mice deficient in TNF-α receptors have greater infarcts (Gary et al., 1998).

Chemokines, which are a subclass of cytokines, are also pivotal mediators of the ischemic inflammatory response and are produced by several kinds of cells, including activated leukocytes and microglia. The primary role of a chemokine is as a chemoattractant, which involves creating a chemokine concentration gradient that recruits inflammatory cells to the inflamed region. Inflammatory cells that are attracted to these gradients express chemokine receptors, which are G-protein linked seven transmembrane domain receptors, and include leukocytes, neurons and glial cells (Kiguchi et al., 2011). There are four subfamilies of chemokines, named according to the arrangement of the first two cysteines located at the N-terminal end: CC, CXC, XC, and CX3C. CC and CXC chemokine pathways are crucial in ischemic stroke. Whereas CXC chemokines (e.g., CXCL1, CXCL8, CXCL12) typically promote neutrophils to leave the bloodstream and enter the inflamed tissue (Mirabelli-Badenier et al., 2011), CC chemokines (e.g., CCL2, CCL3, CCL7, CCL20) are involved in the recruitment of monocytes/macrophages (Mirabelli-Badenier et al., 2011). When inflammatory cells reach the damaged region they leave the bloodstream and infiltrate the tissue, which is a process facilitated by cell adhesion molecules.

Cell adhesion molecules, a group of proteins located on the cell surface, play a pivotal role in the infiltration of inflammatory cells into the damaged tissue. Adhesion molecules facilitate the interaction between inflammatory cells and the vascular endothelium, which is necessary for tissue infiltration. Adhesion molecules are commonly classified into four main protein families: immunoglobulin superfamily, integrins, cadherins, and selectins. For example,

intracellular adhesion molecule-1 (ICAM-1), an immunoglobulin member, is expressed on endothelial cells and binds to integrins (CD11/CD18) present on the cell surface of leukocytes. Following the initial interaction between the inflammatory cell and interior surface of the blood vessel, selectins are commonly involved in mediating the rolling process along the endothelium (Tang et al., 2007). The process of rolling decelerates the inflammatory cell, allowing for a firm adhesion to be formed and transmigration into the tissue.

2.2.2.1.2 Nuclear Factor Kappa B

A central regulator of the inflammatory response following brain ischemia is the transcription factor, NFκB. NFκB regulates the transcription of a wide variety of genes, which can be grouped into three main pathways: (1) inflammation (e.g., cytokines, adhesion molecules, chemokines, matrix metalloproteinase); (2) regulation of apoptosis (e.g., Bcl2 family); and (3) inhibitory component kappa B (IκB) inhibitors, which provides a feedback loop for the NFκB pathway (Harari & Liao, 2010). Overall, most evidence suggests that NFκB activation is neurodegenerative by participating in blood-brain barrier disruption, augmentation of the inflammatory response and contributing to neuronal death (Feuerstein, 2001; Harari & Liao, 2010; Ridder & Schwaninger, 2009). However, there are controversial reports suggesting that NFκB activation does not always result in neurodegeneration following brain ischemia. Previously it has been demonstrated that the activation of NFκB could be a pre-conditioning stimulus. Brief periods of ischemia, below the cell death threshold, can trigger NFκB activation in neurons and promote neuroprotection (Blondeau et al., 2001). Nevertheless, it has been well established that NFκB activation can have detrimental effects following brain ischemia (Feuerstein, 2001; Harari & Liao, 2010; Ridder & Schwaninger, 2009). NFκB signaling is the highest within damaged neurons (Clemens et al., 1997; Schneider et al., 1999; Stephenson et al., 2000); however, activation also takes place in endothelial cells, astrocytes, and microglia (Gabriel et al., 1999; Howard et al., 1998; Terai et al., 1996). There are numerous inducers of NFκB signaling, including various inflammatory mediators (e.g., TNF, IL-1, and IL-2), growth factors (e.g., TGF-β1), bacterial mediators (e.g., lipopolysaccharide [LPS]), and oxidants (e.g., hydrogen peroxide) (Harari & Liao, 2010). Therefore, certain target genes of NFκB are also predominant activators of this transcription factor, resulting in an ongoing cycle that creates an elevated response to stress.

NFκB is constitutively expressed in latent cells and is activated via post-translational modification. There are multiple variations of the NFκB complex, both homodimeric and heterodimeric in nature, composed of two members of the Rel protein family (e.g. p50, p65 [RelA], c-Rel, p52, RelB). However, the most common NFκB form is a p50/p65 heterodimer. In quiescent cells, NFκB is located in the cytoplasm and is bound to an IκB inhibitor, preventing translocation into the nucleus (**Figure 2.1**). Liberation of NFκB occurs when the IκB inhibitor is phosphorylated by an upstream activated IκB kinase (IKK). Activation of IKK can be triggered by various stroke related factors, including ROS, inflammatory cytokines and excessive levels of intracellular calcium. Removal of IκB allows NFκB to translocate into the nucleus and bind to specific domains, known as κB sites, within the promoter region of multiple NFκB target genes (**Figure 2.1**). Thereby, NFκB bound to the DNA sequence promotes the recruitment of an RNA polymerase and initializes transcription. The remaining IκB in the cytoplasm is ubiquitinated and degraded by the 26S proteasome (Grisham et al., 1999).

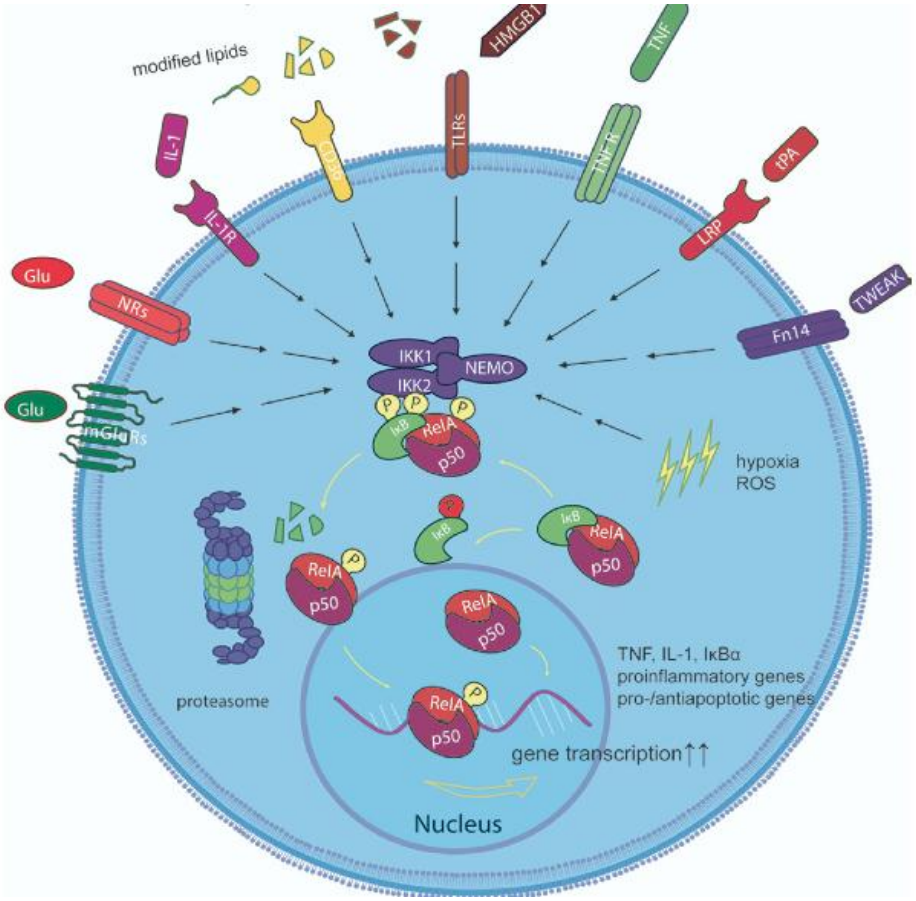

Figure 2.1. Diagram illustrating NFκB signaling in brain ischemia. (From: Ridder DA, et al. Neuroscience. 2009; 158: 995-1006. Copyright © 2009, Elsevier).

2.2.2.1.3 Glial Cells

2.2.2.1.3.1 Introduction

The inflammatory response in the ischemic brain involves three main groups of inflammatory cells and the role of each subclass is greatly dependent on the nature of injury. The predominant responders to ischemic injury are glial cells and in some incidences they are the sole source of inflammatory cells. In response to severe and extensive tissue injury, leukocytes from systemic circulation migrate across the damaged blood-brain barrier and aid in the inflammatory response. Perivascular cells, the third subclass of inflammatory cells involved in the ischemic brain, are constitutive macrophage-like cells located in the vascular wall (Kaur & Ling, 2008). Leukocytes and perivascular cells are not major responders to the type of ischemic brain injury modeled in this thesis, and thus these inflammatory cells have not been reviewed in detail.

2.2.2.1.3.2 Microglial Cells

Microglia are ubiquitously distributed throughout a healthy brain and act as the primary immune defense in the central nervous system. The microglia cell pool makes up approximately 10% of the total number of cells in the mouse brain (Lawson et al., 1990), and is rarely replenished by hematogenous cells due to their stable nature (Lawson et al., 1992). In the presence of abnormal substances, such as LPS and cell necrosis factors, microglia become immediately activated (Liu et al., 2011). Microglia migrate along the chemotaxis gradient to the invaded/injured area and once activated become diversely involved in the inflammatory response by releasing various mediators including cytokines, ROS, proteases, and glutamate (Dheen et al., 2007). Fully activated microglia transform into phagocytes and engulf offending material or cellular debris (Giulian, 1987). Furthermore, microglia secrete growth factors that facilitate tissue repair (Kreutzberg, 1996).

The exact role that a microglial cell plays in the inflammatory response following brain ischemia is completely dependent on the stage of activation. There are three different stages of activation with the first being classified as resting microglia, followed by activated microglia, and lastly phagocytic microglia (Kato, 2001). Microglia activation occurs in a stepwise manner, with each stage having a characteristic cell morphology. Resting microglia are ramified cells, which allow them to constantly survey the area with their long branching processes. In response to various factors, such as pro-inflammatory cytokines, cell necrosis factors and altered

potassium levels, microglia convert from their ramified morphology to an activated form. At this early activated stage the cell body becomes enlarged and the long processes contract, resulting in a rod like appearance. Microglia at this stage are not phagocytic, but instead undergo extensive proliferation to expand the population of microglia. The last stage of activation is comprised of phagocytic microglia, which have the strongest immune response. The characteristic morphology at this final stage of activation is large amoeboid shaped cells, which allows engulfing of foreign material and at the same time production of key inflammatory mediators (Kato, 2001).

2.2.2.1.3.3 Macroglial Cells

Macroglia are another group of glia in the central nervous system. Astrocytes are the most abundant type of macroglia and make up more than 50% of cells in the central nervous system. Astrocytes have a unique star-like structure with long processes extending from a central cell body, which form connections with various cell types including neurons, microglia and other astrocytes. Astrocytes are involved in stabilizing chores, such as maintaining extracellular ion balance and regulating neurotransmitter concentrations (Verkhratsky & Steinhauser, 2000). However, these roles weaken following injury in order for astrocytes to participate in tissue repair, which includes releasing neurotrophic factors, undergoing proliferation and forming glial scars (Liu et al., 2011). The beneficial action of the glial scar is that it suppresses further physical damage to the area; however, it also prevents full recovery by hindering axon regeneration.

Activation of microglia and macroglia happen at different stages of the inflammatory process and involves intricate cross talk between cell types (Liu et al., 2011). Under most pathological conditions, microglia are activated first and release pro-inflammatory cytokines, such as IL-1, to facilitate the activation of astrocytes. In turn, activated astrocytes play a fundamental role in regulating microglia activities by either enhancing or inhibiting the response. Following ischemia, distant microglia become activated by astrocytes via calcium waves (Liu et al., 2011). Conversely, activated astrocytes can have an inhibitory effect on microglia by diminishing the cellular levels of nitric oxide, ROS and pro-inflammatory mediators (Liu et al., 2011). Therefore, astrocytes play a central role in both tissue repair and the regulation of the inflammatory process following brain ischemia.

2.2.2.1.3.4 Assessment of Glial Response

Immunocytochemistry is commonly performed in animal studies to evaluate the presence of glial cells following brain ischemia. To identify astrocytes using immunocytochemistry,

GFAP is frequently targeted and stained. GFAP, an intermediate filament protein that is most commonly expressed by astrocytes, provides mechanical strength for the cell, assists in cellular communication, and aids in the functioning of the blood-brain barrier (Fuchs & Weber, 1994). The positive expression of GFAP is considered one of the universal indicators of central nervous system damage following traumatic injuries. Identification of microglia is commonly by Iba-1, ED-1, and OX-42 immuno-staining, although there are other useful antibodies. Iba-1, expressed by microglia in the brain, is a calcium binding adapter molecule that is upregulated during microglia activation. ED-1, which is also used to mark microglia activation, stains the lysosomal membrane protein CD68 present on phagocytosing activated microglia (Damoiseaux et al., 1994). OX-42 is an antibody that recognizes an antigen (CD11b) associated with the complement receptor 3 protein, which is expressed on amoeboid microglia and is thought to play a role in endocytosis (Ling et al., 1990).

2.2.2.2 Systemic Inflammation

In addition to the neuroinflammatory response to brain ischemia, evidence from clinical studies suggests that systemic inflammation can also be triggered by stroke (Dziedzic, 2008). More specifically, the disruption in homeostasis following cerebral stroke can elicit a systemic reaction, referred to as an acute-phase response (Dziedzic, 2008; Idicula et al., 2009; Smith et al., 2006). The acute-phase response includes a subset of proteins for which plasma concentration changes by a factor of several fold in response to acute and chronic inflammation. Exposure to inflammatory conditions can result in elevated circulating concentrations of various positive acute-phase proteins (e.g. C-reactive protein, A2M, AGP, and haptoglobin), while at the same time causing depleted levels of negative acute-phase proteins (e.g. albumin, transferrin). Positive acute-phase proteins serve various physiological functions, including the role of chemotactic agents, coagulation factors, transport proteins, and regulators of the inflammatory response. The acute-phase response is stimulated and modulated by the release of pro-inflammatory cytokines, including IL-6, IL-1 and TNF-α (Andus et al., 1988). Therefore, concentrations of acute-phase proteins and pro-inflammatory cytokines are commonly measured in stroke patients to assess the extent of systemic inflammation (Dziedzic, 2008).

Numerous clinical studies have assessed the acute-phase response in ischemic stroke and correlated this response to patient outcome. Several factors contribute to the varying degree of acute-phase response observed in stroke patients, which include the severity of stroke, sample

timing, presence of infection, genetic variation, and drug therapy (Smith et al., 2006). Although the acute-phase response after ischemic stroke is variable, increased C-reactive protein levels have been reported in 94% of patients within the first week following stroke onset (Smith et al., 2006). C-reactive protein concentrations begin to rise within hours of a stroke, peak within days, but can remain heightened for months (Dziedzic, 2008). Early serum levels of C-reactive protein, collected within the first 24 hours after stroke onset in patients with no considerable difference in lesion size, showed a significant correlation with 6 month functional outcome (Ryu et al., 2009). Furthermore, C-reactive protein is a predictor of long-term mortality, even when adjusted for age, sex, and stroke severity (Idicula et al., 2009). Similar correlations have been made with other acute-phase markers, including IL-6, IL-1, and fibrinogen (Dziedzic, 2008). Although acute-phase proteins are independent predictors of survival after stroke, it is unknown whether they are active players in tissue destruction or solely reflect the degree of injury.

2.2.2.3 Temperature Regulation and Inflammation

Inflammation is highly temperature sensitive. Post-ischemic hypothermia mitigates damage by, among other mechanisms, directly altering various pathways involved in the inflammatory response. A drop in brain temperature can result in the suppression of neutrophil infiltration, along with a decrease in microglia proliferation and activation (Ishikawa et al., 1999; Silasi & Colbourne, 2011; Wang et al., 2002). Furthermore, mild hypothermia can elicit reduced nuclear NFκB translocation and activation (Webster et al., 2009). Conversely, post-ischemic hyperthermia can significantly aggravate injury, although the mechanisms associated with elevated brain temperatures are less established (Wang et al., 2009). However, there is evidence of an elevated inflammatory response in stroke victims with hyperthermia, as represented by increased plasma levels of IL-6, TNF-α, and ICAM-1 (Leira et al., 2006). These findings have strong clinical relevance, since approximately 50% of patients develop fever during the acute period following a stroke (Wrotek et al., 2011). This common complication of stroke has a severe impact on outcome, with clinical evidence indicating that a fever occurring within 24 hours following an ischemic episode doubles the odds of mortality (Prasad & Krishnan, 2010).

2.2.3 Neuroplasticity

2.2.3.1 Overview

Neuroplasticity refers to the structural and functional changes that can occur within the central nervous system in response to a stimulus (e.g., injury, learning, hormones). Many patients

experience spontaneous recovery following a stroke, with the extent of this response being highly dependent on severity of insult (Jorgensen et al., 1999). Spontaneous recovery during the acute period following stroke often occurs because of resolution of harmful factors (e.g., edema, cell death, raised intracranial pressure, inflammation). Neuroplasticity mechanisms are also involved in spontaneous recovery, and include cell remodeling and neuron production. Surviving neurons undergo extensive repair in an attempt to make new synaptic connections with other functional neurons. Remodeling of synaptic connections, termed synaptic plasticity, involves various growth-related processes including dendritic growth, axonal sprouting, and synaptogenesis (Murphy & Corbett, 2009). In addition to the remodeling of surviving neurons, new neurons are also generated in the ischemic brain through the process of neurogenesis. Cells are typically generated in the dentate gyrus or lateral ventricles and migrate to the damaged region, in an attempt to replenish the neuronal population (Ekdahl et al., 2009). The neuroplasticity mechanisms triggered by brain ischemia are very similar to the processes involved during normal brain development. During brain development, neuronal growth is regulated through the release of particular growth factors, neurotrophins, hormones, and neurotransmitters (Murphy & Corbett, 2009). Expression of these positive factors typically decrease with aging; however, brain ischemia, as well as other forms of trauma, can induce the expression of these proteins with an attempt to return synaptic activity to pre-stroke levels (Murphy & Corbett, 2009).

2.2.3.2 Post-Ischemic Synaptic Plasticity

The mechanisms of neuroplasticity can remain activated for months following brain ischemia and promote restoration of functional ability (Kriz & Lalancette-Hébert, 2009). Spontaneous recovery in the brain involves various growth-related events, including axonal, dendritic and synaptic changes. Within hours following brain ischemia, affected neurons undergo extensive modifications, including a loss in the number and the length of dendritic spines (Kriz & Lalancette-Hébert, 2009). More pronounced changes to the neuronal circuitry occur when neurons die, causing a significant depletion of axons and dendrites, and thus a loss of connections. In an attempt to retain synaptic activity, surviving neurons undergo axonal and dendritic sprouting allowing for the formation of new synapses, and as a result rewiring of the circuitry. Positive factors, including GAP-43 and brain-derived neurotrophic factor (BDNF), are involved in promoting these growth-related-processes (Murphy & Corbett, 2009).

A key player in synaptic plasticity is the intracellular growth associated protein, GAP-43. GAP-43 has been associated with the formation of filopedia, the finger-like projections that develop into neuronal branches (Denny, 2006). GAP-43 is also involved in neurotransmitter release, synaptic vesicle endocytosis, and vesicle recycling. This growth-associated protein is synthesized on free ribosomes, travels by vesicle transport down the axon, and binds to either the growth cone plasma membrane or presynaptic membrane (Denny, 2006). Under steady-state conditions, GAP-43 is found at the highest concentration around the axonal plasma membrane with concentrations estimated at 50-100 μM (He et al., 1997). GAP-43 is vital for brain development, as shown in a mouse model where deletion of GAP-43 resulted in death during the early postnatal period (Strittmatter et al., 1995). Expression of GAP-43 is reduced following development, but retained at low levels throughout most regions of the mature brain (Schmidt-Kastner et al., 1997). However, an exception is within the limbic system, where GAP-43 is expressed at relatively high levels throughout life (Denny, 2006). The most concentrated levels of GAP-43 in the adult rat hippocampus are detected within the cornu ammonis 3 (CA3) region, with these neurons typically having extensive arborization of terminals (Denny, 2006). Continuous GAP-43 expression in the hippocampus may contribute to information storage and more specifically the formation of spatial learning and memory. Following brain ischemia, as well as other forms of trauma, synthesis of GAP-43 is significantly increased within the hippocampus and is associated with the formation of neuronal branches and new synaptic connections. GAP-43-dependent neuronal branching is commonly triggered by neurotrophins, including BDNF, nerve growth factor (NGF) and neurotrophin-3 (NT-3) (Goldberg, 2003).

2.2.3.3 Post-Ischemic Neurogenesis

Neurogenesis, or birth of new neurons, is another component of neuroplasticity that is influenced by brain ischemia. The two regions in the adult brain where neurogenesis occurs are the subgranule zone of the dentate gyrus and the subventricular zone of the lateral ventricles (Gonzalez-Perez, 2012). Progenitor cells generated through the process of neurogenesis undergo neuronal differentiation and are integrated into existing circuits. It has been theorized that adult neurogenesis is required for learning, memory and mood regulation (Ekdahl et al., 2009). Under pathological conditions, including brain ischemia, it is possible for damaged regions to be repopulated with newly born neurons (Ekdahl et al., 2009). Given that the hippocampus is particularly vulnerable to ischemia, neurogenesis has been widely studied in this region. Several

studies have reported spontaneous regeneration within the hippocampal CA1 region following brain ischemia, with evidence suggesting that these newborn neurons originate from both the lateral ventricles and dentate gyrus (Bendel et al., 2005; Salazar-Colocho et al., 2008; Schmidt & Reymann, 2002). It is important to note that newborn neurons may potentially have aberrant function, as the result of abnormal dendritic processes (Langdon et al., 2008). However, there are contradictory findings regarding spontaneous repopulation, with some studies reporting an absence of CA1 neuronal regeneration even when the same brain ischemia models and time-points are used (Colbourne & Corbett, 1995; Silasi & Colbourne, 2011). Another important consideration is that increased cell proliferation in the dentate gyrus does not necessary lead to neuronal regeneration, given that newly generated cells can differentiate into other cell types, such as microglial cells (Silasi & Colbourne, 2011). Newborn cells generated in the dentate gyrus can also migrate to the granule cell layer of the dentate gyrus or to the dentate hilus, where they differentiate into astrocytes (Sharp et al., 2002).

The relationship between neurogenesis and functional recovery following brain ischemia has been examined. Wang et al. (2005) reported a significant reduction in the synaptic field response within the dentate gyrus at 10 days following brain ischemia, with activity recovering at day 35. Within this time period, there was a significant increase in the number of cells generated in the dentate gyrus. Thus, these findings suggest that hippocampal functional recovery following brain ischemia is associated with new cell production in the dentate gyrus. Although it has been theorized that neurogenesis improves recovery after brain ischemia, definite proof of causation has not been convincingly demonstrated. The extent to which neurogenesis improves recovery is most likely dependent on the degree of cell survival. Surprisingly, up to 80% of new neurons generated after stroke die within two weeks of formation (Arvidsson et al., 2002). Cell survival is strongly dependent on the environment, with a major influencing factor being inflammation. The inflammatory response can have either a detrimental or beneficial effect on neurogenesis, depending on if the brain is intact or injured (Ekdahl et al., 2009).

2.2.3.4 The Interaction Between Inflammation and Neuroplasticity

Inflammation strongly influences neuroplasticity and its effects can be both supportive and detrimental. Both pro- and anti-inflammatory cytokines have been highlighted as important regulators of synaptic plasticity; however, the particular role of each cytokine is strongly dependent on their synaptic concentration. Whereas basal levels of inflammatory cytokines are

essential for the maintenance of plasticity (Di Filippo et al., 2008; Vilcek & Feldmann, 2004), over-expression of the same cytokines can deter this response (Bellinger et al., 1995; Katsuki et al., 1990). There is strong evidence that inflammatory cytokines regulate hippocampal long-term potentiation (LTP), which is the prolonged increase in signal transmission that occurs between two neurons. Under physiological conditions, pro-inflammatory cytokines including IL-1, IL-6, and IL-18, are required for hippocampal LTP; however, overexpression of these cytokines impairs synaptic plasticity by inhibiting hippocampal LTP (Di Filippo et al., 2008). Overexpression of these cytokines may result in the blocking of N-methyl-D-aspartate receptor (NMDAR), the glutamate receptor controlling synaptic plasticity (Di Filippo et al., 2008). In addition to the role that cytokines play in signal transmission, there is evidence suggesting that the number of synapses is altered by inflammatory cytokines. Although the results are preliminary, increased neuroinflammation has been associated with synaptic loss, as demonstrated by a decrease in synaptic proteins including synaptophysin and drebrin (Rao et al., 2012). Ultimately, abnormal synaptic signaling that can occur under inflammatory states has been associated with cognitive impairment (Di Filippo et al., 2008).

In addition to the role that neuroinflammation has on synaptic plasticity, neurogenesis is also strongly impacted by inflammatory molecules, including pro-inflammatory cytokines, chemokines, and microglia (Ekdahl et al., 2009). The involvement of each of these molecules is complex and greatly dependent on the extent and timing of the inflammatory response. For example, evidence suggests that TNF-α has an inhibitory effect on progenitor proliferation when expressed during the acute period following brain ischemia; however, the role of TNF-α appears to be reversed at later time-points (Ekdahl et al., 2009). Similar to the inhibitory effects reported with early expression of TNF-α, overexpression of IL-1β and IL-6 can also significantly suppress neurogenesis under various pathological states (Ekdahl et al., 2009). Chemokines also play a central role in neurogenesis through their involvement in recruiting newborn neurons to the site of injury. Stromal cell-derived factor-1α, a chemokine expressed by microglia after brain ischemia, enhances progenitor proliferation and promotes migration of newborn neurons to the affected region (Imitola et al., 2004). Inflammatory cells, particularly microglia, typically interfere with neurogenesis under pathological conditions. A strong microglia reaction, such as the one triggered by LPS, prevents neuronal differentiation and is detrimental to the survival of newborn neurons (Ekdahl et al., 2009). Activated microglia appear to have a similar response

following stroke. Blocking microglia activation in the ischemic brain increases the number of new neurons generated within the dentate gyrus (Liu et al., 2007). It is important to note that the effects of microglia on neurogenesis are strongly dependent on the stage of activation. Unlike the detrimental effects caused by activated microglia, ramified microglia are thought to have a supportive role. Following brain ischemia, chronic accumulation of non-activated microglia within the lateral ventricles is thought to promote progenitor proliferation and differentiation (Ekdahl et al., 2009).

In summary, although neuroinflammation supports various aspects of neuroplasticity under normal conditions, the response is greatly different in the pathological brain. In general, the strong neuroinflammatory response triggered by brain ischemia is unfavorable to both synaptic plasticity and neurogenesis, and as a result may possibly hinder brain recovery.

2.3 Current Advances in Stroke Treatment

Despite years of pre-clinical research focusing on the pathophysiology of ischemic brain injury, there have been challenges in translating much of this work into effective treatments for human stroke victims (Hossmann, 2009). Currently, t-PA, which restores blood flow by dissolving blood clots, is the only validated and approved stroke therapy included in the Canadian Best Practice Recommendations for Stroke Care (Lindsay et al., 2010). T-PA is a serine protease that catalyzes the conversion of plasminogen to plasmin, resulting in the breakdown of blood clots. Administration of this enzyme can be very effective in treating both embolic and thrombotic strokes. However, the 4.5 hour therapeutic time window for drug administration is a major setback for this treatment (Hacke et al., 2008). Consequently, less than 2% of Canadian stroke victims receive this type of intervention (Yip & Demaerschalk, 2007). Given that the current thrombolytic treatment is only administered to a small subset of stroke patients, it is critical to find improved approaches that will allow more rapid reperfusion with an extended therapeutic time window. A second approach of stroke therapies involves administering neuroprotective agents, which target and impede specific aspects of the cellular cascade of ischemic injury. Despite the success of neuroprotectants tested in animal models, this has yet to be translated into effective clinical treatment. Currently, there are no neuroprotective drugs used for the treatment of stroke (Cook & Tymianski, 2011; Fisher et al., 2009).

Various limitations have prevented the successful translation of animal research into clinical stroke treatments. A major setback has been that the majority of studies have targeted

only one part of the ischemic cascade. STAIR has recently highlighted this as a limitation and has recommended that novel neuroprotective therapies target multiple pathways (Albers et al., 2011). Another major limitation is that most research groups do not factor in the effects of common stroke co-morbidity factors, such as malnutrition, hypertension, and hyperglycemia. Given that most human stroke patients are elderly and suffer from underlying diseases, it is crucial to understand what effects these co-morbidity factors have on the ischemic cellular events. Therefore, the major aim of the research described in this thesis was to delve further into the mechanisms by which stroke outcome is impaired by the common co-morbidity factor, malnutrition. Another key reason for this failure of translating animal research into beneficial treatments is that the focus has mostly been on targeting very early events in the cascade of brain injury. Treatments targeting early cellular events, such as radical formation, accumulation of Na^{+}/Ca^{2+} ions, and excitotoxic mediator release, would have to be administered within the first few hours following stroke, which in most cases is unachievable in a medical facility. Since more than half of patients do not seek help within the first 3 hours following stroke (Evenson et al., 2009), targeting cellular events that occur several hours after an ischemic attack will allow for a larger population of stroke patients to receive the pharmacological agent. The inflammatory cascade is a good molecular target since the acute reaction is initiated within hours following brain ischemia and can last for several days, weeks or even months. In addition, brain ischemia triggers a robust neuroplasticity response that remains heightened for at least a month after insult (Krakauer et al., 2012). Therefore, the possibility exists that by targeting the underlying mechanisms of spontaneous recovery, such as through neurorehabilitation, stroke outcome can be improved (Krakauer et al., 2012).

In summary, significant emphasis needs to be placed on expanding our understanding of the cellular cascade of events, examining how these pathways are altered by co-morbidity factors, and ultimately developing new therapies which target long lasting mechanisms including inflammation and neuroplasticity.

2.4 Rodent Models of Cerebral Ischemia

Animal models of stroke are crucial in enhancing our understanding of the pathophysiological changes that occur following ischemia resulting in brain injury. These models have been developed to closely mimic the biochemical changes in brain that occur both during and after human stroke. Currently, there are a wide variety of animal models used to study

cerebral ischemia, with variations such as the species utilized, type of ischemia induced (global or focal) and techniques used to generate insult. There are several criteria that should be satisfied when using an animal model of brain ischemia and they include: (a) the pathophysiological changes replicate that of human ischemic stroke, (b) the ischemic lesion and extent of neuronal damage is reproducible, (c) the surgical procedure is as noninvasive as possible and physiological determinants of ischemic brain injury are maintained within normal ranges, and (d) both histological and functional assessment is performed to evaluate the extent of brain damage (Traystman, 2003).

A wide variety of animals are used to study cerebral ischemia including higher species (e.g., cats, rabbits, dogs, monkeys, pigs) and rodents (e.g., rats, mice, gerbils) (Traystman, 2003). However, the most common animal used in stroke studies is the male rat. The rat is a good candidate for stroke research because of homogeneity within strains, ability to easily monitor physiologic parameters, and the low cost of animal care and availability. The majority of researchers exclusively use male rats to avoid variability caused by female hormones. Previous research has indicated that gender can directly affect ischemic stroke outcome, with female rats presenting with smaller infarctions following brain ischemia (Alkayed et al., 1998; Li et al., 1996). Interestingly, results from multiple studies performed in both female and male rats have shown that estrogen administration has neuroprotective properties (Alkayed et al., 1998; Fukuda et al., 2000; Zhang et al., 1998). Consequently, our understanding of stroke in females is very limited. Another limitation is that the majority of studies are performed on young, growing rats. Ideally, aged rats would be used to better model human stroke victims; however, various surgical complications can arise when using older rats. Furthermore, researchers tend to avoid using aged rats due to the considerable costs of housing animals for extended periods of time. These limitations apply to the research described in this thesis.

Models of ischemic brain injury are classified based on the location of occlusion (focal or global) and the extent of blood blockage (transient or permanent). In human stroke cases, occlusion is commonly seen in the middle cerebral artery or one of its branches (del Zoppo et al., 1992). Therefore, the majority of focal models have been developed to mimic this by inducing ischemia in the middle cerebral artery territory. Alternatively, global ischemia occurs when the cerebral blood flow is drastically reduced throughout most or all of the brain for a brief period of time, followed by reperfusion. Although this model of ischemia replicates the consequences of

cardiac arrest and resuscitation in humans, its use is relevant for the study of focal ischemia (stroke) since it induces many of the same pathophysiological mechanisms (Harukuni & Bhardwaj, 2006; Traystman, 2003). The other type of classification is dependent on whether there is reperfusion (transient model) or no-reflow (permanent model) following occlusion. Transient models of ischemia have two phases of reperfusion, with an initial period of increased blood flow (post-ischemic hyperemia) followed by an extended phase of reduced blood flow (post-ischemic hypoperfusion). Permanent models of ischemia, in which the vessels remain occluded, involve incomplete restoration of blood flow to the damaged tissue resulting in decreased neuronal survival (Iadecola, 1999). In summary, there are numerous animal models of cerebral ischemia that are currently being utilized in the stroke field to investigate ischemic mechanisms, prevention and treatment. Each of these models has potential advantages and disadvantages, which should be considered when designing a pre-clinical experiment.

2.4.1 Focal Ischemia Models

Induction of focal ischemia usually involves the occlusion of one major blood vessel resulting in transient or permanent reduction of blood flow to a specific brain region. Variations in focal models do exist with diverse techniques being commonly utilized to induce focal cerebral ischemia. Some of the current major animal models of focal ischemia include proximal or distal middle cerebral artery occlusion, intraluminal suture, middle cerebral artery embolism, endothelin 1 vasoconstriction, and photothrombosis (Murphy & Corbett, 2009). In brief, for the proximal/distal middle cerebral artery occlusion model, the middle cerebral artery is temporarily occluded using microvascular clips or permanently blocked by cauterization. The intraluminal suture model consists of interruption of blood flow for 30-120 minutes by placing a suture at the carotid artery and middle cerebral artery junction. Longer occlusion periods with this model can cause large infarcts in both hemispheres. Smaller infarct sizes, similar to human ischemic stroke, can be produced using the middle cerebral artery embolism model, in which a blood clot is introduced to occlude the middle cerebral artery. The endothelin 1 vasoconstriction model uses a strong vasoconstrictor, endothelin 1, to occlude various blood vessels. Lastly, photothrombosis involves a vascular injection of a photoactive dye combined with irradiation of a specific area of cortex with a light beam at a given wavelength. Irradiation of the circulating dye, such as rose bengal, produces free radicals and results in platelet aggregation and thrombus formation (Watson et al., 1985). An advantage of this model is the ability to produce a small infarct in a

specific cortical area by selectively illuminating the targeted brain tissue (Tatlisumak et al., 2007). Although these focal models are routinely used in experimental study, there are also disadvantages to using these surgical techniques, as outlined in section 2.4.2.1, which led to the selection of a global ischemia model for the thesis research.

2.4.2 Global Ischemia Models

2.4.2.1 Overview

Global cerebral ischemia models, in which the entire brain is transiently deprived of blood flow, have been designed to mimic cardiac arrest in humans. However, these models are also commonly employed in stroke research, as is the case in this thesis, to provide insight on the regional and cell specific vulnerability to ischemia. Furthermore, global brain ischemia has been shown to induce many of the same pathophysiological mechanisms of focal ischemia, making it a relevant model for studying cerebral ischemia (Harukuni & Bhardwaj, 2006). The advantages of utilizing global cerebral ischemia models are that, in general, they provide more defined and consistent injury, require a lower level of surgical expertise, and have fewer variables that need to be controlled compared to focal ischemia models (Traystman, 2003). In addition, focal ischemia models generally require extensive and invasive surgery (Tatlisumak et al., 2007; Traystman, 2003). Focal ischemia models requiring craniotomy, such as middle cerebral artery occlusion models, are considered especially traumatic. Major invasive surgeries can have pronounced effects on nutritional status, and thus are potentially problematic for the nutritional studies undertaken in this thesis. For these reasons, a global brain ischemia model was considered the most appropriate stroke paradigm for the thesis research. It is important to note, however, that the level of inflammation, and more specifically leukocyte infiltration, differs between global and focal ischemia models. Unlike focal ischemia, in which there is a high level of leukocyte infiltration, global ischemia does not damage the blood-brain barrier and therefore glial cells are the sole source of inflammatory cells (Kato, 2001).

The premise of most global cerebral models is to obstruct blood flow to the entire brain for a short duration (e.g., 5-15 minutes), causing damage to vulnerable neurons and avoiding overt brain infarction. The same subset of vulnerable neurons are injured in response to various experimental models of global ischemia. Reduction of blood flow to the brain results in forebrain structures virtually undergoing complete ischemia, with a rank order of vulnerability throughout the forebrain. Vulnerable brain regions include the hippocampus (CA1, CA2, and hilar neurons),

thalamus, and striatum (Pulsinelli et al., 1982). The most extensive and reliable damage is predominantly in the CA1 region of the hippocampus, becoming apparent within 2-4 days following global ischemia (Kirino et al., 1984). Some regions of the hippocampus, including CA3 pyramidal cells and granule cells of the dentate gyrus, are highly resistant to ischemia. However, damage can occur in these hippocampal areas following global ischemia if the injury is extensive. Therefore, in general, the hippocampus is the main region of interest when assessing damage elicited by global brain ischemia.

