Modelling the Protein-Energy Malnourished Stroke Patient

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By

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ABSTRACT

Little is known about the effects of protein-energy malnutrition (PEM) developing after stroke on brain recovery. The goal of this project was to develop two experimental models in the adult rat to allow evaluation of nutritional effects on post-stroke recovery: (1) a PEM model, and (2) a photothrombotic stroke model.

Experiment 1 examined the hypothesis that *a diet containing either 1% or 0.5% protein will produce an acute state of mild-moderate PEM in adult rats*. Male, Sprague-Dawley rats (16 wk) were trained in the Montoya staircase before being randomized to diets containing 0.5% (n=8), 1% (n=8), or 12.5% protein (n=10 [CON]) for 31d. Both low protein diets increased liver lipid content ($p< 0.001$) and decreased food intake ($p= 0.005$) and body weight ($p< 0.001$) compared to the 12.5% protein diet. The 0.5% protein group best mimicked the stroke patient, as judged by decreased serum albumin ($p= 0.018$) and an acute decrease in mean (±SEM) body weight (g) by d7 (0.5%= 424±15; 1%= 428±14; CON= 477±10; p = 0.011). Increased concentrations of the positive acute phase proteins, alpha-2-macroglobulin and alpha-1-acid glycoprotein, were greatest in the 0.5% group ($p< 0.001$). No differences were observed in the Montoya test on d3, 15, or 30 ($p= 0.26$). Values on d30 were: 0.5%= 109.5±4.4% of pre-diet performance; 1%= 97.2±5.5%; CON= 98.5±10.2%.

Experiment 2 tested the hypothesis that *targeted laser irradiation and 30 mg/kg of rose Bengal injection will cause an infarct in the forepaw region of the cortex with accompanying functional deficits.* Male adult rats trained in the Montoya staircase were randomized to ISCHEMIA (n=15) or SHAM (n=3) surgery. A cortical infarct occurred in 86% of rats, with some misplacement

and variability in volume (5.7-12.8 mm^3). Forepaw impairments were confirmed by decreased performance in the staircase at d3 (34.3±7.3 % of pre-stroke performance, $p<0.001$) and diminished use in the cylinder test (30.3±4.0% affected limb use versus 53.9±1.93% prestroke, $p< 0.001$). At d30, mean recovery was incomplete in the staircase ($p< 0.001$).

These experimental models, with additional refinements, can be used to address the hypothesis that deteriorating nutritional status after a stroke interferes with brain recovery.

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This thesis is dedicated to my inspiring parents,

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the hardest-working people I know.

Genius is one percent inspiration and ninety-nine percent perspiration.

Thomas Alva Edison

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A2M	Alpha-2-macroglobulin
AGP	Alpha-1-acid glycoprotein
AIN-93M	American Institute of Nutrition 1993 Maintenance diet
AMP	Adenosine monophosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CA3	Cornu ammonis 3
DNA	Deoxyribonucleic acid
FGF-2	Fibroblast growth factor-2
GABA	Gamma-aminobutyric acid
GAP-43	Growth-associated protein 43
HSD	Honest significant difference
IGF-1	Insulin growth-factor 1
MAP-2	Microtubule- associated protein-2
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartic acid
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PEM	Protein-energy malnutrition

RNA	Ribonucleic acid
SEM	Standard error of the mean
STAIR	Stroke Therapy Academic Industry Roundtable
t-PA	Tissue plasminogen activator
trkB	Tropomyosin-related kinase B
VLDL	Very low density lipoprotein

CHAPTER 1
INTRODUCTION

1.1 Rationale

Stroke is a leading cause of disabilities worldwide. In Canada, 25% of stroke cases lead to either death or complete recovery, and the remaining 75% of patients are left with some form of impairment (Statistics Canada 2011). The current treatment for acute stroke, the thrombolytic agent tissue plasminogen activator (t-PA), has a very small window of administration (up to 4.5 hours after infarction) (Lindsay, Gubitz et al. 2011). Thus, stroke treatment often cannot be administered, which leaves patients with serious brain damage and severe impairments. Most research to date has attempted to reduce this disability with neuroprotective treatments that target a single pathway in the complex ischemic cascade responsible for brain cell death, and all clinical attempts have, thus far, failed (Ginsberg 2009). Current research strategies include: [1] combination treatments that target multiple pathways responsible for brain cell death (Savitz, Fisher 2007, Ginsberg 2008), and [2] novel approaches that aid brain recovery and repair mechanisms causing functional improvement (Cramer 2008, Murphy, Corbett 2009).

Although a critical component of stroke care, nutritional status and interventions are not often contemplated in stroke treatment and research (Gariballa, Parker et al. 1998a, Shen, Chen et al. 2011). Protein-energy malnutrition (PEM), for instance, develops after stroke in 35-49% of patients (Poels, Brinkman-Zijlker et al. 2006, Finestone, Greene-Finestone et al. 1995), and is characterized by deficient protein and energy status. There have been studies suggesting that PEM negatively affects outcome after human stroke (Martineau, Bauer et al. 2005, Yoo, Kim et al. 2008), indicating that PEM may be an important stroke co-morbidity factor. Our laboratory has shown that some of the mechanisms through which the poor nutritional status affects outcome after brain ischemia involve components of the cascade leading to brain cell death (Bobyn, Corbett et al. 2005). The influence of continued PEM after stroke also includes alterations in plasticity proteins that are important to brain repair (Prosser-Loose, Verge et al. 2010). Even though these studies acknowledge the importance of nutritional status to stroke development, functional outcome, and brain repair, they have all addressed the significance of pre-existing PEM that is already present at the time of stroke. This is a problem that affects approximately 16% of patients (Martineau, Bauer et al. 2005, Davis, Wong et al. 2004). While

the latter is an important issue, the post-stroke PEM is an even more severe problem, considering the dramatic increase in the rate of malnutrition that develops in the post-stroke period (Shen, Chen et al. 2011, Yoo, Kim et al. 2008, Axelsson, Asplund et al. 1988).

Suboptimal nutritional status developing after stroke could be of crucial importance since any spontaneous neurological repair that occurs usually does so within three months (Cramer 2008). During this period, both anatomical and functional brain reorganization occurs, allowing for brain repair and recovery. These changes are known as neuroplasticity or plasticity (Murphy, Corbett 2009, Bergado-Rosado, Almaguer-Melian 2000, McEwen, Chattarji 2004). Mechanisms that contribute to this spontaneous repair include [1] axonal and dendritic growth (by elongating or sprouting mechanisms) of surviving neurons, [2] use of alternative brain circuits, and [3] neurogenesis (Bayona, Bitensky et al. 2005, Kreisel, Hennerici et al. 2007). Repair mechanisms and recovery itself are determined by the cause and the size of the stroke, but also by molecular processes and external factors (Cramer 2008). This period after stroke is, thus, central in the determination of stroke outcome. My overarching hypothesis driving the development of the animal models in this thesis is that PEM that develops after ischemic stroke will impair motor neuroplasticity.

The use of experimental animals is an excellent way to mimick human disorders and to understand the underlying mechanisms, as well as to study novel treatments (Lieschke, Currie 2007). The use of a rat model to simulate the malnourished post-stroke patient would allow for the study of this hypothesis. However, this model does not exist and the development of such a prototype requires the combination of two components:[1] a rat stroke model, such as photothrombotic stroke, that is well-established in the literature (Watson, Dietrich et al. 1985, Dietrich, Busto et al. 1987, Watson 1998, Diederich, Quennet et al. 2012, Jablonka, Burnat et al. 2010, Sulejczak, Ziemlinska et al. 2007), and [2] a model of PEM suitable for mimicking that observed in the post-stroke patient.

Unfortunately, there are no established standards of nutritional status for modelling PEM in rats. To mimick the features of the nutritional status of stroke patients, it is important to consider factors such as diet formulation and type of animal (Woodward 1998). Although there are numerous studies modeling PEM in rats, most use weanling and adolescent rats, which are rapidly growing (Prosser-Loose, Smith et al. 2011, Heard, Frangi et al. 1977, de Belchior, Angeli et al. 2012). However, PEM in the adult rat is not nearly as well characterized. Therefore, a study

was needed to determine the dietary regimen that induces PEM in adult rats that would mimick the clinical situation. The age of rat determines the protein requirement (Reeves, Nielsen et al. 1993). Definition of the level of dietary protein to achieve the desired acute mild-moderate PEM in adult rats was, thus, necessary. This research is described in Chapter 3.

The choice of photothrombosis to produce stroke in the rat was based on several characteristics of this model that allow for reproducible lesions in the cortex, with consequent alterations in sensorimotor function (Dietrich, Busto et al. 1987, Brown, Marlowe et al. 2003). This model shows good consistency for both anatomical (Jablonka, Burnat et al. 2010, Minnerup, Kim et al. 2011) and functional outcomes (Sulejczak, Ziemlinska et al. 2007, Shanina, Schallert et al. 2006, Moon, Alaverdashvili et al. 2009). Also, this model requires less surgical intervention than most experimental models of stroke and no craniotomy (Watson, Dietrich et al. 1985). This is important for studying the effect of PEM on stroke outcome, since there will be fewer metabolic alterations associated with the surgery. After establishing the surgical procedure necessary for this model in our laboratory, the endpoints that needed development were the assessment of infarct volume and function. The use of well-validated behavioral tests to assess motor function at chosen endpoints helps to determine the stroke model reliability (Fisher, Feuerstein et al. 2009) and also will allow future evaluation of the influence of nutritional status on post-stroke functional outcome. Chapter 4 describes the methodological development of these endpoints.

1.2 Hypotheses and Objectives

Experiment 1 (Chapter 3)

Hypothesis: A diet containing either 1% or 0.5% protein will produce an acute state of mild-moderate PEM in adult rats that models post-stroke protein-energy malnutrition.

Objectives:

a) To characterize the protein-energy status of rats fed diets containing 0.5% or 1% protein on the basis of body weight, food intake, liver lipid content, and serum acute phase protein concentrations.

b) To test if PEM causes motor alterations in the rat, which could confound the Montoya staircase task for future assessments of functional abnormalities caused by cortical stroke.

c) To further validate the use of the Montoya staircase for assessing protein-energy malnourished rats by testing if motivation to obtain a sugar pellet is altered as the malnutrition develops.

Experiment 2 (Chapter 4)

Hypothesis: Targeted laser irradiation at an intensity of 25mW for 10 minutes combined with 30 mg/kg of rose Bengal will result in a medium-size infarct located in the region of the cortex corresponding to forepaw function. The resulting infarct will cause motor alterations of the targeted forelimb.

Objectives:

a) To determine the reliability of the model by measurements of infarct volume.

b) To evaluate the extent and consistency of the motor alterations by behavioural assessments.

c) To determine if functional recovery is incomplete after 30 days.

CHAPTER 2
LITERATURE REVIEW

2.1 Ischemic Stroke

2.1.1 Definitions and Prevalence

The term stroke refers to a clinical syndrome that includes infarction and hemorrhage, causing sudden loss of brain function (Whisnant, Bas et al. 1990). In Canada, between 40,000 and 50,000 people have strokes each year; approximately 16,000 die from stroke, and about 29,000 are left with impairments. In 2011, it was estimated that stroke costs the Canadian economy $ 3.6 billion a year, each acute stroke costs about $27,500 (Statistics Canada 2011).

Approximately 20% of strokes are due to bleeding in the brain (Lloyd-Jones, Adams et al. 2009), and this type of stroke is known as hemorrhagic stroke. The clinical characteristics of hemorrhagic stroke depend on the location and severity of bleeding (Choi 1990). Some people may have aneurysms present from early life, which weakens blood vessels, causing them to rupture in the presence of hypertension (Ding, Clark 2006).