2.4.2.2 The Hippocampus

2.4.2.2.1 Overview

The hippocampus belongs to the limbic system and is a paired structure composed of mirror-image halves in both hemispheres of the brain. The elongated c-shaped structure extends from the basal forebrain into the temporal lobe of the rat brain. The hippocampal formation is composed of three distinct regions that include the dentate gyrus, the hippocampus proper (CA1, CA2, and CA3) and the subiculum. These cortical structures all share a similar three-layered appearance with the cellular connectivity mostly being uni-directional (Witter & Amaral, 2004).

2.4.2.2.2 Hippocampus Proper

Characterization of hippocampal neurons is based on cellular morphology and connectivity, with the hippocampus proper being divided into three subfields known as the CA1, CA2 and CA3 regions. All three regions contain a compact layer of excitatory pyramidal neurons (stratum pyramidale) that transmit glutamate; however, the size and arrangement of these neurons differ between subfields. The CA3 region lies proximal to the dentate gyrus and is composed of a layer of relatively large pyramidal cells. Alternatively, the CA1 region lies distal to the dentate gyrus and contains smaller pyramidal neurons that have a more compact arrangement than CA3 neurons. The CA2 region lies between CA3 and CA1 and contains many large pyramidal neurons similar in size to those in CA3, but with a more compact arrangement. The CA2/CA3 border can be hard to discriminate; however, another distinguishing characteristic is that mossy fibers originating from the dentate gyrus only extend to the CA3 neurons and not to CA2 neurons (Witter & Amaral, 2004).

The laminar organization of the hippocampus proper can be categorized into three main layers known as the stratum oriens (SO), stratum pyramidale (SP) and the stratum radiatum (SR) (**Figure 2.2**). The principal layer of the hippocampus proper is the stratum pyramidale, which is

several cells thick and mostly composed of excitatory pyramidal neurons. Stratum oriens is a narrow, relatively cell-free region superior to the stratum pyramidale. This stratum is mostly composed of the basal dendrites of pyramidal neurons, but also contains scattered basket cells and polymorphic neurons. Inferior to the pyramidal layer is the stratum radiatum, which contains projections from pyramidal neurons, basket cells and other interneurons. In addition to the three main layers, there are smaller defined hippocampal strata that include stratum lucidum (SL) and stratum lacunosum-moleculare (SLM) (**Figure 2.2**). Stratum lucidum is only found in the CA3 subfield and is occupied by the mossy fiber axons from the granule cell layer of the dentate gyrus. Stratum lacunosum-moleculare is the deepest stratum of the hippocampus and consists of perforant path fibers that originate from the superficial layers of the entorhinal cortex (Witter & Amaral, 2004).

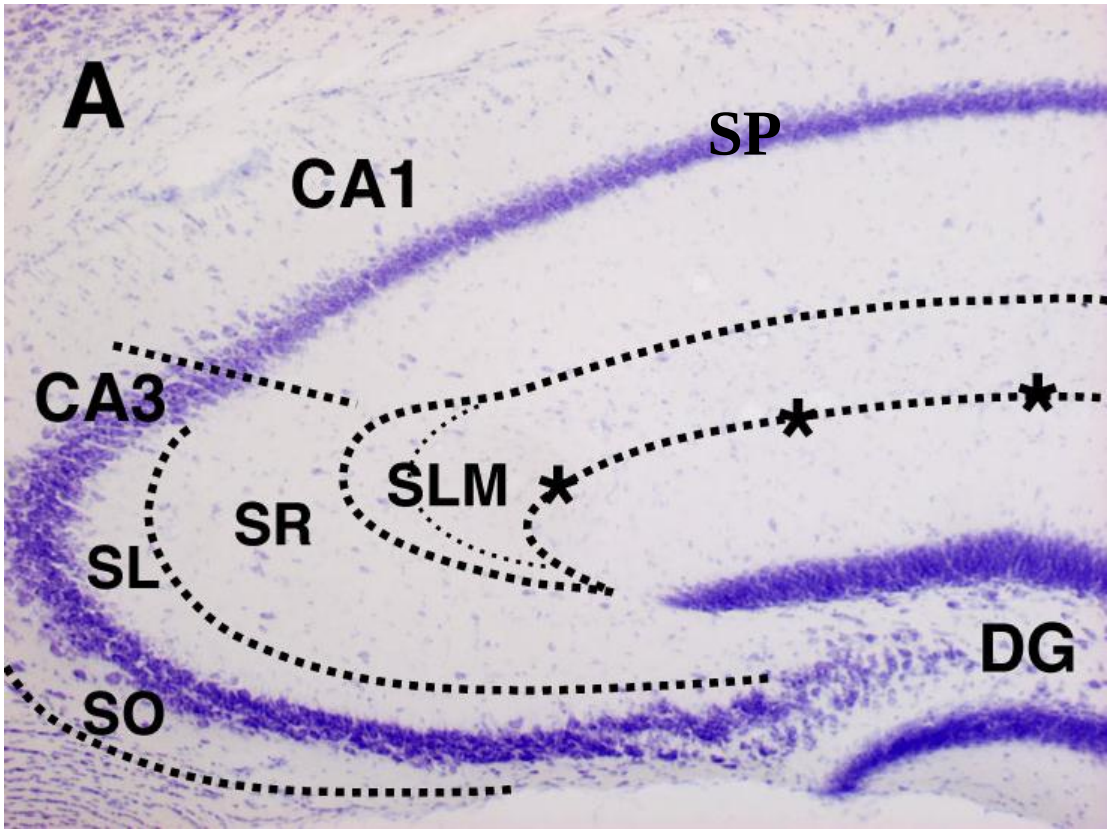

Figure 2.2. Diagram illustrating laminar organization of the hippocampus proper in the rat (SP, stratum pyramidale; SO, stratum oriens; SL, stratum lucidum; SR, stratum radiatum; SLM, stratum lacunosum-moleculare; DG, dentate gyrus). (From: Nishimura-Akiyoshi et al. PNAS. 2007; 104: 14801-14806. © 2013 by The National Academy of Sciences of the USA).

2.4.2.2.3 Hippocampal Circuitry

The hippocampal circuitry is complex and **Figure 2.3** highlights only the major pathways involved in the uni-directional flow of hippocampal information that originates and terminates in the entorhinal cortex (Witter & Amaral, 2004).

The perforant pathway, which is glutamatergic and arises from the entorhinal cortex, is the major input to the hippocampus. The axons of the perforant pathway terminate in all subregions of the hippocampus; however, the dentate gyrus is the predominant target of this pathway. Within the dentate gyrus, the majority of axons synapse on dendritic spines of granule cells. The signal is transferred from the dentate gyrus to the CA3 hippocampal region via the mossy fiber pathway, with glutamate being the primary neurotransmitter released. In particular, axons of dentate gyrus granule cells extend to the CA3 region and synapse on pyramidal neurons. The majority of these synapses occur within the stratum lucidum, the inner region of the CA3 bend. On average, a single mossy fiber has 14 synaptic connections within the stratum lucidum, thus influencing multiple CA3 pyramidal neurons (Witter & Amaral, 2004). CA3 neurons extend their axons to CA1 pyramidal neurons via the Shaffer Collateral pathway. Typically, Shaffer Collateral fibers extend through the CA3 stratum oriens and synapse on dendritic spines of CA1 neurons located within the stratum radiatum. CA3 neurons from the contralateral hippocampus also make connections with CA1 pyramidal neurons, and are termed commissural fibers. In addition, the CA3 region can be innervated by CA3 neurons, by means of CA3-to-CA3 associational connections (Witter & Amaral, 2004). The major output from the hippocampus is the pathway from the CA1 region to the subiculum and back to the entorhinal cortex. CA1 neurons extend their axons through the stratum oriens and synapse on subicular pyramidal neurons. Subsequently, projections from the subiculum terminate on the pyramidal neurons in the entorhinal cortex.

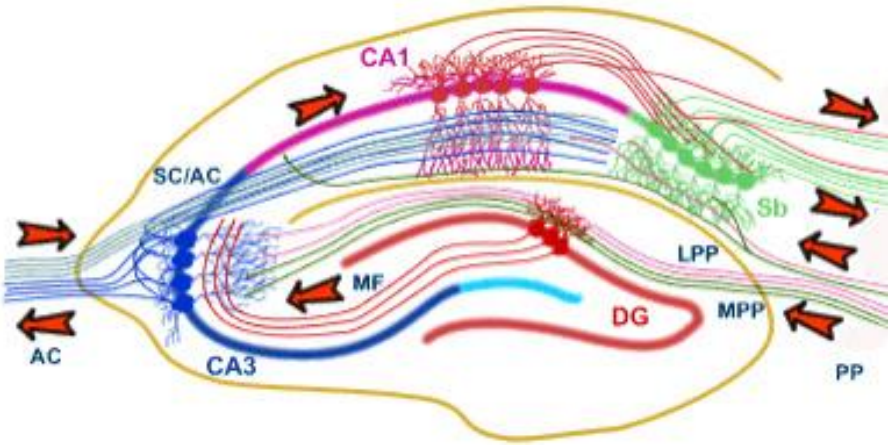

Figure 2.3. The rat hippocampal network. The hippocampus forms a principally uni-directional network, with input from the entorhinal cortex that forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the Perforant Pathway (PP – split into lateral and medial). CA3 neurons also receive input from the DG via the Mossy Fibers (MF). They send axons to CA1 pyramidal cells via the Schaffer Collateral Pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the Associational Commisural (AC) Pathway. CA1 neurons also receive inputs direct from the Perforant Path and send axons to the Subiculum (Sb). These neurons in turn send the main hippocampal output back to the entorhinal cortex, forming a loop. (From: Dr. Andrew Doherty; University of Bristol).

2.4.2.3 Gerbil Bilateral Common Carotid Artery Occlusion Model

The simplest surgical model of global brain ischemia is the bilateral common carotid artery occlusion in the gerbil. This was an excellent model due to the unique cerebrovascular anatomy of the Mongolian gerbil, in which the posterior communicating arteries were absent, thus forming an incomplete circle of Willis (Traystman, 2003). This surgically simple model involved a short occlusion period of approximately 5 minutes, which historically produced very consistent hippocampal CA1 injury (Colbourne & Corbett, 1995; Small & Buchan, 2000). However, over the last decade there has been an alarming increase in variability of ischemic damage (Ji et al., 2008; Laidley et al., 2005; McEwen & Paterson, 2010). To study this phenomenon, the cerebral vasculature of Mongolian gerbils from two suppliers were compared (Laidley et al., 2005). Approximately 61% of gerbils obtained from Charles River, the largest supplier in North America, had posterior communicating arteries forming either a complete (22.7%) or partial (38.6%) circle of Willis. Consequently, this subset of gerbils had significantly less CA1 neuronal damage following bilateral common carotid artery occlusion compared to gerbils with no posterior communicating arteries. The mechanisms leading to the variation in the

circle of Willis are currently unknown, but are most likely due to a genetic component. Consequently, most researchers have abandoned the bilateral common carotid artery occlusion model due to the large portion of gerbils possessing the complete circle of Willis.

2.4.2.4 Rat Global Ischemia Models

Rats are also considered a good candidate for stroke research, given that their relatively large blood volume allows for continuous sampling throughout the surgical period to monitor physiological variables known to influence ischemic injury. Induction of global brain ischemia in rats is commonly via 4-vessel occlusion (4-VO) or 2-VO. The 4-VO model was introduced in 1979 and is a two-step surgical process consisting of permanent occlusion of the vertebral arteries on day one, followed by occlusion of the bilateral common carotid arteries on day two (Pulsinelli & Brierley, 1979). Major disadvantages of this method include the permanent occlusion of the arteries and high occurrence of seizures (up to 40%) following occlusion (McBean & Kelly, 1998). Furthermore, the level of surgical invasiveness with this procedure is problematic for the nutritional studies undertaken in this thesis. The 2-VO model is often used instead of the 4-VO model to reduce the surgical stress on the animal (Smith et al., 1984). The damage induced via the 2-VO model is similar in size and location to that produced with the 4-VO model (McBean & Kelly, 1998). Therefore, the 2-VO model has been widely accepted as a relevant and important model used for identifying the physiological, biochemical and functional mechanisms of the human condition of stroke.

2.4.2.4.1 Rat 2-Vessel Occlusion

The 2-VO model involves occlusion of both bilateral common carotid arteries combined with systemic hypotension, ultimately inducing a rapid onset of ischemia followed by quick reperfusion. Induction of systemic hypotension is necessary, since occlusion of the common carotid arteries alone does not sufficiently decrease the cerebral blood flow below the ischemic threshold (Eklof & Siesjo, 1973). The advantages of this model include a relatively straight-forward surgery that has a lower experimental failure rate than other models, production of high-grade forebrain ischemia, and acceptable consistency in insult severity. The extent of ischemic insult is dependent on successful blood pressure reduction (30-40 mmHg) and complete but temporary occlusion of the carotid arteries (8-10 minutes) (Smith et al., 1984). Depending on the size of rat, systemic hypotension can be induced by withdrawing blood from the jugular vein or tail artery. As well, the tail arterial site can be used to obtain blood samples for physiological

monitoring. Physiologic parameters, including blood gases and glucose, are attained several times throughout the surgery, allowing monitoring of acid-base balance and adjustment of oxygen levels as necessary to achieve appropriate tissue oxygenation.

The severity and homogeneity of ischemic injury induced in rodent stroke models is dependent on several surgical variables, including duration of ischemia, blood pressure, temperature, blood glucose levels and blood gases. The 2-VO model is no exception. Extensive neuronal damage can be obtained with the 2-VO model by using a relatively short occlusion period of 8-10 minutes. The extent of ischemic injury was recently characterized after 8, 9 and 10 minutes of occlusion using the 2-VO model. Histological analysis of tissue collected 7 days following ischemia revealed that all three occlusion periods resulted in extensive CA1 hippocampal injury with no difference in the extent of damage among groups (Clark et al., 2007). Variations in blood glucose levels can contribute to heterogeneity in the 2-VO model, with elevated blood glucose concentrations exacerbating brain injury (Dietrich et al., 1993; Siemkowicz & Hansen, 1978). To prevent potential variability in ischemic injury, animals are commonly fasted to achieve blood glucose levels within a consistent range. Arterial blood samples are generally collected several times throughout the surgical period to allow monitoring of pH, pO_2 and pCO_2. Global ischemia models are relatively insensitive to small changes in blood gases; however, extreme variation from the desired range can cause heterogeneity in ischemic injury (Nowak, 2007). For example, marked reductions in pH during brain ischemia exacerbates tissue damage by activating acid-sensing ion channels resulting in an influx of calcium (Huang & McNamara, 2004). Another surgical challenge is providing adequate oxygen to ensure 100% saturation of the blood (as assessed by arterial blood pO_2), while preventing a surplus of oxygen that would enhance oxidative stress. Hematocrit is routinely monitored as a measure of the percentage of the total blood volume made up by red blood cells, and therefore is a good indicator of the oxygen-carrying capacity of the blood (Kiyohara et al., 1985).

Full characterization of each surgical variable for the given model is necessary to establish the parameter threshold that needs to be obtained to produce consistent and extensive hippocampal CA1 neuronal death. Arvanitidis et al. (2009) recently modified the 2-VO methodology, which was based on that of Smith et al. (1984), providing descriptions of the desirable range for each surgical variable. Nonetheless, it is good practice to establish the desirable range of each variable to induce ischemia under the specific conditions of model usage.

A limitation of the 2-VO model is that some variability in neuronal damage can occur between animals. Unilateral damage is observed in a subset of animals following 2-VO ischemia, even with all surgical parameters being well controlled (Smith et al., 1984). Autoradiographic measurements of local cerebral blood flow have illustrated that in the occasional animal, flow rates are maintained on one side of the brain during carotid artery occlusion (Smith et al., 1984). This phenomenon has been difficult to explain and highlights the importance of histological assessment to confirm extensive ischemic injury.

2.4.2.5 Regulation of Brain Temperature

Brain temperature should be assessed when using any brain ischemia model, given that neuronal damage is highly temperature-sensitive. The persistent lack of attention to temperature regulation is the most common error in experimental stroke research, causing increased variability in neuronal injury. The effects of temperature following global brain ischemia have been comprehensively reviewed, and it is evident that hypothermia has neuroprotective properties by enhancing CA1 survival (Colbourne & Corbett, 1995; MacLellan et al., 2009; van der Worp et al., 2007), while hyperthermia worsens outcome by increasing degeneration of CA1 neurons (Minamisawa et al., 1990; Wang et al., 2009). Attention to intra- and post-ischemic temperature is necessary since hippocampal pathology is prolonged and remains temperature sensitive (Nowak, 2007). Thermocouple wires are commonly used during the 2-VO surgery to record skull or tympanic temperature as estimates of brain temperature. Temperature is maintained within a consistent range with the use of heat lamps and water blankets. Given that core body temperature and brain temperature are usually highly correlated after global ischemia induced by the 2-VO procedure (Clark et al., 2007), implanted core probes are often adequate for monitoring post-ischemic temperatures. Furthermore, a recent report suggests that spontaneous temperature changes do not occur in healthy Sprague-Dawley rats following 2-VO surgery, and thus heating sources are not necessary during the acute post-ischemic period (Clark et al., 2007). However, this response can vary depending on rat strain, surgical sterility, and method used to measure temperature (Clark et al., 2007).

2.4.2.6 Assessment of Ischemic Injury

2.4.2.6.1 Histological Assessment

To accurately assess the extent of ischemic injury, it is necessary to have both histological and functional endpoints. There are various histological techniques that are

commonly used to accurately and objectively analyze the extent of neuronal death. Histological techniques used for global brain ischemia research include hematoxylin and eosin (H&E) staining, nitroblue tetrazolium and Nissl's staining (cresyl violet) (Tatlisumak et al., 2007). Cresyl violet staining is a well established histological stain used for light microscopy (Burnett et al., 1987) to evaluate both early and delayed neuronal death following global ischemia that was used in this thesis research. Cresyl violet stains the nissl substance, known as the rough endoplasmic reticulum, found within the cytoplasm. Early histological endpoints can detect neuronal death within 2-4 days following global brain ischemia, with neurons appearing shrunken (Kirino, 1982; Kirino et al., 1984; Pulsinelli et al., 1982). Cresyl violet staining diminishes as neurons degenerate in the hippocampus. Long-term endpoints are necessary when studying a treatment effect since it might simply delay neuronal death, which if using an early time-point might deliver encouraging but false results (Corbett & Nurse, 1998). Similarly, long-term endpoints are important for the research described in this thesis, given that a detrimental factor such as PEM might exacerbate the slow, long-term increase in CA1 neuronal death.

2.4.2.6.2 Functional Assessment

Functional recovery should be evaluated in combination with histological analysis, given that a neuron exposed to ischemia could appear viable but have aberrant function. Two of the most common techniques incorporated into experimental stroke studies to assess functionality are behavioural tests and electrophysiological assessment. The open field and T-maze test are the best-characterized behavioural tests to assess functional recovery following global ischemia in the gerbil (Babcock & Graham-Goodwin, 1997; Colbourne & Corbett, 1995; Farrell et al., 2001). More sensitive behavioural paradigms are necessary in measuring functional outcome in the rat 2-VO model, given that the behavioural impairments seem to be milder than for the gerbil model. Recently, Langdon et al. (2008) assessed the sensitivity of three commonly used hippocampal-dependent tests; Morris water maze, radial arm maze, and T-maze. It was demonstrated that the radial arm maze, along with more difficult versions of the T-maze, had better sensitivity in detecting CA1 injury than the standard Morris water maze. However, the radial arm maze and T-maze are very time consuming and labour intensive. Increasing the difficulty of the Morris water maze can enhance the sensitivity level. More specifically, a reduction in the number of cues and trials increases the difficulty of the task and allows for

detection of modest functional impairments in rats exposed to global brain ischemia (Langdon et al., 2008).

Electrophysiology is a technique that allows direct observation and recording of the electrical activity of a cell. To analyze the extent of damage to a specific area of the brain, such as the hippocampus, the tissue is excised and analyzed with an electrode. A single-unit recording can be obtained by inserting a micro-electrode into a neuron, which will detect the action potential activity of a single cell (Henderson, 1993). If the neuron is undamaged from ischemia, the recorded resting membrane potential should be between -60 to -80 mV, and reaching +40 mV during an action potential. Another technique, referred to as extracellular field potentials, involves placing a larger electrode in the extracellular space to measure the collective activity of many neurons (Henderson, 1993). Therefore, unlike behavioural tests, which have limited sensitivity of detecting damage to a specific cell group, electrophysiology can effectively measure deficits in localized regions (Corbett & Nurse, 1998). However, the major disadvantage of electrophysiology is that tissue slices are required, and thus afferent inputs outside the plane of the cut are transected. Nevertheless, electrophysiology has been frequently employed to measure injury following global brain ischemia (Corbett & Nurse, 1998). Extracellular field potentials can be evoked in CA1 neurons between 0-48 hours following insult, but then are greatly reduced at later time-points (Jensen et al., 1991). These results illustrate that the morphological and functional changes to CA1 neurons during the acute period after global ischemia follow a similar time course.

2.4.2.7 Synaptic Plasticity Triggered by Global Brain Ischemia

Post-ischemic synaptic plasticity in global ischemia models has not yet been fully characterized, due to limited studies and assessment only at early time-points. Procedures commonly used to assess synaptic plasticity include electrophysiology, Golgi-Cox staining, and immunocytochemical profiles of neuroplastic positive factors (e.g. BDNF, GAP-43) and synaptic vesicle proteins (e.g. synapsin, synaptophysin). Interestingly, increases in proteins involved in plasticity have mostly been detected within the mossy fiber terminals, which is the location where axons of the dentate gyrus synapse on CA3 pyramidal neurons. These findings suggest that global brain ischemia is affecting signaling in ischemia-resistant regions.

Alterations in GAP-43, a molecular marker of neuronal plasticity, have been described in two models of global brain ischemia. Following 4-VO surgery in the rat, mRNA expression of

GAP-43 declined in the CA1 subfield, as the result of cell death, but increased in CA3 neurons and dentate gyrus granule cells (Schmidt-Kastner et al., 1997). GAP-43 protein immunoreactivity after global ischemia in the gerbil increased after 3 days within the dentate gyrus and dying CA1 neurons; however, the signal was lost by day 7 (Prosser-Loose et al., 2010). Similar to the distribution of GAP-43, reports have shown an increase in BDNF expression within the CA3 and dentate gyrus regions following global ischemia (Kokaia et al., 1996; Lee et al., 2002).

Synaptophysin, SNAP-25 (synaptosomal-associated protein of 25 kDa) and Mint1 (munc-18-interacting protein 1) are examples of presynaptic proteins measured to assess changes to synaptic terminals in the ischemic hippocampus. These proteins are found within synaptic vesicles, and thus an increase in concentration suggests enhanced neuronal signaling. Within mossy fiber terminals, protein levels of SNAP-25 are increased as early as 2 days after global ischemia (Marti et al., 1998). Similarly, Mint1 immunoreactivity is significantly increased within the mossy fiber region 3 days following global brain ischemia (Nishimura et al., 2000). Together, these results suggest that increases in synaptic proteins occur in the mossy fiber region near the time of delayed CA1 neuronal death. In the CA1 region, SNAP-25 and synaptophysin levels are significantly depleted by day 2 following global ischemia. However, around surviving CA1 neurons, both of these proteins are considerably increased on days 7 and 14 (Ishimaru et al., 2001). These studies were performed using either a gerbil or mouse model of transient global brain ischemia, and therefore characterization of these synaptic proteins in the rat 2-VO model is required. Nevertheless, the rat 2-VO model was recently used to evaluate whether post-ischemic hypothermia impedes plasticity (Silasi et al., 2012). Although synaptophysin levels were examined in this study, the absence of sham-operated animals prevented analysis of the ischemic effect. Interestingly though, the results suggest that prolonged hypothermia does not alter the neuroplasticity response to global brain ischemia in rats.

2.4.2.8 Inflammatory Response after Global Brain Ischemia

Global brain ischemia results in extensive CA1 neuronal damage within days of insult, which is accompanied by marked glial reactions that involve both microglia and macroglia. Unlike focal ischemia, global ischemia does not usually result in blood-brain barrier damage and therefore immune cells are not recruited from systemic circulation (Kato, 2001). Therefore, microglia are the sole responders since infiltration of leukocytes and activation of perivascular

cells does not occur following global brain ischemia (Kato, 2001). Furthermore, it is currently unknown whether global brain ischemia triggers an acute-phase response, thus highlighting a novel research area that will be assessed in this thesis.

The vulnerability of the hippocampus to global brain ischemia makes it the primary site of inflammation. The initial activation of microglia, which is not confined to one area of the hippocampus but instead is present throughout the entire hippocampus, has been observed within minutes following reperfusion (Kato et al., 1995; Morioka et al., 1991). At day 2-4 following global ischemia, neuronal death becomes apparent which triggers another wave of microglial activation. A significant increase in microglial immunoreactivity occurs within 72 hours following global ischemia in the CA1 region of the hippocampus and between the blades of the dentate gyrus (Webster et al., 2009). Proliferation of microglia within the CA1 region continues until at least the third post-ischemic week, as demonstrated by increased numbers of bromodeoxyuridine (BrdU)/Iba-1 co-labeled cells (Silasi & Colbourne, 2011). The glial population within the CA1 region remains heightened for up to 9 months following global brain ischemia (Langdon et al., 2008). Chronic neuroinflammation suggests that there is continuing injury for months following global ischemia resulting in long-term cell death. In combination with these findings, it has been demonstrated that functional impairments are sustained for at least 9 months following global brain ischemia (Langdon et al., 2008).

The master regulator of the inflammatory response, NFκB, is activated within 12 hours following global brain ischemia (Murakami et al., 2005). NFκB activation occurs at marked levels in the nucleus of degenerating CA1 hippocampal pyramidal neurons. Conversely, activation of NFκB has not been demonstrated in brain regions that are not as vulnerable to global ischemia, such as the cerebral cortex, thalamus, and hypothalamus (Clemens et al., 1997). Activators of NFκB, including TNF-α and IL-Iβ, are vastly increased within hours following global ischemia. Global brain ischemia in the mouse, induced via bilateral common carotid artery occlusion, results in elevated hippocampal TNF-α mRNA expression 3 hours following insult with increased protein levels by 6 hours. Expression levels of TNF-α mRNA remain heightened for 36 hours after global ischemia and return to basal levels by day 4 (Murakami et al., 2005). Furthermore, mRNA expression of the pro-inflammatory cytokine, IL-1β, peaks 8 hours following reperfusion in the hippocampus region (Sairanen et al., 1997). In the CA1 and CA2 regions of the hippocampus, IL-1β mRNA levels remain elevated for up to 24 hours, with

the major cellular source of the protein being from glial cells (Sairanen et al., 1997). Together these results suggest that pro-inflammatory cytokines are rapidly expressed during the acute period following global brain ischemia; however, the cytokine response at longer time-points has not yet been characterized. The inflammatory mechanisms triggered by global brain ischemia could potentially be influenced by various factors, including poor nutrition. The co-morbidity factor, co-existing PEM, could worsen brain ischemia outcome by escalating or prolonging the inflammatory response.

2.5 Protein-Energy Malnutrition

2.5.1 Overview

Mechanisms triggered by brain ischemia can be strongly influenced by a common type of malnutrition called PEM, which is characterized by inadequate protein and energy status. Primary PEM, which results from the consumption of an inadequate diet that is deficient in protein and other nutrients, is predominantly seen in developing countries due to factors such as poverty, famine, and unsanitary living conditions. Primary PEM is often seen among infants and children and is classified as either marasmus, a severe and gradual form of PEM, or kwashiorkor, an acute type of PEM that progresses more rapidly. PEM in the elderly also exists in developed countries and is likely to arise from the combination of suboptimal diet and secondary causes such as co-morbidities. Various underlying diseases or treatments can cause impairments in food digestion, absorption of nutrients, or swallowing, ultimately causing the manifestation of PEM (Torun, 2006). Given that variations of secondary PEM exist, it has been recently proposed that a valid etiology-based diagnosis construct be used in clinical settings to properly identify adult malnutrition (Jensen et al., 2010). One of the key classification criteria proposed for this diagnosis scheme is the presence or absence of a systemic inflammatory response. This has been highlighted as an important component of the diagnosis construct, since the presence of inflammation requires different medical therapy and nutritional intervention. It is imperative that PEM is diagnosed correctly, since it is systemic in nature and can have detrimental effects on almost every organ system in the body (Torun, 2006).

2.5.2 PEM in the Elderly Population

Seniors living in industrialized countries are at high risk for developing PEM, with approximately 15% of community-dwelling and home-bound elderly, 23-62% of hospitalized patients, and up to 85% of individuals residing in nursing homes being categorized as protein-

energy malnourished (Guigoz, 2006). Strong evidence from epidemiologic studies have highlighted PEM as an independent predictor of mortality, regardless of whether the individual is an independent living elderly or a patient in a hospital/nursing home (Campbell et al., 1990; Cederholm et al., 1995; Morley & Silver, 1995). Furthermore, mortality rates are even higher in the cases where PEM is a co-morbidity factor (Chapman, 2006). Given that the elderly population in Canada is on the increase (Turcotte & Schellenberg, 2006), these alarming findings emphasize the importance of research that aims to improve survival rates and quality of life in the malnourished.

The elderly are at increased risk of developing PEM due to social, psychological, and medical factors (Chapman, 2006). Poor eating habits, usually as a consequence of living alone, is the most common underlying cause of primary PEM in seniors. The inability to shop, prepare food, or cook meals are examples of challenges that can result in insufficient food intake. Furthermore, individuals that live alone commonly experience decreased appetite resulting in reductions in daily energy intake (Walker & Beauchene, 1991). Malnutrition in seniors can also be the result of poor dentition, causing difficulties in biting, chewing and swallowing of certain foods. Depression, dementia and alcoholism are examples of other factors that can directly affect eating habits (Chapman, 2006). Additionally, there is a greater likelihood for drug-nutrient interactions in elderly, due to the increased chance of an individual both being of poorer nutritional status and taking medications. Secondary PEM commonly manifests in patients suffering from serious medical conditions, such as cardiac failure, cancer, infection, and stroke, due to physical impairments and long-term treatments. Hence, the research reported in this thesis focused on the effects of both PEM as a co-morbidity factor at the time of stroke and PEM developing as a result of the stroke.

2.5.3 Prevalence of PEM in Stroke Patients

PEM is one of the most prevalent types of malnutrition in North America and is a co-morbidity factor in approximately 16% of stroke cases (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). In addition to the subset of patients with co-existing PEM at the time of stroke, the prevalence rates of PEM double within the first week following a stroke due to inadequate hospital care and physical impairments. Dysphagia, occurring in 24-53% of stroke patients (Foley et al., 2009), is the inability to control food in the mouth and swallow, and is a major precipitating factor of malnutrition. A systematic review demonstrated that there is a

significant relationship between dysphagia and malnutrition following stroke (Foley et al., 2009). In addition to dysphagia, other factors that can contribute to poor nutritional status following a stroke include depression, paralysis of the arm or face, and reduced level of consciousness (Bouziana & Tziomalos, 2011). Therefore, both co-existing PEM and post-ischemic PEM are clinically relevant health conditions. Given that PEM can have adverse effects on stroke outcome, it is crucial that it is correctly diagnosed and monitored during the acute period following stroke. There are numerous methods used to diagnosis PEM in patients, although there are many identified limitations with the current nutritional assessment techniques available.

2.5.4 Diagnosis of PEM

2.5.4.1 Overview

Malnutrition has been associated with poorer survival and functional outcome following a stroke (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). Therefore, it is crucial for health professionals to thoroughly assess the nutritional status of stroke patients so that the appropriate support can be provided. Evaluation of the presence and severity of PEM will assist in determining the effectiveness of nutritional support, and also decrease the risk of complications associated with malnutrition. The traditional methods used in the diagnosis of PEM include dietary, anthropometric, biochemical and functional measurements (Torun, 2006). However, the extent of assessment is commonly dependent on the simplicity and expense of the procedure, time requirements, and availability of measurement instruments. Given that many of these assessment techniques are plagued by difficulties, it is common for PEM diagnosis to be based on a single parameter. Serum albumin is one of the most frequently used single biochemical indices for PEM assessment (Johnson, 1999). However, it has major limitations (see Section 2.5.4.3.2), and a multi-parameter protocol is recommended for a more adequate assessment.

2.5.4.2 Anthropometric Measurements

Weight loss is a common and pronounced symptom of PEM. A reduction in energy intake is commonly compensated by a decrease in energy expenditure. However, if the energy output is still greater than intake, the outcome is weight loss. As malnutrition progresses and energy deficits become more severe, muscle protein catabolism occurs which leads to muscular wasting (Torun, 2006). Anthropometric measurements are generally used to assess body composition, given that they are noninvasive, low in cost, quick and simple. Raw measurements

of weight, height, skinfolds (triceps and subscapular) and waist, hip and arm circumferences are easily attainable and can predict nutritional status. Furthermore, the body mass index, calculated as weight relative to height, is widely used in the assessment of PEM severity in adults. The classification protocol used for PEM assessment states that a body mass index of $\geq$18.5 kg/m^2 is normal, 17.0-18.4 kg/m^2 is mild PEM, 16.0-16.9 kg/m^2 is moderate PEM, and < 16.0 kg/m^2 is severe PEM (World Health Organization, 2006). Although a main clinical characteristic of PEM is weight loss, various nutritional deficits can have similar effects and therefore anthropometric measurements are not sensitive enough to detect specific nutrient deficiencies. Similarly, there are various non-nutritional factors, such as medical conditions and medications, which can affect anthropometric measurements, thus reducing testing sensitivity and specificity. For that reason, relying on only the body mass index assessment is not considered adequate to confirm the diagnosis of PEM, and additional parameters should be measured.

2.5.4.3 Biochemical Measurements

2.5.4.3.1 Overview

PEM can drastically impact protein anabolism and catabolism. PEM most commonly results in a reduction in protein synthesis, and as a result the half-lives of proteins are increased (Torun, 2006). Due to limited amino acid availability, serum concentrations of various proteins, such as albumin and transferrin, are commonly decreased in individuals with moderate to severe PEM and are used as biochemical indices of PEM. As well, suboptimal protein status can cause a decline in urinary excretion of urea nitrogen, creatinine, and hydroxyproline. Measuring total iron binding capacity, white blood cell counts and calculating the ratio of non-essential to essential amino acids in plasma are also examples of laboratory tests that can be included in the screening protocol for PEM (Gibson, 2005; Torun, 2006). Although these biochemical tests can be effective in diagnosing PEM, they are nonspecific in that underlying diseases can alter many of these measured parameters. Therefore, given that these biochemical determinants are non-specific to PEM, they should be included in the full nutritional assessment along with other parameters.

2.5.4.3.2 Serum Albumin

Serum albumin is a valuable indicator of protein production, given that approximately 40% of the total protein produced by the liver and released into circulation is albumin (Charlton, 1996). Depending on the severity of PEM, a shift of albumin from the extravascular system to

the intravascular pool can prevent substantial diminishment in circulating albumin levels. However, with severe PEM the decrease in protein synthesis is too extreme and the adaptive mechanisms are unable to prevent significant depletion of albumin (Torun, 2006). Although serum albumin is one of the most universally used clinical markers of PEM, it is important to highlight that it is not only influenced by dietary amino acid availability but also by the acute-phase reaction (Don & Kaysen, 2004). The effect of protein depletion and inflammation on serum albumin levels can be similar in degree (Qu et al., 1996).

2.5.4.4 Functional Measurements

In many hospitals, functional tests are regularly employed and used in a full nutritional assessment of PEM. Weakening in muscle strength, poor wound healing, fatigue, depression, and disruptions in thermoregulation are all examples of parameters included in full nutritional assessments of PEM to establish the severity of functional impairment (Gibson, 2005). A hand-grip strength test is one of the most frequently used functional assessments of PEM and is a promising indicator of nutritional status (Gibson, 2005). Although most of these assessment tools are simple and inexpensive, one of the major limitations is that they are not yet well standardized. Furthermore, similar to the limitations with anthropometry and biochemical measurements, functionality can be influenced by other nutritional deficits and medical conditions, including stroke. Functional impairment resulting from some types of stroke could impact hand-grip strength, therefore making it difficult to differentiate a post-stroke deficit from a nutritional effect.

2.6 Rat Models of PEM

Minimal information is known about the particular mechanisms altered by PEM in human stroke patients, due to various practical and ethical research challenges. Therefore, animal models of PEM are a valuable approach that can justly mimic clinical PEM and aid in establishing which mechanisms are altered by malnutrition and the impact that this may have on stroke recovery. There is no standard methodological approach used to reproduce PEM in rats, given the differences in nutrient requirements at different ages and the various types of PEM that investigators may wish to mimic. However, there is a vast amount of literature available for the weanling and adolescent rat. Determination of protein-energy status is commonly based on the degree of reduction in food intake, body weight, and serum albumin concentration, as well as the extent of increase in liver lipid levels (Heard et al., 1977; Qu et al., 1996).

PEM is normally achieved by reducing the amount of casein in the diet and feeding animals *ad libitum* (Woodward, 1998). If the protein content is sufficiently low, animals commonly voluntarily reduce their total food intake (Heard et al., 1977; Rozwadowski et al., 1995; Taylor et al., 1992). Consequently, a decrease in food intake causes daily energy intakes to diminish, inducing a condition referred to as mixed PEM. The voluntary reduction of food intake is dependent on many factors including species, age of rat, and severity of the protein restriction. Therefore, in cases where animals do not voluntarily reduce their food intake, researchers may choose to induce mixed PEM by feeding a restricted amount of the low protein diet. Dietary carbohydrate, commonly in the form of cornstarch, is substituted for protein to maintain the protein deficient diet isocaloric relative to the control diet. Although it is important to have the equivalent energy density between diets, the major disadvantage of substituting with carbohydrates is that the ratio of carbohydrates to protein is augmented, which can exert its own effects on the experimental outcome. There is no standard protein-deficient diet that is universally used to model PEM in rats, given that the diet formulation should be specialized to the age of animal and desired duration and severity of PEM.

It is crucial for researchers to verify that the animal nutritional paradigm is correctly mimicking similar features to that of PEM in the clinical population of interest. For example, my laboratory thoroughly characterized a gerbil model of chronic moderate PEM for use in mimicking human stroke patients, in which adult gerbils developed mixed PEM after a 4 week feeding period on a 2% protein diet. Reported findings from studies using this model demonstrated a reduction in food intake (15%) (Bobyn et al., 2005), body weight (17%) (Bobyn et al., 2005), liver reduced-glutathione (49%) (Bobyn et al., 2005), and serum albumin (18%) (Prosser-Loose et al., 2007), and an increase in liver lipids (~65%) (Bobyn et al., 2005).

Since rats fed a low protein diet commonly voluntarily reduce their food intake, collecting data on daily food intake is essential for characterizing models of PEM. A similar phenomenon is commonly seen in clinical PEM in which individuals with kwashiorkor consistently present with symptoms of anorexia (Castiglia, 1996). The mechanisms involved in the suppression of food intake are largely unknown, although it has been suggested that the phenomenon is dependent on the ratio of non-essential to essential amino acids (de Angelis et al., 1978). Evidence indicates an inverse correlation between this ratio and food intake, such that an increase in the ratio results in a decrease in appetite (de Angelis et al., 1978). Further evidence

suggests that anorexia could be regulated by the indispensable amino acid, histidine. Protein malnourished rats commonly experience a systemic increase in histidine, which has shown to be strongly correlated with a depression in food intake. Furthermore, healthy rats exposed to increased levels of histidine produce more hypothalamic histamine and temporarily decrease their food intake (Mercer et al., 1989). Given that histidine is a precursor of the neurotransmitter histamine, it is likely that food depression is neuroregulated. Furthermore, a reduction in body weight is another common indicator of PEM that manifests during the early stages of protein depletion (Balmagiya & Rozovski, 1983; Ling et al., 2004; Lyoumi et al., 1998; Rothwell & Stock, 1987). Measuring body weight is essential in nutritional studies, given that weight loss occurs when protein requirement is not met.

Despite its many limitations (discussed above), serum albumin is the most commonly measured biochemical indicator of PEM (Johnson, 1999), given that its circulating concentration is markedly decreased in response to limited amino acid availability. However, since non-nutritional factors can influence serum albumin levels, it should not be used as a single indicator of malnutrition in patients or rat models of PEM. Although serum albumin is considered a more specific indicator of PEM in animal studies, since health status and environment can be closely regulated, the caveat holds that a decline in serum albumin can also reflect an inflammatory state (reviewed above in Section 2.5.4.3.2).

Elevated liver lipid concentration is another common indicator of PEM. Protein malnourished rats develop fatty livers that have significantly higher levels of triglycerides, monoglycerides, and cholesterol esters (Taylor & Ziboh, 1972). The liver plays a central role in lipid metabolism by synthesizing various compounds such as cholesterol, triglycerides, and lipoproteins. Under normal conditions, cholesterol and triglycerides are distributed throughout the circulatory system within lipoproteins. It has been proposed that PEM causes decreased synthesis of transport proteins, such as lipoproteins, which would ultimately cause a buildup of lipids in the liver. It has been demonstrated that feeding rats a low protein diet does affect lipid storage, as shown by decreased lipoprotein lipase activity and reduced serum levels of very-low-density lipoproteins (VLDL) (Boualga et al., 2000). Fatty liver is also a common characteristic of certain clinical forms of PEM, with excess fat frequently reported in the liver of patients with kwashiorkor but absent in those with marasmus (Agbedana et al., 1979).

2.7 PEM and Cerebral Ischemia

2.7.1 Overview

Recovery from cerebral ischemia can be strongly influenced by malnutrition. Numerous clinical studies have demonstrated that PEM is a common co-morbidity factor of cerebral ischemia and is present in 7-19% of stroke patients upon admission to the hospital (Davis et al., 2004; Martineau et al., 2005; Nip et al., 2011; Yoo et al., 2008). The problem often worsens as a result of post-stroke hospital care to reach prevalence rates as high as 20-35% at one week (Brynningsen et al., 2007; Yoo et al., 2008), and 35-49% by the time of admission to a rehabilitation unit (Finestone et al., 1995; Poels et al., 2006). Clinical evidence, with noted methodological limitations (see below), has demonstrated a correlation between PEM and increased morbidity and mortality following stroke (Davis et al., 2004; FOOD Trial Collaboration, 2003; Martineau et al., 2005; Yoo et al., 2008). A cause-and-effect relationship has been identified with the use of a rodent model of global brain ischemia demonstrating that PEM impairs outcome on both a cellular and functional level (Bobyn et al., 2005; Ji et al., 2008; Prosser-Loose et al., 2010). The objective of the thesis research was to delve further into the mechanisms by which stroke outcome is impaired by either co-existing PEM or compromised protein-energy status that arises after the stroke.

2.7.2 The Clinical and Experimental Evidence that PEM Impairs Stroke Outcome

A strong correlation has been shown to exist between nutritional status and health outcome following stroke. Numerous studies over the last three decades have provided strong evidence that protein-energy malnourished stroke patients have increased risk of morbidity and mortality (Axelsson et al., 1988; Davalos et al., 1996; Gariballa et al., 1998; Pandian et al., 2011). One of the more recent studies highlighting this relationship was performed on 141 stroke patients, with results strongly concluding that malnutrition is an independent predictor of poor outcome (Yoo et al., 2008). Nutritional assessment was performed at the time of admission and one week post-stroke by evaluating several objective nutritional parameters, such as weight loss, body mass index, and serum albumin, transferrin, and prealbumin levels. Undernutrition was diagnosed in 12% of stroke patients at admission and in 20% of patients one week later. Furthermore, assessment at three months post-stroke demonstrated that undernutrition was a significant predictor of poor outcome. Malnourished stroke patients were not able to recover as well from the insult and experienced more post-stroke complications (Yoo et al., 2008).

To determine if nutritional status is a predictor of long-term stroke survival, a large epidemiological study was performed on a Danish cohort of 21,884 stroke patients that were monitored for five years (Olsen et al., 2008). Body mass index was recorded at the time of admission and patients were assigned to one of five categories. Although mortality following a stroke was inversely related to body mass index, the analysis did not establish the latter as an independent predictor. Survival during the five-year follow-up period was the lowest among the underweight patients. Underweight patients had the highest mortality rate at 46%, compared to 25% in normal weight patients and 18% in overweight individuals. These results highlight body mass index as a risk factor marker and demonstrate that poor nutrition significantly increases the risk of mortality within five years following stroke (Olsen et al., 2008).

Evidence that nutritional status adversely affects stroke outcome has been repeatedly demonstrated over the last three decades (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). However, a major limitation of most of these studies is that data were collected from a single tertiary hospital, thus limiting the generalization of these results to other communities. Other limitations of these studies include small sample size, the use of nutritional assessment tools that are limited in sensitivity and specificity, and that some studies were designed to establish correlations but not a cause-and-effect relationship. Therefore, a large multicenter randomized trial was designed, referred to as the FOOD (Feed or Ordinary Food) Collaboration Trial, that had the potential of establishing a causal relationship between nutritional status and functional outcome following stroke (Dennis et al., 2005a; Dennis et al., 2005b). A preliminary analysis was performed on the first 3,012 stroke patients randomized in the FOOD Trial between 1996-2001 using data on nutritional status collected at trial entry and at six months following stroke to assess functional and survival status. Preliminary results showed evidence that patients who were undernourished had reduced functional ability and survival at six months following stroke (FOOD Trial Collaboration, 2003). More specifically, results demonstrated a survival rate of 63% in the undernourished group, whereas a higher survival rate of 80% was reported in the group of well-nourished stroke victims. Malnourished stroke victims were also more likely to have functional impairments and increased risk of developing serious health conditions such as pneumonia and gastrointestinal bleeding (FOOD Trial Collaboration, 2003). Although these results were promising, the completed trial failed to provide a definitive answer on the link between nutritional status and post-stroke recovery due to experimental design flaws, including

the absence of standardized nutritional assessment and poor choice of sampling times (Prosser-Loose & Paterson, 2006). Furthermore, there was inconsistent access to specialized equipment resulting in informal nutritional assessment relying on 'eyeball' estimates of body size and fat distribution. Therefore, due to these major methodological limitations, the FOOD Collaboration Trial failed to address this important question of whether there is a causal relationship between PEM and stroke outcome (Prosser-Loose & Paterson, 2006).

Collective results from various clinical studies strongly imply that PEM predicts a poor stroke outcome, but there are no clinical studies at this time that provide substantive proof of causation. Therefore, my laboratory utilized an animal model of global brain ischemia to determine if a causal relationship exists between co-existing PEM and both functional and histological outcome (Bobyn et al., 2005). Gerbils were randomized to control diet or a low protein diet for 28 days before being subjected to global ischemia or sham surgery. Functional outcome was assessed using an open field test at 3, 7 and 10 days following insult. The open field behavioural test detects deficits in spatial memory, as indicated by an animal's ability to adapt in a novel environment, and therefore provides information about the severity of hippocampal damage (Colbourne & Corbett, 1995; Wang & Corbett, 1990). Results from the open field test demonstrated that protein-energy malnourished gerbils did not habituate appreciably by 10 days after brain ischemia whereas, by day 7, gerbils fed a control diet habituated at a level comparable to the sham controls (Bobyn et al., 2005). However, the impaired function observed in malnourished gerbils occurred without an increase in CA1 hippocampal neuronal death, suggesting that either CA1 neurons remain viable but with aberrant function or that there was injury occurring elsewhere. Given that a relatively short post-ischemic sampling time (10 days) was used for this study and cell death can continue for months (Colbourne & Corbett, 1995), it is possible that long-term cell death could have been increased in the malnourished animals due to elevated inflammation. The latter was suggested by an increase in reactive gliosis in a subset of malnourished gerbils at 10 days after brain ischemia. In summary, the results obtained from this well-controlled animal trial provide support for the relationship found from the preliminary FOOD Trial data (FOOD Trial Collaboration, 2003). As well, this study is the first to conclusively demonstrate the existence of a causal relationship between PEM and poor functional outcome after brain ischemia. Reliance on an animal model of

brain ischemia has the added advantage of being able to examine mechanisms in the brain by which PEM could directly influence ischemic cell death and/or brain remodeling.

2.7.3 Mechanisms By Which PEM Could Alter Ischemic Brain Injury and Brain Remodeling

2.7.3.1 Overview

Although there is considerable evidence that PEM is associated with increased morbidity and mortality following brain ischemia, the exact mechanisms that are altered by PEM are largely unknown. Most likely, co-existing PEM alters various components of the cascade of secondary events responsible for brain injury. It has been suggested that one of the key mechanisms influenced by PEM is the inflammatory response. This notion is based on both the indirect findings that co-existing PEM exacerbates brain inflammation following global ischemia (Bobyn et al., 2005; Ji et al., 2008) and from the evidence that dietary protein depletion can independently stimulate a systemic inflammatory response (Ling et al., 2004; Lyoumi et al., 1998). Additionally, PEM is likely to affect mechanisms involved in brain remodeling following cerebral ischemia, given the previous data demonstrating that various plasticity-associated proteins are altered by co-existing PEM (Prosser-Loose et al., 2010). The aim of this thesis was to more directly address whether PEM exerts some of its deleterious effects on functional outcome by altering post-ischemic inflammation and neuroplasticity. The following sub-sections provide rationale for the mechanisms that were examined in this thesis.

2.7.3.2 Effects of PEM on Inflammation

The relationship between PEM and the immune response has not yet been studied in stroke patients. However, given that both clinical and animal studies have strongly demonstrated that protein malnutrition can have considerable impact on immune system functioning (Torun, 2006), it is speculated that the inflammatory response to stroke is altered by PEM. The effects of PEM on the immune response are broad and when a malnourished individual is confronted with a serious infection or trauma, the inflammatory response could either be inappropriately high or low (Bistrian, 2007). Cell-mediated immunity, phagocyte function, secretory antibody response, acute-phase response, antibody affinity and cytokine production are all examples of mechanisms that can be disrupted by PEM (Amati et al., 2003; Johnson, 1999). Therefore, malnutrition can have varying adverse effects on outcome depending on the particular infection or nature of trauma. While most studies have addressed whether PEM blunts the ability to mount a controlled

inflammatory response to tissue injury or infection, there are data suggesting that dietary protein depletion can be an independent stimulus of low-grade inflammation and an acute-phase response (Dulger et al., 2002; Ling et al., 2004; Lyoumi et al., 1998). A limitation is that the majority of research investigating the effects of PEM on the immune response has been executed in young animals and children. While the effect of PEM on immunity can be strongly influenced by factors such as the age of onset, and the type and severity of PEM (Woodward, 1998), minimal information is known about how PEM influences the immune response in the elderly.

Cytokine production is a major component of the inflammatory response that can be influenced by PEM. Protein deficiency has been shown to trigger atypical expression of various inflammatory cytokines, ultimately hindering immune responsiveness to a stimulus. For example, protein-energy malnourished mice stimulated with LPS had significantly higher circulating levels of IL-10, when compared to control animals (Fock et al., 2008). IL-10 is an anti-inflammatory cytokine and overexpression of this cytokine is often associated with immunosuppression. Furthermore, macrophages from protein-energy malnourished mice have decreased synthesis of pro-inflammatory cytokines, such as TNF-α, in response to LPS stimulation (Fock et al., 2010). Activation of the pro-inflammatory transcription factor, NFκB, can also be altered by PEM. Macrophage NFκB activation after LPS stimulus is significantly lower in protein energy-malnourished mice (Fock et al., 2010), which presumably accounts for the decreased expression of pro-inflammatory cytokines. Together these results demonstrate that the abnormal immune response in malnourished animals following infection is likely the result of elevated levels of anti-inflammatory cytokines, in combination with decreased concentrations of pro-inflammatory cytokines. However, these studies have solely focused on assessing the effects of PEM on the cytokine response stimulated by infection. Therefore, this response may be different in malnourished individuals that are faced with a trauma, such as stroke.

Malnutrition-mediated changes to inflammatory cytokine concentrations can result in an attenuated acute-phase reaction in response to a stimulus. Typically in response to acute inflammation, increased concentrations of various inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, triggers the production of positive acute-phase proteins (e.g., C-reactive protein, A2M, haptoglobin). Interestingly, clinical evidence suggests that malnourished individuals are unable to mount a proper acute-phase response at the time of infection. Reid et al. (2002) examined plasma levels of positive acute-phase proteins in protein-energy malnourished children

that were fighting infection. Although positive acute-phase proteins were elevated in all children at the time of infection, plasma levels of haptoglobin and α1-antitrypsin were the lowest in severely protein-energy malnourished children. These results demonstrate that severely malnourished children are able to mount an acute-phase response to an infection; however, the magnitude of the response is smaller. Similarly, animal models have been used to demonstrate attenuation of the acute-phase reaction in protein malnourished rats in response to an inflammatory stimulus. Jennings et al. (1996) verified that the inflammatory response elicited by turpentine results in an increase in circulating A2M. Interestingly, this response was markedly reduced in protein-deficient rats compared to well-nourished controls. It is possible, then, that malnourished patients might mount an incomplete acute-phase reaction in response to stroke. However, this relationship is likely to be more complex, given emerging evidence that PEM can serve as an independent stimulus of the acute-phase response.

Although there is substantial evidence that protein deficiency impairs the immune response to infection or trauma, certain data suggest that under normal conditions protein malnutrition can be an independent stimulus of systemic inflammation and an acute-phase response. Ling et al. (2004) demonstrated that under basal conditions, serum levels of key inflammatory cytokines, including TNF-α, IL-1, and IL-6, were appreciably increased in rats maintained on a low protein diet for two weeks. Furthermore, serum levels of the positive acute-phase protein, AGP, were also significantly higher in protein-energy malnourished rats than in healthy controls. Similarly, Lyoumi et al. (1998) detected A2M in the serum of severely protein-malnourished rats but not in control animals, inferring higher levels of this acute-phase protein in the malnourished. As well, IL-6 mRNA expression was only detectable in peripheral blood mononuclear cells and the intestine of protein malnourished rats and not in controls.

In addition to the results obtained from animal studies, there is clinical evidence highlighting PEM as an independent stimulus of inflammation. However, it is more challenging to detect the independent effects of malnutrition on systemic inflammation in humans, given that patients with PEM frequently present with concomitant infections. Sauerwein et al. (1997) reported elevated plasma concentrations of IL-6, C-reactive protein, and TNF-receptors in severely malnourished patients. Although patients with obvious signs of serious infections were excluded, subjects presenting with minor infections remained in the study. Therefore, a separate analysis was performed on only those patients presenting with no signs of infections.

Independent of the presence of minor infections, severely malnourished patients showed considerably higher plasma concentrations of TNF-receptors and IL-6. A similar study was performed by Dulger et al. (2002), which compared the inflammatory response in children with either kwashiorkor (primarily caused by protein deficiency) or marasmus (primarily caused by energy deficiency). It is important to note that this study did not evaluate the presence of underlying infections. Children with kwashiorkor had significantly lower serum albumin levels, as compared to the marasmus and control groups. In addition, serum IL-6 concentrations were considerably increased in both the kwashiorkor and marasmus groups, as compared to healthy patients. Collectively, results from both clinical and animal studies strengthen the notion that PEM can independently stimulate a systemic inflammatory response.

As the relationship between PEM and the acute-phase response appears to be complex, one cannot predict the relationship following brain ischemia. An animal model of PEM was thus used in this thesis to confirm the previous findings showing that PEM is an independent stimulus of an acute-phase response. A subsequent objective of the thesis research was to determine whether post-ischemic PEM blunts the ability to mount a controlled acute-phase response to global brain ischemia.

2.7.3.3 Effects of PEM on the Neuroinflammatory Response to Global Brain Ischemia

Previous studies from my laboratory have inferred that the previously reported adverse effects of malnutrition on outcome following brain ischemia are in part due to increased neuroinflammation. A major finding from these studies has been that co-existing PEM increased activation of NFκB in the hippocampus of gerbils exposed to global ischemia (Ji et al., 2008). In addition, results from electrophoretic mobility shift assays (EMSA) showed that the effect of PEM was independent of brain ischemia. That is, increased activation of this transcription factor was also present in the hippocampus of sham-operated and non-operated malnourished gerbils. Therefore, given that pro-inflammatory mediators are predominantly regulated at the level of transcription by NFκB, these results suggest that PEM can independently induce hippocampal inflammation (Ji et al., 2008). However, there is yet no direct evidence that PEM enhances the expression of NFκB pro-inflammatory target genes following brain ischemia in experimental stroke models or stroke patients.

Other evidence supporting the hypothesis that PEM increases the inflammatory response after global ischemia came from the observation that a subset of malnourished gerbils subjected

to global ischemia experienced marked reactive gliosis (Bobyn et al., 2005). The latter was evident on routine histological staining in one-third of malnourished gerbils following global brain ischemia. Interestingly, these animals also presented with the most severe functional impairment (thigmotaxis in the open field test), and greater than average hippocampal CA1 neuron death. A limitation of this study, however, was that the glial cell type was not characterized by immunocytochemical analysis. Another perplexing finding was that the pronounced reactive gliosis, while never apparent after global ischemia in control diet fed gerbils, was evident in only a subset of malnourished animals. A possible contributor to the variability in reactive gliosis is reduced reliability in the hippocampal injury due to the previously reviewed developing problem of altered brain vasculature in the gerbil. Accompanying the exacerbated glial response in malnourished gerbils was an increase in the oxidative stress response following global ischemia. Protein thiols, which are susceptible to oxidation and therefore used as a marker of oxidative stress, were significantly decreased by PEM. Similarly, the data suggested that PEM could alter components of anti-oxidant defense, as shown by a trend for glutathione reductase activity to be decreased following brain ischemia. Given that oxidative stress is a strong activator of NFκB in ischemic stroke (Harari & Liao, 2010), a likely outcome of PEM-induced oxidative stress is an exacerbated inflammatory response. Nevertheless, a major limitation of this study, possibly contributing to variability in reactive gliosis and the extent of ischemic injury, is that post-ischemic temperature was not monitored (although intra-ischemic tympanic temperature was strictly controlled). Given that spontaneous variation in post-ischemic temperature is known to occur in the gerbil (Colbourne & Corbett, 1994), the occurrence of either hypothermia or hyperthermia cannot be ruled out.

In summary, my laboratory has provided indirect evidence of an altered glial and inflammatory response following global ischemia in protein-energy malnourished rodents. This research was done using a co-existing PEM model, which mirrors those patients who are already malnourished at the time of brain ischemia. A major objective of this thesis was to obtain direct evidence that PEM exacerbates or prolongs the inflammatory response to global brain ischemia. To date, the additional clinical problem of PEM developing after and because of the stroke (post-ischemic PEM) has not been modeled experimentally. Thus, this thesis work examined the effects of both co-existing and post-ischemic PEM on the inflammatory response to brain ischemia.

2.7.3.4 Effects of PEM on Neuroplasticity

In addition to malnutrition-mediated alterations to inflammation, there is evidence suggesting that PEM also induces neuroplastic deficits. PEM-induced changes to these two processes may be related, given that inflammatory mediators are involved in regulating neuroplastic mechanisms (Kriz & Lalancette-Hébert, 2009). It is well established that malnutrition during brain development can result in structural and functional deficits (Alamy & Bengelloun, 2012). More specifically, malnutrition during brain maturation affects neurogenesis, cell migration and differentiation, and synaptogenesis (Alamy & Bengelloun, 2012). Consequently, changes to the organization of the brain can cause functional deficits, such as impaired learning and retention (Alamy & Bengelloun, 2012). Although there is sound evidence that protein malnutrition is detrimental to the developing brain, the effects of malnutrition started during adulthood are not as clear.

The majority of studies assessing the effect of protein deprivation during adulthood have characterized changes to the hippocampus, given that this region of the brain is one of the most affected by low dietary protein (Andrade et al., 1996). Within the CA3 mossy fiber region of the hippocampus, adult protein deprivation causes a 30% decrease in the number of synapses (Andrade et al., 1991; Andrade et al., 1995; Lukoyanov & Andrade, 2000). These synaptic deficits were present in adult rats after lengthy periods on a low protein diet lasting between 6-18 months. A decrease in the number of mossy fiber synapses is most likely the combined result of changes to neuronal structure and the number of viable neurons, both of which have shown to be affected by protein deprivation. Rats started on a low protein diet during adulthood and continued for 6 months had reduced numbers of dendritic branches within the CA3 and dentate gyrus hippocampal subfields (Andrade et al., 1996); however, the changes to dendritic branches are not uniform throughout the hippocampus (Andrade et al., 1996). Protein deprivation during adulthood also affects hippocampal cell number, with considerable loss of granule cells and CA1 and CA3 pyramidal neurons reported after prolonged periods on a low protein diet (Lukoyanov & Andrade, 2000; Paula-Barbosa et al., 1989). Progressive cell loss may potentially be caused by changes to neurotrophin levels. Levels of BDNF, and its receptor tropomyosin-related kinase B (trkB), were notably reduced within the granular layer of adult rats submitted to protein deprivation for 6 months (Mesquita et al., 2002). Ultimately, synaptic and neuronal loss within the hippocampus is associated with behavioural impairments. Rats that were protein deprived

during adulthood exhibited impaired habituation in the open field test, suggesting deficits in hippocampal functioning (Lukoyanov & Andrade, 2000).

In summary, malnutrition during development has severe and wide-spread effects on the brain; however, protein deprivation during adulthood can also cause marked structural alterations, which have mostly been characterized within the hippocampus (Alamy & Bengelloun, 2012). It is important to note that these studies have highlighted the effects of chronic protein deprivation on neuroplastic mechanisms. A major limitation of these studies is that nutritional status of the animal was poorly characterized and several of these reports lacked evidence of malnutrition. Therefore, the immediate effect of adult PEM (e.g. a few weeks on low protein diet), as used in this thesis, upon the structure of the hippocampus is mostly unknown. In addition, the effects of PEM on the neuroplasticity response triggered by brain injury, such as stroke, have not yet been well characterized. Therefore, a previous study performed by my research group examined whether the neuroplasticity response to global brain ischemia is altered by co-existing PEM.

2.7.3.5 Effects of PEM on the Neuroplasticity Response to Global Brain Ischemia

Promising findings from my laboratory have demonstrated that co-existing PEM alters plasticity mechanisms important to brain recovery (Prosser-Loose et al., 2010). A gerbil model of global brain ischemia was employed to examine the effects of co-existing PEM on post-ischemic expression of various plasticity-associated proteins that included BDNF, its receptor trkB, and GAP-43 (Prosser-Loose et al., 2010). In contrast to the underlying hypothesis that PEM would reduce plasticity in the hippocampus following global ischemia, the most striking finding was a more robust GAP-43 response with PEM. Co-existing PEM enhanced and prolonged GAP-43 expression within the CA1 hippocampal region following brain ischemia. Additionally, PEM exacerbated GAP-43 expression within the CA3 and hilar regions at 3 days post-ischemia. Protein expression of trkB within the CA1 hippocampal region was also exacerbated by co-existing PEM at 7 days post-ischemia. Unlike trkB and GAP-43, BDNF was unaltered by PEM, although these results were not considered definitive due to a number of methodological limitations. Elevated GAP-43 protein levels in malnourished gerbils could possibly be explained by an augmented inflammatory response, given that expression of this gene can be directly induced by inflammation (Hossain-Ibrahim et al., 2006). Although enhanced expression of GAP-43 is generally associated with a protective neuroplastic response, it was

proposed that excessive GAP-43 signal could instigate excessive sprouting resulting in hyper-excitability. However, electrophysiological recordings to assess whether PEM altered the electrical activity of the surviving neurons were not obtained to support this hypothesis. While the influence of PEM on post-ischemic brain plasticity was expanded upon in this thesis, a different research question was addressed. It was investigated whether PEM developing in the first few days after global brain ischemia would exert effects on plasticity.

2.7.3.6 Effects of PEM on Thermoregulation

PEM may exert some of its effects on outcome after brain ischemia by altering key physiological determinants of ischemic brain injury. Brain temperature is a major influencing factor on brain ischemia outcome, given that hypothermia can drastically reduce brain injury (MacLellan et al., 2009; van der Worp et al., 2007), while hyperthermia can aggravate injury (Wang et al., 2009). More specifically, an increase in brain temperature can directly exacerbate both the glial and inflammatory responses following global ischemia (Ceulemans et al., 2010). Therefore, it is imperative to understand the effects of PEM on thermoregulation so that the correct measures can be made during post-stroke hospital care. It is difficult to predict the effects on the basis of experimental studies, since there are considerable discrepancies in the literature, with both cooling (Duran et al., 2008) and warming (Balmagiya & Rozovski, 1983; Castanon-Cervantes & Cintra, 2002) responses reported in rodents experiencing varying states of protein and/or energy deficiencies.

Although clinical studies addressing the effects of malnutrition on body temperature are scarce, and data do not exist for malnourished stroke patients, most of the literature suggests that poor nutrition impairs thermoregulatory function and results in mild hypothermia. Allison et al. (2000) reported a relationship between undernutrition and impaired thermoregulation, with malnourished elderly patients experiencing lower core temperatures than well-nourished individuals. Brooke et al. (1972) also reported mild hypothermia in a group of malnourished Jamaican children. Rectal temperatures were measured every four hours during the first week of hospital treatment in 137 malnourished children. Mild hypothermia (<35°C) was evident in 20% of malnourished children, with the highest prevalence in patients with marasmus and marasmic kwashiorkor. Similar statistics were reported by Devi et al. (1980), in which 26% of children with PEM experienced mild hypothermia during their hospital stay. Although the results from

clinical studies are thus generally in agreement, a major drawback is that the majority of data in this area of research has come from young patients and not from the elderly.

Minimal information is known about how PEM alters thermoregulation in the elderly, and reports on how malnourished elderly patients respond to the stress of stroke are nonexistent. Given that the majority of clinical data suggests that PEM causes hypothermia, it could be speculated that this in turn is protective at the time of stroke. However, this is contradictory to the reports cited above that malnourished stroke patients have impaired outcome. A potential explanation is that the absolute changes in temperature are too small to directly influence ischemic brain injury. Similarly, if the drop in temperature is small, PEM, acting through other mechanisms, could induce adverse effects even in the presence of mild hypothermia. However, a more likely explanation is that the effect of PEM on temperature regulation is different in the presence of stroke. This notion is supported by numerous studies that have consistently demonstrated that malnourished humans and rodents experience disruptions in thermoregulatory homoeostasis when confronted with a challenge (Balmagiya & Rozovski, 1983; Fellows et al., 1985). Given that a common complication of stroke is fever, which occurs in approximately 50% of cases (Wrotek et al., 2011), it can be postulated that malnourished stroke victims are among this subset of patients due to their inability to finely regulate body temperature. Consequently, elevated temperatures experienced by malnourished stroke patients could cause an exacerbated inflammatory response leading to impaired outcome. However, to establish whether this mechanism contributes to PEM-induced increases in morbidity and mortality following brain ischemia, a more detailed evaluation of thermoregulatory function is necessary.

Since the effects of PEM on temperature are unclear, there is a demand for more comprehensive assessment of thermoregulatory function in the malnourished. Chronic and frequent temperature measurements obtained using implantable temperature probes are useful in exemplifying the daily rhythmicity of the temperature diurnal cycle. More specifically, chronic temperature readings make it possible to assess specific parameters commonly utilized to characterize the pattern of a rhythm. These include the mean, fluctuation/amplitude, period, acrophase, and robustness (Refinetti, 2006). To measure the central tendency of a daily rhythm, the most frequent method is by calculating the arithmetic mean. Fluctuation and amplitude are the two most common terms to express the daily range of oscillation. Whereas daily temperature fluctuation is the full range of oscillation over a 24 hour cycle, amplitude refers to half the range

of oscillation (Refinetti, 2006). However, in most cases the real data is noisy and is filtered prior to amplitude computation. As a result, the amplitude of a fitted curve is typically considerably smaller than half the range of oscillation. The term period refers to the time it takes to complete a daily cycle, which under normal conditions is 24 hours (Refinetti, 2006). Furthermore, acrophase is the time at which the daily rhythm peaks. Another parameter used to describe diurnal cycles is robustness, which refers to the strength of the rhythm. If the robustness is low, then the cycle is rather variable, while a stronger robustness refers to a more consistent cycle (Refinetti, 2006). These parameters were used in this thesis to characterize the diurnal rhythm of temperature in malnourished rats. Ultimately, the research conducted in this thesis investigated how PEM affects temperature and its relationship to the inflammatory response to brain ischemia.

CHAPTER 3:
PROTEIN-ENERGY MALNUTRITION ALTERS THERMOREGULATORY HOMEOSTASIS AND THE RESPONSE TO BRAIN ISCHEMIA

This chapter has been published as follows:
Smith SE, Prosser-Loose EJ, Colbourne F, Paterson PG. Protein-energy malnutrition alters thermoregulatory homeostasis and the response to brain ischemia. Current Neurovascular Research 2011; 8: 64-74.
Publisher and Copyright Holder: Bentham Science Publishers Ltd.

3.1 Abstract

Co-existing protein-energy malnutrition (PEM), characterized by deficits in both protein and energy status, impairs functional outcome following global ischemia and has been associated with increased reactive gliosis. Since temperature is a key determinant of brain damage following an ischemic insult, the objective was to investigate whether alterations in post-ischemic temperature regulation contribute to PEM-induced reactive gliosis following ischemia. Male Sprague-Dawley rats (190-280g) were assigned to either control diet (18% protein) or PEM induced by feeding a low protein diet (2% protein) for 7 days prior to either global ischemia or sham surgery. There was a rapid disruption in thermoregulatory function in rats fed the low protein diet as assessed by continuous recording of core temperature with bio-electrical sensor transmitters. Both daily temperature fluctuation and mean temperature increased within the first 24 hours, and these remained significantly elevated throughout the 7 day pre-ischemic period ($p < 0.027$). In the immediate post-surgical period, PEM decreased body temperature to a greater extent than that in well-nourished controls ($p = 0.003$). The increase in daily temperature fluctuation caused by PEM persisted throughout the 7 day post-surgical period ($p < 0.001$), and this interacted with the effects of global ischemia on days 8 ($p = 0.018$) and 11 ($p = 0.021$). The astrocytic and microglial responses induced at 7 days after global ischemia were not influenced by PEM, but this preliminary analysis needs to be confirmed with a more reliable global ischemia model. In conclusion, exposure to a low protein diet rapidly impairs the ability to

maintain thermoregulatory homeostasis, and the resultant PEM also diminishes the ability to thermoregulate in response to a challenge. Since temperature regulation is a key determinant of brain injury following ischemia, these findings suggest that the pathophysiology of brain injury could be altered in stroke victims with co-existing PEM.

3.2 Introduction

Despite years of animal research focused on understanding the pathophysiology of ischemic brain injury, there have been challenges in translating much of this work into effective neuroprotective treatments for human stroke victims (Hossmann, 2009). Our laboratory has instead focused on investigating the implications of compromised nutritional status as a possible stroke co-morbidity factor. Since a significant proportion of stroke patients have poor nutritional status at the time of insult (Crary et al., 2006; Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008), it is crucial to understand what impact this may have on the outcome. Thus, differences in the intracellular events following stroke caused by malnutrition need to be identified as the basis for designing targeted nutritional interventions.

The extent of neuronal damage following an ischemic insult can be strongly influenced by poor nutritional status. PEM, characterized by deficits in both protein and energy status, is present in 12-19% of stroke patients upon admission to the hospital (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). The problem often worsens as a result of post-stroke hospital care to reach prevalence rates as high as 20-35% at one week (Brynningsen et al., 2007; Yoo et al., 2008), and 35-49% by the time of admission to a rehabilitation unit (Finestone et al., 1995; Poels et al., 2006). Clinical evidence, with noted methodological limitations, has demonstrated a correlation between PEM and increased morbidity and mortality following stroke (Davis et al., 2004; FOOD Trial Collaboration, 2003; Martineau et al., 2005; Yoo et al., 2008). A cause-and-effect relationship has been identified with the use of a rodent model of global brain ischemia demonstrating that PEM affects outcome on both a cellular and functional level (Bobyn et al., 2005; Ji et al., 2008; Prosser-Loose et al., 2010).

The extent of neuronal injury following global ischemia is highly temperature-sensitive. Post-ischemic hypothermia can drastically reduce ischemic brain injury (MacLellan et al., 2009; van der Worp et al., 2007), whereas hyperthermia can aggravate injury (Wang et al., 2009). Chronic malnutrition has been reported to impair the ability to maintain constant thermoregulatory homeostasis (Balmagiya & Rozovski, 1983; Castanon-Cervantes & Cintra,

2002; Conradi et al., 1988), especially when animals are confronted with thermoregulatory challenges (Balmagiya & Rozovski, 1983). Therefore, an altered thermoregulatory response induced by PEM could explain some of the changes in neuronal and glial response following global ischemia previously reported by our laboratory (Bobyn et al., 2005; Ji et al., 2008).

Neuronal damage progresses during the acute period following ischemia by activation of several interrelated secondary mechanisms. Reactive gliosis and inflammation are among two of these processes within a complex cascade of intracellular and extracellular events (Ceulemans et al., 2010; Lai & Todd, 2006). These two processes are also strongly interrelated, since pro-inflammatory mechanisms modulate the activation of glial cells, which in turn produce more inflammatory cytokines (Ceulemans et al., 2010; Lai & Todd, 2006). Inflammation is an orchestrated response following an ischemic insult that can contribute both to neuronal death and modulation of repair processes (Kriz & Lalancette-Hébert, 2009). Since the post-ischemic inflammatory response is only partly understood, there has been much debate about its overall effect on outcome. That immunodeficient mice present with less stroke injury (Hurn et al., 2007) suggests that inflammation is more detrimental than beneficial. Temperature regulation can directly alter both the glial and inflammatory responses following global ischemia (Ceulemans et al., 2010). One mechanism by which hypothermia attenuates neuronal damage after global ischemia is by reducing microglial activation and NFκB activation (Webster et al., 2009). The mechanisms associated with hyperthermia are less established (Wang et al., 2009); however, stroke victims with hyperthermia have increased plasma levels of pro-inflammatory mediators when compared to normothermic patients (Leira et al., 2006).

We have previously obtained indirect evidence of an altered glial and inflammatory response following global ischemia in protein-energy malnourished rodents (Bobyn et al., 2005; Ji et al., 2008) that appeared to be related to impaired functional outcome as assessed by the open field test (Bobyn et al., 2005). Since this impaired function occurred without an increase in hippocampal CA1 neuronal death, it suggests either that the CA1 neurons remained viable but with aberrant function or that there was injury occurring elsewhere. Given that a short post-ischemic sampling time (10 days) was used for this study and cell death can continue for months (Colbourne & Corbett, 1995), it is possible that long-term cell death would have been increased in the malnourished animals due to elevated inflammation. Unusually dramatic reactive gliosis was evident on routine histological staining in one-third of the malnourished gerbils (Bobyn et

al., 2005). These animals were also the ones with both the most severe functional impairment (thigmotaxis) and greater than average hippocampal CA1 neuron death, leading to the hypothesis that PEM enhanced the inflammatory response. A limitation, however, was that the glial cell type was not characterized by immunohistochemical analysis. Another perplexing finding was that the pronounced reactive gliosis, while never apparent after global ischemia in control diet fed gerbils, was evident in only a subset of malnourished animals (Bobyn et al., 2005). While intra-ischemic tympanic temperature was strictly controlled in this study, a possible contributor to variability in reactive gliosis and the extent of ischemic injury is post-ischemic temperature regulation. Since spontaneous variation in post-ischemic temperature occurs in the gerbil bilateral carotid artery model of global ischemia (Colbourne & Corbett, 1994), we cannot rule out the occurrence of either hypothermia or hyperthermia.