The remaining 80% of strokes, known as ischemic strokes, are caused by an interruption of blood flow to the brain (Lloyd-Jones, Adams et al. 2009) and are the subject of this thesis. The accumulation of atherosclerotic plaque (calcium, scar tissue and lipid materials) narrows the arteries that supply blood to the brain and can interfere with or completely block normal flow. Hyperlipidemia and inflammation are major contributors to the development of atherosclerosis. Hypercholesterolemia, for instance, helps to promote migration of circulating monocytes into the atherosclerotic lesion, where they help to form and develop the plaque as well as destabilize it. This endothelial pathophysiology is the main trigger of ischemic stroke (Libby, Ridker et al. 2011). An ischemic stroke is caused by either a thrombus or an embolism. Thrombotic stroke is caused by a clot that forms in an artery supplying the brain. When blood flow decreases in the affected artery, the collateral circulation maintains brain function. When the compensatory mechanism of collateral circulation fails, perfusion is compromised, leading to decreased blood flow and the death of neurons. Embolic stroke occurs when the clot forms elsewhere in the body and is carried through the blood stream to lodge in cerebral vessels (Adams, Bendixen et al. 1993). Microemboli can break away from a plaque from cardiac

sources, such as in atrial fibrillation (Warlow, Dennis et al. 2001).

2.1.2 Mechanisms of Brain Injury

The pathogenesis of ischemic stroke involves a complex cascade of mechanisms that lead to brain injury. The severity of the stroke depends on the region of infarct, the length of blood cessation and other secondary insults caused by reperfusion (reviewed in Doyle, Simon et al. 2008).

Neuronal and glial cell death contribute to the pathology of stroke-induced brain injury. Two main categories are defined to describe brain cell death: [1] necrosis, when there is a loss of ionic homeostasis, swelling of the cell and its organelles, and loss of membrane integrity; [2] apoptosis, which presents with diminished cell volume, maintenance of organelle structure, and cytoplasmic and nuclear condensation and fragmentation (Fink, Cookson 2005). Morphological characteristics of necrotic neurons can be seen after brain ischemia, for instance plasma membrane failure and swelling of organelles and cell bodies. Apoptotic features are also apparent, including chromatin condensation, decreased cell size and deoxyribonucleic acid (DNA) fragmentation (Lee, Zipfel et al. 2000).

The acute ischemic cascade responsible for neuronal death following stroke happens when the brain lacks glucose and oxygen in the area supplied by the occluded artery. This causes adenosine triphosphate (ATP) depletion, depolarization of membranes, glutamate excitotoxicity, calcium toxicity, oxidative stress and an inflammatory response (Hossmann 2006). Ischemia causes depolarization of the neuronal membranes, and an increase in neurotransmitter release. In the case of glutamate, it overstimulates the N-methyl-D-aspartic acid (NMDA)-type, as well as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate-type ionotropic receptors present in the pre- and post-synapses. This causes an increased influx of calcium that causes an overload of mitochondrial calcium and activation of Ca^{2+}-dependant enzymes (Choi 1988). Metabotropic glutamate receptors activate inositol triphosphate dependant signal transduction pathways. Likewise, metabotropic receptors are a part of the regulatory cascade in glutamate release and re-uptake via cyclic- adenosine monophosphate (AMP) signalling. Because cyclic-AMP needs ATP to function, and in ischemia there is ATP depletion, glutamate regulation is impaired (Hazell 2007). Another glutamate regulator is the astrocyte. This type of glial cell takes up extracellular glutamate via ATP- dependent receptors present on the

membranes. Due to excessive intracellular Ca^{2+}, in the ischemic brain, there is a reversal of these receptors that causes additional glutamate efflux, contributing to the excitotoxicity (Hazell 2007). Metabolic demand is increased in the area of the infarct, and ATP is further depleted, leading to additional glutamate liberation. This pathway results in neuronal necrosis, since neurons swell due to influx of Na^{+}, Ca^{2+}, Cl^{-} and H_2O (reviewed in Lee, Zipfel et al. 1999). Further NMDA activation leads to mitochondrial failure due to the Ca^{2+} overload. Proteases, endonucleases and phospholipases are activated since they are Ca^{2+}-dependent enzymes. Calpain-1, a protease activated by calcium, leads to breakdown of structural proteins in the brain (Choi 1990). Endonucleases cause DNA cleavage, and the activated phospholipases break down membrane phospholipid and produce reactive oxygen species (Hazell 2007). Nitric oxide production, which results from nitric oxide synthase activity, is also increased in neurons in response to the Ca^{2+} overload and that also augments the production of reactive oxygen species (Hazell 2007).

Reperfused areas, in which blood flow has been re-established, or areas with low blood perfusion are responsible for secondary damage since the blood brings with it high oxygen content and an accumulation of inflammatory cells (Ding, Clark 2006). Formation of reactive oxygen species causes injury to the plasma membrane and organelles. The injury to the mitochondria provokes release of cytochrome C that, once freed, promotes the apoptotic cell death cascade (Doyle, Simon et al. 2008, Fink, Cookson 2005).

Inflammation also follows ischemic injury and contributes to its progression. Pro-inflammatory cytokines such as Interleukins- 1, 6 and 10 are secreted after glial cells are activated (Durukan, Tatlisumak 2007). Neurons and endothelial cells also produce inflammatory cytokines in response to ischemia that further injure neuronal tissue. Since there is evidence of increased cytokine concentrations in the brain and in the circulation hours after ischemia, they are believed to be the initiators of the inflammatory response after stroke (Ahmad, Graham 2010). Under normal conditions, the blood-brain barrier prevents the infiltration of inflammatory molecules. However, after stroke, the brain becomes susceptible to inflammatory reactions (Danton, Dietrich 2003). The infiltration of neutrophils allows adhesion to the microvasculature and an increase in the endothelial permeability that can disrupt the blood-brain barrier and allow monocytes, macrophages and neutrophils to enter. In the brain, these cells can occlude the microvasculature and produce nitric oxide, reactive oxygen species and prostanoids, all toxic to the brain (Ding, Clark 2006).

Conversely, some inflammatory cells can be beneficial to the brain after stroke. They can remove cellular debris, repair tissue, and prevent additional neuronal damage. Astrocytes also play a protective role. They respond to stroke by a process referred to as reactive gliosis, which involves changes in their molecular expression and morphology, as well as a scar formation. Recent studies have shown that the glial scar may have a range of essential neuroprotective and repair-related functions, such as controlling the dissemination of inflammation (reviewed in Sofroniew 2009). The role of inflammation in brain injury is dependent on the severity of inflammation and the timing after stroke. Interleukin-10 for instance, has been shown to have neuroprotective effects on the ischemic brain (Grilli, Barbieri et al. 2000, Froen, Munkeby et al. 2002). Microglia can also have protective effects since they have a role in removing dead neurons, even though they also produce reactive oxygen species and cytokines (Lai, Todd 2006).

The mechanisms and molecular pathways that lead to either type of cell death after stroke have formed the basis for developing pharmacological neuroprotective treatment to be administered immediately after stroke, a strategy that has largely failed (Faden 2002, Albers, Goldstein et al. 2011).

2.1.3 Advances in Acute Stroke Treatment

Currently, the only successful treatment for ischemic stroke is the tissue-plasminogen activator, a thrombolytic agent, which breaks up the clot and allows the reestablishment of the blood flow, or reperfusion (Fisher, Feuerstein et al. 2009). However, to be effective, this treatment has to be administered within 4.5 hours after the stroke (Lindsay, Gubitz et al. 2011). Unfortunately, patients do not always go to the hospital at the onset of symptoms, or they do not recall the exact time when symptoms started. After the patients are admitted to the hospital, they are assessed and undergo blood tests and brain imaging. Several tests are required prior to the intravenous injection of t-PA to exclude the possibility of hemorrhagic stroke, which is a critical exclusion criterion (Brott, Bougousslavsky 2000). The physician then explains the situation and the drug to the patients and families, and must obtain their understanding and approval before administering the drug. There is often not enough time to safely treat the patients with t-PA (Liang, Lew et al. 2008). The current acute stroke-management thus leaves the vast majority of patients untreatable. It was estimated in 2007 that, in Canada, less than 2% of stroke

patients actually received t-PA (Yip, Demaerschalk 2007).

Outside the area of minimum blood flow in the infarct core, there is an area composed of tissue with less reduction in blood flow that can be salvaged if blood flow can be re-established; this is called the penumbra. There have been decades of research attempting to develop neuroprotective agents that could reduce brain damage by protecting neurons in the penumbra. Nonetheless, the achievements made in this field with experimental animal models have not successfully translated to clinical trials with positive outcomes (Ginsberg 2009). A commonly accepted criticism is that most neuroprotective strategies have failed because they target a single pathway in a very complex ischemic cascade, whereas novel neuroprotective approaches that target multiple pathways are likely to be more successful (Albers, Goldstein et al. 2011). Other reasons for the unsuccessful translation of neuroprotective approaches include: [1] failure to include essential design features in clinical trials, such as adequate dose, adequate follow-up period with significant outcomes, and early time window to initiate treatment; [2] clinical trial testing of agents for which preclinical efficacy was not replicated; and [3] use of agents at such high doses that they could not be safely used in humans (Mergenthaler, Meisel 2012).

2.1.4 Neuroplasticity, Spontaneous Brain Repair and Post-Stroke Therapy

After stroke, the brain is capable of some spontaneous recovery, and that is partly due to resolution of edema and recovery of tissue function in areas that were not destroyed. This part of recovery occurs within a few days. Beyond this point, recovery is possible through neuroplasticity (reviewed in Hallett 2001). The term neuroplasticity refers to both anatomical and functional alterations of the brain in response to the ischemic insult, which allow for repair and recovery. Plastic changes can occur in either the contralateral or ipsilateral hemisphere or in both of them simultaneously. The ipsilateral hemisphere refers to the side where the infarct occurs, whereas the term “contralateral” refers to the other hemisphere.

Improved functional performance does not necessarily reflect true recovery. After stroke, it is common to see behavioural compensation (Whishaw 2000, Finger, Almli 1985, Levin, Kleim et al. 2009). In rats, for example, impairments in grasping ability and supination of the forelimb after cortical stroke are counterbalanced by adjustments in posture, allowing the animal to perform similarly to pre-stroke levels (Moon, Alaverdashvili et al 2009). True recovery

is possible when the lesion is very small and the neurons needed for that function are saved from damage, or the residual brain tissue can support rewiring of the circuits responsible for a particular function (Moon, Alaverdashvili et al. 2009, Friel, Nudo 1998, Plautz, Milliken et al. 2000). Another possibility for true recovery is when there is replacement of the injured circuitry, which is only possible with novel treatments such as the use of stem-cells, which is under intense investigation and still needs further research to answer questions with respect to cell line, timing of transplantation, route of administration, and safety (Kokaia, Martino et al. 2012, Kolb, Gibb 2007, Lee, Kim et al. 2006). Thus, the term recovery refers to not only true recovery, but is also commonly used to describe mechanisms that improve performance through compensatory behavior (Buurke, Nene et al 2008). The plasticity mechanisms described below consider recovery simply as improved performance, and refer to both true recovery and compensation.

It has been suggested that new stroke research should focus on understanding the mechanisms that lead to functional recovery, because of the possibility of enhancing the naturally occurring capabilities of the brain to recover (Murphy and Corbett 2009). However, mechanisms of plasticity are not completely understood because brain function is a complex system. Thus, recovery after a stroke comprises changes in cells and in the entire network (Jiang, Zhang et al 2010). The reorganization of neuronal routes following ischemia relies upon some of the same mechanisms occurring in the development of the brain and during changes related to experience, such as learning (Murphy and Corbett 2009).

One plasticity mechanism after stroke is called unmasking. Neurons have a larger anatomical connectivity than utilized functionally, and the zones not in use are kept inhibited. When other areas fail, there is cessation of that inhibition and a new area of interest is unmasked (Jacobs, Donoghue 1991). Also, synaptic connections can be strengthened after stroke by processes of long-term potentiation or long-term depression. Some synaptic remodelling also occurs. Synaptic plasticity results in the sculpting of synaptic connections via an activity-dependent process, which includes changes in the molecular level, as well as formation of new synapses. AMPA receptors at a synapse can control the coupling strength between neurons in the pre- and post-synapse, and thus serve as control for neural function. The presence of these receptors at a synapse has been shown to represent recent plasticity (Takahashi, Svoboda et al 2003). Neuroplastic events are also assisted by chemical changes in neurotransmitters, growth factors, hormones and neurotrophins that promote survival and growth of neurons, as well as

remodelling of synapses (Johansson 2000).