Other evidence supporting the hypothesis that PEM increases the inflammatory response came from the observation that malnourished gerbils have increased activation of the transcription factor NFκB in the hippocampus (Ji et al., 2008). Pro-inflammatory molecules are predominantly regulated at the level of transcription by the critical intracellular mediator, NFκB, which also plays a role in glial cell function (Mattson, 2005). NFκB activation rapidly occurs following ischemic stroke, ultimately escalating inflammation by altering pro-inflammatory gene expression (Harari & Liao, 2010; Ridder & Schwaninger, 2009). Although there is yet no direct evidence that PEM enhances the expression of NFκB pro-inflammatory target genes following brain ischemia in experimental stroke models or stroke patients, it appears that PEM itself causes a low-grade inflammatory response (Ling & Bistrian, 2009; Ling et al., 2004).

In the current study, we extend our previous findings to the influence of PEM on global ischemia modeled by 2-VO in the rat. The first objective was to investigate if the astrocytic (GFAP) and microglial (ED-1 and OX-42) responses after global ischemia were altered by PEM. Second, we assessed whether PEM modifies pre- and post-ischemic temperature regulation with the intent of clarifying whether such alterations contribute to PEM-induced reactive gliosis following ischemia.

3.3 Materials and Methods

3.3.1 Animals

Forty-three male Sprague-Dawley rats (30-32 day old) (Charles River Canada, QC, Canada) were acclimatized on rat chow for 2 days and then placed on a purified control diet (see

below for composition) for 5-6 days. The rats were caged in groups of 2-4 and had food and water available *ad libitum*. Animals were housed in a temperature and humidity controlled room with a 12-hour light/dark cycle. All animal procedures were in compliance with guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Committee on Animal Care and Supply.

3.3.2 Core Temperature Bio-electrical Sensor Transmitter Implantation

After acclimation, calibrated bio-electrical sensor transmitters encapsulated in biocompatible silicone (*SubCue*™ Dataloggers), pre-programmed to record core body temperature every 30 minutes, were implanted aseptically into the abdominal cavity. Rats were anaesthetized with isoflurane (4% induction, 2% maintenance with 1 L/min oxygen) and placed on a heated water blanket. A 2-3 centimeter mid-abdominal incision through the skin and linea-alba was made, allowing insertion of the sterilized, calibrated temperature sensor into the peritoneal cavity. Following suturing of the incision site, a single subcutaneous injection of Ketaprofen (5mg/kg) was given. Temperature sensors remained *in situ* until the end of the experiment.

3.3.3 Diet Assignment

At 5-7 days following temperature sensor implantation, the rats (190-280g) were randomly assigned to either a protein adequate control diet (**CON**, 18% protein) or a protein-deficient diet (**PEM**, 2% protein) (Dyets, Inc., PA, USA) (Prosser-Loose et al., 2011) (see Table 4.1 for diet composition) and remained on the respective diet regimen until euthanasia. Rats of this age fed a 2% protein diet voluntarily reduce food intake, resulting in mixed PEM (Prosser-Loose et al., 2011). Diets were modified from the American Institute of Nutrition-93G diet (Reeves et al., 1993) to not contain the antioxidant, tertiary-butylhydroquinone. Food intake was recorded daily and body weight recorded weekly. The day of assignment to diet was considered experimental day 0, and the experimental timeline is shown in **Figure 3.1**.

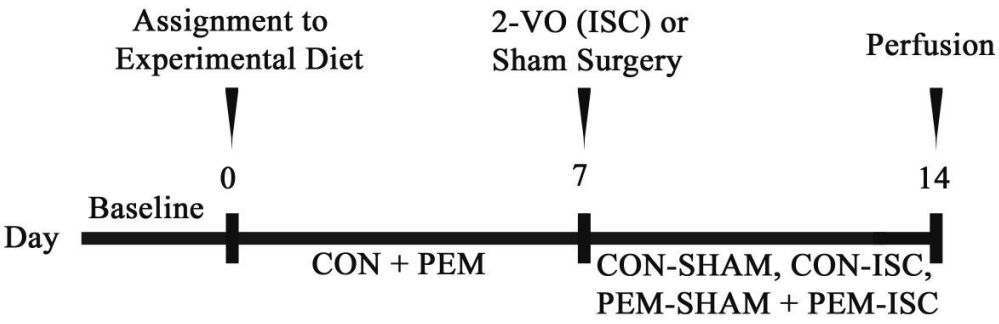

Figure 3.1. Experimental design and timeline. Baseline core temperature readings were collected for a 3 day period with all animals on control diet prior to placement on experimental diet. On experimental day 0, rats were randomly assigned to either PEM or a CON diet. Following surgery on experimental day 7, four experimental groups were present (CON-SHAM, CON-ISC, PEM-SHAM, PEM-ISC).

3.3.4 Global Forebrain Ischemia

On experimental day 7-8, rats were subjected to either global forebrain ischemia via the 2-VO model of bilateral common carotid artery occlusion combined with hypotension (**ISC**) or sham surgery (**SHAM**), as previously modified (Arvanitidis et al., 2009) from Smith et al. (1984). All surgical procedures were performed using aseptic technique. Prior to surgery, animals were fasted (16-20 hours) to achieve blood glucose levels within a consistent range. Animals were anaesthetized with isoflurane (~2% maintenance in 70% N_2O and 30% O_2) and placed on a heated water blanket. During surgery, brain temperature was estimated using a tympanic probe (IT-18 flexible probe; Physitemp Instruments Inc., NJ, USA) and maintained near 37.5°C with an overhead heating lamp outfitted with an infrared bulb (250W). The heating lamp was controlled by an automated feedback temperature controller (CN9500; Omega Engineering Inc., CT, USA) attached to the tympanic probe. Both common carotid arteries were isolated via a 2 centimeter ventral midline incision. The jugular vein was cannulated for blood withdrawal and infusion to induce hypotension during the ischemic period. The tail artery was cannulated for insertion of a blood pressure sensor (PressureMAT PDKTP4-PCS; PendoTech, NJ, USA) for measurement of mean arterial blood pressure (MABP). Arterial blood samples (100 µL) were obtained from the tail artery for measurement of blood gases, hematocrit, and glucose concentration. For induction of ischemia, once blood pressure had reached 35 mmHg,

micro-aneurysm clips (S&T Vascular Clamps HD-S; Fine Science Tools, BC, Canada) were applied to both carotid arteries for 10 minutes. During this period, blood was withdrawn into a heparinized syringe warmed by the heating lamp or infused as necessary to sustain MABP at 35-40 mmHg. Clips were removed following the 10 minute occlusion, carotid artery reperfusion was visually verified, and blood was slowly re-infused via the jugular vein. All incisions were sutured, and a bupivacaine dose (2mg/kg) was divided equally among the 3 incision sites and subcutaneously injected. Sham rats were treated identically except that carotid arteries were not occluded and hypotension was not induced. Thus, the four experimental groups generated at this time were: CON-SHAM (n=10), CON-ISC (n=10), PEM-SHAM (n=12), and PEM-ISC (n=11).

3.3.5 Immunohistochemistry

On experimental day 14 (7 days following surgery), rats were anaesthetized under isoflurane and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde. Intact heads were stored at 4°C overnight in paraformaldehyde. Brains were removed and refrigerated for an additional 24 hours in paraformaldehyde. Extracted brains were submerged into a 20% sucrose solution for 3-5 days. Fixed brains were stored at -20°C until sectioning. Coronal sections (14 µm) were taken from the anterior hippocampus and immunolabeled for GFAP, ED-1 and OX-42. To ensure accurate assessment of relative immunohistochemical changes among experimental groups, one section from each of the experimental groups was mounted on the same slide. Thus, each slide contained 4 sections, and all experimental groups were processed under identical conditions.

The protocol used for each of the glial markers was identical, except that the sections for OX-42 staining underwent citrate antigen retrieval. Sections were washed in phosphate-buffered saline (PBS) and exposed to 1.0% H_2O_2. Sections were blocked with normal goat serum (5%; Invitrogen, CA, USA), followed by an overnight incubation at 4°C with either polyclonal rabbit anti-GFAP (1:1000, Z0334; DakoCytomation, ON, Canada), monoclonal mouse anti-rat CD68 (ED-1, 1:1000, MCA341R; Serotec, NC, USA), or monoclonal mouse anti-rat CD11b (OX-42, 1:500, MCA275G; Serotec). Slides were washed in PBS and incubated in goat anti-mouse biotinylated secondary antibodies (GFAP, 1:500; ED-1, 1:1000; OX-42, 1:1000; Vector Laboratories, CA, USA). Sections were treated with extravidin (10 µg/ml; Sigma –Aldrich, ON, Canada) and reacted with diaminobenzidine (Vector Laboratories). Quantification was performed by measuring the integrated density value (IDV=sum of pixel values in the region of

interest) (AlphaEaseFC Imaging Software, Alpha Innotech) of photographs taken under identical conditions at 400x magnification. The IDV for OX-42 and ED-1 was determined for an isolated region of the hippocampal CA1 pyramidal cell layer (pixel area of 59,985) for both the right and left hemispheres and averaged. The same method was used to obtain the IDV for GFAP, except the stratum oriens and stratum radiatum areas were included with the hippocampal CA1 pyramidal cell layer (pixel area of 367,845). The staining in the CA1 region was normalized against background staining measured as an IDV for the corpus callosum by calculating the ratio, IDV of CA1/IDV of corpus callosum.

3.3.6 Core Temperature Analysis

At the time of euthanasia, the temperature sensors were retrieved and logged temperature data were obtained using the *SubCue*™ analyzer software. Alterations in thermoregulatory function resulting from experimental diet and surgical assignment were assessed by examining differences in: a) daily mean temperature, b) daily temperature fluctuation calculated by deducting the lowest core temperature from the highest temperature measured over a 24 hour diurnal cycle, and c) lowest core temperature observed within 8 hours of anaesthetic induction for the surgery.

3.3.7 Statistical Analysis

Statistical analyses were conducted using SPSS 17.0 for Windows. All data are presented as mean ± SEM. Pre-surgical temperature data were analyzed using an independent t-test. Two groups were analyzed during the baseline period when all rats were on control diet to demonstrate that there were no initial differences in the groups randomly selected to receive different experimental diets. All post-surgical temperature and immunohistochemical data were analyzed by two-factor ANOVA, and LSD post-hoc tests were performed where indicated. Correlations between hippocampal CA1 injury and the lowest core temperature observed within 8 hours of anaesthetic induction were analyzed by Pearson's correlation coefficient for data obtained for all rats exposed to ischemia (PEM-ISC + CON-ISC combined) as well as for individual ischemic groups. A probability value of ≤ 0.05 was considered to be statistically significant.

3.4 Results

Three rats (2 PEM-SHAM, 1 PEM-ISC) were excluded from the study due to surgical complications involving arterial cannulation and maintenance of anaesthesia. Data collected

from these experimental rats to assess protein-energy status (food intake, body weight, and serum albumin), intra-ischemic tympanic temperature and blood pressure, the physiological response during surgery (blood gases, blood glucose, and hematocrit), hippocampal CA1 neuron loss, expression of microtubule-associated protein-2, and serum corticosterone concentration are presented in a separate publication (Prosser-Loose et al., 2011). These results formed part of the thesis of another graduate student in the laboratory.

3.4.1 Thermoregulation

Figure 3.2 shows diurnal rhythm for core temperature as influenced by both PEM and exposure to global ischemia.

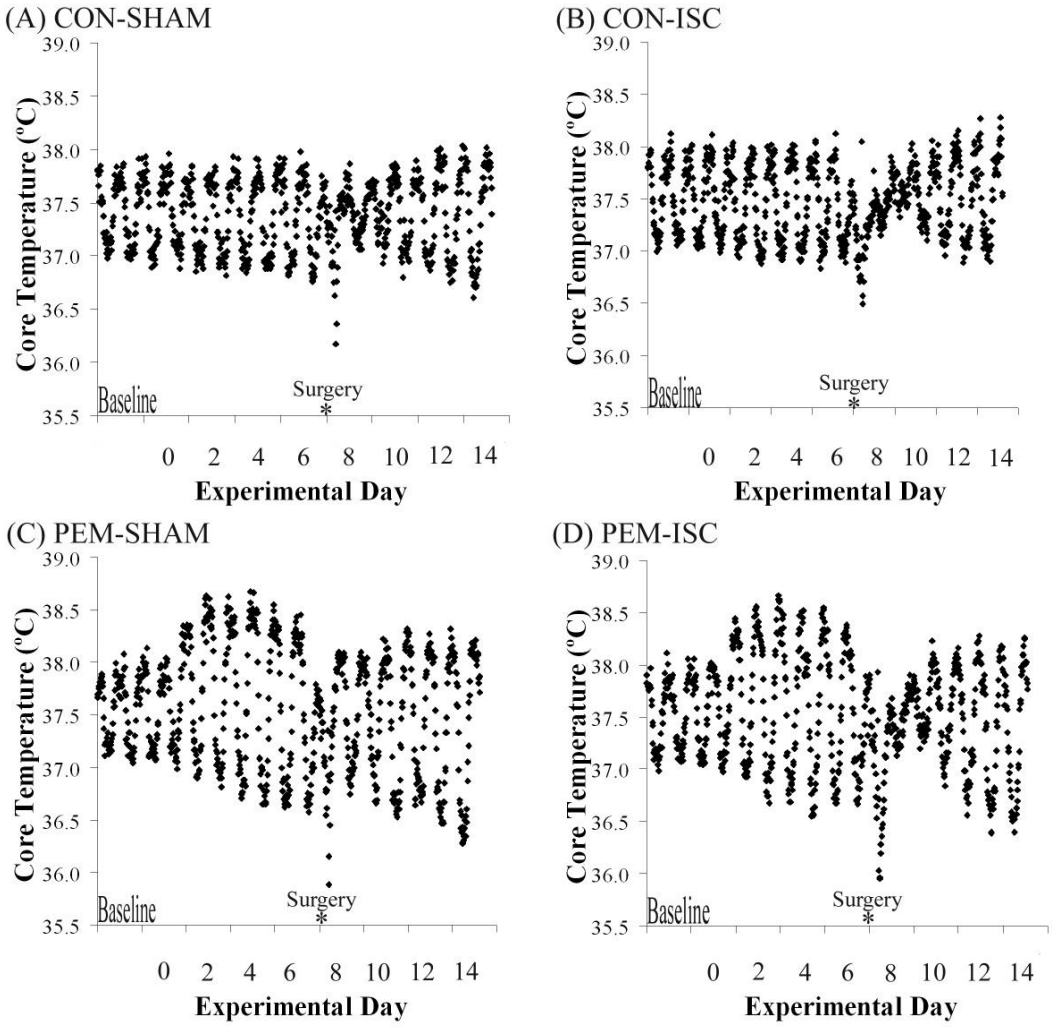

Figure 3.2. Diurnal rhythm of core temperature as influenced by both PEM and global ischemia. Values are mean core temperature readings taken at 30 minute intervals (n=10). Baseline data were collected for 3 days before assignment to experimental diet (day 0). The pre-surgical fasting occurred on days 6-7, and surgery occurred on day 7.

3.4.1.1 Pre-Surgical Period

There were no significant differences in baseline mean daily core temperature (p=0.063) and daily temperature fluctuation (p=0.37) when all animals were still on the CON diet (**Figure 3.3**). After commencement of the diet regimen, mean daily core temperature significantly increased in the group assigned to PEM within the first 24 hours (p=0.006), and remained significant for each of the following days during the pre-surgical period (**Figure 3.3A**) (p<0.024). PEM also significantly increased the daily core temperature fluctuation within 24 hours (p=0.027), which remained elevated until surgery day (**Figure 3.3B**) (p<0.027). The malnourished rats were warmer than the CON rats during the 12-hour dark period of each day and cooler during the 12-hours of light (**Figure 3.2**).

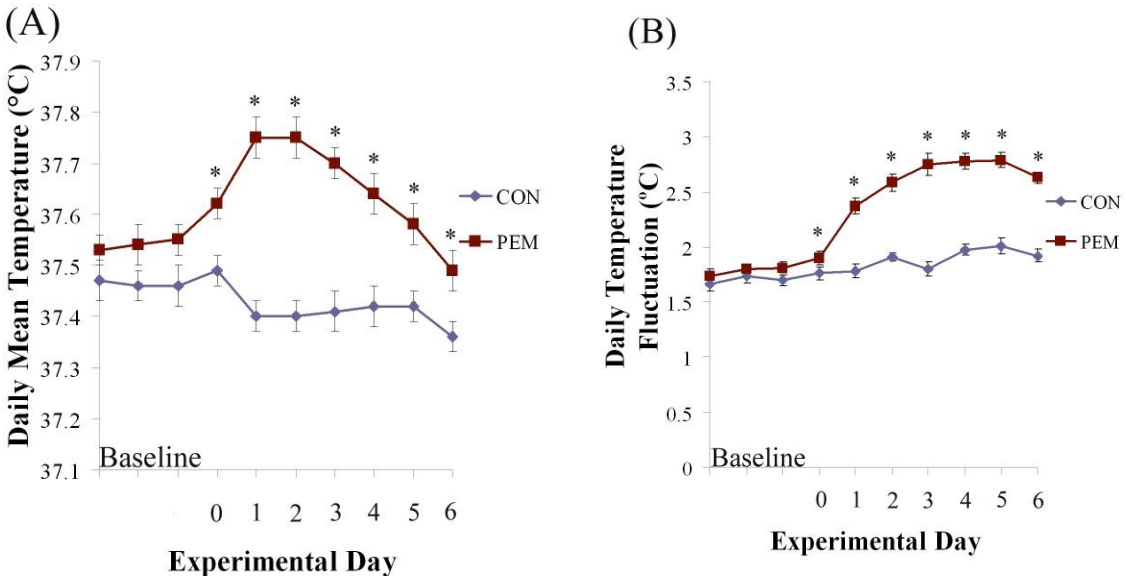

Figure 3.3. Mean (±SEM) daily core temperature (**A**) and daily core temperature fluctuation (**B**) for CON and PEM rats during the pre-surgical period calculated for each 24-hr diurnal cycle (n=20). Baseline data were collected for 3 days before assignment to experimental diet (day 0). Day 6 represents the beginning of the fasting period (~6 PM). *Indicates a significant effect of diet based on an independent t-test (p<0.05).

3.4.1.1 Immediate Pre- and Post-Surgical Periods

The mean core temperatures for PEM and CON animals during 12 hours (8 PM-8 AM) of the 16-20 hour fasting period before surgery are shown in **Table 3.1**. The fasted protein-energy malnourished rats were significantly warmer prior to surgery (p= 0.002). Although tympanic

temperature was maintained at ~37.5°C throughout the surgical period for all 4 treatment groups, core temperature was altered by dietary treatment in the immediate post-surgical period. **Table 3.1** shows that, irrespective of surgical treatment, PEM independently caused a decrease in the mean lowest core temperature measured within 8 hours following anaesthetic induction (p=0.003). There was no effect of ischemia (p=0.75) nor was there an interaction between diet and ischemia (p=0.24).

Table 3.1. Core temperature measurements in the immediate pre- and post-surgical periods

Pre-Surgery	**CON**		**PEM**	
12-hour Fasting Mean Temperature (°C)	37.4 ± 0.1		37.7 ± 0.1*	
Post-Surgery	**CON-SHAM**	**CON-ISC**	**PEM-SHAM**	**PEM-ISC**
Lowest Core Temperature Within 8 hours After Anaesthetic Induction (°C) $^{\alpha}$	35.9 ± 0.1	36.1 ± 0.1	35.6 ± 0.2	35.3 ± 0.3

Data are mean (±SEM). n=20 for pre-surgical data, and n=10 for post-surgical groups. *Indicates a significant difference from CON group by independent t-test (p=0.002). α Indicates a significant independent effect of diet (p=0.003) by 2-factor ANOVA.

3.4.1.2 Post-Surgical Period

The only significant difference in mean daily temperature in the post-surgical period was an independent decrease on day 7 (p=0.004) and an increase on day 9 caused by ischemia (p=0.02) (**Figure 3.4A**). There were no effects of PEM nor an interaction on any experimental day (p>0.05). The daily temperature fluctuation remained significantly increased by PEM throughout the 7 day post-surgical period (**Figure 3.4B**). This was an independent effect of PEM (p<0.001), with the exception of an interaction on days 8 (p=0.018) and 11 (p=0.021). On day 8, the PEM-SHAM animals had a significantly greater temperature fluctuation compared to the other three experimental groups (p<0.001). On day 11, ischemia increased the temperature fluctuation only when the rats were malnourished (p<0.015). There was also an independent decrease caused by ischemia on day 9 (p=0.002).

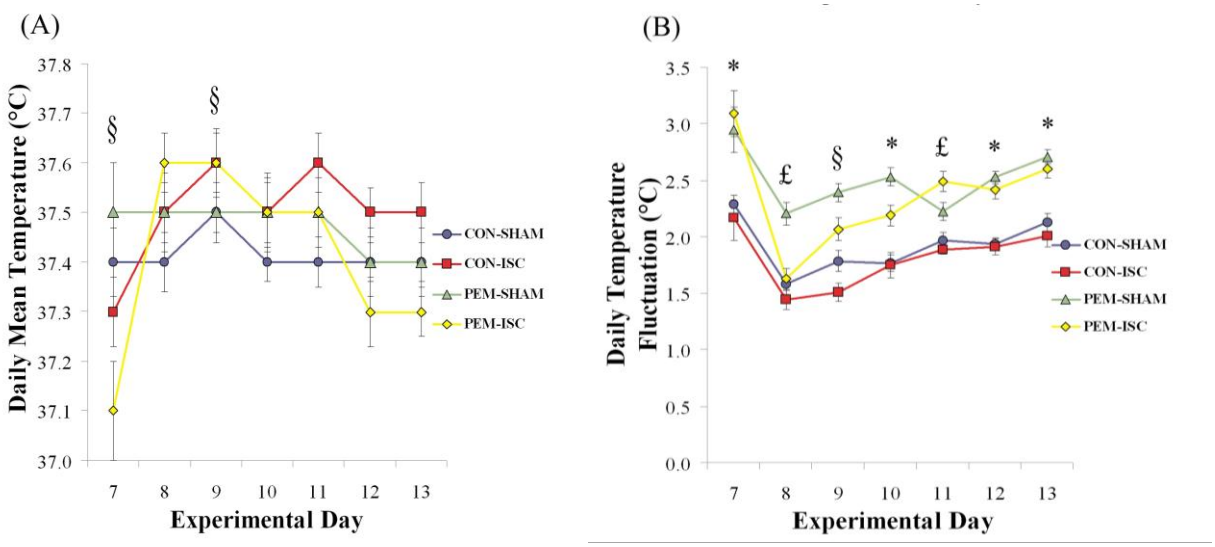

Figure 3.4. Post-surgical mean (± SEM) core temperature (**A**) and daily core temperature fluctuation (**B**) for CON-SHAM, CON-ISC, PEM-SHAM, and PEM-ISC (n=10). Experimental day 7 is 0-24 hours after surgery. For mean daily core temperature, an independent effect of ischemia occurred on days 7 and 9 (§). For daily temperature fluctuation, there was an independent effect of PEM (*), with the exception of an interaction on days 8 and 11 (£) and an independent effect of ischemia on day 9 (§).

Figure 3.5A shows representative data to illustrate the influence of the extent of hippocampal injury on the diurnal rhythm of core temperature during the post-ischemic period in well-nourished rats. In general, CON-ISC rats presenting with unilateral hippocampal damage tended to have diurnal cycles similar to those of CON-SHAM rats (**Figure 3.5A**). With extensive bilateral hippocampal CA1 injury, there was a disruption in the diurnal cycle of CON-ISC rats (**Figure 3.5B**). The disruption lasted for 1-2 days, at which time the cycle returned to a normal pattern. This pattern of disruption in relation to extent of injury was observed in some of the PEM-ISC group, but the observation was not as consistent.

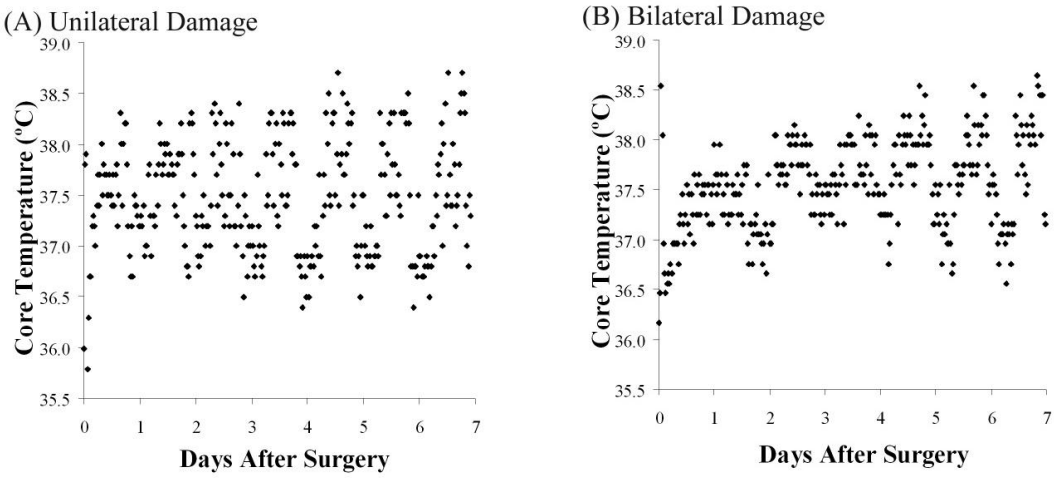

Figure 3.5. Post-ischemic diurnal pattern for core temperature as influenced by extent of hippocampal injury in rats exposed to control diet and global ischemia. Representative patterns are shown for a CON-ISC rat with unilateral hippocampal damage (**A**) and a CON-ISC rat with extensive bilateral damage (**B**).

3.4.2 Immunohistochemistry

A separate report describes hippocampal CA1 cell counts from the animals in this study (Prosser-Loose et al., 2011). Extensive bilateral hippocampal CA1 injury after 2-VO surgery was evident in 7 CON-ISC animals, with 3 rats demonstrating unilateral hippocampal injury. Surprisingly, only 5 rats in the PEM-ISC group had bilateral hippocampal CA1 injury, while 1 rat demonstrated unilateral CA1 damage and 4 rats sustained no CA1 injury (Prosser-Loose et al., 2011).

There were no differences in background staining in the corpus callosum caused by surgical treatment ($p>0.05$) or diet assignment ($p>0.05$) for any of the three glial markers (GFAP, ED-1, OX-42).

Due to the unexpected variability in the CA1 neuronal damage, only rats with bilateral hippocampal damage were included in analysis to provide a preliminary assessment of the effect of PEM. **Figure 3.6** shows images of the CA1 region immunostained for GFAP, ED-1 and OX-42 on experimental day 14 for all four experimental groups. The images for the CON-ISC and PEM-ISC groups are representative of sections from rats sustaining bilateral CA1 injury in which CA1 cell counts are similar. Semi-quantification analyses revealed that global ischemia significantly increased GFAP ($p<0.001$; n=10 CON-SHAM, n=7 CON-ISC, n=10 PEM-SHAM, n=5 PEM-ISC), ED-1 ($p<0.001$; n=10 CON-SHAM, n=7 CON-ISC, n=10 PEM-SHAM, n=5

PEM-ISC) and OX-42 ($p<0.001$; n=7 CON-SHAM, n=5 CON-ISC, n=7 PEM-SHAM, n=3 PEM-ISC) staining, but were unaffected by dietary treatment (GFAP p=0.61; ED-1 p=0.20; OX-42 p=0.17), nor was there an interaction (GFAP p=0.056; ED-1 p=0.15; OX-42 p=0.13).

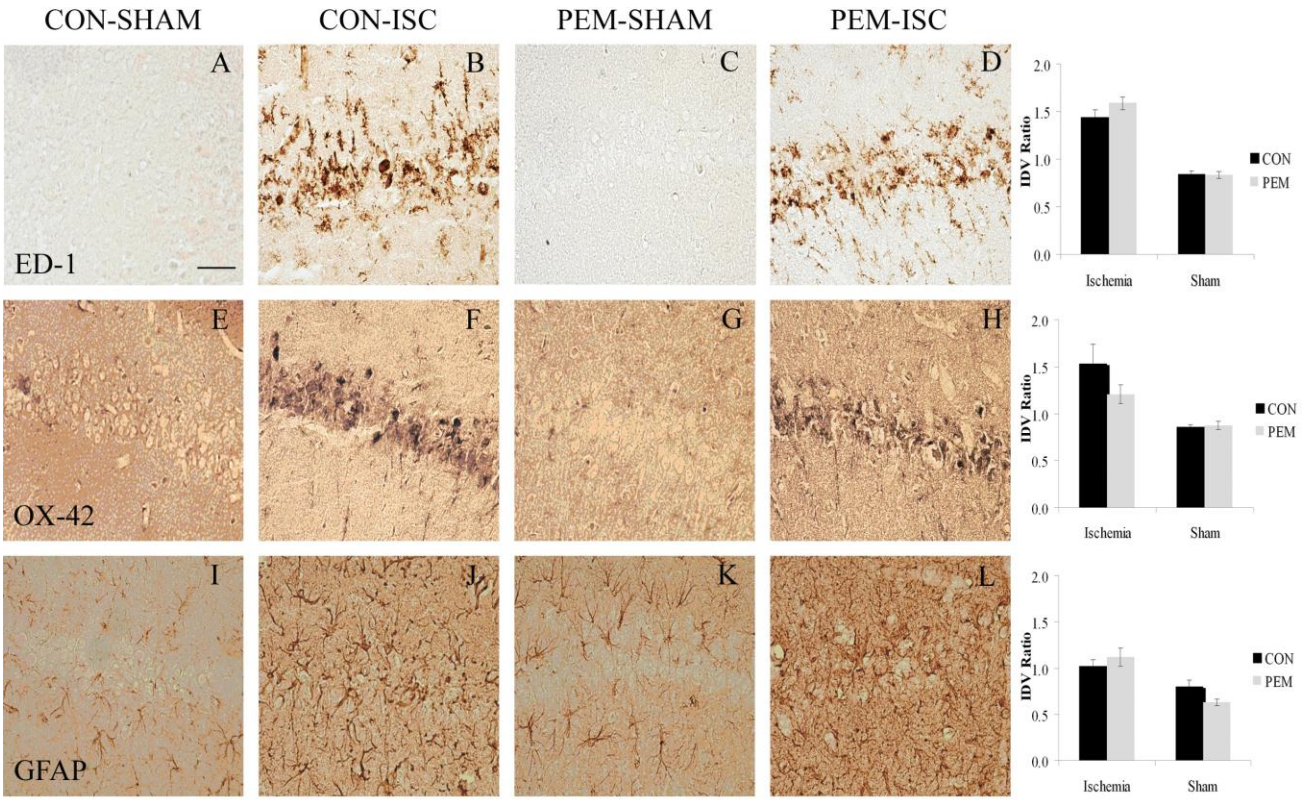

Figure 3.6. Representative images from brains with bilateral hippocampal damage to highlight the immunohistochemical characterization of the glial response in the CA1 region of CON-SHAM (**A,E,I**), CON-ISC (**B,F,J**), PEM-SHAM (**C,G,K**) and PEM-ISC (**D,H,L**) rats. There was a significant independent increase ($p<0.001$) in integrated density values (IDV) for ED-1 (**A-D**), OX-42 (**E-H**) and GFAP (**I-L**) caused by global ischemia with no effect of dietary treatment. Scale bar = 50 μm.

3.4.3 Correlations

Correlation analysis was performed to determine if the inconsistency in CA1 hippocampal injury was related to a post-ischemic drop in core temperature. The lowest core temperature within 8 hours of anaesthetic induction was correlated with total CA1 cell counts reported in (Prosser-Loose et al., 2011) for: 1) CON-ISC and PEM-ISC groups combined, and 2) CON-ISC and PEM-ISC groups separately as shown in **Table 3.2**. Results from correlation

analysis detected a significant negative correlation between hippocampal injury and the drop in temperature when only CON-ISC animals were included in the analysis ($r = -0.897$, $p < 0.001$). Thus, the decrease in neuronal damage was limited to the CON-ISC animals that experienced a drop in core temperature.

Table 3.2. Correlation of post-ischemic hippocampal CA1 total cell counts with post-surgical decline in core temperature*

	CON-ISC + PEM-ISC (n=20)		CON-ISC (n=10)		PEM-ISC (n=10)	
	r	p	r	p	r	p
Core Temperature (°C) $^{\Delta}$	-0.352	0.127	-0.897	< 0.001	0.010	0.978

*Detected by Pearson's Correlation Coefficient. ΔLowest core temperature observed within 8 hours following surgical anaesthetic induction.

3.5 Discussion

The major finding from this study was the rapid effect of a change in experimental diet on thermoregulation. Both daily temperature fluctuation and daily mean temperature were increased consistently within the first 24 hours after assignment to the low protein diet. As malnutrition developed, both parameters remained significantly higher than those of control diet-fed rats throughout the pre-ischemic period. During this period, the greatest differences between the two experimental groups were 0.90ºC for temperature fluctuation on experimental day 3 and 0.35ºC for mean temperature on day 1.

This disruption in thermoregulatory response is in agreement with previous reports. However, the extent of the response and the trend in temperature patterns vary, with both warming (Balmagiya & Rozovski, 1983; Castanon-Cervantes & Cintra, 2002) and cooling (Allison et al., 2000; Brooke, 1973; Duran et al., 2008) responses reported. These discrepancies are likely due to differences in species, method used to measure temperature, age, severity of malnutrition, extent to which protein and/or energy were limiting, and stage of adaptation to the nutrient deficiency. Most studies in malnourished rodents have been well-controlled, although findings cannot be specifically attributed to protein-energy status in those studies in which the control and deficiency diets are not matched for all other nutrients (Duran et al., 2008). The

technique by which temperature data are obtained is a key factor, since most studies rely on single periodic measurements that fail to highlight changes in diurnal cycles. Furthermore, when conclusions have been drawn from acute rectal monitoring in rodents, this type of measurement can yield artificial temperature data by triggering stress-induced hyperthermia (Clark et al., 2003). An advantage of implantable temperature transmitters, as used by us and others (Castanon-Cervantes & Cintra, 2002; Duran et al., 2008), is that accurate chronic readings highlighting diurnal cycles can be obtained in unanaesthetized animals without inducing a stress response.

Our core temperature results are comparable to the findings from Castañón-Cervantes et al. (2002), who observed chronic occipital-cortex temperature regulation in rats exposed to protein malnutrition during brain development and into adulthood. Analogous to our core temperature findings, malnourished Sprague-Dawley rats presented with an altered circadian rhythm characterized by higher-amplitude daily cortical temperature rhythms. Mean daily cortical temperatures were also higher in young malnourished rats. However, this altered thermoregulatory response was not significant in malnourished old rats, demonstrating that age is another variable that may explain discrepancies among studies.

We found a prominent difference between the effects of the low protein diet on the daily temperature fluctuation and mean temperature during the pre-ischemic period. During the first three days after exposure to a low protein diet, rats increased both their daily temperature fluctuation and mean temperature. Following this initial period, the increase in temperature fluctuation remained constant and appeared to be permanent, whereas the increase in mean temperature began to decline after the second day. During the initial three day period, malnourished animals were warmer than control animals while awake, but had similar core temperatures while asleep. As time advanced, malnourished rats became cooler during the sleep period, resulting in more normalized mean core temperatures. This suggests that malnourished rats were able to adapt to the initial warming period by adjusting their temperature fluctuation. The increase in amplitude of daily temperature fluctuation was sustained throughout the post-surgical period. In contrast, the malnutrition-induced increase in mean daily temperature did not re-establish after surgery, suggesting that it was an acute response. Due to the short post-surgical period, we cannot rule out the possibility that a change in mean core temperature would re-establish with chronic PEM. Although the long-lasting effects of PEM on thermoregulation are

rarely studied, one previous study revealed a persistent effect of chronic protein malnutrition on circadian rhythm for 28 days (Castanon-Cervantes & Cintra, 2002).

Our study did not address the mechanisms responsible for the temperature changes observed in the protein-energy malnourished rat. Body temperature is tightly regulated, with afferent thermal sensing receptor input sent from the skin surface, deep abdominal and thoracic tissues, spinal cord, and brain to the major thermoregulatory regulator, the hypothalamus (Kurz, 2008). Temperatures exceeding the warm- or cold-response thresholds activate the thermoregulatory defense (efferent) responses of sweating, vasodilation or vasoconstriction, non-shivering thermogenesis, and shivering. Behavioural responses, such as altering locomotion, are another form of defense. We propose that the effects of PEM on thermoregulatory defense are complex, involving heat production and loss, and are dependent upon the characteristics of the deficiency as discussed above.

The immediate temperature effects of the low protein diet may reflect a behavioral stress response similar to what has been described with rat handling (Briese, 1998). We hypothesized that the immediate increase in mean core temperature is contributed to by an acute stress-induced rise in locomotor activity reflecting increased foraging caused by exposure to suboptimal diet. The data indirectly support this, since during the initial period of exposure to low protein diet, the largest temperature change in malnourished rats was during the awake cycle. Simultaneous measurements of the activity and temperature circadian rhythms would address this hypothesis. The sustained increase in amplitude of the diurnal temperature cycle, however, suggests that alterations in thermoregulatory defense are also related to the adaptive metabolic and behavioral adjustments that occur as malnutrition develops (Torun, 2006).