Other mechanisms that contribute to brain plasticity include axonal and dendritic changes related to surviving neurons. Axonal plasticity, which occurs under physiological conditions, also contributes to brain repair. It includes molecular changes in transmission and structural changes, such as sprouting and regeneration (Leenders, Sheng 2005). Because several molecules are involved in the regulation of vesicle exocytosis for neurotransmitter release across the synapse, many of them have roles in the plasticity of the pre-synapse (Leenders, Sheng 2005). Protein kinase C and protein kinase A are examples of enzymes believed to be involved in pre-synaptic plasticity. Their role is to phosphorylate proteins involved in vesicle liberation (Leenders and Sheng 2005). There is also the growth of axonal branches following stroke. Growth factors and neurotrophins enhance the formation of side branches from axons to a new target (Gogolla, Galimberti et al 2007). Pre-synaptic function is highly altered because of the increase in intracellular Ca^{2+} after stroke. Pre-synaptic proteins involved in the regulation of synaptic function cause a decrease in the number of vesicles for neurotransmission (Kovalenko, Osadchenko et al 2006).

Plasticity of dendrites is related to changes in structure, shape, and changes in receptor type (Luscher, Nicoll et al 2000). Structural changes related to recovery of function after stroke are also associated with changes in structure and shape of dendrites (Kolb, Muhammad et al. 2011, Kolb, Teskey et al. 2010). One study showed an increase in dendritic length after ischemia, which was suggested to be due to sprouting of new dendrites rather than extension of existing ones (Ruan, Lei et al 2009). Structural proteins are involved in recovery of synaptic transmission by restructuring of damaged dendrites after the ischemic insult (Briones, Woods et al 2006). Induction of microtubule-associated protein-2 (MAP-2) in regions adjacent to an infarct is believed to be a protective response of the brain. Also, increased MAP-2 expression is observed in the penumbra, which indicates regrowth and restructuring of dendrites after stroke. Therefore, MAP-2 is considered a marker of dendritic plasticity (Li, Jiang et al 1998).

Besides altering the existing neurons and synapses, stroke also induces the proliferation of endogenous neural progenitor cells and an increase in the number of immature neurons (Jin, Minami et al 2001). New cells, adjacent to the infarct, will most likely mature as neurons. It has been observed that these cells express markers of mature neurons, including the

neuronal-specific nuclear protein (Parent, Vexler et al 2002). There is migration of neuroblasts into the injured area with the help of reactive astrocytes and blood vessels (Yamashita, Ninomiya et al 2006). One group showed that angiogenesis is linked to neurogenesis (Carmichael 2005; Tsai, Ohab et al 2006). Blood vessels upregulate angiopoietin 1 and stromal-derived factor 1, and their actions recruit immature neurons into the infarcted cortex. Upregulated angiopoietin 1 and stromal-derived factor 1 recruit immature neurons into the injured area of the cortex and cause improved behavioral recovery in the period after stroke (Carmichael 2005).

Although studies of plasticity mechanisms are extremely valuable for understanding brain repair, all of these trials have ignored the potential influences of co-morbidity factors commonly present in stroke patients, such as PEM. The presence of PEM could affect post-stroke functional outcome or alter the efficacy of rehabilitation strategies through direct effects on neuroplasticity mechanisms. This topic is directly addressed below in Section 2.2.4. Therefore, the research described in this thesis is aimed at the methodological development required to address such questions in a well-controlled rat model of the malnourished stroke patient.

2.2 Protein-Energy Malnutrition in the Stroke Patient

2.2.1 Overview

The extent of brain damage caused by stroke and the ability of the brain to recover is influenced by co-morbidity factors, and malnutrition is hypothesized to be one of them. Lack of or insufficient intake of nutrients leads to malnutrition (World Food Programme (WFP), Centers for Disease Control and Prevention (CDC) 2005). The body adapts to a reduced intake with a corresponding decrease in activity and increase in the use of energy reserves (muscle and fat). Therefore, a malnourished status will result in thinner individuals (Prudhon 2002). The most common form of malnutrition is protein-energy malnutrition (PEM), which refers to a combined deficiency of both protein and energy. All forms of PEM can occur as primary or secondary conditions (Woodward 1998).

Acute diseases, such as stroke, can have an important effect on patients' nutritional status. Poor nutritional status has been associated with poor survival and functional outcome following stroke (Shen, Chen et al. 2010, Chai, Chu et al. 2008). This relationship between acute stroke and PEM is very much influenced by patient age, since the highest prevalence of stroke

exists in the elderly. The Centers for Disease Control and Prevention (CDC) analyzed data from the 2005 Behavioural Risk Factor Surveillance System (BRFSS) survey in a sample of 356,112 people across the United States and reported that 8.1% of respondents aged over 65 years reported a history of stroke, compared with 0.8% of persons aged 18-44 years (Centers for Disease Control and Prevention (CDC) 2007). The risk of PEM also increases with increasing age. Because of their reduced reserves and diminished food intake (Gariballa, Parker et al. 1998b), elderly patients are more prone to being malnourished even if they do not present with any illness (Manandhar 1995). Development of illnesses, which are common in this age group, is another risk factor for the progression of malnutrition (Mowe, Bohmer et al. 1994).

Upon admission to a hospital for acute stroke, it was estimated in the 1990's that at least 16% of elderly patients were already malnourished (Gariballa, Parker et al. 1998b, Davalos, Ricart et al. 1996). Recent studies show that the statistics have not improved (Shen, Chen et al. 2011, Yang, Wang et al. 2009, Davis, Wong et al. 2004). In Taiwan, a study assessed the functional outcome of stroke patients related to their nutritional status, and showed that 19.7% were malnourished at admission (Shen, Chen et al. 2010). One study provided evidence that patients that were malnourished on admission to the hospital had reduced functional ability and survival at 6 months after stroke; however, the study design did not allow for testing of a cause and effect relationship (FOOD Trial Collaboration 2003). A recent study reported an association between malnutrition and poor stroke outcome (Babu, Kaul et al. 2013). However, in this study, malnutrition was solely measured as low serum albumin concentrations, which is problematic because albumin is not a specific marker of malnutrition. There are numerous limitations with the existing clinical research studies that have attempted to link nutritional status with stroke outcome. These include the heterogeneity of nutritional assessments and diagnosis and the inability to isolate important variables in order to establish if nutritional status directly affects stroke outcome (Meijers, van Bokhorst-de van der Schueren et al. 2010, Soeters, Reijven et al. 2008). There are many variables which cannot be controlled for in the clinical reality. Animal studies can overcome this limitation because of highly controlled conditions, in which it is possible to study single variables affecting outcome (Soeters, Reijven et al. 2008). One such study established that pre-existing PEM can impair cognitive recovery after brain ischemia, but this study had the limitation of relying on a global brain ischemia model that does not exactly mimick stroke (Bobyn, Corbett et al. 2005).

Although pre-existing PEM is an important clinical problem, PEM is even more prevalent after and as a result of a stroke. Several studies have shown that nutritional status worsens during hospital stays after stroke (Gariballa, Parker et al. 1998b, Axelsson, Asplund et al. 1989, Crary, Humphrey et al. 2013). After one or two weeks of hospitalization, 26-35% were diagnosed as malnourished (Brynningsen, Damsgaard et al. 2007), and by the time of admission to a rehabilitation setting, 35-49% received the same diagnosis (Poels, Brinkman-Zijlker et al. 2006, Finestone, Greene-Finestone et al. 1995). Little is known about the effects of PEM that develops during the recovery period following stroke. The experimental models developed in this thesis will make it possible in future to address the hypothesis that deteriorating nutritional status that develops because of the stroke interferes with brain reorganization that promotes recovery. Section 2.2.4 proposes mechanisms by which this could occur.

2.2.2 Causes of PEM after Stroke

In 1974, Butterworth (2005) had already highlighted a number of practices which contributed to the decline of nutritional status in acute care patients. They persist in the system today and include: [1] diffusion of responsibility for patient care; [2] the longer than needed use of saline or glucose-based parenteral nutrition; [3] the poor observation and even poorer documentation of patients' dietary intake; and [4] failure to diagnose and treat malnutrition (Hogan, Healey et al. 2012). Studies have revealed that hospital routines can lead to insufficient nutrient intake (Dupertuis, Kossovsky et al. 2003), and that is proposed to be due to lack of training and awareness of hospital staff (Kondrup, Johansen et al. 2002). Patients are often prescribed 'nil by mouth' without being fed by any other route, and often patients are taken to an exam or investigation prior to food being served. Another related problem is the multiple episodes of fasting prior to exams. Also, the palatability of the food prepared and served is a major issue (Sullivan, Sun et al. 1999, Incalzi, Gemma et al. 1996, Dutta, Josiah et al. 2013). The post-stroke patient is particularly vulnerable to developing PEM because of additional feeding challenges related to the stroke.

There are many causes for the development of PEM after stroke, and they involve: [1] the patient, [2] the health professionals and the health system, and [3] the acute disease (stroke) itself. The greatest reason for post-stroke patients to become malnourished is that they often cannot eat by themselves. The reasons are physical or mental incapacities, communication

and perception problems (Gariballa, Parker et al. 1998b), and the result is a reduced nutrient intake and a consequent malnourished status (Unosson, Ek et al. 1994). Many patients develop swallowing problems (dysphagia) which delay nutritional supply after a stroke (Hayes 1998). Dysphagia that develops after stroke can also be permanent, in which case alternative feeding has to be considered (Coyle 2012). However, the relationship is complex since although early tube feeding might reduce fatality in dysphagic stroke patients, it has been shown to increase the proportion of those surviving with poor outcome (Dennis, Lewis et al. 2005). Others are paralysed on their dominant side of the body, or have taste and smell perceptions altered (Akner, Cederholm 2001). There are also those who become depressed or have cognitive changes that affect eating behaviours (Richards, Malouin et al. 2009). All of these factors contribute to weight and muscle mass loss that will eventually result in PEM.

Stroke patients do not present with the elevated resting metabolic rate and protein requirements that occurs in stress states such as traumatic brain injury; this is an important point when analyzing their nutritional requirements (Finestone, Greene-Finestone et al. 2003). However, there are other aspects related to stroke that could lead to alterations in caloric and protein requirements, such as infection, age, severity of stroke, medications, ventilator status, activity levels, mobility and nutritional status prior to the stroke. For the determination of nutritional diagnosis and requirements in the stroke patient, it is thus essential to have a complete assessment (Corrigan, Escuro et al. 2011).

2.2.3 Diagnosis of PEM – Nutritional Assessment

The prevalence of PEM developing after stroke varies greatly among published studies (22-62%) (Finestone, Greene-Finestone et al. 1995, Axelsson, Asplund et al. 1988, Davalos, Ricart et al. 1996, Unosson, Ek et al. 1994, Shen, Chen et al. 2010b, Choi-Kwon, Yang et al. 1998). The reason for the differences in prevalence could be patients' characteristics. However, since there is no gold standard for nutritional assessment and no one single definition of malnutrition accepted unanimously, the greatest problems with these studies are methodology and timing of the assessment. Most studies choose one tool for the nutritional diagnosis and fail to perform a comprehensive assessment. Since some sequelae after stroke can mimick signs of malnutrition, this obstructs the process of detection, especially if there is only one tool being

used for diagnosis (Foley, Salter et al. 2009). However, it is important to note that different studies, using different assessments and protocols, still report high, although different, prevalence of PEM after stroke. This shows that post-stroke PEM is truly an existing problem.

The potential consequences of PEM on the outcomes of stroke justify the need for a complete nutritional screening of stroke patients upon admission and at regular intervals after the stroke. The assessment of nutritional status can be used to support the decisions regarding nutritional support and will diminish complications associated with malnutrition (reviewed in Green, Watson 2006). Ideally, a multi-parameter assessment should be performed prior to the diagnosis, and it should include biochemical, anthropometric, and functional assessments, as well as a nutritional history. Screening for malnutrition should be top priority (Gibson 2005) and parameters of diagnosis should be well defined.

2.2.3.1 Nutritional History

The most important information to collect from the stroke patient is history of weight loss. One study showed that weight loss after the age of 50 years is associated with increased mortality. Information regarding weight at different times can be compared to present weight (Bush, Horenkamp et al. 1996). Evaluation of dietary intake should also be performed, since stroke patients tend to consume less than estimated requirements (Ha, Hauge et al. 2010). Different techniques can be applied with the objective of reviewing food preferences, number and frequency of meals, portions and sizes, ability to prepare food, place of preparation and consumption, and presence of other people. There are published studies regarding validity and reproducibility of dietary assessment. However, in the stroke patient, dietary assessment alone cannot be used in the diagnosis of malnutrition (Nip, Perry et al. 2011).

2.2.3.2 Anthropometric Measurements

Weight loss is the first identifiable sign of PEM. The simplest and cheapest methods to assess body composition are anthropometric measurements and thus they are generally used in the diagnosis of malnutrition if appropriate equations are available (Chumlea, Roche et al. 1984). Anthropometric measurements include weight, height, mid-upper arm circumference and skinfold thickness.

Weight is a fundamental part of anthropometric analysis. It should be obtained with

attention to edema, ascites and clothing. Paired with height, weight measurement allows for body-mass index calculation (BMI) which is widely used in the assessment of PEM severity. The protocol classifies a BMI of ≥ 18.5 kg/m^2 as normal, 17-18.4 kg/m^2 as mild PEM, 16-16.9 kg/m^2 as moderate PEM, and <16 kg/m^2 as severe PEM (World Health Organization 2006). One study showed that BMI closely correlates with other sophisticated measurements in estimating nutritional status (Blaum, O'Neill et al. 1997). Other nutritional deficits can also cause weight loss and muscle wasting, and thus anthropometry cannot be used alone to diagnose PEM. Certain

medical conditions and medications can have an effect on anthropometric measurements and therefore reduce sensitivity and specificity of the measurements. Biochemical assessment can be used to complement the history and anthropometry in the diagnosis of PEM (Blaum, O'Neill et al. 1997).

2.2.3.3 Biochemical Assessment

The decrease in protein intake and reduction in skeletal muscle associated with PEM can interfere with protein breakdown and synthesis. The result is limited amino acid availability and reduced protein synthesis (Torun 2006). The concentrations of certain proteins in the serum can be considered markers for the body protein pool and thus are used as diagnostic tools. Albumin, transferrin, and retinol-binding protein are examples of serum proteins that can be measured. Chronic nutritional changes are better tested by proteins with a long half-life, whereas acute settings require proteins with a short half-life (reviewed in Omran, Morley 2000). Lymphocyte counts and white blood cell counts are also indicators of PEM (Gibson 2005). It is important to note, nonetheless, that since these tools lack specificity, there are limitations to using biochemical markers to diagnose PEM.

Serum Albumin

In the past, serum albumin concentration was a major tool in the diagnosis of PEM, whereas its limitations are currently more widely appreciated. Many stroke studies used serum albumin as a tool in the diagnosis of PEM (Babu, Kaul et al. 2013, Unosson, Ek et al. 1994, Dziedzic, Slowik et al. 2004). Although serum albumin has a number of limitations for assessing

protein-energy status, it is of note that it is a good predictor of post-stroke mortality, and thus can be used to assess risk (Gillum, Ingram et al. 1994, Gariballa, Parker et al. 1998a).

The usefulness of serum albumin in diagnosing PEM relies on the fact that protein deficiency depresses the ability of the liver to synthesize albumin because of limited substrate. Serum albumin concentration reflects hepatic synthesis, plasma distribution and protein loss. Around 40% of all protein produced by the liver is released into the bloodstream as albumin. However, it is important to account for the poor specificity of assessing serum albumin because it shifts location under a stress state. More than 60% of albumin is in the extravascular pool but it is mobilized to the intravascular space in conditions such as stress and disease to maintain circulating levels (Charlton 1996).

In PEM, there is a decrease in protein synthesis, and the circulating levels of albumin cannot be maintained. An important limitation, however, is that serum albumin can also be influenced by the acute-phase reaction, which is characterized by the increased synthesis of

positive acute-phase proteins and decreased synthesis of negative acute phase proteins by the liver in response to the secretion of cytokines by inflammatory cells in response to injury (Don, Kaysen 2004). Thus, serum albumin is a nonspecific tool in that reduced concentration cannot be solely attributed to dietary amino acid availability (Kirsch, Fhrit et al. 1968). Serum albumin is a negative acute-phase reactant that is diminished in inflammatory conditions. The liver reduces albumin synthesis during inflammation while increasing synthesis of positive acute- phase proteins that can perform roles in leukocytosis, clotting and tissue repair (Don, Kaysen 2004; Kirsch, Fhrit et al. 1968). In fact, Qu et al. (1996) have demonstrated in a rat model that the effects of dietary protein depletion and inflammation on serum albumin levels can be similar in degree.

Liver Lipid Content

Although not a part of conventional nutritional assessment in humans, liver lipid content is an indicator of PEM. The liver is very affected in protein-energy malnourished individuals. Fatty infiltration of the liver is seen in both humans (Thurnham 1990) and animals (Kirsch, Brock et al. 1968) with PEM. Liver steatosis with an increase in triacylglycerol is used as a specific indicator of PEM. Rats fed low-protein diets have lipid accumulation in the liver possibly because there is an impaired transport of triacylglycerol by very-low-density

lipoproteins (VLDL). VLDLs are reduced due to a reduction in the synthesis of VLDL apolipoproteins by the liver in the malnourished individual (Seakins, Waterlow 1972, Lamri, Meghelli-Bouchenak et al. 1995).

2.2.3.4 Inflammatory Markers

Recent data suggest that PEM can independently stimulate an acute-phase response (Ling, Smith et al. 2004, Smith, Andrade Ramos et al. 2013). Serum inflammatory markers of positive and negative acute-phase proteins could be useful in diagnosing and/or characterizing PEM. Alpha-1-acid glycoprotein (AGP) is a major positive acute-phase reactant in both humans and rats. Although the biological function of AGP is still unknown, studies have shown immunomodulating effects and ability to bind and carry certain drugs. Its serum concentration increases in response to inflammation, and this elevation is correlated with increased hepatic synthesis. Gene expression of AGP is controlled by glucocorticoids and certain cytokines such as tumor necrosis factor-α and interleukin-6 (reviewed in Fournier, Medjoubi-N et al. 2000). One study suggested a low-grade systemic inflammatory response to PEM on the basis of increased serum levels of AGP and the cytokines, tumor necrosis factor-α and interleukin-1β (Ling, Smith et al. 2004).

Another positive acute-phase reactant that can be measured in serum is alpha-2-macroglobulin (A2M). The synthesis of this protein is stimulated by cytokines of the interleukin-6 family. A2M is responsible for the transport of cellular growth factors and it modulates immunological response by inhibiting the activity of natural killer cells and reducing the antibody-dependent cell-mediated cytotoxicity (reviewed in Jura, Koj 2011). One study demonstrated elevated serum A2M levels and interleukin-6 messenger ribonucleic acid (mRNA) in peripheral blood mononuclear cells and intestine of severely protein-malnourished rats (Lyoumi, Tamion et al. 1998).

2.2.3.5 Functional Assessments

PEM causes decrease in muscle strength, poor wound healing, depression, and excessive tiredness, all of which are considered functional abnormalities. For a complete nutritional assessment and a well-defined nutritional diagnosis, the use of parameters to assess function is an asset. Some practices use examinations such as the hand-grip test that allows for

strength evaluation and an estimation of muscle loss. However, in the stroke patient, it is difficult to define with the hand-grip test if strength is related to a motor deficit caused by stroke or if PEM is a contributor (Ha, Hauge et al. 2010). Psychological screening, as well as a thorough wound observation are other tools that aid PEM diagnosis (Gibson 2005).

The problem with functional assessments is the lack of standardization and the fact that other deficits can alter function. If used in combination with anthropometry, biochemistry and history, however, the functional assessment can assist the diagnosis of PEM.

2.2.4 Proposed Mechanisms by which PEM Could Affect Brain Plasticity and Functional Outcome after Stroke

There is a correlation between nutritional status and outcome after stroke. Stroke patients diagnosed as protein-energy malnourished appear to have an increased risk for disability and mortality (Gariballa, Parker et al. 1998a, Davalos, Ricart et al. 1996). However, the clinical studies have placed more focus on the general health outcomes of PEM, such as infection rates, motivation for rehabilitation and development of pressure sores. For example, Davalos and colleagues (1996) associated malnutrition, after 1 month of stroke onset, with greater incidence of infection and pressure sores, and increased risk of death. While such outcomes could clearly be important determinants of patient recovery, the potential for PEM to directly influence brain remodelling after stroke is not as well appreciated.

Despite the fact that the mechanisms by which post-stroke PEM could influence recovery have not been extensively explored, the experimental data suggest that there could be direct effects on neuroplasticity. PEM in adult rats has been reported to reduce expression of the neurotrophin, brain-derived neurotrophic factor (BDNF) and its major receptor, tropomyosin-related kinase B (trkB), in surviving neurons (Mesquita, Pereira et al. 2002); these molecules are believed to be important in cell survival, proliferation, differentiation and plasticity in the nervous system (Hennigan, O'Callaghan et al. 2007). Also, brain-derived neurotrophic factor is increased by environmental enrichment and rehabilitation exercise after stroke. This increase in BDNF contributes to the benefit of rehabilitation in promoting recovery after stroke (Maclellan, Keough et al. 2011). Recent research from my laboratory showed that PEM beginning 3 days after global ischemia (widespread forebrain ischemia), decreased the ischemia- induced increase in protein expression of synaptophysin and growth-associated protein 43 (GAP- 43), two

plasticity-related proteins, in the cornu ammonis 3 (CA3) region of the hippocampus (Smith, 2013 *unpublished observations)*. However there have been no studies of this in preclinical rat models that better mimick stroke. A second study from my laboratory demonstrated a different pattern of alteration in key plasticity proteins when PEM pre-existed with the onset of global brain ischemia, providing additional evidence that protein-energy status has the potential to alter post-stroke plasticity (Prosser-Loose, Verge et al. 2010). However neither of these studies attempted to relate the alterations in these proteins to functional outcome.

Neurogenesis, which is another type of plasticity, could also be affected by PEM. Several studies have linked diet to the modulation of adult neurogenesis in the hippocampus (reviewed in Zainuddin, Thuret 2012), but exact mechanisms have yet to be described. One may be alteration of release of growth factors, which are thought to be involved in neural sprouting and endogenous progenitor cell migration and differentiation in the brain (Cairns, Finklestein 2003). Some growth factors are decreased in the serum because of poor nutritional status (Alves, Torrinhas et al. 2012). Malnutrition has been shown to decrease, for example, insulin growth-factor 1 (IGF-1) in the serum of neonatal rats (Donovan, Atilano et al. 1991). Insulin growth-factor 1 is an anti-apoptotic factor, and has been shown to play a role in promotion of proliferation and differentiation of neuronal progenitors (reviewed in Kriz, Lalancette-Habert 2009).

Brain inflammation after stroke appears to exert an important influence on neuroplasticity; whether inflammation is beneficial or deleterious to the ischemic brain is still controversial (Kriz, Lalancette-Habert 2009). Reports from my laboratory have been inconsistent as to whether PEM increases brain inflammation following cerebral ischemia (Bobyn, Corbett et al. 2005, Prosser-Loose, Smith et al. 2011, Ji, Nazarali et al. 2008, Smith, Prosser-Loose et al. 2011). Additional limitations are that these studies are restricted to analysing the influence of pre-existing PEM on global ischemia, but not stroke. However, more recently, we have demonstrated that PEM causes an acute phase response, which may be indicative of a systemic inflammatory stimulus (Smith, Andrade Ramos et al. 2013; Smith, 2013 *unpublished observations).* This may be important to the malnourished stroke patient, since increased levels of acute-phase proteins are associated with poor outcome after stroke (Whiteley, Jackson et al. 2009).

This thesis research is based on the underlying hypothesis that deteriorating protein-

energy nutritional status that develops after and because of the stroke interferes with brain reorganization, repair and recovery. Well-characterized experimental models of stroke and PEM need to be developed in order to test this hypothesis, and are thus the subject of this thesis.