PEM may exert direct effects on central regulation of temperature by altering specific hypothalamic nuclei that sense nutrient level and alter feeding and metabolic rate (Tabarean et al., 2010). In addition, the altered thermoregulatory amplitude suggests that the low protein diet could be interacting with the hypothalamic suprachiasmatic nucleus (SCN). The latter regulates the circadian rhythms for many processes including sleep-wake cycles, feeding, body temperature, and locomotor activity; an alteration in one cycle can influence that of another (Weinert, 2005). Increases in rapid eye movement (REM) sleep, which attenuates thermoregulatory responsiveness (Horne, 2009), have been reported with chronic malnutrition (Durán et al., 1999). The latter can also modify the phase relation of core temperature to the

motor activity oscillation (Castanon-Cervantes & Cintra, 2002). It has even been suggested that a low protein diet can modify the cellular composition of the SCN, resulting in a weaker coupling force among oscillators (Aguilar-Roblero et al., 1997). However, unlike our investigation, all of these studies initiated protein deficiency during brain development. Nonetheless, influences could also be indirectly exerted through modifications in neuroactive peptides and hormones. PEM induces a host of endocrine changes that regulate heat production and basal metabolic rate, including increased glucocorticoids (Torun, 2006). Although we did not detect elevated corticosterone levels in these malnourished rats (Prosser-Loose et al., 2011), the unphysiologically high values measured are likely indicative of sampling stress, which can mask an increase caused by malnutrition (Shipp & Woodward, 1998).

Protein-energy malnourished rats also showed a greater decrease in core temperature immediately following anaesthesia, and this was independent of the type of surgery. These data demonstrate that extra precautions need to be taken when studying PEM coupled with any surgical model. Rodents are highly susceptible to hypothermia induced by anaesthesia (Taylor, 2007). Despite the use of a circulating water blanket and heat lamp in the present study, all four experimental groups experienced a decrease in core temperature either during or immediately after anaesthesia. The greater tendency in the malnourished group may have been related to decreased heat conservation resulting from reduced thermal insulation from adipose tissue. None of the rats were able to seek a warmer environment (that is, huddle with cage-mates) due to single-housing during the recovery period. This finding is in agreement with others' reports on the inability of malnourished rats to cope with environmental temperature stressors. Sprague-Dawley rats fed a protein-restricted diet for 6 weeks were not able to respond to the same level as control animals when subjected to a mild cold challenge. Following a 90-minute exposure period to an 18-19ºC environment, the malnourished animals were on average 2.5ºC cooler and took approximately twice the length of time to recover from the stressor (Balmagiya & Rozovski, 1983). A similar trend has been reported in malnourished elderly patients (Allison et al., 2000).

Unlike a previous report (Clark et al., 2007), exposure to global ischemia induced by 2-VO also affected temperature regulation during the post-surgical period. Following the immediate cooling period, there was a distinct recovery phase, which differed depending on experimental group and the extent of neuronal damage. Independent of diet, SHAM animals returned to regular diurnal oscillations immediately following the anaesthetic induced cooling

period. CON-ISC animals with extensive hippocampal damage tended to present with distorted diurnal rhythms that lasted approximately 48 hours whereas CON-ISC rats with unilateral hippocampal damage regulated temperature similarly to SHAM animals during the recovery phase. This relationship between disrupted diurnal cycle and extent of hippocampal injury was more ambiguous in the PEM-ISC rats, presumably because of the independent effects of PEM on thermoregulation.

An unfortunate and unexpected finding of the current study reported in detail elsewhere (Prosser-Loose et al., 2011) is that co-existing PEM increased the inconsistency of the 2-VO model of global ischemia. This may be a consequence of PEM altering multiple physiological variables, since there was no one clear contributor (Prosser-Loose et al., 2011). The decline in core temperature in the immediate post-anaesthetic period presented here also does not offer an explanation. The mean lowest core temperature in PEM-ISC rats during the 8 hours following anaesthetic induction was 0.8ºC lower than the corresponding mean of CON-ISC animals. However, a negative correlation between temperature and the number of surviving CA1 neurons, while detected for the CON-ISC animals, was not evident in the malnourished rats exposed to ischemia. Thus, the decrease in CA1 neuronal damage was not explained by a drop in core temperature. Multiple variables can affect the efficacy of post-ischemic hypothermia for neuroprotection, including the duration, rate and severity of temperature drop, as well as the type of stroke (MacLellan et al., 2009). The majority of protein-energy malnourished rats experienced a brief post-ischemic drop in temperature, and it is unlikely that the temperatures were low enough to provide significant neuroprotection. Greater and more prolonged drops in temperature are needed to significantly mitigate injury (MacLellan et al., 2009).

This failure to achieve a consistent model of global ischemia (Prosser-Loose et al., 2011) has confounded the assessment of glial response and prevents us from drawing conclusions on whether PEM exacerbates reactive gliosis after global ischemia through alterations in temperature regulation. A preliminary assessment was conducted exclusively on rats presenting with extensive bilateral hippocampal damage. The extent of gliosis appeared to mirror the degree of neuronal injury, but the striking increase in reactive gliosis previously observed in protein-energy malnourished gerbils after global ischemia (Bobyn et al., 2005) was not observed. While the glial response significantly differed between SHAM and ISC groups, PEM had no effect on GFAP, ED-1 and OX-42 expression. Since the model of brain ischemia was confounded and

evaluating only those rats with bilateral hippocampal injury can introduce bias, our preliminary analysis of the effects of malnutrition on the glial response is inconclusive and should be re-addressed in future with a reliable ischemia model.

Exposure to a low protein diet rapidly impairs the ability to maintain constant thermoregulatory homeostasis. Furthermore, when confronted with the thermoregulatory challenge associated with anaesthesia, protein-energy malnourished rats were less able to adapt than well-nourished animals and thus presented with a greater transient drop in core temperature. Since temperature regulation is a key determinant of brain injury following ischemia, these findings suggest that the pathophysiology of brain injury in malnourished stroke victims could be altered and that these patients might require distinct treatment. Although our preliminary analysis of the influence of PEM on the glial response to global ischemia is inconclusive, this question should be re-addressed in a different model of brain ischemia since other evidence suggests that PEM lowers the setpoint for inflammation.

3.6 Experimental Progression of Thesis

The overarching objective of this thesis was to investigate the effects of PEM on the inflammatory response to brain ischemia. Given that there is a strong relationship between inflammation and temperature, it was imperative to follow-up on the temperature findings obtained in this first study. Although it is well known that food availability influences body temperature, this is the first report to highlight the immediate changes in body temperature in response to a protein-deficient diet. However, the long-term independent effects of PEM on temperature were not clear from this study, and therefore a comprehensive assessment of core temperature in chronically malnourished rats was performed in the second study. It was hypothesized that the immediate elevation in core temperature following placement on the suboptimal diet was an acute stress response characterized by hyperactivity. Thus, this relationship was examined in the second study by measuring the motor activity levels of rats on initial exposure to a low protein diet. Lastly, the findings from this study suggested that the clinical problem of co-existing PEM could not be reliably assessed using the rat 2-VO model. Therefore, this research question was abandoned. Instead, the third study in this thesis examined the consequences of the equally clinically relevant situation of PEM developing after brain ischemia.

CHAPTER 4:
PROTEIN-ENERGY MALNUTRITION INDUCES AN ABERRANT ACUTE-PHASE RESPONSE AND MODIFIES THE CIRCADIAN RHYTHM OF CORE TEMPERATURE

This chapter has been published as follows:
Smith SE, Ramos RA, Refinetti R, Farthing JP, Paterson PG. Protein-energy malnutrition induces an aberrant acute-phase response and modifies the circadian rhythm of core temperature. Applied Physiology, Nutrition and Metabolism. Accepted March 2013.

4.1 Abstract

Protein-energy malnutrition (PEM), present in 12-19% of stroke patients upon hospital admission, appears to be a detrimental comorbidity factor that impairs functional outcome, but the mechanisms are not fully elucidated. Since ischemic brain injury is highly temperature-sensitive, the objectives of this study were to investigate whether PEM causes sustained changes in temperature that are associated with an inflammatory response. Activity levels were recorded as a possible explanation for the immediate elevation in temperature upon introduction to a low protein diet. Male, Sprague-Dawley rats (7 wk old) were fed control diet (18% protein) or a low protein diet (PEM, 2% protein) for either 7 or 28d. Continuous core temperature recordings from bio-electrical sensor transmitters demonstrated a rapid increase in temperature amplitude, sustained over 28d, in response to a low protein diet. Daily mean temperature rose transiently by d2 ($p=0.01$), falling to normal by d4 ($p=0.08$), after which mean temperature continually declined as malnutrition progressed. There were no alterations in activity mean ($p=0.3$) or amplitude ($p=0.2$) that were associated with the early rise in mean temperature. Increased serum alpha-2-macroglobulin ($p<0.001$) and decreased serum albumin ($p\leq0.005$) combined with a decrease in serum alpha-1-acid glycoprotein ($p<0.001$) suggest an atypical acute-phase response. In contrast, a low protein diet had no effect on the signaling pathway of the pro-inflammatory

transcription factor, NFκB, in the hippocampus. In conclusion, PEM induces an aberrant and sustained acute-phase response coupled with long-lasting effects on body temperature.

4.2 Introduction

PEM, characterized by deficits in protein and energy status, is frequently associated with critical illnesses and chronic disease states, ultimately contributing to increased morbidity and mortality (Omran & Morley, 2000). We have been investigating the implications of PEM to stroke outcome, since 12-19% of stroke patients are protein-energy malnourished at the time of hospital admission (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008), and this proportion rises to 35% by the time of admission to a rehabilitation unit (Brynningsen et al., 2007; Poels et al., 2006). Numerous clinical studies, the largest being the multi-centre FOOD trial (FOOD Trial Collaboration, 2003), have associated suboptimal nutritional status with adverse clinical outcomes after stroke. However, since these studies had numerous methodological shortcomings that precluded establishing a causal relationship, we addressed this more directly in a rodent model of global brain ischemia in which the hippocampal CA1 region is most vulnerable. Co-existing PEM was found to impair functional outcome (Bobyn et al., 2005) and alter mechanisms underlying both neuronal death (Bobyn et al., 2005; Ji et al., 2008) and brain repair (Prosser-Loose et al., 2010). Here, we seek to characterize other pathophysiological features of malnutrition that could explain PEM-mediated changes in brain function following cerebral ischemia.

Co-existing PEM may exert some of its effects on post-stroke functional outcome by altering key physiological determinants of ischemic brain injury. Stroke pathophysiology is highly temperature-sensitive (MacLellan et al., 2009; van der Worp et al., 2007; Wang et al., 2009), and we recently employed bio-electrical sensor transmitters to observe a rapid perturbation in the circadian rhythm of core temperature in rats exposed to a low protein diet (Smith et al., 2011). Within the first day, daily temperature amplitude (half of the range of daily temperature oscillation over the 24hr diurnal cycle) increased markedly, and there was a small but clearly defined increase in mean temperature. Unfortunately, the long-term independent effects on mean temperature were not clear from this study, since the design exposed rats to the thermoregulatory stress of anaesthesia and surgery after only 7d on the low protein diet, which lowered core temperature. However, the increased amplitude was sustained over the two weeks of testing, during which the rats voluntarily reduced food intake and became protein-energy

malnourished (Prosser-Loose et al., 2011). There is considerable discrepancy in the related reports of others, with both cooling (Bastow et al., 1983; Brooke, 1973; Duran et al., 2008) and warming (Balmagiya & Rozovski, 1983; Castanon-Cervantes & Cintra, 2002) responses reported in humans and rodents experiencing varying states of protein and/or energy deficiencies. Reliance on single periodic rectal monitoring accounts for some of the inconsistencies in rodent studies, since this can trigger stress-induced hyperthermia (Clark et al., 2003), and changes in the diurnal cycle cannot be detected. Previous studies have not investigated the thermoregulatory response to PEM as it evolves over time.

Since prolonged hyperthermia is associated with both an exacerbated inflammatory response and aggravated ischemic brain injury (Wang et al., 2009), it is clinically important to characterize the duration and severity of temperature changes that occur with the chronic PEM characteristic of patients admitted with stroke. The first objective of the current study was therefore to investigate whether the increase in daily temperature amplitude induced by exposure to a protein-deficient diet would be sustained over a 28d period. We further hypothesized that exposure to a low protein diet would cause an initial elevation in core temperature associated with increased foraging behavior, and that the higher temperature would persist due to the induction of inflammation (Ji et al., 2008; Ling et al., 2004; Lyoumi et al., 1998). Contradictory reports have described either an increase (Duran et al., 2008) or no change (Castanon-Cervantes & Cintra, 2002) in motor activity in rats exposed to low dietary protein since conception; however, these studies offer limited evidence of the protein-energy status under study.

Since temperature alterations can modulate brain inflammatory pathways (Ceulemans et al., 2010; Wang et al., 2009; Webster et al., 2009), chronic PEM may trigger an exacerbated inflammatory reaction to brain ischemia through changes in thermoregulatory capacity. The hippocampal CA1 subregion of the brain is selectively vulnerable to global brain ischemia (Corbett & Nurse, 1998), and indirect evidence from our laboratory suggests that PEM could exacerbate the inflammatory response in this brain region (Bobyn et al., 2005; Ji et al., 2008). However, an additional intriguing finding was that PEM not only increased activation of the transcription factor, NFκB, in the ischemic hippocampus, but also in the hippocampus of sham and non-surgically treated malnourished gerbils (Ji et al., 2008). As NFκB directly regulates pro-inflammatory gene expression (Hayden & Ghosh, 2011), this suggests that PEM can independently induce hippocampal inflammation. Therefore, in the current study, we aimed to

further characterize the influence of both acute and chronic PEM on the hippocampal NFκB pathway. We hypothesized that PEM would increase mRNA expression of pro-inflammatory genes regulated by the NFκB cascade.

A final study objective was to investigate the degree to which a systemic inflammatory response is induced by PEM, as the extent of the acute-phase response predicts outcome in stroke patients (Dziedzic, 2008). While most studies have addressed whether PEM blunts the ability to mount a controlled inflammatory response to tissue injury or infection, there is also evidence that malnutrition can be an independent stimulus of inflammation and an acute-phase response. Serum albumin is a major negative acute-phase protein that decreases with inflammation (Qu et al., 1996). Positive acute-phase proteins produced at high levels with inflammation in the rat include alpha-1-acid glycoprotein (AGP) and alpha-2-macroglobulin (A2M); these are regulated by inflammatory cytokines such as IL-6, IL-1β and TNF-α (Andus et al., 1988). Ling et al. (2004) reported a systemic inflammatory response to PEM on the basis of increased serum concentrations of TNF-α, IL-1β, IL-6 and AGP. Similarly, Lyoumi et al. (1998) demonstrated elevated serum A2M levels and IL-6 mRNA in peripheral blood mononuclear cells and intestine of severely protein-malnourished rats. In fact, increased inflammatory cytokines reported with PEM (Dulger et al., 2002; Ling et al., 2004) may constitute a mechanism for irregular thermoregulatory homeostasis, since the major endogenous pyrogens, including IL-6 and TNF-α, migrate to the brain and increase the thermoregulatory set-point in the hypothalamus (Biddle, 2006).

We thus hypothesized that PEM elicits hippocampal and systemic inflammation that is associated with elevated core temperature. The first study was designed to investigate the effects of acute (7d) and chronic (28d) exposure to a low protein diet and the evolution of PEM on the hippocampal NFκB signaling pathway and the systemic acute-phase response, as reflected by serum A2M, AGP and albumin concentrations. Chronic core temperature readings were obtained with bio-electrical sensor transmitters to characterize disruptions in body temperature induced by PEM as it evolved over 28d. In a second experiment, a cross-over design was used to study the short-term effects of exposure to low protein diet on the relationship between the daily rhythms of locomotor activity and body temperature.

4.3 Materials and Methods

4.3.1 Animals

Twenty-eight male Sprague-Dawley rats (Experiment 1, n=24; Experiment 2, n=4) obtained from Charles River Canada (QC, Canada), 30d in age, were acclimatized on rat chow for 2d before placement on a purified control diet (see below for composition). Rats were housed in groups of 2-4 (Carefresh bedding; Absorption Corp., Washington, USA) until temperature sensor implantation surgery and maintained on a 12hr light (07:00-19:00)/12hr dark (19:00-07:00) cycle in a temperature controlled room (~22ºC) with free access to food and water. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.3.2 Core Temperature Bio-Electrical Sensor Transmitter Implantation

At 7d following arrival, calibrated bio-electrical sensor transmitters encapsulated in biocompatible silicone (*SubCue*™Dataloggers, AB, Canada) were aseptically implanted into the abdominal cavity. Sensor transmitters were pre-programmed to record core body temperature at set times as described under individual experiments. Rats were placed on a heated water blanket and anaesthetized with isoflurane (4% induction, 2% maintenance with 1 L/min oxygen). A 3-centimeter mid-abdominal incision was made, allowing intra-peritoneal implantation of the sterilized, calibrated temperature sensor. Following the suturing of the muscle wall and skin incisions, a subcutaneous injection of Ketoprofen (5mg/kg) was given.

4.3.3 Experimental Diets

A protein adequate control diet (CON, 18% protein) and a low protein diet (PEM, 2% protein) (Dyets, Inc., PA, USA) were used in both Experiments 1 and 2 (**Table 4.1**). Rats of this age fed a 2% protein diet voluntarily reduce food intake, resulting in mixed PEM (Prosser-Loose et al., 2011). The experimental diets were modified from the American Institute of Nutrition (AIN)-93G diet (Reeves et al., 1993) to not contain the antioxidant tertiary-butylhydroquinone.

Table 4.1. Composition of control and low protein diets[§]

Component	Adequate Protein*(CON) g/kg	Low Protein** (PEM) g/kg
Vitamin Free Casein	200	22.4
L-Cystine	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 α	35	0
Mineral Mix β	0	35
Calcium Phosphate, dibasic	0	12.4
Calcium Carbonate	0	3.36
Vitamin Mix γ	10	10
Choline Bitartrate	2.5	2.5

[§] Experimental diets obtained from Dyets Inc. (PA, USA). *Protein adequate control diet was formulated to contain 18% protein. ** Low protein diet, used to induce PEM, was formulated to contain 2% protein. [#] Tertiary-butylhydroquinone. α AIN-93G mineral mix (Reeves et al., 1993). β AIN-93G modified mineral mix, without calcium and phosphorus, potassium citrate$\cdot H_2O$ increased from 28 to 226.55 g/kg, sucrose increased from 209.806 to 618.256 g/kg mineral mix. γ AIN-93G vitamin mix (Reeves et al., 1993).

4.3.4 Experiment 1

Experiment 1 assessed whether PEM induces an acute-phase response and evaluated the effects of PEM on circadian rhythm of core temperature and the hippocampal NFκB signaling pathway. Two time-points were used to characterize both the acute (7d) and chronic (28d) response to a low protein diet.

4.3.4.1 Diet Assignment

Rats (47d old) were housed in groups of 3 and were randomly assigned to either continue on the CON diet (fed since d2 after arrival) or a low protein diet at 10d following temperature sensor implantation, which was sufficient time for full recovery from surgery and Ketoprofen

washout. Animals were fed their respective diets *ad libitum* for either 7 or 28d. Food intake was recorded daily and body weight recorded weekly. The four experimental groups generated in Experiment 1 were (n=6): CON-7d, PEM-7d, CON-28d, and PEM-28d.

4.3.4.2 Tissue Collection

Following either 7 or 28d on the assigned diet, rats were anaesthetized with isoflurane (100% O_2), and blood samples were collected via cardiac puncture. Blood samples were allowed to clot for 30min at room temperature and then centrifuged at 1,500 x g for 10min. Serum was removed and stored at -80°C. Following transcardial perfusion with saline, liver was collected, immediately transferred to liquid nitrogen, and stored at -80°C. Intact hippocampus was excised from both hemispheres on an ice-cold dissection stage and stored at -80°C in RNA*later* RNA Stabilization Reagent (Qiagen, CA, USA). Temperature sensors were retrieved from the peritoneal cavity.

4.3.4.3 Indices of PEM

For analysis of liver lipid levels, liver was homogenized in 1 mL of 0.15 mol/L NaCl and added to 5 mL of chloroform:methanol (2:1). Samples were centrifuged at 3,200 x g for 10min, followed by dehydration of the isolated liver lipid pellet with anhydrous Na_2SO_4. The contents were filtered with chloroform, followed by evaporation of any remaining chloroform. To determine the liver lipid content of each sample, liver lipid weight was expressed as a percent of liver wet weight.

4.3.4.4 Hippocampal NFκB Signaling Pathway

Hippocampal RNA was prepared using the Qiagen RNeasy Midi kit following the manufacturer's protocol (Qiagen). Reverse transcription was carried out with the RT^2 First-Strand kit (SABiosciences, MD, USA) using 500 ng of RNA. The Rat NFκB Signaling Pathway RT^2 Profiler PCR Array (SABiosciences) was used to measure the transcript levels of 84 genes involved in NFκB-mediated signal transduction and appropriate housekeeping controls (Rplp1, Hprt1, Rpl13a, Ldha, Actb). Gene detection and quantification was carried out using the AB1 7300 Applied Biosystems Real-Time PCR Detection System. Data analysis was performed using the RT^2 Profiler PCR Array Data Analysis program obtained from SABiosciences. Only the genes that had average delta cycle threshold (Ct) values differ by more than two cycles between treatment groups were included in the statistical analysis.

4.3.4.5 Serum AGP, A2M and Albumin

To study the systemic inflammatory response resulting from exposure to a low protein diet, serum levels of positive acute-phase proteins were determined using the rat AGP ELISA kit and the rat A2M ELISA kit (Immunology Consultants Laboratory, Inc., OR, USA). Serum albumin concentration was determined by the bromocresol green method (Doumas et al., 1971). A volume of 25 μL of sample serum or standard solution was added to 5.0 mL bromocresol green reagent (0.15 mmol/L bromocresol green, 0.075 mol/L succinate buffer, 30% Brij-35). After 30min, absorbance was measured spectrophotometrically at 628 nm (Biochrom Ultrospec 3100 Pro). The albumin concentration of the sample was determined by linear regression. For all serum assays, samples were run in triplicate.

4.3.4.6 Core Temperature Analysis

Temperature probes were pre-programmed to record core body temperature every 15min. *SubCue*™ analyzer software (*SubCue*™Dataloggers, AB, Canada) was used to obtain logged temperature data. Alterations in body temperature resulting from exposure to a low protein diet were assessed by examining the mean temperature and temperature amplitude (half of the range of oscillation of a fitted curve) measured over each 24hr diurnal cycle. The software program COSINOR 2.3 was used to calculate daily temperature mean and amplitude (Refinetti et al., 2007).

4.3.4.7 Statistical Analysis

Statistical analyses were conducted using SPSS 18.0 for Windows. All data are presented as mean ± SEM. Body weight, food intake, liver lipid percentage, serum protein concentrations and NFκB PCR arrays were analyzed using an independent-sample Student's *t* test. When multiple comparisons were made, the level of significance was adjusted using Bonferroni correction. Differences were considered significant at $p \leq 0.05$. Variability is presented as SEM; 95% confidence intervals around the means were also calculated for food intake because of the small sample size (n=2 cages [3 animals/cage]).

Analysis of daily mean temperature and amplitude was performed using a repeated measures ANOVA. To assess the immediate and longer-term effects of the low protein diet, data from the acute (d1-7) and chronic (d8-28) phases of malnutrition were analyzed separately for both temperature mean and amplitude. To allow for increased power with the sample size of n=6, a diet group X time ANOVA with repeated measures was performed using data from each day

during the acute phase (diet [2] X time [7]) and every other day during the chronic phase (diet [2] X time [10]). The Huynh-Feldt test was used to correct for violations of sphericity. For post-hoc comparisons, independent-sample Student's *t* tests were performed among groups on d2, 3 and 4. Bonferroni correction was used when multiple comparisons were made to adjust the level of significance.

4.3.5 Experiment 2

To determine the extent to which low protein diet-induced changes in core body temperature were influenced by alterations in locomotor activity, 4 male, Sprague-Dawley rats were utilized in a cross-over design as described below.

4.3.5.1 Locomotor Activity Monitoring

At 7d following temperature sensor implantation, 4 cages with 1 rat per cage were placed in the Opto-M3 sensor cage system (Columbus Instruments, Ohio, USA). Detection of locomotor activity was based on disruptions of the 8 X 8 infrared beam grid. A count was accumulated every time a new beam was broken, so that stationary activity such as scratching, grooming and digging was excluded. Activity data were collected in 15min sampling intervals. Activity monitors were connected to a computer, and the CI Multi-Device Interface software (Columbus Instruments) was used to control the Opto-M3 device. Activity recording started at 4d after placement in the activity monitoring setup to allow for acclimatization to the new environment prior to recording.

A cross-over design was utilized to study the relationship between daily rhythms of activity and body temperature under CON diet (18% protein) conditions versus low protein (2%) diet. For the first 7d of activity monitoring, rats remained on the purified CON diet provided at the time of arrival. Following 1 transition day, rats were given the low protein diet and locomotor activity was recorded for an additional 7d. To ensure that stress related to cage and animal handling did not alter activity readings, cage changes and body weight measurements were performed on the day before initiating activity monitoring on CON diet, the transition day between diets, and at the end of the low dietary protein period. That is, recording of activity was always initiated 24hr after manipulation.

4.3.5.2 Temperature and Activity Analysis

At the time of euthanasia, temperature sensors, pre-programmed to record temperature every 30min, were retrieved from the abdominal cavity. Logged temperature data were obtained

using the *SubCue*™ analyzer software. Parameters measured for both temperature and activity rhythms included: daily mean, amplitude and robustness (strength of the rhythm). In addition, we examined the effect of a low protein diet upon the acrophase, the time at which the peak of a rhythm occurs. Acrophase and robustness were calculated for the combined 7d while on each experimental diet. Acrophase difference (APH-D) was calculated by subtracting the acrophase of temperature from the acrophase of activity for each animal. Since the daily rhythms of activity and temperature are known to be closely associated (Refinetti, 1997), calculating the APH-D can identify any differences in the rhythmic pattern between the low protein and CON diet periods. Computation of all parameters was by means of the software program COSINOR 2.3 (Refinetti et al., 2007).

4.3.5.3 Statistical Analysis

Statistical analyses were conducted using SPSS 18.0 for Windows. All data are presented as mean ± SEM. To assess the effects of a low protein diet on locomotor activity, both daily activity mean and amplitude were compared for each respective day on experimental diet (e.g. CON d1 versus low protein diet d1) using repeated measures ANOVA with two within-subject factors (diet [2] and time [7]). This approach was used to best examine the effects from the cross-over design. The Huynh-Feldt test was used to correct for violations of sphericity, and the significance set at $p \leq 0.05$. Statistical analyses of rhythm robustness and acrophase for both temperature and activity were conducted using paired-sample Student's *t* tests.

4.4 Results

4.4.1 Experiment 1

4.4.1.1 Indices of PEM

There were no significant differences in initial body weight between CON-28d and PEM-28d rats before commencement of the diet regimen ($p > 0.4$) (**Figure 4.1A**). A marked reduction in body weight compared to control rats was observed after 7d on the low protein diet ($p < 0.001$); body weight remained significantly decreased throughout the 28d period ($p < 0.001$). Whereas rats fed the CON diet gained 72% of their initial body weight after 28d of feeding, malnourished rats lost 5% of their initial body weight. As shown in **Figure 4.1B**, food intake decreased by wk 2 in the low dietary protein-fed group compared to control rats ($p=0.02$). Food consumption remained significantly lower in malnourished rats for each of the subsequent weeks ($p=0.01$). The 95% confidence interval around the mean food intake for each week is as follows:

Wk 1 – CON: 475.3 to 555.7, PEM: 412.3 to 543.9; Wk2 - CON: 508.1 to 585.9, PEM: 341.9 to 395.4; Wk3 – CON: 484.5 to 592.9, PEM: 244.9 to 305.3; Wk4 – CON: 521.6 to 604.0, PEM: 201.7 to 307.3. Body weight and food intake results were similar for the 7d of data collected in CON-7d and PEM-7d rats (n=6, data not shown). Liver lipid percentage was significantly elevated in protein-energy malnourished rats on d28 ($p < 0.001$) but not by d7 ($p = 1.00$) (**Figure 4.1C).**

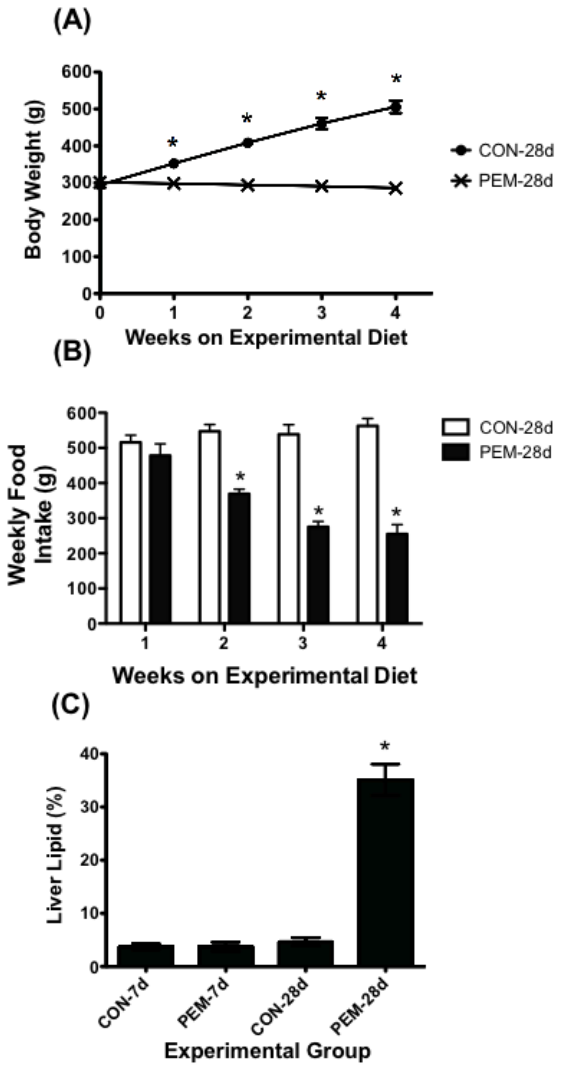

Figure 4.1. Effects of feeding a low protein diet for 28d on body weight (**A**), food intake (**B**) and liver lipid levels (**C**). Data are shown as mean ± SEM (some SEM bars are not visible due to small variability). Sample size for body weight and liver lipid is n=6. Food intake is based on cage data (n=2 cages [3 animals/cage]) and is presented as total food intake for each experimental week. *Indicates a significant difference between groups as detected by independent *t* test ($p < 0.05$).

4.4.1.2 Effects of a Low Protein Diet on the Acute-Phase Response

Serum albumin concentration was significantly decreased in malnourished rats at d7 ($p=0.005$) and remained significantly lower than CON rats on d28 ($p<0.001$) (**Figure 4.2A**). Conversely, serum A2M levels were significantly heightened by the low protein diet at both the 7 and 28d time-points ($p<0.001$) (**Figure 4.2B**). The low protein diet did not alter serum AGP concentration after 7d ($p=0.9$), but serum AGP concentration was decreased 3.5-fold in protein-energy malnourished rats by d28 ($p<0.001$) (**Figure 4.2C**).

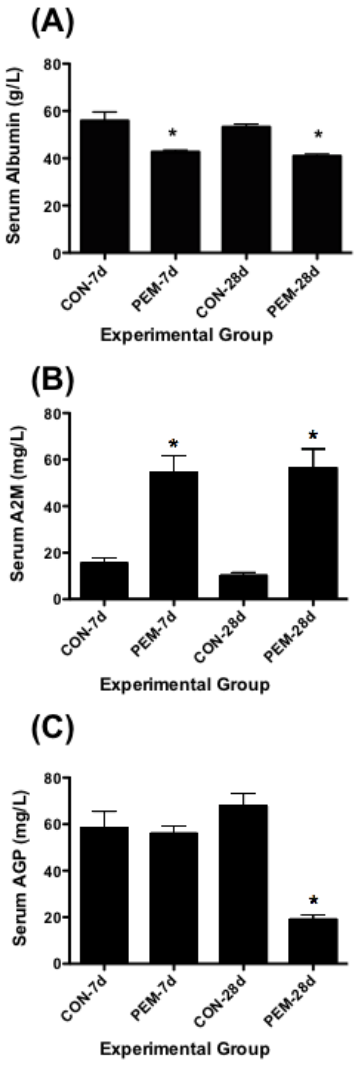

Figure 4.2. Decreased serum albumin (**A**) and increased serum A2M (**B**) concentrations are evident by d7 on a low protein diet, whereas serum concentrations of AGP are decreased by d28 (**C**). Data are shown as mean ± SEM, n=6. Rats received either CON or a low protein diet for 7 or 28d. *Indicates a significant difference from control group at the given time-point as detected by independent *t* test ($p < 0.05$).

4.4.1.3 Effects of a Low Protein Diet on the Hippocampal NFκB Signaling Pathway

The complete list of the 84 genes examined by PCR arrays is provided in **Table A.1**. Gene expression of NFκB signaling molecules was compared between diet groups at both time-points (7d, n=5; 28d, n=6). Out of the 84 genes examined, only 3 genes at 7d (Atf1, Smad3, and TNF) and 2 genes at 28d (Ifna1 and Nalp12) met the defined cutoff value of greater than a 2-cycle difference in average delta Ct between experimental groups and were included in the statistical analysis. Independent *t* tests demonstrated that neither acute nor chronic exposure to a low protein diet significantly altered the expression levels of these NFκB signaling genes.

4.4.1.4 Effects of a Low Protein Diet on Circadian Rhythm of Core Temperature

Figure 4.3 shows daily mean temperature and temperature amplitude as influenced by PEM over a 28d period. The acute (d1-7) and chronic (d8-28) phases of low protein diet were analyzed separately for CON-28d and PEM-28d rats. During the acute period, there was a significant interaction between diet and time for both daily mean temperature ($p=0.001$) and daily temperature amplitude ($p=0.001$). The small increase in daily mean temperature was transient, becoming significant by d2 on low protein diet ($p=0.01$), and normal temperatures were again observed by d4 on the diet regimen ($p=0.08$). Temperature amplitude for the group fed the low protein diet first became significantly greater than that of CON rats on d3 ($p=0.002$). These data were similar in the CON-7d and PEM-7d rats (n=6, data not shown).

As malnutrition progressed during the chronic phase (d8-28), the interaction between diet and time remained significant for both daily mean temperature ($p < 0.001$) and daily temperature amplitude ($p=0.05$). As shown in **Figure 4.3A**, after the initial increase in daily mean temperature during the acute phase of malnutrition, there was a continual decline in mean temperature throughout the chronic phase of PEM which eventually reached a plateau. In contrast, the increase in temperature amplitude appeared to be relatively constant in the malnourished rats throughout the 28d test period (**Figure 4.3B**).

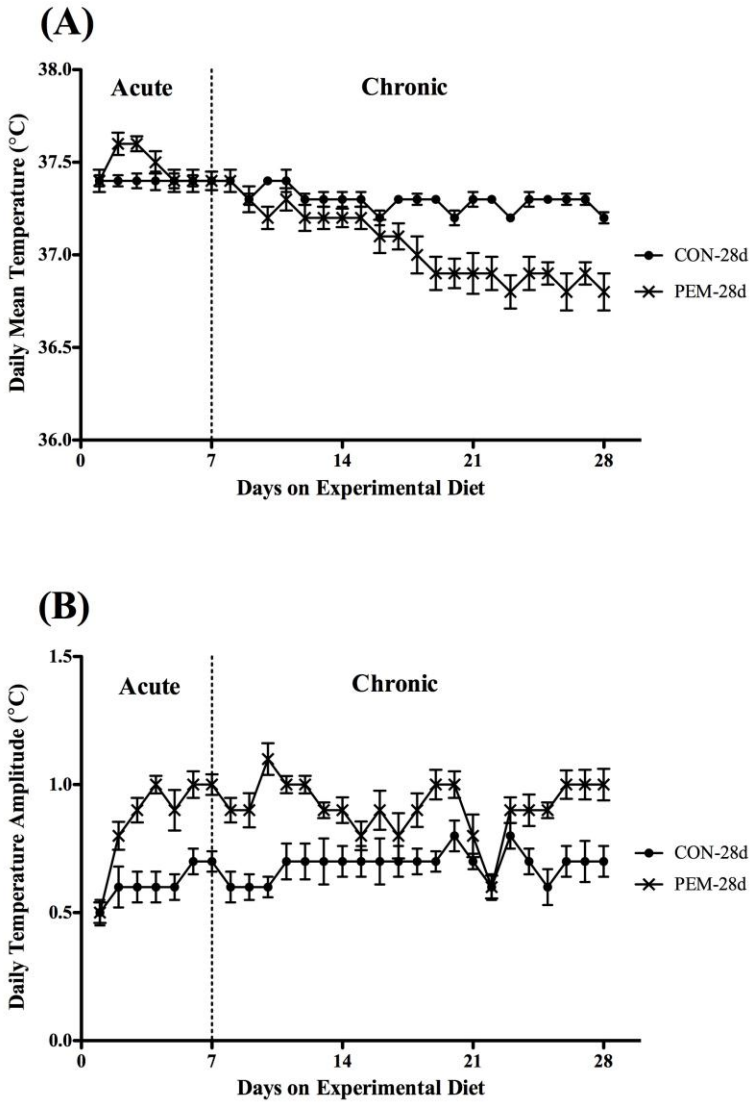

Figure 4.3. Daily mean temperature (**A**) and daily temperature amplitude (**B**) as influenced by chronic feeding of a low protein diet. Data are shown as mean ± SEM, n=6. Rats received either a CON or low protein diet for 28d. Results from repeated measures ANOVA demonstrated a significant interaction between diet and time for both the acute (1-7d) and chronic (8-28d) phases of malnutrition for daily mean temperature and daily temperature amplitude ($p \leq 0.05$). Independent-sample Student's *t* tests with Bonferroni correction were used for post-hoc comparisons on d2, 3, and 4. Rats on a low protein diet had a significantly higher daily mean temperature by d2 (p=0.01) to d4 (p=0.08); after this spike, mean temperature declined and eventually reached a plateau. Significantly greater daily temperature amplitude in the low protein-fed group developed by d3 (p=0.002).