2.3 Experimental Models to Study Post-Stroke PEM

Animal models would have advantages for investigating whether post-stroke PEM affects neuroplasticity while avoiding ethical barriers associated with human investigation and exerting the strict study control that has not been achieved in the clinical studies to date. The study of mechanistic effects, co-morbidity factors, and novel therapies is not only possible, but a common baseline undertaken before clinical trials. The development of such animal models is the object of this thesis research.

2.3.1 Stroke Models

Modeling human disorders in experimental animals allows for a better understanding of mechanisms and provides the basis for the development of current and novel treatments (Lieschke, Currie 2007). The advantage of using a cerebral ischemia model is to understand the various mechanisms of injury, and later find potential sites for neuroprotection or enhance understanding of neuroplastic mechanisms that underlie rehabilitation strategies. Several criteria should be considered when using an animal stroke model: (1) the pathophysiology should resemble that of human ischemic stroke; (2) the procedure should be non-invasive and physiological variables monitored and kept within normal range; (3) the lesion should be reproducible; and (4) histological and functional assessments are to be used in the evaluation of stroke severity (Traystman 2003). There is international consensus on how these models should be run to be reliable. The Stroke Therapy Academic Industry Roundtable (STAIR) published recommendations to improve the quality of stroke studies, and they include: (1) using a well-characterized model and long survival times to ensure reliability and test the permanence of any protective effects; (2) including comorbidity factors along with stroke, to better mimick the clinical reality, and (3) run studies in older animals, to both mimick the clinical situation and see the effects of age (Albers, Goldstein et al. 2011). These recommendations were made because the majority of animal stroke studies have been done in adolescent animals, and many of these suffer from poor control of physiological variables, short survival times and the absence of

functional assessments.

There are many models available, all of which reduce glucose and oxygen supply to the brain, producing ischemic injury by impairing the energy necessary to maintain normal functioning of the brain (Traystman 2003). The rat is the animal most commonly used in stroke studies. There is homology in both behaviour and anatomy between humans and rodents, and this renders the rat as an excellent animal for neurological studies (Cenci, Whishaw et al. 2002). However, rats lack gyrencephalic brains, which makes them different than humans in behaviour and sensorimotor integration (Uylings, Van Eden 1991). In the rat, it is possible to monitor physiological variables. Rats have homogeneity within strains, and have relatively low cost of care and availability, which are other positive factors to consider when choosing animals as models of human disease (Lieschke, Currie 2007, Bacigaluppi, Comi et al. 2010).

Brain ischemia models are categorized as focal or global ischemia on the basis of location of the ischemia. Global ischemia models best mimick brain damage caused by cardiac arrest, and occur when blood flow to the brain is reduced in most or all of the tissue. Focal ischemia models more closely mimick ischemic stroke, and thus are the subject of this thesis; the reduction in blood flow is restricted to a specific part of the brain (Bacigaluppi, Comi et al. 2010). Models can be further classified as transient or permanent occlusion. In transient occlusion models, there is reperfusion, whereas in permanent occlusion models, there is no-reflow following. Without reperfusion, there is incomplete restoration of blood flow and the result is fewer surviving neurons (reviewed in Doyle, Simon et al. 2008).

2.3.1.1 Focal Ischemia Models

There are many models of focal ischemia, and most of them involve occlusion of one major cerebral blood vessel. The middle cerebral artery occlusion (MCAO) is the most common

(Hossmann 1991). The result of this occlusion is the reduction in blood flow to the cortex. Whether the brain injury also extends beyond the cortex to the striatum depends on the duration of occlusion, the site of occlusion and the amount of blood flow collateral to the occlusion area. These models are intended to resemble human thromboembolic stroke (Garcia 1984).

The major rat focal ischemia models can be classified as the: [1] endothelin 1

vasoconstriction model, [2] MCAO (proximal or distal) model, [3] intraluminal suture model, [4] middle cerebral artery embolism, and [5] photothrombotic model (Murphy, Corbett 2009).

[1] Endothelin 1 vasoconstriction

Endothelin is an endogenous peptide that causes vasoconstriction, and when it is applied to the middle cerebral artery (MCA), cerebral blood flow decreases in that territory, causing an ischemic lesion (Windle, Szymanska et al. 2006). This has been reported to yield considerable variability among different laboratories, which is a disadvantage of the model.

[2] Proximal or distal MCAO

Different surgical methods can be used to promote MCAO. Cauterization results in permanent occlusion, and clipping or snares allow for reperfusion. Variations include the occlusion of different arteries. A disadvantage is the invasiveness of the procedures and the need for good surgical skills (Murphy, Corbett 2009).

[3] Intraluminal suture model

In this model, occlusion is produced by inserting a suture into the internal carotid artery to block blood flow to the MCA, by transection of the external carotid artery. It is a technique suitable for neuroprotective drug experiments, because it produces a good penumbra. The suture can also be withdrawn to allow studies of the aspects of reperfusion. However, subarachnoid haemorrhage secondary to suture-induced arterial rupture is likely to happen. The endothelium can also be damaged, which complicates reperfusion, and the procedure affects muscles related to mastication and swallowing, leading to a decrease in food intake and weight loss (Bederson, Pitts et al. 1986). The biggest problem with this model, and the reason why many laboratories have rejected this technique, is that it often causes hypothalamic injury together with the stroke. This can complicate histological and behavioural outcomes because motivation and temperature regulation also change. Also, extensive damage such as the one produced by this model normally causes death or untreatable infarcts in human stroke, which renders the model unsuitable for studying the treatable stroke patient (reviewed in Murphy, Corbett 2009).

[4] Middle cerebral artery embolism

This model requires the introduction of a blood clot, via the internal carotid artery, to occlude the MCA. It mimicks human ischemic stroke very well because the stroke tends to be smaller than those produced by other models. A limitation is that there is high variability and

mortality associated with this model since the clots can undergo spontaneous thrombolysis and cause multiple infarcts (Murphy, Corbett 2009).

[5] Photothrombotic model

With this model, which has been chosen for the thesis research, a cortical infarct is induced by injection of a photoactive dye that is irradiated by a light beam transmitted through the skull. There is oxidative damage to the endothelium caused by the altered dye, which leads to platelet aggregation in the irradiated areas. It is a less surgically invasive procedure that results in reproducible infarcts that can be targeted to any cortical location (Murphy, Corbett 2009). A detailed discussion of the photothrombotic stroke model is found in Chapter 4.

2.3.1.2 Sensorimotor Cortex

Rat stroke models, including the photothrombotic model, often have the goal of inducing injury in the sensorimotor cortex, and thus a brief anatomical review is included here. Sensorimotor deficits in the rat after stroke display many similarities with those in humans who are left with motor impairments after stroke (Cenci, Whishaw et al. 2002). Depending on the type of stroke, patients may present with altered functional abilities that include loss of mobility, manual disability, psychomotor and cognitive function, and paralysis (Chen, Rimmer 2011).

The photothrombotic stroke model can be used to produce infarction in preselected cortical regions, which facilitates the analysis of functional, as well as structural consequences of acute stroke. Impairments in skilled and gross motor performance are enduring disabilities after stroke in primate and non-primate animals (Wolf, Catlin et al. 2001). The motor abnormalities after stroke are associated with damage to cortical (e.g. motor cortex) and subcortical areas (e.g. basal ganglia, the caudate nucleus) involved in control and execution of motor performance. The neural organization of the motor system is similar in rats and primates. As defined through electrophysiological mapping, the rat has two forelimb regions in its motor cortex that may be homologous to the primary and supplementary motor cortex of primates (Kleim, Barbay et al. 1998). Therefore, rodent model systems are created and used to study stroke associated motor abnormalities. Well-validated models of stroke in the rat induce ischemia in the forelimb and/or hindlimb regions of the cortex (Ploughman, Windle et al. 2009, Boyko, Zlotnik et al. 2010). Functional tests such as the ladder test, cylinder test, Montoya staircase test, and single pellet

reach test are used to measure the deficits observed in both skilled and gross motor performance (Jackson 2009).

In the earliest study of cortical control of skilled reaching, Peterson and Francarol (1951) delineated the region of the cortex in which stroke produced a change in paw preference previously established by training. They found that the most effective region for producing a change in handedness is the motor cortex. The photothrombotic model of focal ischemia in rats, as used in our studies, can promote occlusion of the distal part of the middle cerebral artery (MCA) and arterioles supplying cortical areas involved in skilled motor behavior (Moon, Alaverdashvili et al. 2009, Metz, Whishaw 2009, Alaverdashvili, Moon et al. 2008).

Motor representations in rats have been localized by surface and intracortical stimulation of the cortex. Parcellation of primary motor and sensory cortices is relatively challenging in rats because of the overlap of sensory and motor areas. Nevertheless, juxtaposition of the findings from histological and electrophysiological studies revealed that frontal cortical areas that (1) lack a distinct granular layer IV i.e. an agranular area (Kreig 1946) and (2) require low microstimulation currents to elicit movement corresponds to primary motor cortex. Primary motor cortex is localized mainly in the lateral agranular field. Nevertheless, there is a part of the motor cortex that overlaps with sensory cortex that lies in the medial agranular field that requires high microstimulation currents to evoke movements. Therefore, this area has not been considered as primary motor cortex. The majority of the motor cortex topographic representation is employed by areas that stimulate movement of hindlimb, forelimb, jaw, lips and tongue (Kolb, Wishaw 1983).

Rat motor cortex receives afferents from cortical somatosensory areas, cortical and subcortical motor control structures and some subcortical nuclei. It sends the efferents to the spinal cord, brain stem and red nucleus, the regions in the central nervous system that directly control muscle activity (Donoghue, Wise 1982).

The deficit in skilled upper extremity functions is the most severe and enduring motor abnormality after stroke (Richards, Malouin et al. 2009, Hoffman, Strick 1995). In this research thesis, the photothrombotic model of focal ischemia was thus used to target the cortical region involved in forelimb function as a means of mimicking the human condition.

Two discontinuous forelimb regions have been identified in rat motor cortex (Neafsey, Sievert 1982) [1] a large caudal area that begins at bregma; and [2] a small rostral

area, near the frontal pole. The small rostral area is located in the lateral agranular field region, whereas the large caudal area lies both in the lateral agranular field and the sensory cortex (Neafsey, Sievert 1982).

There is not very clear separation on different parts of hand segments in rostral vs. caudal forelimb areas (Lemon, Griffiths 2005). Differences in movements are observed when each area is targeted. When the caudal area is stimulated, wrist, elbow, or shoulder is targeted. When the rostral zone is stimulated, digit movement as well as elbow movements are elicited. Within the rostral zone, digit movements are evoked more laterally while elbow movements are seen medially (Neafsey, Bold et al. 1986).

2.3.1.3 Assessment of Brain Injury

Stroke, as well as neuroplasticity, alters the brain morphologically as well as functionally. A complete assessment of motor alterations, in addition to observation of anatomic changes, is necessary to accurately assess reliability and consistency of the alterations and extent of brain injury.

Histology

The main objective in using histology to assess ischemic injury in experimental models of stroke is to localize and measure infarct volume. Several stains are used to identify the infarct after stroke, including hematoxylin and eosin staining, nitroblue tetrazolium, and Nissl staining (reviewed in Tatlisumak, Li et al. 2007). Nissl staining (cresyl violet) is commonly used in stroke studies because the Nissl granule is found in neurons. These granules are rough endoplasmic reticulum and they are stained in the cresyl violet method because neuronal cytoplasm has acidic components (such as extranuclear RNA granules) that bind to the basic stain (Alvarez-Buylla, Ling et al. 1990). Cresyl violet does not specifically stain neurons but the high concentration of Nissl substance in neurons gives its blue characteristic color (Türeyen, Vemuganti et al. 2004). Nissl bodies change in pathological conditions and cell death. Neurons after stroke can appear shrunken at early time-points. Longer time-points will show diminished cresyl violet staining in the infarct area, because of chromatolysis (Türeyen, Vemuganti et al. 2004).

Behavioural Assessment

It is essential to compare histological results with functional (behavioural) endpoints since neurons exposed to ischemia can appear normal on histological analysis but have abnormal function. A number of behavioural tests can be used to assess functional recovery after focal ischemia. Two well-characterized tests, the Montoya staircase and the cylinder task, were chosen for evaluation of functional impairments and recovery for this thesis research.

The task developed by Montoya et al (1991) can provide a complex objective assessment of the functional deficit caused by cortical ischemia in the rat. It provides a measurement of skilled forepaw use, and reaching and grasping abilities can be measured quantitatively. The Montoya apparatus consists of a rectangular Plexiglas box in which the animal is positioned in a central platform. On each side of the platform, there is a 7 step staircase, each with a well to hold sugar pellets. The presentation of pellets gives the rat 7 degrees of reaching difficulty; the lower the step, the harder it is for the animal to retrieve pellets. This task provides an objective measure of side bias, forelimb extension and grasping ability (Montoya, Campbell-Hope et al. 1991).

Numerous studies have validated the use of this task for different strains of rats (Pagnussat, Michaelsen et al. 2009, Webb, Gowribai et al. 2003, Colbourne, Corbett et al. 2000, MacLellan, Gyawali et al. 2006, Klein, Sacrey et al. 2012). Advantages of using this task to assess functional deficits of the forelimbs are the simplicity of the method and the low rate of animal exclusion due to inability to train, since most animals are able to reach the minimum criteria (Montoya, Campbell-Hope et al. 1991). After stroke, rats are expected to greatly decrease the number of pellets eaten because they are unable to reach from the affected side (contralateral to that of stroke placement) (Ploughman, Windle et al. 2009). Functional deficits depend on severity of stroke, localization and type of infarct, day of post-stroke testing and the overall condition of the animals (e.g. stress, wound healing). Functional recovery is also expected after stroke in the rat, and typically progresses over time. Very small lesions result in full functional recovery of the affected limb. Medium to large lesions will result in improvements in reaching ability but not full recovery (Moon, Alaverdashvili et al. 2009, Soleman, Yip et al. 2010, Rasmussen, Overgaard et al. 2011). Some authors have also noted that animals can become more proficient after stroke at using the ipsilateral (unaffected) paw to retrieve pellets (Grabowski, Brundin et al. 1993). The number of trials after stroke will dictate whether the task works as a

functional test or a rehabilitative therapy, since the Montoya staircase test can have beneficial effects on early rehabilitation, affecting neuroplasticity and functional recovery (Clarke, Mala et al. 2009).

The cylinder test involves a Plexiglas cylinder that is wide enough for the rat to vertically explore. Animals rear and touch the walls of the cylinder by using one or both paws for postural support. Preferred paw use for support can be observed by analyzing video recordings taped from below via an angled mirror (Gharbawie, Whishaw et al. 2004). After stroke, rats are expected to decrease the number of wall touches with the affected paw (Minnerup, Kim et al. 2011, Milgram 2002). One group was able to distinguish differences in the cylinder test between rats exposed to sham surgery and stroke for up to one month (Schallert, Fleming et al. 2000). Various recent studies have used the cylinder test to assess motor recovery after ischemic stroke in the rat (Minnerup, Kim et al. 2011, Ploughman, Windle et al. 2009, Diederich, Frauenknecht et al. 2012, Yanev, Jolkkonen et al. 2010, Diederich, Quennet et al. 2012).

2.3.2 Protein-Energy Malnutrition Models in the Rat

Although there are no established standards for modelling PEM in rats, there is extensive literature describing models of PEM in rapidly growing weanling and adolescent rats (Heard, Frangi et al. 1977, de Belchior, Aucelia C. S., Angeli et al. 2012, Lago, Teodosio et al. 1993). However, there is little information regarding such models in the adult rat. It is important to develop this model to mimick PEM arising after stroke. Factors such as diet formulation, severity of malnutrition and whether the aim is acute or chronic malnutrition need to be taken into consideration in the model development. The parameters to diagnose PEM in the rat should include body weight, food intake, biochemical markers of malnutrition, such as liver lipid content and serum albumin concentration. The rationale and the details for the development of the PEM model in adult rats to mimick the acutely malnourished stroke patient are discussed in Chapter 3.

CHAPTER 3
MODELLING POST-STROKE PROTEIN-ENERGY MALNUTRITION

3.1 Introduction

PEM commonly arises following stroke. Although prevalence estimates show some variability, partly related to methodological limitations, up to 35-49% of stroke patients are believed to be affected (Finestone, Greene-Finestone et al. 1995, Poels, Brinkman-Zijlker et al. 2006). Many factors contribute to weight and muscle mass loss after stroke. These are related to age, eating difficulties, delayed nutritional supply and motor impairments that affect eating habits. The older population is more prone to the development of malnutrition after stroke (Mowe, Bohmer et al. 1994) because of their reduced reserves and diminished food intake (Gariballa, Parker et al. 1998b). Often, stroke patients cannot eat by themselves due to both physical and mental disabilities. The need to be fed greatly increases the risk for the development of malnutrition (Gariballa, Parker et al. 1998b). Swallowing problems after brain infarction delay nutritional supply until a medical assessment authorizes alternative feeding routes (Hayes 1998). Even after tube feeding has been prescribed, patients are frequently left in a fasted state for long periods due to shortage of staff or lack of proper training (Mould 2009, Unosson, Ek et al. 1994). Some patients suffer paralysis on the dominant side of the body or have altered taste and smell perceptions (Akner, Cederholm 2001). There are also those who become depressed or have cognitive changes that affect eating behaviours (Richards, Malouin et al. 2009).

Clinical data have shown poor nutritional status to be associated with poor survival and functional outcome following stroke (Shen, Chen et al. 2010, Chai, Chu et al. 2008). However, these studies have not been designed to examine if there is a direct causal relationship, which reinforces the need for experimental studies to address this question. The exact mechanisms by which PEM could affect brain plasticity and remodelling are similarly not understood. Such questions could be investigated by combining an adult rat model of stroke with acute, moderate PEM that mimicks what occurs in patients after stroke (Shen, Chen et al. 2011, Nip, Perry et al. 2011).

Well-established methods exist for inducing PEM in young, rapidly growing weanling and adolescent rats. These rely on feeding diets of low protein composition (0.5-5%) that cause an accompanying voluntary reduction in food intake, resulting in moderate to severe PEM (Swenne, Borg et al. 1992, Coward, Whitehead et al. 1977, Ling, Smith et al. 2004, Taylor,

Bauman et al. 1992, Prosser-Loose, Smith et al. 2011). Data also exist for inducing PEM in aged rats that have a lower protein requirement. One study compared protein needs of 15 and 25 month-old rats and reported that both groups were still gaining weight when fed a 2% protein diet for 21 days (Fischer, Canolty 1983). However, there is limited information on models to generate different types of PEM in the adult rat. Since developing a model of PEM depends on the energy and protein requirements for the age and sex of rat under study as well as diet composition (Woodward 1998, Reeves, Nielsen et al. 1993), the first objective of this study was to develop a dietary PEM model for the adult rat.

One challenge is to estimate protein requirement for the 16-20 week old adult rat to be used in our studies. Protein requirements are influenced by age and growth rate (Lewis, Ullrey et al. 2006), and protein requirements decrease with age until rats reach about 1 year of age (Hartsook, Mitchell 1956). For instance, a protein restriction of 5% will induce PEM in 8 week old but not 24 week old rats (Kahn, Bender 1979). Twelve % protein is the level at which nitrogen balance is maintained at 10 weeks of age (Archer, Vernon Rayner et al. 2003). However, 10 week old rats have a higher growth rate and protein requirement than the 16 week old rats to be used in our studies, for which 4-6% protein is believed to maintain nitrogen balance (Levin, Keesey 1998). The energy concentration of the diet and the source and quality of protein, which influence the biological availability of the amino acids, also influence protein requirements (Lewis, Ullrey et al. 2006). The National Research Council guidelines suggest that for adult rats, if high-quality protein is used, protein requirement is 5% of total energy intake, whereas when the diet is made of poor-quality proteins, requirement increases to 7% of total intake (National Research Council 1995).

Another important consideration when modeling PEM is whether acute or chronic malnutrition is the goal. Acute PEM in humans causes faster weight loss and a more dramatic change in the biochemical markers of malnutrition, such as serum albumin concentration, than does chronic PEM. After stroke, human patients suffer a rapid drop in weight, of about 2-4% in 2 weeks, which results in an acute state of mild-moderate PEM (Shen, Chen et al. 2011, Unosson, Ek et al. 1994, Jönsson, Lindgren et al. 2008). After 1 month of stroke onset, the range is 2-11% loss of body weight (Jönsson, Lindgren et al. 2008, Gariballa, Parker et al. 1998b, Finestone, Greene-Finestone et al. 1995). To achieve acute PEM in rats, the protein content of the diet has to be lower than that used to induce chronic PEM. Based on the available literature, it was

hypothesized that a American Institute of Nutrition 1993 Maintenance diet (AIN-93M diet) (Reeves, Nielsen et al. 1993) modified to contain either 1% or 0.5% protein of high biological value would produce an acute state of mild-moderate PEM in adult rats, and that the diet containing 0.5% protein would have the more rapid effects. We predicted that both diets would be accompanied by a voluntary reduction in total food, and thus energy, intake. The PEM was characterized on the basis of food intake, body weight, serum acute phase protein concentrations, and liver lipid content.

In characterizing the PEM, a second objective of the study was to investigate the extent to which an acute phase response is induced, since systemic inflammation may worsen stroke outcome (Vila, Filella et al. 1999, McColl, Rothwell et al. 2008). This was assessed on the basis of serum concentrations of two positive acute phase proteins, alpha-1-acid glycoprotein (Fournier, Medjoubi-N et al. 2000) and alpha-2 macroglobulin (Gauthier, Mouray 1976), and the negative acute phase reactant, serum albumin (Don, Kaysen 2004). Ling et al. (2004) have reported increased inflammatory markers such as interleukin-6, interleukin-1 and tumor necrosis factor-alpha in the serum of young malnourished rats. Another group showed that PEM in 6 week-old rats increases a positive acute-phase protein, serum alpha-2 macroglobulin (Lyoumi, Tamion et al. 1998). Our laboratory has previously measured elevated serum alpha-2 macroglobulin concentrations in protein-energy malnourished adolescent rats (Smith, Andrade Ramos et al. 2013). In humans, serum analysis of protein-energy malnourished children showed increased interleukin-6 concentration (Dulger, Arik et al. 2002). Additionally, serum albumin concentration, which often rapidly declines with PEM, can provide evidence for an inflammatory stimulus. Serum albumin is a major negative acute-phase protein (Don, Kaysen 2004), but is also influenced by decreased dietary amino acid supply for synthesis, preventing its use as a specific marker of inflammation (Qu, Ling et al. 1996; Omran, Morley 2000). One study in rats showed that the effect of protein depletion and inflammation on serum albumin levels can be similar in degree (Qu, Ling et al. 1996).

Motor disabilities are among the various impairments that occur after stroke. Functional alterations caused by paralysis are very common and can last a lifetime (Kreisel, Hennerici et al. 2007). Whether post-stroke PEM worsens these motor impairments is unknown and should be addressed in a rat pre-clinical stroke model. Among behavioural tests used to assess functional impairment and recovery in rat stroke models, the well-validated Montoya

staircase is sensitive for detecting motor deficits (Montoya, Campbell-Hope et al. 1991, Pagnussat, Michaelsen et al. 2009, Rasmussen, Overgaard et al. 2011, Clarke, Mala et al. 2009). The task developed by Montoya et al (1991) measures skilled forepaw use by assessing the ability to reach and grasp for a desirable food such as sugar pellets. The third study objective was to test whether the development of PEM in the adult rat independently causes any motor alterations in this task, which could confound future use of the test for assessing the effect of nutritional status on post-stroke motor deficits.

The final objective was to further validate the Montoya staircase for use in protein-energy malnourished rats, by testing whether rats sustained motivation to obtain sugar pellets as PEM developed. An imbalance in the protein/carbohydrate ratio, as is the case with a low protein diet, is known to affect the amount and type of foods that a rat chooses to eat (Theall, Wurtman et al. 1984). For instance, if fed below protein requirement, the rat will voluntarily decrease carbohydrate intake, when given the choice, in order to re-establish the normal protein/carbohydrate ratio (Li, Anderson 1982, Anderson 1979). Thus, we assessed whether a low protein diet and the development of PEM over 31 days would render rats less motivated to reach for sugar pellets.