4.4.2 Experiment 2

4.4.2.1 Body Weight

During the 7d recording period on CON diet, rats on average (± SEM) gained 96 ± 12 grams (35% body weight increase). The subsequent 7d on low protein diet resulted in stunting of growth (0.4% body weight decrease).

4.4.2.2 Activity and Temperature Response to Low Protein Diet

Activity and temperature rhythms are shown for both dietary intervals for 1 rat in **Figure 4.4,** which is representative of the pattern observed in all rats. Analogous to our findings from Experiment 1 (reported in Figure 4.3), there was an immediate increase in mean and amplitude of daily temperature after placement on low protein diet. The two-factor ANOVA with repeated measures demonstrated that there were no differences in daily mean activity (p=0.3) (**Figure 4.5A**) or daily activity amplitude (p=0.2) (**Figure 4.5B**) between the two diet regimens when assessed throughout the 7d feeding intervals.

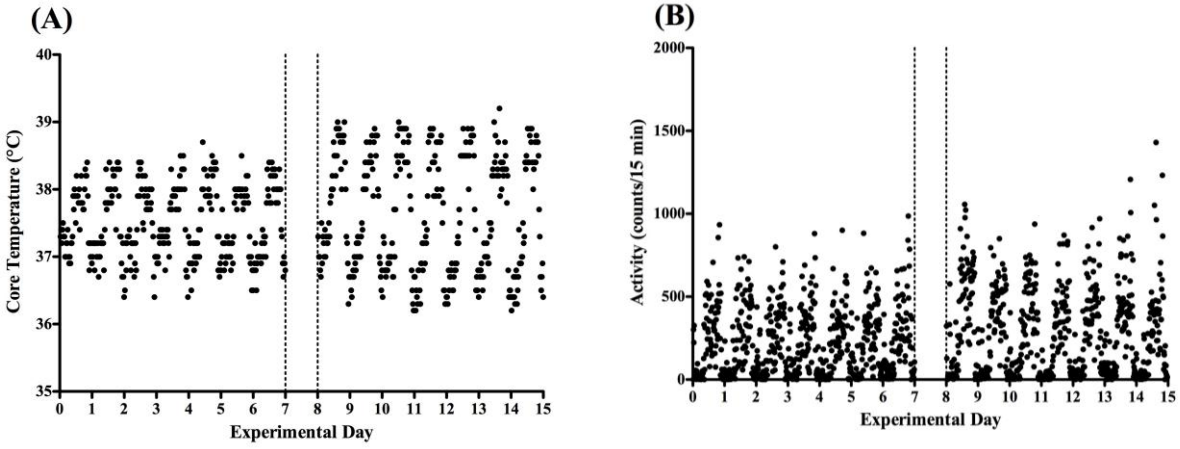

Figure 4.4. Representative patterns are shown for 1 rat during periods of CON diet (d0-7) and low protein diet (d8-15). Circadian rhythms of temperature (**A**) and activity (**B**) are displayed to highlight the immediate effect of a low protein diet on temperature that is not accompanied by an alteration in activity level. Core temperature readings were taken at 30 min intervals and activity at 15 min intervals. The dashed lines illustrate the transition day between experimental treatments.

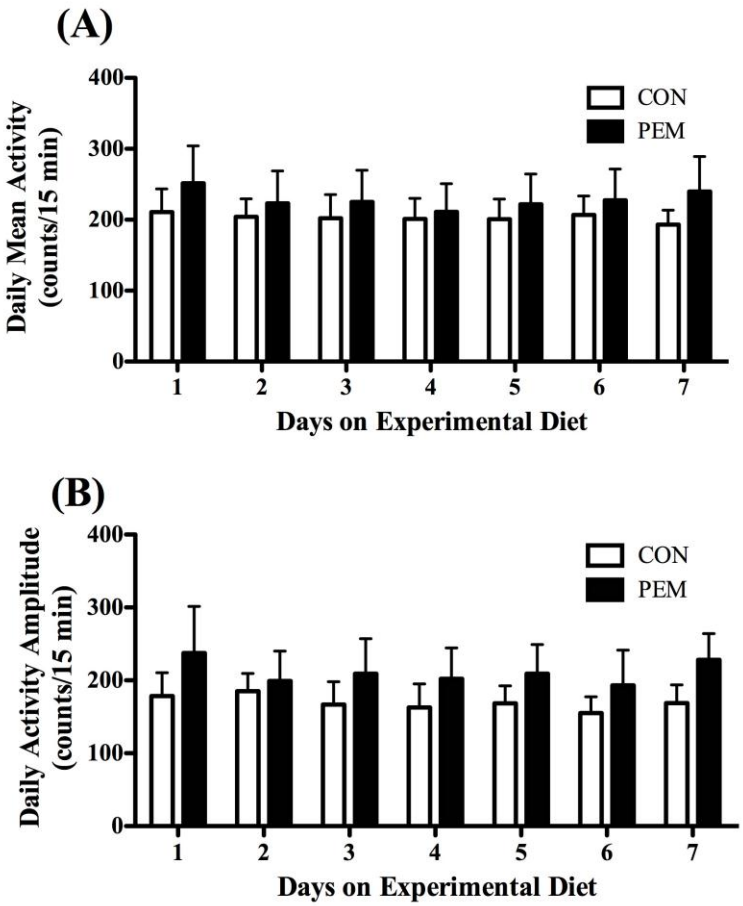

Figure 4.5. Exposure to a low protein diet for 7d had no effect on daily mean activity (**A**) and daily activity amplitude (**B**). Data are shown as mean ± SEM, n=4. Rats received CON diet for 7d and then, after a transition day, were transferred to a low protein diet (PEM) for the subsequent 7d. No effect of diet was detected by repeated measures ANOVA ($p > 0.05$).

4.4.2.3 Effect of PEM on Internal Synchronization

Acrophase and robustness were computed for the combined 7d on CON diet and compared to the 7d on low protein diet to determine whether acute malnutrition affects the internal synchronization of daily temperature and activity rhythms. As shown in **Table 4.2**, exposure to a low protein diet increased the robustness of both the rhythms of body temperature ($p=0.001$) and activity ($p=0.02$). Acrophase analysis, which calculates the peak time of the daily rhythm, showed that exposure to low protein diet significantly alters the acrophase of the activity rhythm ($p=0.02$) but not the temperature rhythm ($p=0.1$). More specifically, rats on low protein diet experienced an acrophase-advancement of the activity oscillation such that the peak of the daily cycle occurred approximately 1hr earlier. Although this resulted in a trend for activity and

temperature rhythms to be more superimposed, as identified by computing the APH-D (calculated time between the peak of the activity cycle and the peak of the temperature cycle), this was not statistically significant (p=0.06).

Table 4.2. Acrophase, robustness and acrophase difference of temperature and activity rhythms between periods of control and low protein diets

	CON	PEM
Temperature		
Acrophase (hr)	1.2 ± 0.3	0.9 ± 0.1
Robustness (%)	61 ± 4	75 ± 4*
Activity		
Acrophase (hr)	2.1 ± 0.3	1.2 ± 0.1*
Robustness (%)	28 ± 4	42 ± 5*
Acrophase Difference		
APH-D (hr)	1.1 ± 0.3	0.2 ± 0.1

Acrophase and robustness were calculated for the 7d on CON diet and low protein diet (n=4). Values are expressed as mean ± SEM. Acrophase difference (APH-D) is the calculated time between the daily activity acrophase and temperature acrophase. *Indicates a significant difference between groups as detected by a paired sample *t* test ($p < 0.05$).

4.5 Discussion

Our study was designed to determine if PEM, which often co-exists with stroke (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008), has sustained effects on physiological determinants of ischemic brain injury. PEM was induced by feeding a 2% protein diet. Total food intake and body weight over the 28d experimental period were depressed by 36% and 43%, respectively, and liver lipid content was increased by 647%. The early effects of the suboptimal protein intake were evident by d7 as a reduction in growth. However, the energy status of the rat was not yet compromised, given that voluntary reduction in food intake occurred during the second week on the low protein diet.

The findings provide evidence that PEM can stimulate an acute-phase response, as demonstrated by an increase in serum A2M, a positive acute-phase protein, which is indicative of inflammation (Cray et al., 2009). The decrease in serum albumin, a major negative acute-phase protein, provides further evidence, with the caveat that the additional influence of decreased

dietary amino acid supply for synthesis prevents its use as a specific marker of inflammation (Qu et al., 1996). Thus, dietary protein depletion appears to act as an independent inflammatory stimulus, and the effects occur rapidly. One week on a low protein diet was sufficient time to induce alterations in both serum A2M and albumin. The evolution of PEM sustained alterations in these acute-phase reactants for 28d, which further suggests that the inflammation is chronic in nature.

However, not all acute-phase proteins responded similarly to PEM. In response to systemic inflammation, the liver generally produces abundant levels of positive acute-phase proteins. In contrast to the influence of PEM on serum A2M, AGP was decreased after 28d, suggesting an aberrant acute-phase response. The inability to mount a complete acute-phase response is most likely due to limited amino acid availability for protein synthesis. Lyoumi et al. (1998) also observed varying effects of severe protein malnutrition on the induction and modulation of the acute-phase response in rats. Whereas AGP gene expression was not influenced by severe protein deficiency, A2M expression was elevated in the liver. These results suggest that A2M production has precedence over AGP synthesis when the animal is confronted with either acute or chronic exposure to low protein diet. The differential acute-phase protein response may be due to the specific inflammatory cytokine milieu, including IL-1 and IL-6, which modulate the acute-phase response. IL-1 mainly regulates AGP, a class 1 acute-phase protein, whereas A2M belongs to class 2 and is generally induced by IL-6 (Baumann et al., 1989). A limitation of our study is the absence of serum cytokine measurements, which would have provided more direct evidence for inflammation and assisted interpretation of the variability in the acute-phase protein response. Previous study results indicate that PEM can trigger an elevation in circulating IL-6 (Ling et al., 2004), which could drive increased A2M production.

Our data suggest that PEM-induced systemic inflammation does not extend to the hippocampus. No effects on the hippocampal NFκB signaling pathway were detected by measuring the expression of 84 genes encoding members of the Rel, NFκB, and IκB families, receptors activating NFκB signaling, and NFκB-responsive genes. These results were unexpected, given our previous findings of augmented NFκB activation in the hippocampus of protein-energy malnourished gerbils (Ji et al., 2008). The previous study relied on an electrophoretic mobility shift assay to assess the transcriptional activity of NFκB by measuring the extent of binding of nuclear extracts to NFκB consensus sequences (Ji et al., 2008). To

address whether this would alter transcription regulation, real time PCR arrays were used in the current study to measure NFκB-responsive gene expression. There are several possible explanations for the apparent discrepancy in the findings. Although there may be increased localization of NFκB to the nucleus with augmented transcription of target genes, this may be undetectable with PCR if mRNA stability is influenced by PEM, resulting in either rapid translation or degradation of the sequence. Another possible explanation is poor sensitivity of the microarray technique. Alternatively, hippocampal NFκB activation may have been unaltered by the malnutrition paradigm used in the current study design. The 2% protein AIN-93G-based diet fed in the current study to rapidly growing adolescent rats generates a different degree of PEM than the previously used 2% protein AIN-93M-based diet fed for 28d to the slowly growing adult gerbil (Ji et al., 2008). Other inconsistencies in the literature might also be related to differences in the extent of malnutrition and tissue or cell type. Increased NFκB activation has been reported in peritoneal macrophages from protein-energy malnourished mice, whereas this effect disappeared when the cells were stimulated ex vivo with LPS (Fock et al., 2010). In contrast, NFκB activation was unaltered in unstimulated peritoneal macrophages from severely protein-deficient mice (when the reduced energy intake was controlled for), while protein deficient cells showed decreased NFκB activation when treated with LPS. Li et al. (2002) reported increased liver NFκB activation in protein-energy malnourished mice stimulated in vivo with LPS. Further research is necessary to confirm whether PEM is an independent stimulus of NFκB-mediated signaling in brain and whether co-existing malnutrition would exacerbate brain inflammation triggered by stroke. Given that the hypothalamus is likely responsible for the disturbances in core temperature discussed below, future investigations should examine the effects of PEM on hypothalamic NFκB-mediated signaling and inflammation.

A striking pattern of change in core temperature emerged as malnutrition evolved from an acute to chronic state. This observation was possible by the use of implantable temperature probes that monitor diurnal cycles in unanaesthetized animals without triggering a stress response. Exposure to a low protein diet caused an immediate small and transient increase in daily mean temperature and a larger sustained increase in temperature amplitude. Throughout the 28d test period, chronically malnourished rats experienced warmer temperatures during the active period of the day and cooler temperatures during the sleep period, resulting in a persistent increase in daily amplitude. Following the initial ~2d increase, mean core temperature gradually

descended to below normal values as malnutrition progressed, resulting in a difference of 0.4-0.5°C during the final week of malnutrition. Given the small magnitude, the temperature changes are not likely to alter ischemic brain injury (MacLellan et al., 2009). The absence of a clear febrile pattern in the presence of systemic inflammation is perplexing, but we hypothesize that the relationship between temperature and inflammation is lost when the inflammatory response is less pronounced.

It is difficult to compare our chronic temperature results against the other two studies that have used telemetry probes to accurately measure core temperature changes in response to suboptimal diet (Castanon-Cervantes & Cintra, 2002; Duran et al., 2008). The low protein diets (6% casein) administered in the previous studies were fed from pregnancy through to adolescence or adulthood, and the malnutrition was poorly characterized. Castanon-Cervantes et al. (2002) reported short-term higher daily mean cortical temperature and amplitude in malnourished rats of comparable age to the rats used in our study. A decrease in mean core (Duran et al., 2008) but not cortical (Castanon-Cervantes & Cintra, 2002) temperature was observed when these rats were studied until ~550d of age, suggesting a dissociation of temperature regulation. If this is the case, the common practice of using core probes to estimate brain temperature changes after brain ischemia in rodent models of stroke (DeBow & Colbourne, 2003) may be inaccurate for studies of post-ischemic malnutrition.

Simultaneous measurements of body temperature and activity in the second study refuted the hypothesis that the initial transient rise in body temperature on exposure to low protein diet might be explained by hyperactivity associated with increased foraging behavior. The results clearly demonstrate that daily activity mean and amplitude were unaffected during the 7d on low protein diet, a finding in agreement with that from a study of more mildly malnourished adolescent mice (Dietrich et al., 2004). The transient rise in core temperature on exposure to the low protein diet may, in fact, be caused by the increased postabsorptive metabolic rate (thermogenesis) occurring with low dietary protein-to-energy ratio (Rothwell & Stock, 1987). Our data indirectly support this since rats initially experienced higher than normal temperatures during the active period of the day when food consumption is greatest, but experienced only slight decreases in temperature during the inactive phase. Daily fluctuation in temperature adjusted over time, as shown by cooler temperatures during the sleep cycle, resulting in normal

mean temperature. As malnutrition progressed further, core temperature gradually fell below normal.

Given that our study was designed to measure the immediate response to presentation of a low protein diet and measurements were obtained for 7d only, we cannot rule out the possibility that chronic PEM causes internal desynchrony between activity and temperature rhythms. In fact, exposure to low dietary protein did alter the timing of the activity circadian rhythm. Acrophase for the activity cycle occurred approximately 1hr earlier during the 7d interval on low protein diet than during the 7d on control diet. However, the phase relationship between activity and temperature was not significantly altered. A surprising finding was the stronger rhythm robustness for both activity and temperature when rats were on low protein diet. Since neurotransmitter and circadian systems interact (Ciarleglio et al., 2011; Meijer et al., 2010), the effects of the low protein diet on the circadian rhythms might be mediated by altered amino acid availability for neurotransmitter production. Although malnutrition-induced alterations in neurotransmitter systems, such as γ-aminobutyric acid and serotonin, have been most studied in the developing brain (Wiggins et al., 1984), there is also evidence for effects of a low protein diet provided during adolescence and adulthood (Andrade & Paula-Barbosa, 1996; Yokogoshi et al., 1987).

In summary, exposure to low dietary protein with subsequent development of PEM has rapid and sustained effects on variables known to be key determinants of ischemic brain injury. This was demonstrated by a systemic inflammatory response and disruptions in the circadian rhythm of core temperature. Whereas an acute-phase response could contribute to poorer outcome when PEM co-exists with stroke, the persistent effects of protein-energy depletion on body temperature are small in magnitude and thus unlikely to directly influence ischemic brain injury. The current results suggest that the PEM-induced inflammation does not extend to the hippocampus, although other key brain regions such as hypothalamus should be studied. Finally, hyperactivity does not explain the immediate increase in core body temperature that occurs on exposure to a low protein diet.

4.6 Experimental Progression of Thesis

A major finding from this study was that PEM induces an atypical acute-phase response, which could be a mechanism by which PEM adversely affects outcome after brain ischemia (Dziedzic, 2008; Whiteley et al., 2009). However, the effects of PEM on the acute-phase

response may differ following brain ischemia. Previous evidence indicates that PEM can hinder the ability to mount a complete acute-phase response to injury or infection (Jennings & Elia, 1996; Reid et al., 2002). A similar pattern may emerge when the stimulus is global brain ischemia or the associated surgical manipulations. Therefore, this relationship was assessed in the third study by determining whether the effects of PEM on the acute-phase response persist in the presence of global brain ischemia. In order to address this question, serum levels of acute-phase proteins were measured in rats exposed to global brain ischemia and fed a low protein diet. In addition to the three acute-phase proteins reported in Chapter 4, haptoglobin was also assessed since it has been shown to be a sensitive and useful marker of acute inflammation in the rat (Giffen et al., 2003).

Although no pronounced changes to the NFκB cascade were detected in the hippocampus of protein-energy malnourished rats, the effects of PEM on neuroinflammation could be much greater in response to brain ischemia. Therefore, this relationship was assessed in the final study. Microglia and macroglia are the major inflammatory cells following global brain ischemia and remain activated in the hippocampus for extended periods of time (≥ 270d) (Langdon et al., 2008). Therefore, the glial response was assessed in the third study to determine whether PEM that develops after global brain ischemia augments the chronic neuroinflammatory response and affects the neuroplastic response in the hippocampus.

CHAPTER 5:
PROTEIN-ENERGY MALNUTRITION DEVELOPING AFTER GLOBAL BRAIN ISCHEMIA INDUCES AN ATYPICAL ACUTE-PHASE RESPONSE AND HINDERS NEUROPLASTICITY

5.1 Abstract

Protein-energy malnutrition (PEM), characterized by inadequate protein and energy status, is a common stroke co-morbidity that is associated with poor outcome. PEM is present in 12-19% of stroke patients upon admission, with prevalence rates rising to 20-35% at one week and reaching 35-49% by the time of admission to a rehabilitation unit. PEM induces a systemic acute-phase response, and there is indirect evidence that co-existing PEM exacerbates neuroinflammation after global brain ischemia, both of which may adversely affect ischemic outcome. Since a heightened inflammatory response can hinder neuroplasticity, this effect of PEM may impede brain-remodeling processes following global brain ischemia. Therefore, the aim of this study was to investigate the degree to which post-ischemic PEM (developing after brain ischemia) alters neuro- and systemic inflammation and determine what impact this may have on neuroplasticity. Male, Sprague-Dawley rats (~9 wk old) were subjected to global forebrain ischemia via the 2-vessel occlusion model and either placed on a control (18% protein) or low protein (PEM, 2% protein) diet at 3d following surgery. A third group was exposed to control diet and sham surgery. To assess the immediate and longer-term effects of a low protein diet, samples were collected at either 5 or 21d post-surgery. No systemic acute-phase reaction attributable to global ischemia was detected, as reflected by serum concentrations of positive acute-phase proteins (alpha-2-macroglobulin [A2M], alpha-1-acid glycoprotein [AGP], and haptoglobin) and a negative acute-phase protein (albumin) ($p > 0.54$). Acute exposure to a low protein diet after global ischemia decreased the serum concentrations of albumin ($p<0.001$) and haptoglobin ($p<0.001$) at 5d, with both proteins remaining significantly decreased at 21d.

Whereas serum concentrations of AGP were not influenced by PEM ($p>0.26$), A2M levels were significantly higher in malnourished rats at 21d ($p=0.001$). GFAP and Iba-1 immuno-labeling illustrated a strong hippocampal glial response to global brain ischemia, which was unaltered by PEM. Conversely, post-ischemic PEM decreased GAP-43 and synaptophysin within the CA3 mossy fiber terminals at 21d following global brain ischemia. Thus, these findings demonstrate that exposure to PEM following global brain ischemia induces a persistent but aberrant acute-phase response. Post-ischemic PEM hinders some key neuroplasticity mechanisms triggered by brain ischemia, but these changes do not appear to be related to an exacerbation of reactive gliosis.

5.2 Introduction

PEM, a common type of malnutrition characterized by inadequate protein and energy status, has been associated with poorer survival and functional outcome following stroke (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). Approximately 1 out of 6 stroke patients are protein-energy malnourished at the time of admission to the hospital (Davis et al., 2004; Martineau et al., 2005; Yoo et al., 2008). Nutritional status often worsens during post-stroke hospital stay, with prevalence rates reaching as high as 20-35% at one week (Brynningsen et al., 2007; Yoo et al., 2008) and 35-49% by the time of admission to a rehabilitation unit (Finestone et al., 1995; Poels et al., 2006). No mechanistic studies have assessed the effects of PEM when it develops after brain ischemia (post-ischemic PEM) on post-stroke brain repair. Thus, with the use of a rat model of global brain ischemia, a major goal was to investigate whether post-ischemic PEM influences the inflammatory response and determine the extent to which this affects neuroplasticity.

The neuroinflammatory response to brain ischemia is thought to both contribute to neuronal injury and affect neuroplasticity (Kriz & Lalancette-Hébert, 2009). Microglia and macroglia, the predominant responders to cerebral ischemic injury, are triggered within hours following global brain ischemia (Morioka et al., 1991; Webster et al., 2009). The glial response is persistent and can remain heightened within the hippocampal CA1 subregion for up to 270 days after global brain ischemia in the rat (Langdon et al., 2008). Previous findings suggest that co-existing PEM (PEM that is present at the time of insult) can exacerbate the glial response to global brain ischemia in the gerbil (Bobyn et al., 2005) and increase activation of the predominant pro-inflammatory transcription factor, NFκB (Ji et al., 2008). When PEM does not

develop until after brain ischemia, the effects may be quite different, since there is less potential to affect the acute inflammatory response to brain ischemia. Nevertheless, since neuroinflammation following brain ischemia can have a marked chronic component (Langdon et al., 2008), it was hypothesized that post-ischemic PEM would exert adverse effects on cellular function by exacerbating and/or prolonging the inflammatory response. Interestingly, the effects of PEM on NFκB activation, independent of global ischemia, have also been investigated, with inconsistencies in the findings. Although PEM independently increased NFκB activation in the hippocampus of malnourished gerbils (Ji et al., 2008), the study described in Chapter 4 detected no alteration in NFκB transcription regulation in the hippocampus of the protein-energy malnourished rat.

Post-ischemic inflammation extending through the brain recovery phase can play a major role in regulating the innate repair processes that are engaged in the injured brain, known as neuroplasticity (Kriz & Lalancette-Hébert, 2009). Activated glial cells express and secrete various biomolecules that are capable of altering dendritic growth, synapse number and synaptic structure. Secretion of growth factors by microglia can promote neuroplasticity (Banati, 2002), while over-expression of pro-inflammatory cytokines can impair recovery processes (Bellinger et al., 1995; Katsuki et al., 1990). If the hypothesis that post-ischemic PEM will augment the inflammatory response to brain ischemia is correct, this in turn could impede brain remodeling. Chronic protein deprivation has been reported to induce neuroplastic deficits within the hippocampus. Protein deprivation during adulthood in rats can reduce the number of CA1 and CA3 pyramidal neurons, synapses within the CA3 mossy fiber region and CA3 dendritic branches (Andrade et al., 1991; Andrade et al., 1995; Andrade et al., 1996; Lukoyanov & Andrade, 2000; Paula-Barbosa et al., 1989). One previous study demonstrated increased hippocampal expression of the plasticity-associated proteins, GAP-43 and trkB, when PEM co-existed with the onset of global brain ischemia (Prosser-Loose et al., 2010). While these findings do not support the hypothesis that PEM will hinder plasticity mechanisms, it is proposed that the effects will be different when PEM develops during the acute phase after brain ischemia, when brain remodeling is initiated. Also, whereas the previous study addressed only short post-ischemic sampling times (1, 3 and 7d), the current study had the objective of assessing both early and later stages of brain remodeling. GAP-43 and synaptophysin were chosen as markers of brain remodeling, given their roles as plasticity-associated proteins that are up-regulated in the

hippocampus following global brain ischemia (Ishimaru et al., 2001; Prosser-Loose et al., 2010; Schmidt-Kastner et al., 1997).

In addition to the detrimental role that neuroinflammation can have on neuronal function, the systemic response to brain ischemia may also influence outcome. The acute-phase response, which is systemic in nature, includes a subset of proteins whose plasma concentrations change by a factor of several fold in response to acute and chronic inflammation. Exposure to inflammatory conditions can result in elevated concentrations of various positive acute-phase proteins (e.g., alpha-2-macroglobulin [A2M], haptoglobin, alpha-1-acid glycoprotein [AGP], c-reactive protein), while at the same time causing depleted levels of negative acute-phase proteins (e.g., albumin, transferrin). Cerebral ischemia can trigger an acute-phase reaction, although considerable variation in this systemic response exists between stroke patients (Dziedzic, 2008; Idicula et al., 2009; Ryu et al., 2009). Although clinical findings suggest that the extent of the acute-phase reaction predicts outcome in stroke patients (Dziedzic, 2008; Idicula et al., 2009; Ryu et al., 2009), it is unclear whether this response plays a detrimental role in the ischemic cascade or merely reflects insult severity. In contrast to stroke, the extent to which global brain ischemia stimulates an acute-phase response has not been characterized.

The influence of declining nutritional status after brain ischemia on systemic inflammation is likely to be complex. While evidence from both clinical and animal studies suggests that PEM can blunt the ability to mount a controlled inflammatory response to injury or infection (Jennings et al., 1992; Jennings & Elia, 1996; Reid et al., 2002), evidence is also emerging that PEM can serve as an independent stimulus of systemic inflammation. Animal models have demonstrated increased serum levels of TNF-α, IL-1β, IL-6, AGP and A2M in rats fed a low protein diet (Ling et al., 2004; Lyoumi et al., 1998). Recent findings reported in Chapter 4 demonstrate that acute and chronic malnutrition can stimulate an acute-phase response; however, the effect of PEM on the production of individual acute-phase proteins is variable (Smith et al., 2013). Thus, the final objective was to investigate whether the effects of PEM on the acute-phase response would persist after global ischemia. This was examined using the rat 2-VO global brain ischemia model, in which any acute-phase reaction triggered by brain ischemia or the associated surgical manipulations has yet to be characterized.

Given the high prevalence of PEM developing after stroke, the aim of this study was to address whether post-ischemic PEM alters brain and systemic inflammation and determine what

impact this may have on neuroplasticity. Global brain ischemia was modeled by 2-VO in the rat, with a low protein diet being introduced at 3 days post-surgery. Time-points of 5 and 21 days were used to investigate both the immediate response to a low protein diet, as well as the chronic effects of PEM. Serum levels of albumin, A2M, AGP, and haptoglobin were measured to assess the acute-phase response. Iba-1 and GFAP were measured to assess the glial response, while GAP-43 and synaptophysin were examined as markers of neuroplasticity.

5.3 Materials and Methods

5.3.1 Animals

Sixty-four male Sprague-Dawley rats (52-55d old) (Charles River Canada, QC, Canada) were acclimatized on rat chow for 2d before placement on a protein adequate control diet (CON, 18% protein) (Smith et al., 2013). Rats were housed in groups of 2-4 and maintained on a 12hr light/dark cycle in a temperature controlled room with free access to food and water. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

5.3.2 Global Brain Ischemia Surgery

Following a 4-6d acclimation period on CON diet, rats were subjected to either sham surgery (**Sham**) or transient forebrain ischemia via the 2-VO model (**ISC**), as previously described (Smith et al., 2011) and modified from Smith et al. (1984). Surgical procedures were performed aseptically under isoflurane anaesthesia (1.75-4% in 70% N_2O and 30% O_2). Before surgery, rats were fasted for ~16 hr to achieve consistent blood glucose levels. Following anaesthesia induction, animals were placed on a heated water blanket and a tympanic probe was inserted to estimate brain temperature (IT-18 flexible probe; Physitemp Instruments Inc. NJ, USA). Temperature was maintained close to 37.5°C with the use of an overhead infrared lamp (250W) controlled by an automated feedback temperature controller (CN9500; Omega Engineering Inc., CT, USA). Tail artery cannulation allowed for continuous measurement of mean arterial blood pressure (PressureMAT PDKTP4-PCS; PendoTech, NJ, USA) and collection of arterial blood samples (100 μL) for measurement of blood gases, hematocrit, and glucose concentration. Both common carotid arteries were isolated and the right jugular vein was cannulated. Blood was withdrawn via the jugular vein into a warmed heparinized syringe until blood pressure reached ~35 mmHg. At this time, micro-aneurysm clips (S&T Vascular Clamps HD-S; Fine Science Tools, BC, Canada) were applied to both carotid arteries for 10 min. Blood

pressure was maintained at 35-40 mmHg throughout the occlusion period by withdrawing or infusing blood as needed. After releasing the clamps, blood was slowly reinfused and incisions were sutured. A bupivacaine dose (2mg/kg) was divided equally for injection around the 2 incision sites. Sham rats were treated identically except that carotid arteries were not occluded and hypotension was not induced. Rats were singly housed until post-surgical d3, at which time the rats were placed back with their original cage mates.

5.3.3 Diet Assignment

At 3d following surgery, rats were randomly assigned to either a protein-deficient diet (PEM, 2% protein) or remained on the CON diet (18% protein) (Dyets, Inc., PA, USA). Diet composition is shown in Table 4.1. Rats of this age fed a 2% protein diet voluntarily reduce food consumption, thereby causing a deficit in both energy and protein intake (Prosser-Loose et al., 2011; Smith et al., 2013). Diets were modified from the American Institute of Nutrition-93G diet (Reeves et al., 1993) to not contain the antioxidant, tertiary-butylhydroquinone (Smith et al., 2013). Body weight and food intake were recorded on surgery day (d0), d1, and d2. Following diet assignment (d3), food intake was recorded daily and body weight recorded weekly and on perfusion day. The six experimental groups generated were: CON-Sham5d (n=9), CON-ISC5d (n=13), PEM-ISC5d (n=12), CON-Sham21d (n=8), CON-ISC21d (n=11), and PEM-ISC21d (n=11).

5.3.4 Euthanasia and Histology

At 5 or 21d post-surgery, rats were anaesthetized under isoflurane (5% in 100% O_2) and blood samples were collected via cardiac puncture. Rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Intact heads were submerged into 4% paraformaldehyde and stored overnight at 4°C. Brains were removed and post-fixed in paraformaldehyde for an additional 24 hr. Following fixation, brains were submerged into a 20% sucrose solution and stored at 4°C for 3d to prevent ice crystal formation and tissue cracking. Fixed brains were placed in plastic cryomolds and embedded in Optimal Cutting Temperature (OCT) compound. Samples were flash frozen using dry ice-cooled isopentane and stored at -80°C until sectioning. For each rat, the coronal section (14 μm) corresponding to -3.8 mm from Bregma was stained with cresyl violet, while adjacent sections were used for immunofluorescence. Assessment of cell counts and immunofluorescence semi-quantification was blinded.

Cresyl violet stained sections were examined under a light microscope (400x) using a 200μm square (10x10) microscope grid. Viable-looking pyramidal neurons, with intact nuclear and cellular membranes, were counted bilaterally in the medial, middle, and lateral sectors of the CA1 region (-3.8 mm relative to Bregma) (Paxinos & Watson, 1998). CA1 cell counts from the right and left hemisphere were summed to generate the total neuron count for each rat.

5.3.5 Immunofluorescence

Identical protocols were performed using Shandon Sequenza Immunostaining Racks (Thermo Scientific, CHS, UK) to immuno-label coronal sections for astrocytes (GFAP), activated microglia (Iba-1), and neuroplasticity markers (synaptophysin and GAP-43). Sections were blocked with normal goat serum (5%; Sigma-Aldrich, ON, Canada) and incubated at 4°C overnight in a primary antibody either for astrocytes (Rabbit anti-GFAP, 1:400; Z0334, DakoCytomation, ON, Canada), microglia (Rabbit anti-Iba-1, 1:1000; 019-19741, Wako Chemicals, VA, USA), synaptophysin (Mouse anti-synaptophysin, 1:200; Millipore, clone SY38, Millipore, MA, USA) or GAP-43 (anti-GAP-43, 1:2500; 9-1E12 Ascites, Dr. David Schreyer). Following a wash step with PBS, slides were incubated in a secondary fluorescent antibody (Goat-anti-mouse AlexaFluor488, 1:200, 115-545-166, Jackson Laboratories, PA, USA; Goat-anti-rabbit AlexaFluor 594, 1:500, A11012, Molecular Probes, Invitrogen) in the dark for 2 hr. Slides were washed with PBS and cover slipped with ProLong Gold Antifade Reagent with DAPI (P36931, Molecular Probes, Invitrogen) in order to visualize cell nuclei. Negative control sections were treated identically, except that the sections were not incubated in the primary antibody.

Semi-quantification of each marker was completed using one section per hemisphere from every rat. The integrated density value (IDV=sum of pixel values in the region of interest) (ImageJ, U.S. National Institutes of Health, MD, USA) was measured for each region of interest using high magnification photographs captured from a fluorescent microscope. All densitometry measurements were adjusted for background staining, as calculated from negative control sections. GFAP and Iba-1 were measured within the CA1 hippocampal region by placing a box (pixel area = 265816) that included the CA1 pyramidal layer, stratum oriens and stratum radiatum. A second box was placed in the CA3 mossy fiber terminals (pixel area = 265816). Synaptophysin was predominantly within the CA3 region and therefore semi-quantification was only performed in the mossy fiber terminals (pixel area = 78402). GAP-43 was semi-quantified

in both the CA1 and CA3 hippocampal subregions. For stratum oriens and stratum radiatum analysis within the CA1 region, a box (pixel area = 45360 each) was placed along each layer and the values were summed. In the CA3 region, a box was placed in the mossy fiber terminals (pixel area = 141778) and another box was placed along the CA3 stratum oriens layer (pixel area = 141778). For each marker and time-point, individual densitometry measurements were normalized by expressing them as a ratio to the mean IDV of the respective CON-Sham treatment group. Values from the right and left hemispheres were averaged. The photographs shown below were taken under identical conditions at higher magnification.

5.3.6 Serum Acute-Phase Proteins

Blood samples collected at the time of euthanasia were allowed to clot for 30 min at room temperature and then centrifuged at 1,500 x g for 10 min. Following centrifugation, serum was collected and stored at -80°C. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the serum concentrations of A2M, AGP, and haptoglobin (Immunology Consultants Laboratory, Inc., OR, USA). The bromocresol green method was utilized to measure serum concentration of albumin (Doumas et al., 1971). A volume of 25 μL of blank, serum sample, or standard solution was added to 5.0 mL bromocresol green reagent (0.15 mmol/L bromocresol green, 0.075 mol/L succinate buffer, 30% Brij-35). After 30 min, absorbance was measured spectrophotometrically at 628 nm (Biochrom Ultrospec 3100 Pro). Linear regression was used to determine the albumin concentration of the sample.

5.3.7 Statistical Analysis

Statistical analyses were conducted using SPSS 20.0 for Windows. All data are presented as mean ± SEM and the significance set at $p<0.05$. Body weight and food intake differences between sham-operated and ischemic rats were analyzed on post-surgical d0, 1, 2, and 3 using an independent-sample Student's t-test. Following assignment to experimental diet, food intake was analyzed for rats fed the low protein diet or CON diet on d4, 5, 6 and 7 using an independent-sample Student's t-test. Significant differences in body weight, CA1 neuronal cell counts, and serum acute-phase protein concentrations among the three treatment groups were determined for each time-point using a 1-factor ANOVA and Tukey's test where indicated. Immunofluorescence data were analyzed using an independent-sample Student's t-test.