3.2 Materials and Methods

3.2.1 Experimental Design

Adult, male, Sprague-Dawley rats, 13 weeks old (N=26) were caged in groups of 2. Housing facilities were maintained at 22°C and had a 12hr light/dark cycle. Rats were acclimatized to the facility for 3 days with *ad libitum* access to control diet (see details below) and water. On the fourth day, animals were removed from the colony to the behavioral room where they were acclimated for 30 minutes in their cages. After that time, they were handled for 10 minutes each. Daily handling was done for 1 week, during which the rats were also weighed daily to allow them to acclimate to the procedure. Food intake on a cage basis was measured to permit calculation of the food restriction required for the Montoya staircase training period. Over the next 15 days, they were exposed to training in the Montoya staircase 2 times/day. On the following day, rats were randomized to 1 of 3 diet groups for 31 days: 0.5% protein (n=8), 1% protein (n=8) or control diet (n=10).

To assess if diet affected sensorimotor function, the rats were tested in the Montoya staircase test on days 3, 15 and 30 post-diet assignment. The test to assess motivation to eat sugar

pellets was done on days 2, 14, and 29. During this period, body weight was recorded 2 times/week and food intake daily. On day 31, rats were humanely euthanized. Blood and liver samples were collected for biochemical analysis to assess protein-energy status**.** The details of each step of the experimental design are provided below.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.

3.2.2 Training in the Montoya Staircase

This task assesses skilled reaching and grasping abilities (Montoya, Campbell-Hope et al. 1991). All training for the Montoya staircase test was done during the dark phase with three 40 watt red lamps, since this is the rat's active period. All rats were food restricted by 15% during the first week of training and 10% during the second week of training to provide sufficient motivation to reach for the 45 mg banana flavored sucrose pellets (TestDiet, Richmond, IN).

Before starting the task, the rats were allowed to acclimate in the behaviour room for 30 minutes. The Montoya box was modified to accommodate the adult rats used in this experiment and had 3 adjustable heights for rats of varying sizes (Langdon, Clarke et al. 2011).This task consists of placing the rat into the box, on top of a central platform that has a staircase on either side. Each step of the 7-step staircase has a well that holds 3 sugar pellets. The stair arrangement and the platform on which the rats are positioned do not allow the rat to retrieve dropped pellets, and the animal is only able to reach for the right steps with the right paw and for the left steps with the left paw (Biernaskie, Chernenko et al. 2004). Training consisted of 15 days, during which the rat was left in the chamber for 15 minutes and the total number of pellets eaten, dropped and remaining on the steps was recorded. This procedure was repeated after 2 hours; that is, each rat was trained 2 times per day. In between sessions, the rat remained in the testing room, in the dark. The number of pellets eaten per side was used as a measure of forelimb reaching ability (Montoya, Campbell-Hope et al. 1991). Between individual rat sessions, the boxes were cleaned with 70% ethanol and wiped dry with a paper towel.

Baseline performance in the Montoya staircase was collected on the night before assignment to experimental diets at the end of the training period, under the same conditions as used for training days. Animals were tested twice for 15 minutes each, and the interval between

trials was of at least 2 hours. Only rats that were able to eat with one forepaw 12/21 pellets with a standard deviation of ≤ 2 over a period of 8 trials were included in the Montoya staircase testing period. Rats that did not meet these criteria were assigned to an experimental diet group and used for all other assessments except the Montoya test. After baseline assessment, paw preference was determined as the side in which the rat retrieved more pellets. All analysis was done with the preferred paw.

3.2.3 Motivation for Performance in the Montoya Staircase

To evaluate test validity, a motivation test was performed on the night before assignment to experimental diets to determine baseline motivation to retrieve sucrose pellets. Drive to eat banana-flavored sugar pellets was determined by placing the rat into a standard size rat cage (35cm X 25cm X 24 cm) that contained sucrose pellets and timing how fast the pellets were eaten (Baunez, Amalric et al. 2002). The 10 sucrose pellets were scattered randomly in the cage; the rat was then placed in the cage, the lid was closed and the timer started. The rat was returned to his home cage after all pellets were eaten. The test cage was wiped with 70% ethanol before and after each rat was tested for motivation.

3.2.4 Experimental Diets

After 15 days of training and baseline testing, rats were randomly assigned to 1 of 3 diet groups. The diets were modified from the AIN-93M diet (Reeves, Nielsen et al. 1993). The control diet (12.5% protein) meets the nutritional requirements for this age of rat, and contained a protein: carbohydrate: lipid ratio of 12.5: 72.1: 4 on a weight basis (Table 3.1). The ratios for the 1% and 0.5% protein diets were 1: 83.5: 4 and 0.5: 84.1: 4, respectively. Animals had *ad libitum* access to food and water (except for the Montoya staircase testing days - see below), and were fed the specific diets for 31 days. Food intake on a cage basis was measured daily and individual body weight twice a week.

3.2.5 Testing in the Montoya Staircase and Motivation Test

On days 3, 15 and 30 post-diet assignment, rats were tested 2 times/day for 15 minutes each in the Montoya staircase, with an interval of at least 2 hours in between trials. The methods were identical to those described above for the training period except that rats were not

chronically food-restricted. The total number of pellets eaten, dropped and remaining on the steps was counted. The mean of the two tests was used for analysis. Rats were fasted for 10 hours before the first test of the day, to ensure sufficient motivation to perform the task.

To ensure that experimental diet did not alter motivation to reach for sugar pellets in the Montoya staircase, thus confounding the test results, the motivation test was performed on days 2, 14 and 29 post-diet assignment, as described above.

Table 3.1 Composition of experimental diets*

Components	Adequate Protein (Control) (g/kg)	1% Protein Diet (g/kg)	0.5% Protein Diet (g/kg)
Vitamin Free Casein	140.00	11.32	5.67
L-Cystine	1.80	0.15	0.07
Sucrose	100.00	100.00	100.00
Cornstarch	465.688	551.158	556.842
Dextrinized Cornstarch	155.00	184.00	184.00
Soybean Oil (without tBHQ)	40.00	40.00	40.00
Cellulose	50.00	50.00	50.00
Mineral Mix[£]	35.000	0	0
Mineral Mix[¶]	0	35.000	35.000
Calcium Phosphate, dibasic	0	12.821	12.989
Calcium Carbonate	0	3.070	2.931
Vitamin Mix[κ]	10.000	10.000	10.000
Choline Bitartrate	2.500	2.500	2.500

*Diets were purchased from Dyets Inc. (Bethlehem, PA). [#]L-Cystine added proportionally to amount of casein. Amounts vary to maintain a constant ratio of total protein to total sulphur amino acid content. [£]AIN-93M mineral mix (Reeves, Nielsen et al. 1993). [¶] AIN-93M modified mineral mix with calcium and phosphorus deleted, potassium citrate • H_2O increased from 28 to 226.55 g/kg, sucrose increased from 209.806 to 618.256 g/kg mineral mix. [κ]AIN-93M vitamin mix (Reeves, Nielsen et al. 1993).

3.2.6 Characterization of the Malnutrition

3.2.6.1 Tissue Collection

On day 31, rats were deeply anesthetized (5% isofluorane) and humanely euthanized for tissue collection. Blood samples were collected via cardiac puncture before perfusion for later measurement of serum albumin, alpha-1-acid glycoprotein, and alpha-2 macroglobulin concentrations. Following transcardial perfusion with saline, liver was dissected on ice, immediately transferred to liquid nitrogen, and stored at -80°C. Blood samples were allowed to clot at room temperature for 30 minutes after collection, followed by centrifugation at 1,500 x g for 10 minutes (Eppendorf Centrifuge 5424). Serum samples were stored at -80°C.

3.2.6.2 Serum Albumin Concentration

Serum albumin concentration was determined by the bromocresol green method (Dumas, Watson et al. 1997). A volume of 25 µL of sample serum, blank, or standard was added to 5.0 mL bromocresol green solution (0.15mM bromocresol green, 0.075 M succinate buffer [pH 4.2], 30% Brij-35) in triplicate. After 30 minutes, absorbance was measured spectrophotometrically at 628 nm (Biochrom Ultrospec 3100Pro). A standard curve (2-6 g/dL) was made in which the concentration of serum albumin was positively correlated to measured absorbance. The albumin concentration of the sample was determined by linear regression, after calculating the mean of the triplicate samples.

3.2.6.3 Liver Lipid Content

Liver (~ 0.5 g) was homogenized in 1 mL of 0.15 M NaCl and added to 5 mL of chloroform:methanol (2:1). Samples were centrifuged at 3,200 x g (IEC Centra-HN) for 10 minutes and the liver lipid pellet was isolated. To dehydrate the pellet, 1 g of anhydrous Na_2SO_4 was added. The contents were filtered and washed with chloroform. The remaining chloroform was evaporated using a Concentrator 5301 (Eppendorf) with a Vac V-500 (Buchi) attached to a Vacuum Controller V-850 (Buchi). The tubes and liver samples were weighed to determine the lipid content of each sample. Analysis was run in duplicate.

3.2.6.4 Alpha-1-Acid Glycoprotein (AGP) and Alpha-2 Macroglobulin (A2M) Concentrations

Serum concentrations of AGP and A2M were determined using double sandwich ELISA kits (Immunology Consultants Laboratory, Inc., OR, USA). Serum samples (100 μL) were diluted either 1:1000 for determining AGP concentration or 1:500 for measuring A2M concentration. Diluted serum was pipetted in triplicate into wells that had been coated with anti-AGP and anti-A2M antibodies, respectively. The microtiter plates were covered and incubated for 60 minutes. The contents of the wells were manually washed 4 times to remove unbound proteins. Anti-AGP or anti-A2M antibodies conjugated with horseradish peroxidase were added and the antibodies complexed with the previously bound markers (AGP or A2M). A chromogenic substrate, containing 3,3′,5,5′- tetramethylbenzidine and hydrogen peroxide, was added after 4 washes and incubated for 10 minutes in the dark. Sulfuric acid (0.3M) was added to stop the reaction. Since the quantity of bound enzyme varies directly with the concentrations of AGP and A2M in the sample, the absorbance at 450 nm is a measure of these markers in the samples. The concentrations of AGP and A2M were interpolated from the standard curve (five-parameter logistics curve) [GraphPad Prism, 5.04, California] and corrected for the dilution factor. All standards were run in duplicates and all samples in triplicates.

3.2.7 Statistical Analysis

Statistical analyses were performed using SPSS 18.0. Univariate Analysis of Variance was conducted to determine significant differences in serum albumin, AGP and A2M concentrations and liver lipid percentages. Tukey's Honestly Significant Difference (HSD) was used whenever Post-Hoc testing was required to determine significant differences among specific experimental groups. Differences were considered statistically significant at $p < 0.05$. Mixed-designs ANOVA was used to compare results on the Montoya staircase, motivation task, body weight and food intake among the diet groups over time. For body weight analysis, further testing with univariate ANOVA was performed on days 3 and 7. Since the results were statistically significant on day 7, Tukey's HSD was used as the post-hoc test to determine differences among specific groups. Differences in body weight were considered significant at the $p < 0.017$ level because of Bonferroni adjustment. For the analysis of food intake, data from every third day [beginning on day 3] was analyzed to allow for sufficient power to run the mixed

designs ANOVA. No post-hoc test was performed for food intake.

3.3 Results

3.3.1 Assessment of Protein-Energy Status

3.3.1.1 Body Weight

Body weight decreased significantly in both 0.5% and 1% protein groups ($F(16,184) = 77.25$, $p < 0.001$), showing a group-time interaction (Figure 3.1). The difference from the control group started being significant on day 7 ($F(2,23) = 5.55$, $p = 0.01$), but the 0.5% and 1% protein groups were not statistically different from one another (Tukey's HSD; $p = 0.97$). Over 31 days on experimental diet, the control group gained an average of 20.3% of initial body weight, while the 0.5% protein group lost 10.7% and the 1% protein group lost 5.9%. On day 31, the body weight of the 1% protein group was 23.2% lower than that of the control group. For the 0.5% protein group, body weight was 25.4% lower than that of the control group.