5.4 Results

5.4.1 Physiological Parameters Measured During Surgery

Table 5.1 is a summary of the physiological parameters obtained during all 2-VO and sham surgeries. The three treatment groups are shown separately, in order to illustrate baseline data prior to treatment assignment, although all rats were still on CON diet at the time of surgery. Mean pre- and post-ischemic values for all physiological parameters were within the target range. Tympanic temperature and blood pressure were tightly regulated throughout the 10 min occlusion period and recorded at 1 min intervals. The resulting mean values for tympanic temperature and blood pressure in the two groups exposed to global ischemia were within the desired range; however, several individual rat values fell out of range for 1-2 consecutive readings. Five additional rats were excluded from the study due to surgical complications (e.g. blood pressure readings fell out of range for an extended period of time, complications with tail artery cannulation) or poor recovery (e.g. severe weight loss).

5.4.2 Impact of Surgery on Food Intake and Body Weight

Mean initial body weight on surgery day was not significantly different between sham and ischemic treatment groups, when all rats were on CON diet ($p = 0.71$; Sham, n=17; ISC, n=47). Following surgery, there was a significant difference in body weight on d1, 2 and 3 ($p < 0.001$), with rats exposed to the 2-VO surgery weighing less than sham animals. Individual food intake was monitored on post-surgical d1, 2, and 3, which was possible due to individual rat housing (Sham, n=17; ISC, n=47). Daily food intake was significantly decreased in the ischemic treatment group compared to sham-operated rats on d1, 2 and 3 ($p < 0.041$).

Table 5.1. Physiological parameters measured during 2-VO and Sham surgeries

		CON-ISC	PEM-ISC	CON-Sham	Desired Range
Pre-Ischemic Values	pH	7.42±0.01	7.42±0.01	7.43±0.01	7.25-7.45
	pCO2 (mmHg)	38±1	37±2	35±1	35-45
	pO2 (mmHg)	130±2	130±2	125±2	125-140
	Hct (%)	38.1±0.6	40.0±0.7	37.8±0.7	~ 40
	Glucose (mmol/L)	5.6±0.2	5.6±0.2	5.4±0.2	4-8
Post-Ischemic Values	pH	7.37±0.01	7.33±0.02	7.42±0.01	7.25-7.45
	pCO2 (mmHg)	38±1	42±2	36±1	35-45
	pO2 (mmHg)	135±3	135±3	131±3	125-140
	Hct (%)	35.0±0.8	36.1±0.9	38.9±0.9	~ 40
	Glucose (mmol/L)	5.5±0.2	5.1±0.2	5.5±0.1	4-8
Intra-Ischemic Values	Tympanic Temperature (°C)	37.3±0.01	37.4±0.02	37.5±0.01	37.5±0.2
	Blood Pressure (mmHg)	36±0.1	35±0.1	86±3	35-40 (ISC)

Data are expressed as mean ± SEM (CON-ISC, n=24; PEM-ISC, n=23; CON-Sham, n=17). Mean pre- and post-ischemic values for all measured physiological parameters fell within the desired range for all surgical groups.

5.4.3 Indices of PEM

Figure 5.1A shows the pattern of body weight change starting on post-surgical d3, as a result of experimental diet assignment for rats in the 21d treatment groups (CON-Sham21d, n=8; CON-ISC21d, n=11; PEM-ISC21d, n=11). At 7d post-surgery (4d after diet assignment), there was a significant difference in body weight ($p < 0.001$), with PEM-ISC rats weighing significantly less than CON-Sham ($p < 0.001$) and CON-ISC ($p < 0.001$) rats; this continued throughout the 21d post-surgical period ($p < 0.001$). Malnourished rats gained 6% of their initial body weight by d21, compared to a 59% increase observed in both the CON-ISC and CON-Sham treatment groups. This resulted in a difference in final body weight between the PEM-ISC group and both CON-fed groups of 33%.

Figure 5.1B shows mean (± SEM) daily food intake starting on post-surgical d4 (1d after diet assignment) for rats fed the low protein (PEM-ISC21d, n = 5 cages [2-3 rats/cage]) or CON diet (CON-Sham21d + CON-ISC21d, n = 8 cages [2-3 rats/cage]). Mean food intake was

calculated based on cage data (daily cage food intake/number of rats per cage). Since CON-ISC and CON-Sham rats were housed together, differences between these groups could not be analyzed. This also potentially biased the analysis due to the unequal sample sizes. A significant decrease in food intake in the PEM-ISC group was first reported on post-surgical d7 (4d after diet assignment), as compared to the other two CON groups combined ($p = 0.026$). Total food intake over 18 days on experimental diet was depressed by 21% in PEM-ISC rats. Rats in the 5d treatment groups presented with similar body weight and food intake patterns (data not shown).

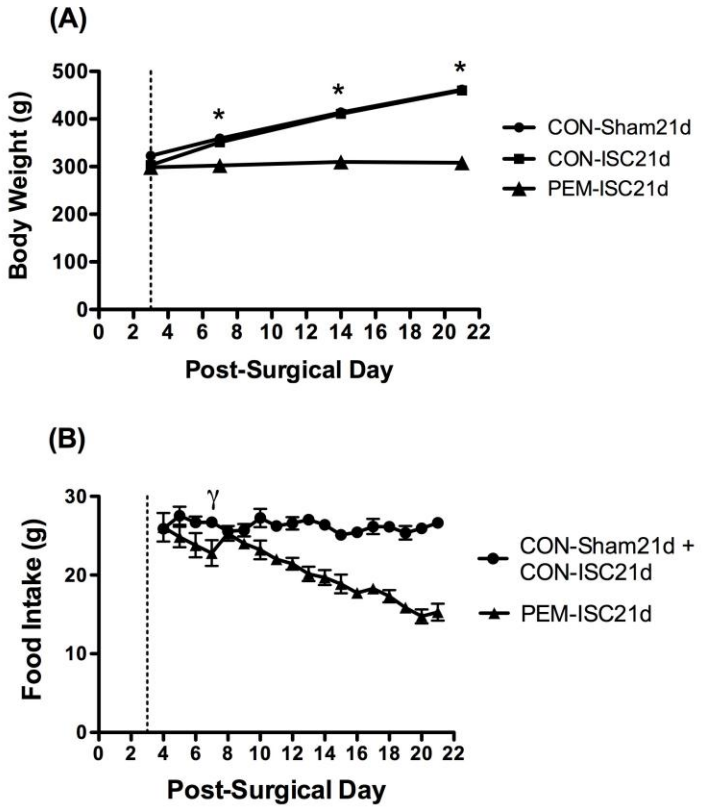

Figure 5.1. The protein-deficient diet introduced on d3 after global brain ischemia depressed body weight (**A**) and food intake (**B**). Data are shown as mean ± SEM for the 21d treatment groups. The dashed lines illustrate the day on which rats were assigned to experimental diet. Food intake was collected daily on a cage basis (CON-Sham21d + CON-ISC21d, n = 8 [2-3 rats/cage]; PEM-ISC21d, n = 5 cages [2-3 rats/cage]). Food intake was calculated as daily cage food intake/number of rats per cage. Body weights are shown for d3, d7, d14 and d21 (CON-Sham21d, n=8; CON-ISC21d, n=11; PEM-ISC21d, n=11). *Indicates a significant effect of experimental diet on body weight (PEM-ISC compared to CON-Sham and CON-ISC groups) as detected by 1-factor ANOVA and Tukey's Test ($p<0.05$). **γ**Indicates the first day on which PEM-ISC rats experienced a significant reduction in food intake, when compared to the other two CON groups combined, as detected by an independent-sample Student's t-test ($p<0.05$).

5.4.4 CA1 Neuronal Death

Hippocampal CA1 neuronal counts are shown in **Table 5.2**. At both 5 and 21d post-ischemia, there was extensive CA1 neuronal death in PEM-ISC and CON-ISC rats compared to CON-Sham rats ($p < 0.001$), but no significant differences between the PEM-ISC and CON-ISC rats ($p > 0.87$). Since the 2-VO model of global brain ischemia yields some variability in ischemic cell death, this statistical analysis excluded those rats exposed to 2-VO surgery that showed CA1 neuronal damage that was unilateral or minimal (<50% reduction in CA1 neurons in either hemisphere). This was done after first verifying that there were no significant differences in neuronal loss between the PEM-ISC and CON-ISC groups. That is, it was first demonstrated that excluding rats with incomplete forebrain ischemia did not bias the results by masking an effect of PEM on neuronal death at either time-point (t-test; $p > 0.16$). Out of the 47 rats that underwent the 2-VO surgery, 9 rats showed unilateral CA1 neuronal death and 1 rat had no apparent damage (CON-ISC5d, n=2; PEM-ISC5d, n=4; CON-ISC21d, n=3; PEM-ISC, n=1). Analyses of the acute-phase response and immunofluorescence data were conducted exclusively on rats presenting with extensive bilateral hippocampal damage at 5d (CON-Sham, n=8; CON-ISC, n=11; PEM-ISC, n=8) and 21d (CON-Sham, n=8; CON-ISC, n=7; PEM-ISC, n=10).

Table 5.2: PEM initiated at 3d after global brain ischemia does not exacerbate hippocampal CA1 neuronal cell death

	CON-Sham	**CON-ISC**	**PEM-ISC**
5d	258 ± 10 (n=8) **a**	46 ± 7 (n=11) **b**	41 ± 7 (n=8) **b**
21d	241 ± 10 (n=8) **a**	27 ± 7 (n=7) **b**	22 ± 4 (n=10) **b**

Data are presented as mean ± SEM. Only those rats demonstrating bilateral hippocampal injury were included in analysis. Groups within a row with different superscripts are significantly different by 1-factor ANOVA and Tukey's Test ($p<0.001$).

5.4.5 Glial Response

Iba-1 was examined by immunofluorescence at 5 and 21d following global brain ischemia within the CA1 and CA3 subregions of the hippocampus (**Figure 5.2A-B)**. At 5d following surgery, Iba-1 signal was increased by ~3.4 fold in the CON-ISC group (versus CON-

Sham) within the CA1 hippocampal region, with no significant difference observed between the two ischemic experimental groups ($p = 0.85$). By d21, the effect of ischemia on Iba-1 signal within the CA1 region was more pronounced (~6.4 fold difference between CON-ISC and CON-Sham), but dietary treatment had no effect (PEM-ISC versus CON-ISC; $p = 0.95$). Iba-1 was less increased by global ischemia in the CA3 subregion (1.8 fold difference between CON-ISC and CON-Sham at d21), with no significant differences between the two ischemic groups at either time-point ($p > 0.49$).

Figure 5.2C-D shows the results from GFAP immunofluorescence at 5 and 21d post-surgery within the CA1 and CA3 subregions of the hippocampus. At post-surgical d5, GFAP immuno-staining was increased within the CA1 region by 1.8 fold in the CON-ISC group (versus CON-Sham), with no differences between the two ischemic experimental groups ($p = 0.98$). A similar response was present at the later time-point, with a fold increase of 1.7 in the CON-ISC group (versus CON-Sham), but dietary treatment had no effect (PEM-ISC versus CON-ISC; $p = 0.91$). Within the CA3 subregion, the effects of ischemia on GFAP signal was marginal (1.2 fold difference between CON-ISC and CON-Sham at d21), and exposure to a low protein diet after brain ischemia did not alter the GFAP response at either time-point as compared to the CON-ISC group ($p > 0.59$).

Figure A.1 shows the raw un-normalized data for Iba-1 and GFAP within the CA1 hippocampal subregion, which corresponds to the normalized data (normalized to respective mean of CON-Sham) presented in Figure 5.2. The data are presented as the IDV (mean ± SEM) for CON-Sham, CON-ISC and PEM-ISC treatment groups at 5 and 21d.

Representative photographs are presented in **Figure 5.3** showing the distribution of Iba-1 and GFAP within the CA1 hippocampal region for each experimental treatment group at 5 and 21d.

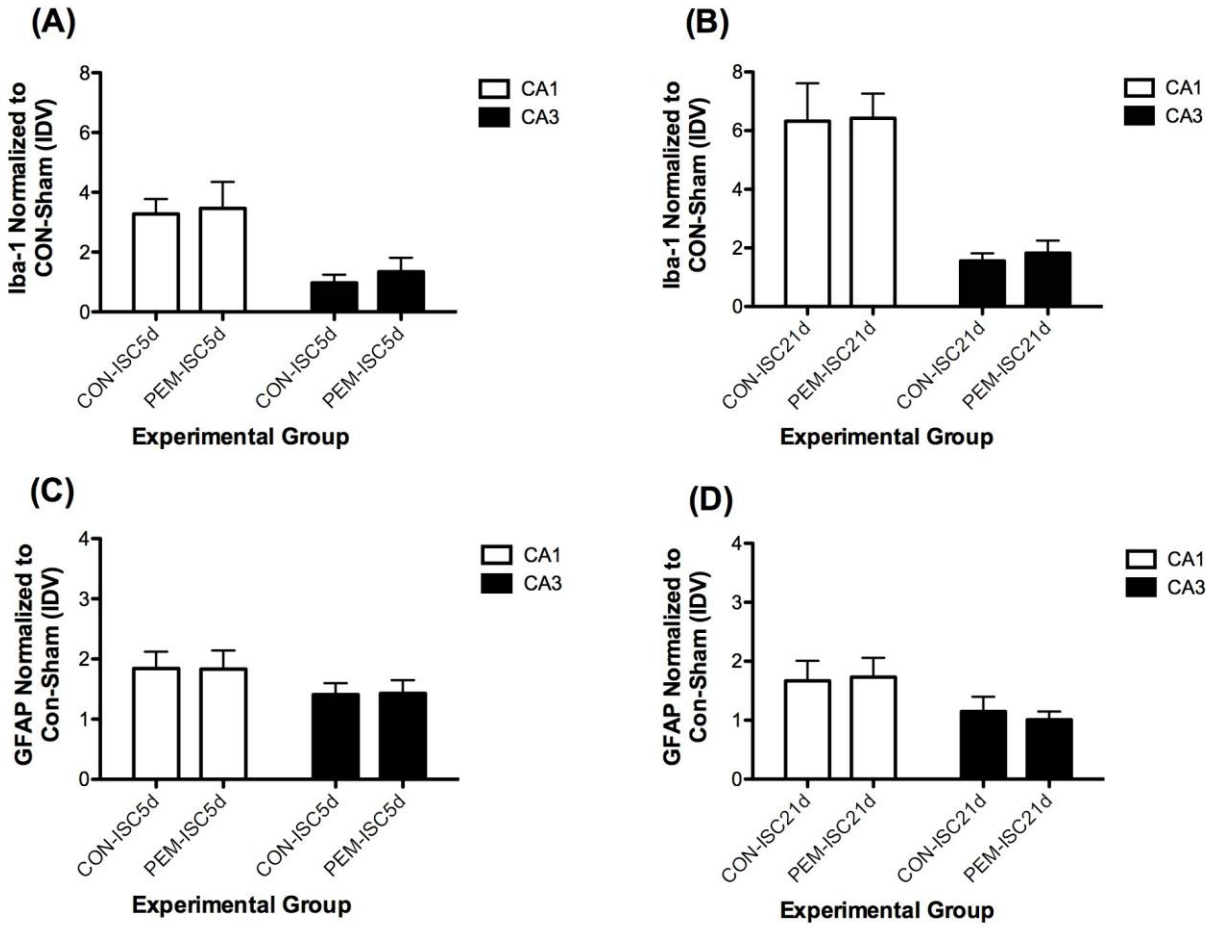

Figure 5.2. PEM introduced at 3d post-surgery did not alter the hippocampal glial response that is elicited by global brain ischemia. The glial response was evaluated in the hippocampal CA1 and CA3 subregions by immuno-labeling for Iba-1 at 5d (**A**) or 21d (**B**) and GFAP at 5d (**C**) or 21d (**D**). Results are expressed as change in fluorescence relative to the respective mean of the CON-Sham treatment group. Results are shown as mean ± SEM integrated density value (IDV) for each hippocampal region examined.

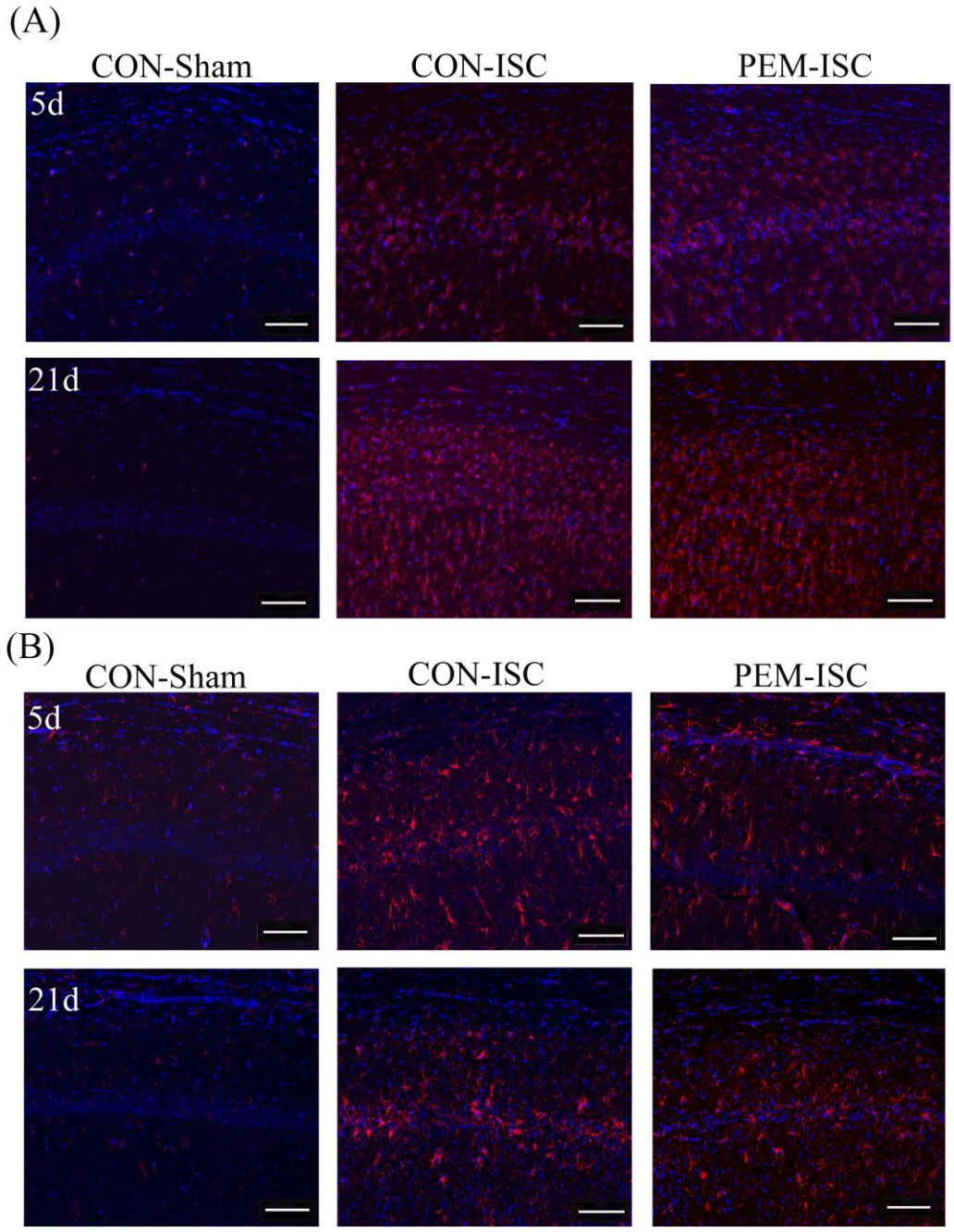

Figure 5.3. PEM introduced at 3d following global brain ischemia did not significantly alter Iba-1 (**A**) or GFAP (**B**) within the CA1 hippocampal region. Representative photographs of the CA1 hippocampal region for each experimental treatment group at 5 and 21d show DAPI (blue; staining of cell nuclei) and either Iba-1 (red; activated microglia marker) or GFAP (red; astrocytic marker). Scale bar = 100 μm.

5.4.6 Neuroplasticity

GAP-43 was examined by immunofluorescence at 5 and 21d following global brain ischemia within the CA1 and CA3 subregions of the hippocampus (**Figure 5.4.A-B).** GAP-43 signal within the CA1 hippocampal subfield was not different between the two ischemic groups at either time-point ($p > 0.39$). GAP-43 immuno-staining was predominantly within the CA3 stratum oriens, but there was no difference between CON-ISC and PEM-ISC rats at 5d ($p = 0.73$) and 21d ($p = 0.20$). Within the CA3 mossy fiber terminals, GAP-43 did not differ between the two ischemic groups at 5d ($p = 0.52$). However, GAP-43 signal within the CA3 mossy fiber region at d21 was significantly decreased in the PEM-ISC rats when compared to CON-ISC animals ($p = 0.017$).

Results from semi-quantification of synaptophysin immunofluorescence are shown in **Figure 5.4C-D**. At 5d, there were no significant differences in synaptophysin immuno-staining within the CA3 mossy fiber terminals between the two ischemic experimental groups ($p = 0.47$). However, by d21, synaptophysin signal within the CA3 mossy fiber terminals was significantly depressed in PEM-ISC rats as compared to the CON-ISC group ($p = 0.042$).

Figure A.2 shows the raw un-normalized data for GAP-43 and synaptophysin within the CA3 mossy fiber region, which corresponds to the normalized data (normalized to respective mean of CON-Sham) presented in Figure 5.4. The data are presented as the IDV (mean ± SEM) for CON-Sham, CON-ISC and PEM-ISC treatment groups at 5 and 21d.

Representative photographs are presented in **Figure 5.5** showing the distribution of GAP-43 and synaptophysin within the CA3 hippocampal region for each experimental treatment group at post-surgical d5 and 21.

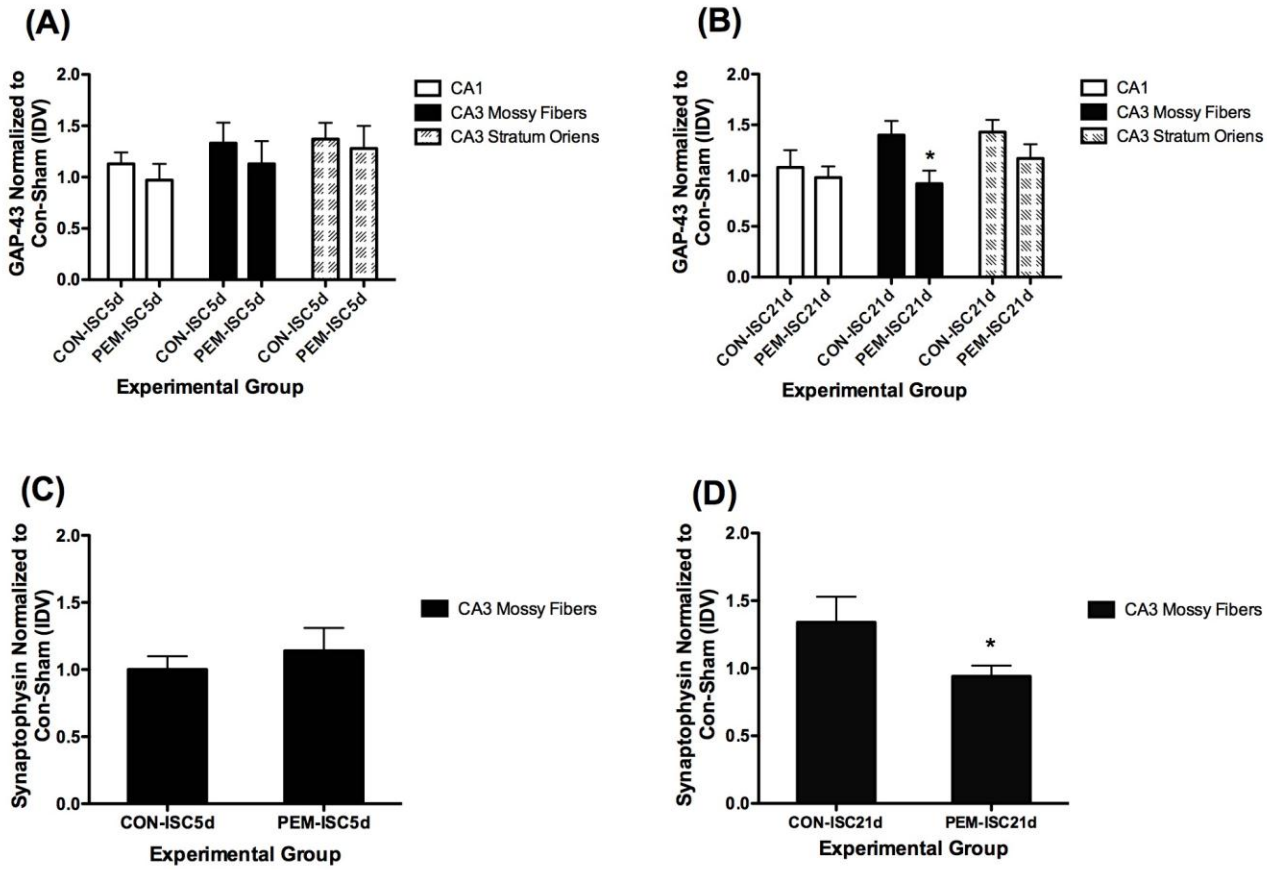

Figure 5.4. PEM introduced at 3d post-surgery decreased GAP-43 and synaptophysin within the CA3 mossy fibers at d21 following global brain ischemia. GAP-43 was evaluated in both the CA1 and CA3 hippocampal subregions at 5d (**A**) and 21d (**B**). Synaptophysin was evaluated in the CA3 mossy fibers at 5d (**C**) and 21d (**D**). Results are expressed as change in fluorescence relative to the respective mean of the CON-Sham treatment group. Results are shown as mean ± SEM integrated density value (IDV) for each hippocampal region examined.

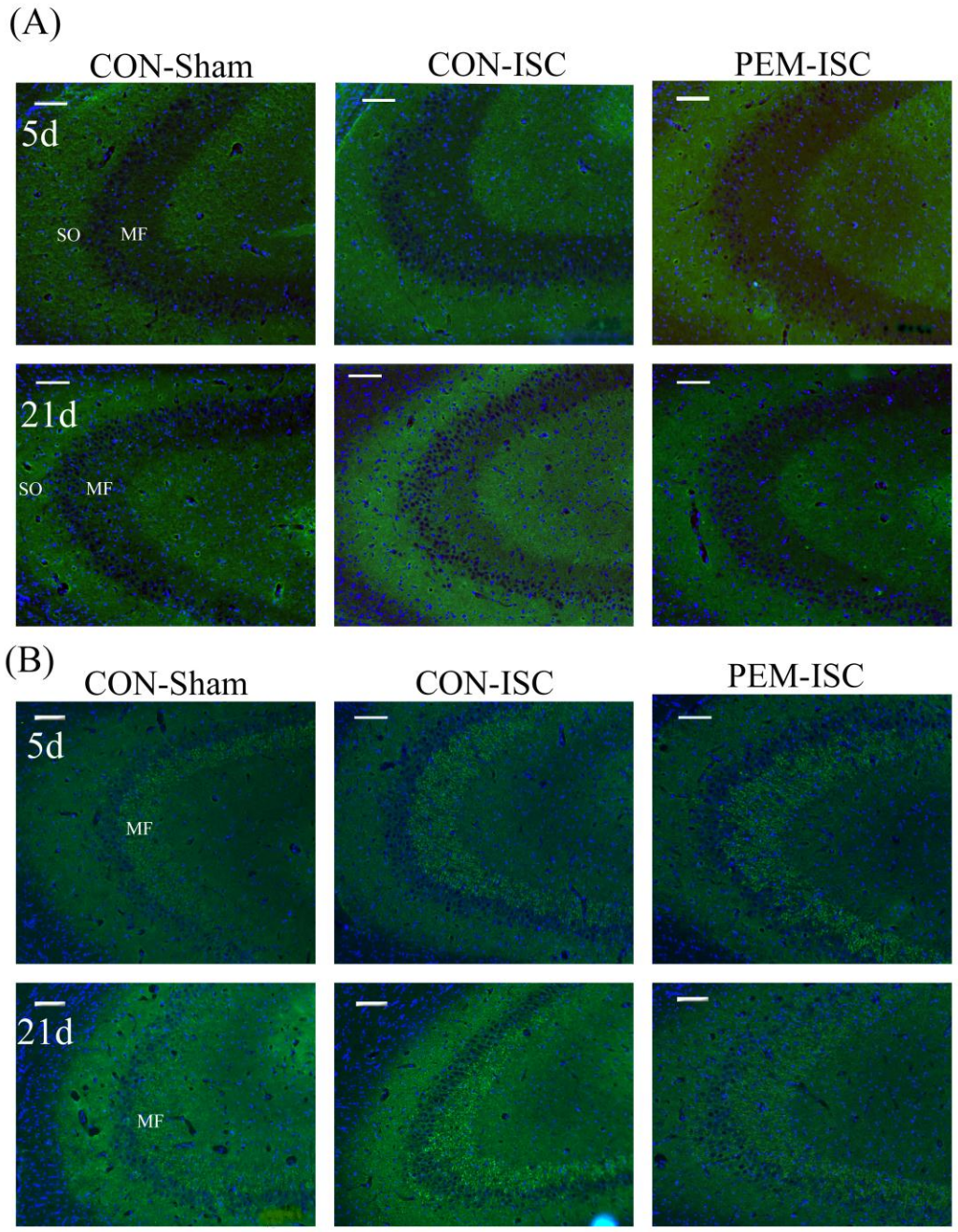

Figure 5.5. PEM developing after global brain ischemia significantly decreased GAP-43 (**A**) and synaptophysin (**B**) within the CA3 mossy fiber bundles at 21d. Representative photographs of the CA3 hippocampal region for each experimental treatment group at 5 and 21d show DAPI (blue; staining of cell nuclei) and either GAP-43 (green) or synaptophysin (green). Semi-quantification of immuno-staining was performed for the regions indicated (SO, stratum oriens; MF, mossy fibers). Scale bar = 100 μm.

5.4.7 Acute-Phase Response

Figure 5.6 shows the serum concentrations of the acute-phase proteins at 5 and 21d post-surgery. Analysis of CON-ISC and CON-Sham rats for each time-point showed no significant differences between these two treatment groups for any protein, suggesting that this model of global brain ischemia does not induce an acute-phase response ($p > 0.40$). Two days of exposure to a low protein diet after global ischemia had no significant effect on serum A2M concentration compared to the CON-ISC group ($p = 0.20$), but levels were significantly increased at 21d in PEM-ISC rats ($p < 0.004$). The short-term exposure to the protein deficient diet following global brain ischemia caused significant decreases in serum concentrations of haptoglobin ($p < 0.01$) and albumin ($p < 0.001$) relative to CON-ISC levels. At d21, concentrations of both of these proteins remained low in the PEM-ISC group, but albumin concentration was significantly different between CON-ISC and PEM-ISC ($p = 0.001$), whereas haptoglobin concentration was not ($p = 0.35$). Serum AGP concentrations in the PEM-ISC group were not significantly altered relative to CON-ISC levels, at either time-point ($p > 0.53$).

The raw data for the four acute-phase proteins are presented as scatter plots in **Figure A.3** showing the serum concentrations for individual animals. Data from individual rats are plotted to highlight the inter-animal variability within each treatment group. Inter-animal variability for serum albumin is much lower relative to the three positive acute-phase proteins measured. Although the results were not statistically analyzed by time, an apparent increase in A2M and haptoglobin at d5 in the CON-Sham and CON-ISC treatment groups, which was diminished by d21, suggests a surgical response.

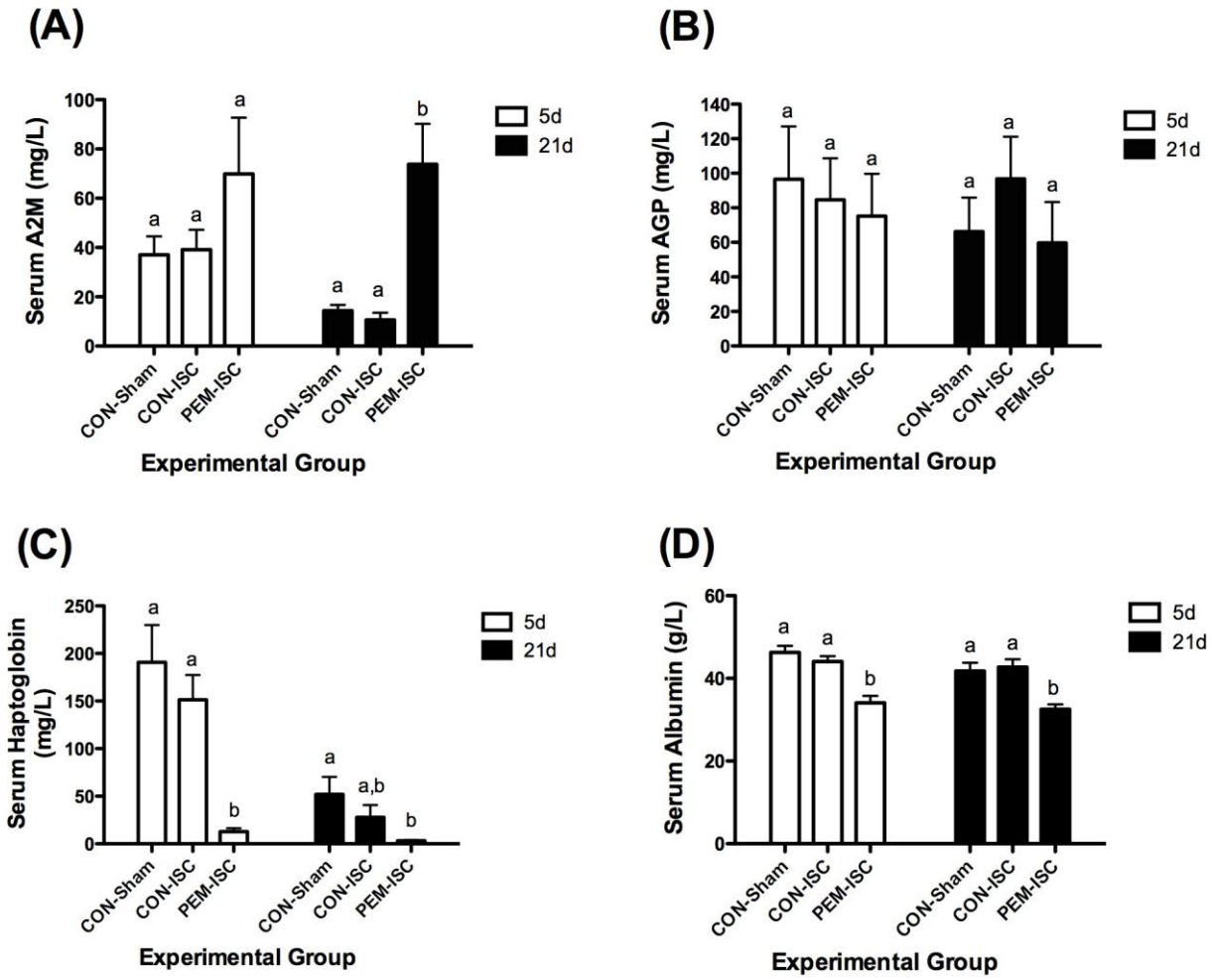

Figure 5.6. PEM introduced at 3d following global brain ischemia causes an atypical acute-phase response. Data are shown as mean ± SEM. Alpha-2-macroglobulin (A2M) **(A)**, alpha-1-acid glycoprotein (AGP) **(B)**, and haptoglobin **(C)** are positive acute-phase proteins, while albumin **(D)** is a negative acute-phase protein. Experimental groups with different superscripts at the same time-point are significantly different, as detected by 1-factor ANOVA and Tukey's test ($p<0.05$).

5.5 Discussion

The major findings from this study were the effects of post-ischemic PEM on both the neuroplasticity and acute-phase responses following global brain ischemia. To assess the effects of post-ischemic PEM, a low protein diet was introduced at 3 days after surgery. Unlike the study described in Chapter 4 showing that healthy rats voluntarily reduce their food intake by week 2 on a diet containing 2% protein (Smith et al., 2013), the current study demonstrated that this response occurs more quickly when diet is introduced within days of brain ischemia. A significant decrease in food intake and body weight was evident by post-surgical day 7 (after 4 days on low protein diet). Total food intake and body weight over the 18 day period on low protein diet were depressed by 21% and 33% respectively. The early effects of the suboptimal diet suggest that the malnourished rats were under additional stress during the acute period following brain ischemia. This theory is supported by the data demonstrating that exposure to the low protein diet for 2 days was sufficient time to significantly alter the serum concentrations of the acute-phase proteins, albumin and haptoglobin.

Stroke triggers an acute-phase reaction in a subset of patients, with considerable variation in the response existing between individuals (Dziedzic, 2008; Idicula et al., 2009; Ryu et al., 2009; Smith et al., 2006). The rat model of global brain ischemia employed in the current study, however, does not appear to trigger an acute-phase response, since there were no differences between CON-ISC and CON-Sham rats for any of the serum acute-phase reactants measured. However, it is inferred that the surgical procedures required to induce brain ischemia do trigger an acute-phase response, given the considerably higher A2M and haptoglobin concentrations in the CON-Sham and CON-ISC groups at 5 days after surgery compared to the levels measured on post-surgical day 21. To definitively confirm that a surgical effect exists, serum levels of these acute-phase proteins would have to be compared with those of un-operated rats. This finding also raises an important limitation in using the currently available rat models of stroke to study an acute-phase response, since all major pre-clinical stroke models require a surgical approach.

A major goal of this study was to address whether PEM would induce an acute-phase response when the experimental diet was introduced following global brain ischemia. Findings from this study demonstrate that the introduction of a low protein diet following global brain ischemia does cause both immediate and persistent changes to the circulating concentrations of certain acute-phase proteins. Serum albumin levels were appreciably lower in the PEM-ISC

group at 5 days following global ischemia. As malnutrition progressed, serum albumin levels remained decreased in PEM-ISC rats while serum concentrations of the positive acute-phase protein, A2M, were increased. However, not all acute-phase proteins responded similarly to PEM. Whereas AGP serum levels were not influenced by PEM, the positive acute-phase protein, haptoglobin, was immediately decreased by exposure to low dietary protein. This pattern was sustained up to day 21, with haptoglobin barely detected in PEM-ISC rats at this time. Given that haptoglobin is a positive acute-phase protein, these results suggest that post-ischemic PEM induces an aberrant acute-phase reaction. The latter results suggest that acute dietary protein deprivation and the evolution of PEM can diminish an acute-phase response to surgery, which is in agreement with reports that PEM can dampen the ability to mount a controlled inflammatory response to stimuli such as injury or infection (Jennings et al., 1992; Jennings & Elia, 1996; Reid et al., 2002). However, the addition of a PEM-Sham treatment group would have more clearly defined the interactions among global ischemia, surgery, and PEM on the acute-phase response.

These findings are comparable to those observed for PEM independent of exposure to global ischemia (reported in Chapter 4) (Smith et al., 2013). Both studies reported decreased serum albumin concentrations and increased serum A2M levels in malnourished rats. Although the decrease in AGP previously reported with 28 days of feeding was not observed in the current study, it is speculated that a similar finding would have been obtained if the same time-point were used. Results from both studies suggest that when mounting an acute-phase reaction in response to PEM, A2M production has precedence over both AGP and haptoglobin synthesis. This notion is strengthened by the findings from Lyoumi et al. (1998), which reported an elevation in A2M expression within the liver of malnourished rats, while AGP production was unaltered.

A limitation of the study is the absence of data on regulatory inflammatory cytokines. The aberrant acute-phase response observed in protein-energy malnourished rats in two independent studies (Chapter 4 and Chapter 5) is most likely associated with alterations in the circulating concentrations of regulatory inflammatory cytokines. Both AGP and haptoglobin are type 1 acute-phase proteins, which are mainly regulated by IL-1 and TNF-α, while A2M is a type 2 acute-phase protein predominantly stimulated by IL-6 (Petersen et al., 2004). Together, the data reported here and in Chapter 4 suggest that production of type 2 acute-phase proteins has precedence over the synthesis of type 1 acute-phase proteins in malnourished rats. It is

speculated that exposure to a low protein diet triggers an elevation in IL-6, which in turn stimulates A2M synthesis. This notion is strengthened by the findings of Ling et al. (2004), who reported augmented IL-6 serum concentrations in protein-energy malnourished rats. Given that amino acid availability is limited in protein-energy malnourished rats, it is hypothesized that this reduction in specific type 1 acute-phase proteins is an attempt to conserve amino acids for producing proteins such as A2M more efficiently.

Although post-ischemic PEM caused a systemic acute-phase reaction, the strong hippocampal glial response elicited by global brain ischemia appears to be unaffected by a low protein diet and the evolution of PEM over 18 days. Given that glial cells are the sole source of inflammatory cells following global brain ischemia (Kato, 2001), these findings lead to the conclusion that PEM arising after global brain ischemia does not exacerbate brain inflammation. Nevertheless, findings from this study assist in the characterization of the glial response elicited after global brain ischemia in the 2-VO model. Our results suggest that microglia and astrocytes remain within the CA1 subregion for up to 21 days after global brain ischemia. Given that Iba-1 is a protein expressed by activated microglia (Suter et al., 2007), it is speculated that this population of inflammatory cells is comprised of phagocytic microglia participating in a strong immune response. Similarly, Silasi et al. (2011) demonstrated that microglial proliferation continues for at least 3 weeks within the CA1 hippocampal region following global brain ischemia. Furthermore, the hippocampal microglial response has been detected for up to 270 days following global brain ischemia (Langdon et al., 2008), although it is unclear whether these inflammatory cells are in an activated state.

PEM developing acutely after brain ischemia does appear to hinder neuroplasticity mechanisms typically triggered by global brain ischemia, since the increased signal for GAP-43 and synaptophysin within the mossy fibers of CON-ISC rats after 21 days was not present in PEM-ISC rats. Notably, these alterations were not triggered by a greater extent of hippocampal CA1 or CA3 (data not shown) neuronal death in PEM-ISC rats, and the trigger does not appear to be increased brain inflammation. These findings suggest that post-ischemic PEM causes a loss of synapses in CA3 mossy fibers following global brain ischemia. A similar response has been reported in adult rats subjected to dietary protein deprivation. Chronic exposure to a low protein diet initiated during adulthood has been shown to cause a 30% reduction in the number of synapses within the CA3 mossy fiber terminals (Andrade et al., 1991; Andrade et al., 1995;

Lukoyanov & Andrade, 2000). Furthermore, protein deprivation in adult rats can result in decreased numbers of dendritic branches within the CA3 and dentate gyrus hippocampal subfields (Andrade et al., 1996), along with considerable loss of granule cells and pyramidal CA1 and CA3 neurons (Lukoyanov & Andrade, 2000; Paula-Barbosa et al., 1989). These hippocampal deficits were present in adult rats after prolonged periods on a low protein diet lasting between 6-18 months. Results from the current study, however, suggest that exposure to a low protein diet for a few weeks is sufficient time to cause alterations in the neuroplasticity response to brain ischemia. Together, these reports suggest that malnutrition could impede synaptic remodeling that occurs after brain ischemia. Ultimately, since plasticity promotes recovery from stroke damage, disruptions to these mechanisms could result in impaired outcome.

In summary, exposure to a low protein diet within days of global brain ischemia triggers an aberrant acute-phase response. Dietary protein depletion after global ischemia caused serum levels of albumin and haptoglobin to decrease, while the circulating concentration of A2M was increased. In addition, post-ischemic PEM may be detrimental to recovery by diminishing the neuroplasticity mechanisms triggered by brain ischemia. However, these changes do not appear to be triggered by an increase in brain inflammation.

CHAPTER 6:
GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Introduction

Collectively, the three studies described in this thesis provide strong evidence that PEM alters key determinants of ischemic brain injury and brain recovery. To characterize the effects of PEM on global brain ischemia, three states of malnutrition were assessed and described in this thesis: PEM co-existing with global brain ischemia (Study 1), effects of PEM independent of brain ischemia (Study 2), and PEM developing after global brain ischemia (Study 3). Although this thesis is centered around the effects of PEM on the inflammatory response to global brain ischemia, mechanisms related to inflammation were also assessed, including effects on core temperature and neuroplasticity.

6.2 Summary of Findings

The study described in Chapter 3 was the first experiment performed in my laboratory exploring the effects of co-existing PEM on outcome from global brain ischemia induced by 2-VO in rats. In light of this, it was necessary to verify that the effects of co-existing PEM could be reliably studied with the 2-VO model. An unfortunate and unexpected finding was that co-existing PEM increased the inconsistency of the 2-VO model of global brain ischemia (Prosser-Loose et al., 2011). As a result, the failure to achieve a consistent model of global brain ischemia prevented us from drawing conclusions on whether co-existing PEM exacerbates reactive gliosis.

Despite the inability to assess the nutritional influence on reactive gliosis associated with global brain ischemia, this study yielded novel data highlighting the immediate effect of a low protein diet on core temperature. Within 24 hours of exposure, both mean temperature and fluctuation significantly increased and remained heightened throughout the 7 day pre-ischemic testing period. At the time of surgery, protein-energy malnourished rats were less able to maintain core temperature when exposed to anaesthesia. This was reflected by a greater decrease

in core temperature during the immediate post-surgical period, which was independent of the type of surgery (sham or ischemia). These findings strongly suggest that protein-energy malnourished rats are unable to finely regulate their body temperature when faced with an environmental temperature stressor. To our knowledge, this is the first report demonstrating the immediate effect of a low protein diet on core temperature.

Given that temperature is a key determinant of ischemic brain injury, core temperature recordings were collected for 28 days in the study described in Chapter 4 to provide a comprehensive assessment of the effects of PEM on the diurnal cycle of temperature. Continuous core temperature recordings demonstrated striking changes in temperature as malnutrition evolved from an acute to chronic state. Similar to the response observed in the first study, exposure to a low protein diet caused an immediate increase in temperature mean and temperature amplitude. Whereas the increase in temperature amplitude was sustained over 28 days in the malnourished group, the rapid increase in mean temperature was a transient response. Following the initial peak, mean temperature declined as malnutrition progressed. During the final week of testing, malnourished rats were approximately 0.5 ºC cooler than rats fed a control diet. Although others have reported a cooling response in malnourished rodents and humans (Bastow et al., 1983; Brooke, 1973; Brooke, 1972; Duran et al., 2008), this is the first report to fully characterize the temperature response to PEM as it evolves over time. It was hypothesized that the immediate change in mean core temperature reflected a behavioural stress response to a suboptimal diet. However, there was no acute increase in activity that correlated with the rise in body temperature immediately following placement on a low protein diet. Thus, it is not clear what is triggering the immediate change in temperature.

Since temperature and inflammation are closely related, the independent effects of PEM on neuro- and systemic inflammation were assessed in the study described in Chapter 4. Results from Real Time PCR arrays, which measured the expression levels of 84 genes involved in the NFκB cascade, demonstrated that this hippocampal pathway was unaltered by either acute or chronic exposure to a low protein diet in the rat. To address whether PEM is an independent stimulus of systemic inflammation, the circulating concentration of various acute-phase proteins were also measured. PEM can stimulate an acute-phase response, as demonstrated by an increase in the positive acute-phase protein, A2M, and a decrease in the negative acute-phase protein, albumin. These changes were observed at day 7 and day 28 on the low protein diet, illustrating

that the acute-phase response is both immediate and persistent. However, this response appears to be aberrant, given that the positive acute-phase protein, AGP, was considerably decreased in the malnourished rats. These findings may have clinical significance, given that the extent of the acute-phase response predicts outcome in stroke patients (Dziedzic, 2008). Therefore, one objective of the final study was to determine if the ability of PEM to induce an acute-phase response differs following global brain ischemia.

The third and final study of this thesis examined whether PEM arising after global brain ischemia altered the neuro- and systemic inflammatory responses and what impact this may have on neuroplasticity. The experimental design was intended to mimic the PEM that develops after and because of a stroke. To address these objectives, the 2-VO rat model of global brain ischemia was used, with a low protein diet being introduced at 3 days after brain ischemia. Findings from this study demonstrated that global brain ischemia induced by 2-VO does not elicit an acute-phase response; however, in rats fed control diet, the data suggested an acute-phase response related to the surgical manipulations required for both sham surgery and global brain ischemia. Interestingly, malnourished rats exposed to the surgery required to induce global ischemia responded differently. These rats presented with an aberrant acute-phase response similar to what was observed in the second study without global brain ischemia. Exposure to a low protein diet following global brain ischemia caused a decrease in serum albumin and an increase in serum A2M. In addition, a decrease in the positive acute-phase protein, haptoglobin, strengthened the evidence that PEM causes an atypical acute-phase reaction. Finally, although a strong glial response was elicited by global brain ischemia within the CA1 hippocampal subfield, immunofluorescence staining of Iba-1 and GFAP demonstrated no effect of PEM. However, the neuroplasticity response triggered by global brain ischemia did appear to be influenced by malnutrition, as GAP-43 and synaptophysin were significantly lower within the CA3 mossy fiber terminals at day 21. Therefore, it is speculated that PEM developing after brain ischemia could impair brain remodeling, resulting in fewer functional synapses.

In summary, the three studies described in this thesis highlight the effects of PEM on core temperature, the systemic acute-phase reaction and the neuroplasticity response to global brain ischemia. However, many unanswered questions remained, that are discussed in some detail below.

6.3 Limitations and Future Directions

6.3.1 PEM and an Acute-Phase Reaction

The research described in the thesis provides strong evidence that PEM induces an aberrant acute-phase response; however, a limitation of this work is that serum concentrations of regulatory inflammatory cytokines were not measured. IL-1 mainly regulates the synthesis of type 1 acute-phase proteins, including AGP and haptoglobin, while IL-6 stimulates the production of type 2 acute-phase proteins, such as A2M (Baumann et al., 1989; Petersen et al., 2004). Given that the production of type 2 acute-phase proteins appears to have precedence over the synthesis of type 1 acute-phase proteins in malnourished rats, it is speculated that the stress response triggered by a low protein diet causes the upregulation of IL-6. To verify this theory, future studies should examine the concentrations of additional type 2 acute-phase proteins, such as fibrinogen and α1-antitrypsin, as well as measure IL-6 concentration in the serum and liver of protein-energy malnourished rats.

During the second study described in this thesis, an attempt was made to measure IL-6 serum concentrations with a commercially available ELISA kit. Unfortunately, the ELISA assay was not sensitive enough to quantify IL-6 levels reliably. Although ELISA assays are commonly employed to measure serum concentrations of inflammatory cytokines, it is important to note that this method of quantification can provide an inaccurate measurement of total cytokine concentration. A major limitation of the ELISA technique is that it only measures unbound cytokines (Malone et al., 2001). Given that most cytokines in the blood are bound to carrier proteins, the concentration measured from an ELISA is generally an underestimate of the actual total cytokine concentration (Malone et al., 2001). Therefore, to more accurately measure the serum concentration of IL-6 in protein-energy malnourished rats, future studies should use a competitive binding immunoassay. A major advantage of this technique is that it measures both free and bound cytokines. Malone et al. (2001) compared the standard ELISA technique with the competitive binding immunoassay and demonstrated that the new alternative method yielded IL-1, IL-6 and IL-10 concentrations that were 10- to 500-fold higher than the ELISA measurements. An additional limitation of the ELISA technique is that it does not provide insight into the bioactivity of a cytokine. This information can be obtained through the use of bioassays, which, similar to competitive binding immunoassays, yield cytokine concentrations considerably higher than that obtained from the standard ELISA technique (Hillyer & Woodward, 2003).

In addition to selecting the appropriate quantification technique, it is also crucial that the correct sampling times are used. Inflammatory cytokines are short half-life molecules (Gruys et al., 2005), and therefore it is important to select the proper sampling time to capture the peak of the response. The circulating concentration of A2M is heightened within the first 48 hours of being on a low protein diet, when introduced during the acute period following global brain ischemia (Chapter 5). Therefore, future studies should use sampling time-points during this 2 day period to illustrate whether exposure to the suboptimal diet immediately triggers an increase in IL-6. Several time-points should be used to fully characterize the influence of PEM on cytokine concentrations, including sampling at later time-points since IL-6 also stimulates the production of acute-phase proteins under states of chronic inflammation (Gabay, 2006). In general, acute-phase proteins have a half-life of several days (e.g. transferrin [2d; rat] (Schreiber et al., 1982), fibrinogen [1d; rat] (Ruckdeschel et al., 1972), albumin [2.5d; rat] (Schreiber et al., 1982), AGP [1.3d; rat] (Schreiber et al., 1982)). Therefore, it is speculated that changes to the acute-phase response observed after weeks of exposure to low protein diet were not merely the remnants of the initial acute-phase response. Instead, a more likely explanation is that the stress caused by the suboptimal diet is persistent and continually stimulates the production of A2M. To determine whether A2M synthesis is continually increased in malnourished rats, future studies could measure the concentration of this acute-phase protein in the liver, in addition to serum concentrations.

6.3.2 PEM and NFκB-mediated signaling

A major limitation of the second study presented in this thesis is that NFκB activity was not directly measured. The assumption was made that NFκB activity is increased in the hippocampus of malnourished rats; however, this phenomenon had not yet been shown in rats. The original study showing that PEM increases NFκB activity was performed in the gerbil (Ji et al., 2008), and therefore it is possible that PEM affects NFκB signaling differently among species. Thus, future studies should first attempt to replicate these findings by employing electrophoretic mobility shift assays (EMSA) to measure hippocampal NFκB activity in both non-operated malnourished rats and malnourished rats exposed to global brain ischemia. If positive results are obtained from EMSA analysis, then the effects of PEM on NFκB -mediated gene expression can be re-examined using PCR. However, unlike the second study, which assessed the whole hippocampus, future experiments should assess hippocampal subfields

separately to avoid a dilution effect. Given that NFκB is most commonly activated in injured neurons, astrocytes and microglia (Clemens et al., 1997; Gabriel et al., 1999; Schneider et al., 1999; Stephenson et al., 2000), it is speculated that activation of this transcription factor is the most pronounced within the CA1 hippocampal subfield following global brain ischemia. Thus, the CA1 hippocampal subfield should be the primary region of interest in future Real-Time PCR experiments. Furthermore, experimental sample size should be increased, in an attempt to increase the power of the study.

6.3.3 PEM and Neuroplasticity

In an attempt to strengthen the neuroplasticity results from Chapter 5 for publication, additional brain sections will be labeled for other neuroplastic markers, including SNAP-25, postsynaptic density-95 (PSD-95), and microtubule-associated protein-2 (MAP-2). Similar to synaptophysin, SNAP-25 is located within the presynaptic terminals of CA3 mossy fibers (Oyler et al., 1989) and is thought to play a crucial role in synaptic vesicle exocytosis (Rizo & Sudhof, 2002). The postsynaptic protein, PSD-95, is a major scaffolding protein localized within glutamatergic synapses and is involved in the anchoring of synaptic proteins (Keith & El-Husseini, 2008). MAP-2, which is present within the cell body and dendrites of neurons, stabilizes dendritic structural microtubules and plays a role in dendritic branching (Shafit-Zagardo & Kalcheva, 1998). Examining additional synaptic markers, as well as a dendritic marker, will provide a more in-depth analysis of the effects of post-ischemic PEM on the repair processes occurring within CA3 mossy fibers following global brain ischemia.

Although the immunofluorescence approach relied on in the thesis research provides valuable details about the chemical features of a neuron, a limitation of this procedure is that it does not highlight the morphological characteristics of a cell. Therefore, future studies should perform Golgi-Cox impregnation to assess whether PEM alters the morphology of neurons following brain ischemia. Golgi-Cox staining is a valuable technique, which can be used to quantify the number and density of dendritic spines. This technique has been used in combination with the 2-VO global brain ischemia model to demonstrate that a marked decrease in spine density occurs in the CA1 hippocampal subfield within 48 hours following insult (Nagy et al., 2011). The combined use of these two techniques will further our understanding of whether PEM developing after brain ischemia hinders dendritic regeneration within mossy fiber bundles, resulting in decreased numbers of functional synapses.

The largest limitation of the final study was the absence of functional testing to determine if the changes in plasticity-associated proteins translated into reduced functional recovery. To relate mechanistic findings to functional outcome, behavioural testing is considered the gold standard in stroke research. Although functional testing is achievable in the 2-VO model, rats appear to be rather resistant to cognitive changes and therefore sensitive behavioural paradigms are required (Langdon et al., 2008). Given the challenges with behavioural testing, electrophysiology should be considered for future studies to determine whether the extent of PEM-induced changes to plasticity markers is great enough to alter cell function. Extracellular field potentials should be measured to assess the collective activity of CA3 neurons. It is hypothesized that the electrophysiological studies will reveal deficits in the CA3 hippocampal region in protein-energy malnourished ischemic rats, which will correspond with the decrease in synaptic markers observed in the final study.

6.3.4 Studying the Effects of PEM in a More Clinically Relevant Model of Stroke

The 2-VO global brain ischemia model, along with the adolescent age of rat required for its use, is a major limitation of this thesis work. Although global brain ischemia induces many of the same pathophysiological mechanisms of human stroke, it is a better representation of what occurs following cardiac arrest and resuscitation. The 2-VO ischemia model is commonly used for preliminary assessment and was selected for this thesis work since the surgical model is well characterized and the level of invasiveness has been reported as relatively minor. However, direct experience with the 2-VO global ischemia model has illustrated that the required surgery is quite invasive. Furthermore, only 70-80% of rats present with extensive bilateral damage in the CA1 hippocampal region following 2-VO surgery. Despite these limitations, findings from global ischemia models have yielded important information highlighting the effects of co-existing PEM and post-ischemic PEM on brain biochemistry (Bobyn et al., 2005; Ji et al., 2008) (Chapter 3 and Chapter 5), repair mechanisms (Prosser-Loose et al., 2010) (Chapter 5) and functional outcome (Bobyn et al., 2005). Ultimately, these findings will be used to form the basis of hypotheses for future studies using a more clinically relevant model of stroke.

Given that there are a wide variety of animal models currently used to study cerebral ischemia (Murphy & Corbett, 2009), it is important for researchers to diversify and replicate their findings in other stroke models. Therefore, future studies should employ a focal ischemia model to re-examine the effects of PEM on the inflammatory response. The rat photothrombotic

stroke model has recently been established in my laboratory and will be used in upcoming studies to assess the effects of PEM on stroke outcome. Unlike the 2-VO model, which generates extensive damage to regions of the hippocampus proper, the photothrombotic model produces a localized infarct in a specific cortical region. The photothrombotic model involves a vascular injection of a photoactive dye combined with irradiation of a specific area of cortex, using a light beam at a given wavelength. Irradiation of the circulating dye, such as rose bengal, produces free radicals and results in platelet aggregation and thrombus formation (Murphy & Corbett, 2009). Since there are notable differences between these two models, the effects of PEM following focal brain ischemia may be different from the findings reported in this thesis.

Invasive surgical procedures are problematic for the nutritional studies undertaken in my laboratory, and therefore extra caution had to be made when selecting an appropriate brain ischemia model. Photothrombosis does not require a craniotomy, is relatively non-invasive, and has few surgical variables that need to be controlled, thus making it a suitable model for assessing our research objectives. Furthermore, aged rats can be used with the photothrombotic model, which better mimics human stroke victims. This focal model generates cerebral infarcts that are reproducible in size and location (Tatlisumak et al., 2007). Thus far, my laboratory has generated infarcts located within the motor cortex using the photothrombotic model and have assessed both histological and functional endpoints. Compared to the mild behavioural (cognitive) impairments commonly reported following 2-VO global brain ischemia (Langdon et al., 2008), functional (motor) deficits are more severe using the photothrombotic model and therefore easier to detect with behavioural tests. Widely used behavioural tests, such as the Montoya staircase test and cylinder test (Kleim et al., 2007), have been established and will be employed in future studies to assess the impact of PEM on functional recovery following focal ischemia. Another advantage of the photothrombotic focal model, as compared to the 2-VO model, is that it triggers an inflammatory response that is more comparable to the human condition of stroke (Schroeter et al., 1997; Schroeter et al., 2001; Tatlisumak et al., 2007).

The effects of PEM on the inflammatory response to focal brain ischemia have not yet been characterized. Since photothrombosis is accompanied by damage to the blood-brain barrier resulting in the recruitment of systemic immune cells (Tatlisumak et al., 2007), these features make it a good model for studying the nutritional effects on neuroinflammation. Leukocyte recruitment appears to be delayed following photothrombosis (Schroeter et al., 2001), and

therefore this response may be influenced by both co-existing PEM and PEM that develops after brain ischemia. PEM will most likely have a greater impact on the inflammatory response to brain ischemia when there is systemic recruitment of immune cells. This hypothesis is based on the findings reported in Chapter 4 and 5 demonstrating that PEM stimulates a systemic acute-phase response. The acute-phase reaction in malnourished rats is most likely triggered by a systemic inflammatory response, given that inflammatory cytokines regulate the synthesis of acute-phase proteins (Cray et al., 2009). Thus, it is speculated that a systemic inflammatory response in malnourished rats could in turn result in exacerbated neuroinflammation following focal ischemia. This theory should be assessed in future studies by immuno-labeling glial cells and leukocytes to determine whether the recruitment of resident and systemic immune cells is augmented in malnourished rats following focal ischemia.

6.4 Clinical Implications

Although it is well recognized that co-morbidities, such as malnutrition, can have deleterious effects on patient recovery following stroke (FOOD Trial Collaboration, 2003), they are not being adequately managed by current post-stroke care. Therefore, it is imperative that pre-clinical research is performed to clarify if there is truly a cause-and-effect relationship between malnutrition and poor outcome. If so, this would emphasize to practitioners the importance of targeted nutritional interventions. The overarching aim of the thesis research was to investigate underlying mechanisms altered by PEM that could affect the extent of brain damage and recovery following brain ischemia. Together, the three studies provide considerable evidence that PEM alters body temperature, the acute-phase response, and mechanisms of neuroplasticity, all of which have clinical importance.

Given that temperature is a key determinant of brain injury following stroke (MacLellan et al., 2009; Silasi & Colbourne, 2011; van der Worp et al., 2007; Wang et al., 2009), the findings in this thesis suggest that the pathophysiology of brain injury in malnourished stroke patients may be altered. While the PEM-induced temperature changes reported in Chapter 3 and Chapter 4 are not sizeable enough to alter ischemic brain injury, the effect of PEM on temperature may be amplified in the presence of stroke. PEM impairs the ability to finely regulate body temperature (Chapter 3), and therefore it is speculated that malnourished victims are among those patients that experience a fever during the acute period following stroke. Although the presence of a fever in stroke patients often coincides with an underlying infection

(e.g. urinary infection, sepsis, bronchopulmonary infection), noninfectious fevers have been reported in approximately 40% of hyperthermic stroke patients (Castillo et al., 1998). Post-ischemic hyperthermia can significantly aggregate injury (Wang et al., 2009), with clinical evidence suggesting that the presence of a fever within 24 hours following a stroke doubles the odds of mortality (Prasad & Krishnan, 2010). Therefore, temperature should be closely monitored in all stroke patients, especially in malnourished individuals, and treated immediately with antipyretics if a fever develops. Another important consideration is whether the presence of PEM could interfere with therapeutic hypothermia. It has been repeatedly demonstrated in various models of global and focal brain ischemia that prolonged hypothermia is neuroprotective (Colbourne & Corbett, 1994; MacLellan et al., 2009; Silasi & Colbourne, 2011; van der Worp et al., 2007). Furthermore, therapeutic hypothermia improves neurologic outcome after cardiac arrest (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study Group, 2002). Thus, therapeutic hypothermia is one of the most promising neuroprotective treatments for stroke patients. However, the therapeutic effects of hypothermia may vary among the patient population, and factors such as malnutrition may interfere with the beneficial effects of hypothermia. Therefore, it should be examined whether therapeutic hypothermia can be induced safely and effectively in protein-energy malnourished stroke victims. Also, further research should be employed to assess whether the mechanisms decreased by therapeutic hypothermia, such as inflammation (Silasi & Colbourne, 2011; Webster et al., 2009), are in fact reversed by PEM.

The model of PEM used in the thesis research induces an aberrant acute-phase reaction, with a similar response being demonstrated in a small number of clinical studies (Dulger et al., 2002; Sauerwein et al., 1997). However, the relationship has never been studied in malnourished stroke patients. It is hypothesized that the acute-phase response triggered by PEM is persistent during states of stress, such as after a stroke. Given the clinical findings showing that an acute-phase reaction is present in a subset of individuals following cerebral ischemia (Dziedzic, 2008; Idicula et al., 2009; Ryu et al., 2009; Smith et al., 2006), it is speculated that protein-energy malnourished patients are among this population. Since the presence of an acute-phase response following cerebral ischemia is associated with poorer outcome (Dziedzic, 2008; Idicula et al., 2009), these patients may require targeted interventions. Improving the nutritional status of a stroke patient may in turn subside the acute-phase response, and as a result improve outcome.

Although this relationship has not yet been explored, it has been demonstrated that intervening with the appropriate nutritional therapy can considerably improve the quality of life in undernourished stroke patients (Ha et al., 2010).

International standards for conducting experimental stroke research has recommended including the influence of clinically relevant co-morbidity factors when investigating potential stroke treatments (Fisher et al., 2009). The research conducted in this thesis stresses the importance of this issue by highlighting the considerable impact that malnutrition has on various interrelated physiological systems. In addition, these results emphasize the challenges of studying co-morbidity factors in combination with stroke models. Findings from the first study demonstrate that clinically relevant stroke co-morbidity factors can interfere with well-validated experimental models of stroke (Prosser-Loose et al., 2011). Thus, stroke researchers need to be cautious when designing experiments and confirm that including a co-morbidity factor in the study design does not compromise the experimental stroke model.

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APPENDIX

Table A.1. Effects of feeding a low protein diet for 7 and 28d on NFκB-mediated signaling in the hippocampus.

Gene Symbol		
Akt1	IL10	Nfkb2
Atf1	Il1a	Ippk
Atf2	IL1b	Ripk2
Bcl10	IL1r1	Slc20a1
Bcl3	IL6	Smad3
C3	Irak2	Smad4
Card10	Irf1	Stat1
Casp1	Jun	Tgfbr1
Casp8	Kcnh8	Tgfbr2
Ccl2	Tbk1	Tlr1
Cflar	Tnfsf14	Tlr2
Chuk	Tlr7	Trl3
Crebbp	Irak1	Trl4
Csf2	Lta	Tlr6
Csf3	Ltbr	Tlr9
Lpar1	Map2k3	Tnf
Egr1	Map3k1	Tnfrsf10b
F2r	Mapk3	Tnfrsf1a
Fadd	Myd88	Tnfrsf1b
Fos	Nalp12	Cd40
Gja1	Nfkb1	Tnfsf10
Htr2b	Nfkbia	Faslg
Icam1	Pcaf	Tnip2
Ifna1	Ppm1a	Tollip
Ifng	Eif2ak2	Tradd
Ikbkb	Raf1	Traf2
Ikbke	Rel	Traf3
Ikbkg	Rela	Zap70

Expression levels of 84 genes were determined using the Rat NFκB Signaling Pathway RT^2 Profiler PCR Array (7d, n=5; 28d, n=6). No effect of diet was detected at either time-point by independent *t* test with Bonferroni correction.

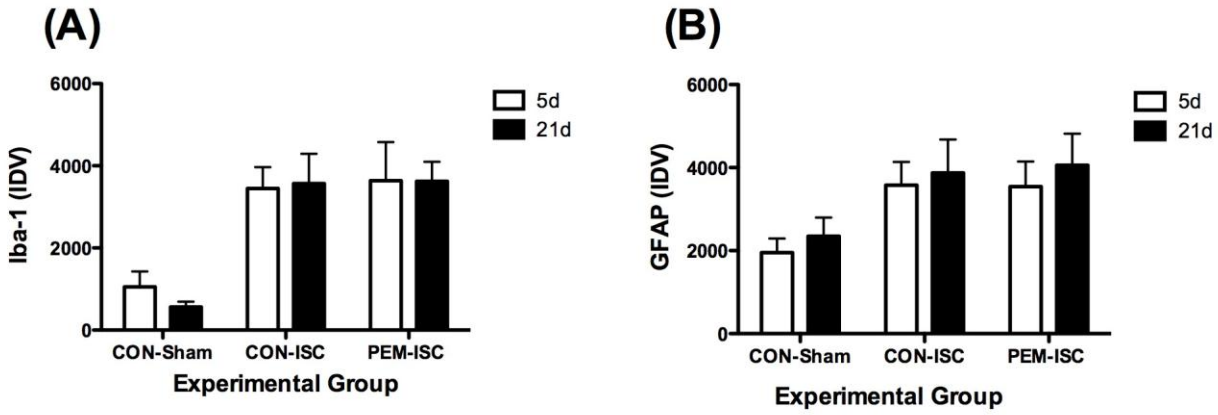

Figure A.1. Raw data for Iba-1 **(A)** and GFAP **(B)** immuno-staining within the CA1 hippocampal subregion. Data uncorrected for processing variability (i.e. not divided by the CON-Sham group mean) is shown to highlight the differences among the three treatment groups at 5 and 21d. Results are shown as mean ± SEM integrated density value (IDV) for the CA1 hippocampal subfield.

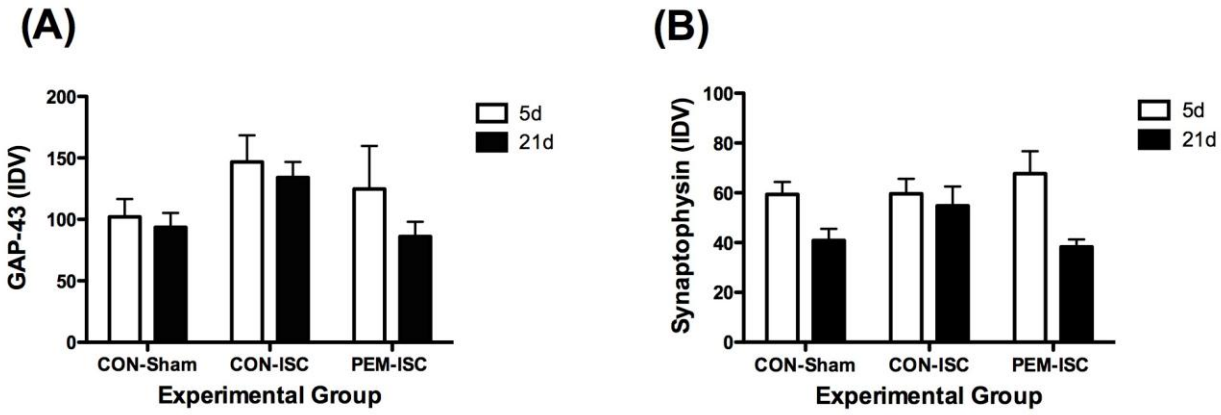

Figure A.2. Raw data for GAP-43 **(A)** and synaptophysin **(B)** immuno-staining within the CA3 mossy fiber region. Data uncorrected for processing variability (i.e. not divided by the CON-Sham group mean) is shown to highlight the differences among the three treatment groups at 5 and 21. Results are shown as mean ± SEM integrated density value (IDV).

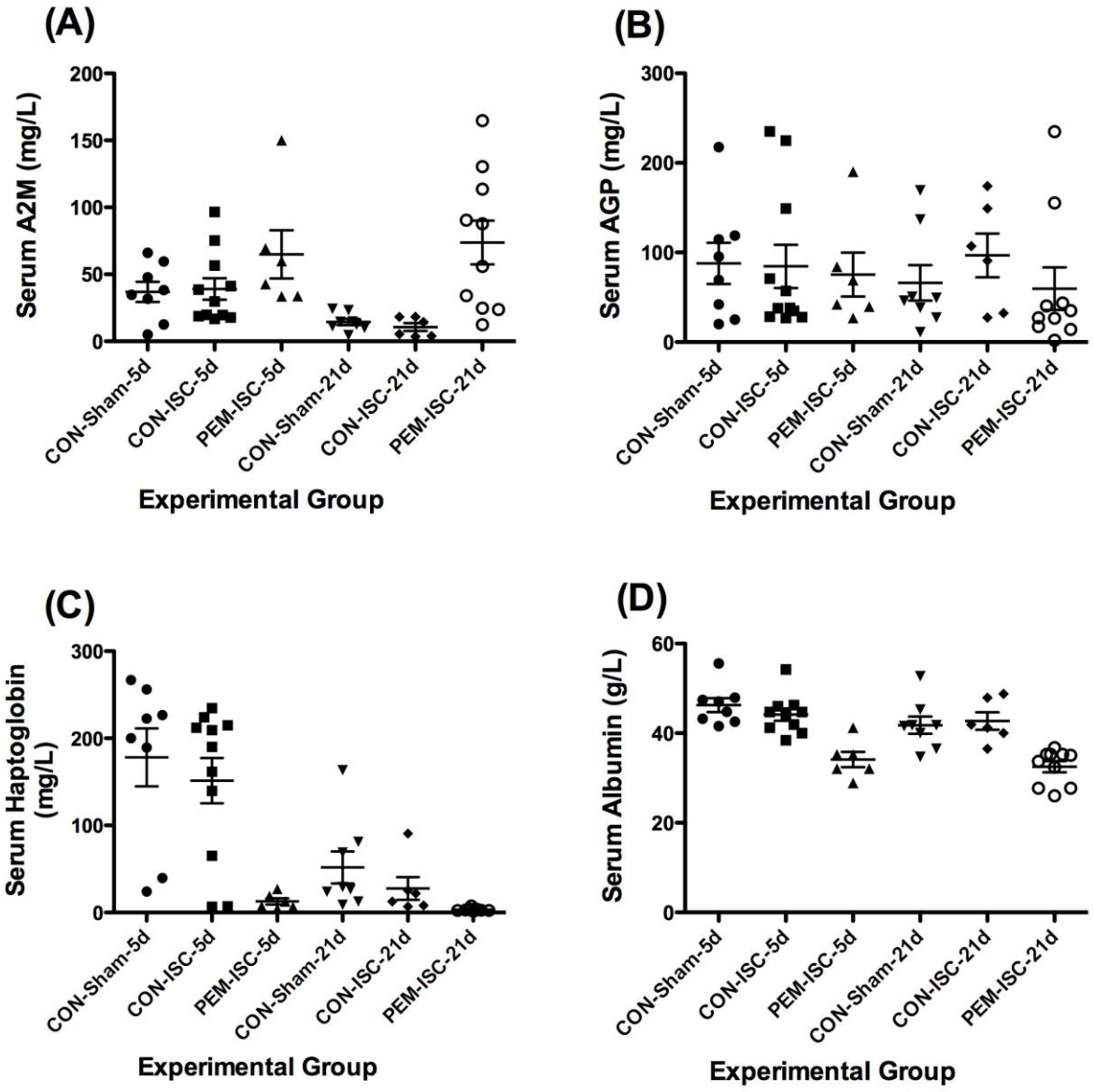

Figure A.3. Scatter plots of the individual serum concentrations of alpha-2-macroglobulin (A2M) **(A)**, alpha-1-acid glycoprotein (AGP) **(B)**, haptoglobin **(C)**, and albumin **(D)**. Data from individual animals are presented to highlight inter-animal variability within treatment groups. Mean ± SEM are represented by a horizontal line and error bars.