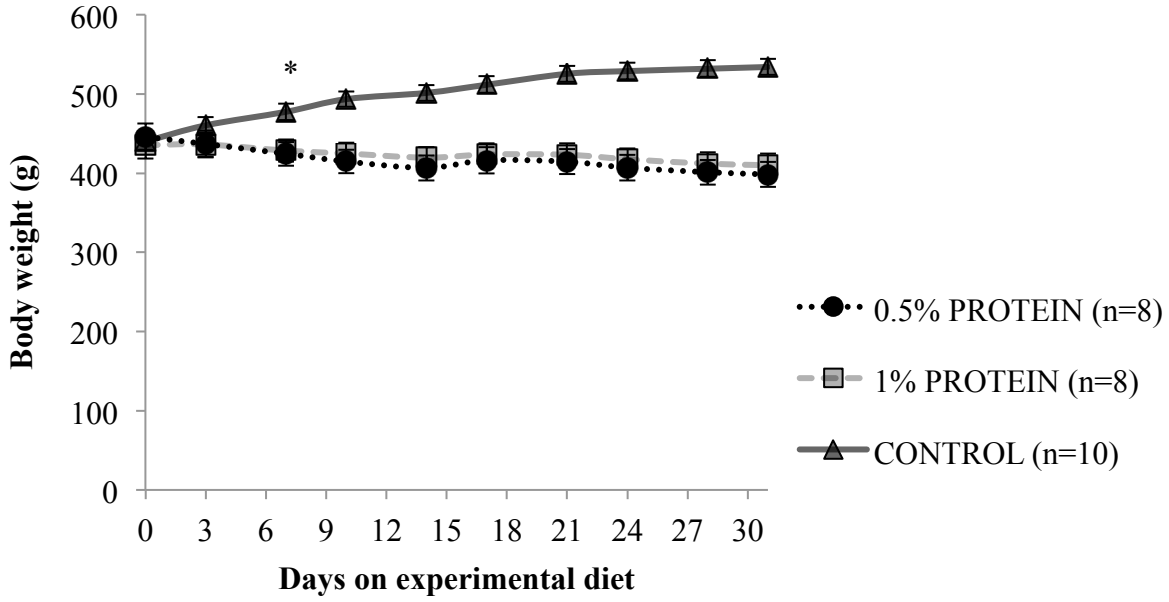


Figure 3.1: Mean (±SEM) body weight (g) measurements showed a group x time interaction ($F(16,184) = 77.25$, $p < 0.001$). *The difference between the low protein groups and the control group first became significant on day 7, as determined by one-way ANOVA analysis ($F(2,23) = 5.55$, $p = 0.01$). The dietary protein deficient groups were not significantly different from each other (Tukey's HSD; $p = 0.97$).

3.3.1.2 Food Intake

Mean ± standard error of the mean (± SEM) food intake measured daily on a cage

basis (n = 4-5 [2 rats/cage]) is shown in Figure 3.2. Since food intake was elevated on the first 2 days after the food restriction period in the control group, mixed-designs ANOVA was started on day 3. No interaction was demonstrated, but significant main-effects were observed for time ($F(8,80) = 4.87$, $p < 0.001$) and experimental diet group ($F(2,10) = 9.58$, $p = 0.005$). A decrease in food intake occurred in the 0.5 and 1% protein groups. Compared to control intake, the decrease was of 20.6% and 15.9%, for 0.5 and 1% protein groups, respectively. No further testing was performed due to insufficient statistical power.

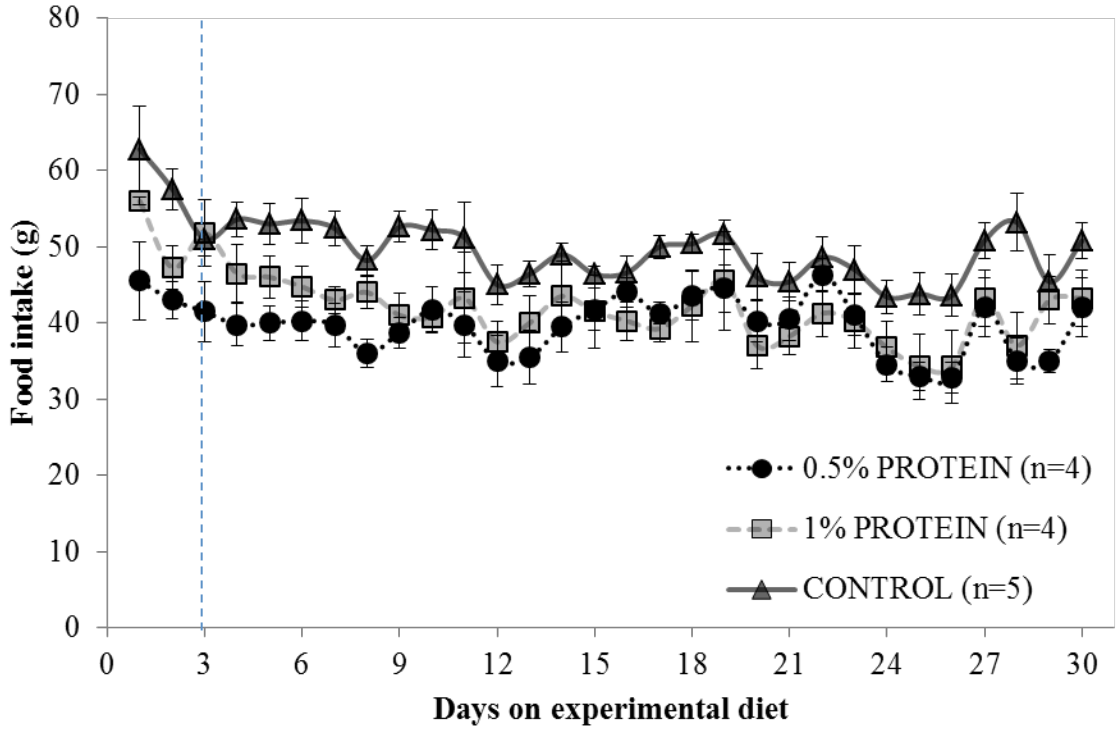


Figure 3.2: Food intake (mean ± SEM) of rats fed low protein diets was lower than that of control fed rats. Food intake was decreased 20.6% by feeding a 0.5% protein diet and 15.9% by feeding a 1% protein diet. Food intake was measured daily, based on cage data (n = 4-5 [2 rats/ cage]). Food intake changed as time progressed ($F(8,80) = 4.27$, $p < 0.001$). There was a significant difference in food intake among diet groups ($F(2,10) = 9.58$, $p = 0.005$). Dashed line on day 3 indicates when analysis was started.

3.3.1.3 Liver Lipid Content

Figure 3.3 demonstrates that liver lipid content was altered by experimental diet ($F(2,23) = 61.83$, $p < 0.001$). Both the 0.5% protein and 1% protein diet groups increased liver lipid relative to the control group (Tukey's HSD, $p < 0.001$). There was no significant difference between the 0.5% and 1% protein groups ($p = 0.13$).

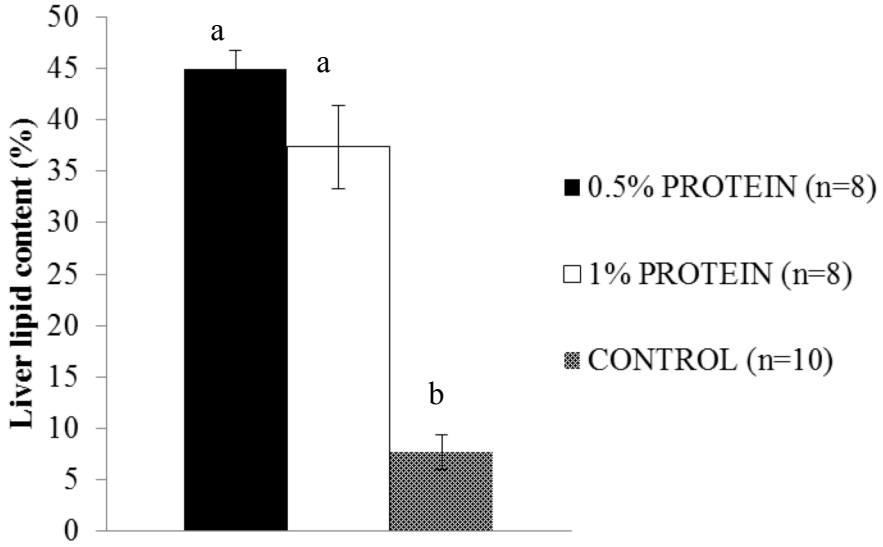


Figure 3.3: Liver lipid content was increased by both low protein diets. Data are presented as mean (±SEM). Liver lipid weight was calculated as a percentage of liver wet weight (0.5% protein = 44.9 ± 1.8%; 1% protein = 37.3 ± 4%; Control = 7.7 ± 1.2%). One-way ANOVA showed a significant difference among groups ($F(2,23) = 61.83$, $p < 0.001$). Experimental groups not sharing a common superscript letter were significantly different by Tukey's HSD ($p < 0.001$).

3.3.1.4 Serum Albumin

Mean (± SEM) serum albumin concentration shown in Figure 3.4 was significantly influenced by diet ($F(2,23) = 4.81$, $p = 0.018$). Only the 0.5% protein diet decreased serum albumin concentration significantly relative to the control group (Tukey's HSD, $p = 0.015$). The 0.5% and 1% protein groups did not differ from one another ($p = 0.53$), nor did the 1% protein and control groups ($p = 0.16$). Serum albumin concentration was, on average, reduced by 22.2% in the 0.5% protein group when compared to control values.

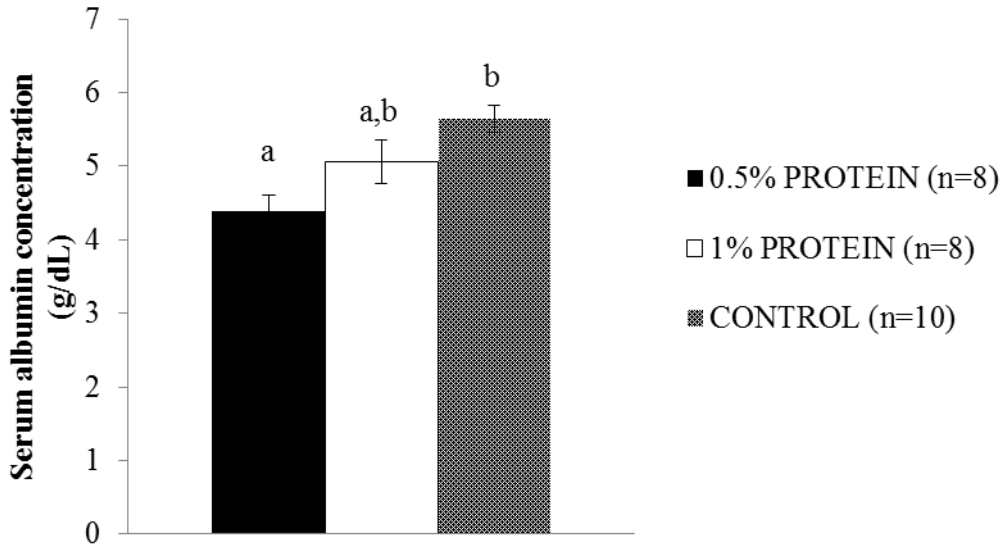


Figure 3.4: Serum albumin concentration (mean ± SEM) was decreased by the 0.5% protein diet (0.5% = 4.4 ± 0.2 g/dL; 1% = 5.1 ± 0.3 g/dL; Control = 5.6 ± 0.2 g/dL). One way ANOVA showed a significant difference among groups ($F(2,23) = 4.81$, $p = 0.018$). Experimental groups not sharing a common superscript letter were significantly different by Tukey's HSD. The mean reduction in serum albumin concentration was 22.2% in the 0.5% protein group.

3.3.1.5 Serum Alpha-1-Acid Glycoprotein

Figure 3.5 shows that serum AGP concentration was increased by feeding low protein diets ($F(2,23) = 35.84$, $p < 0.001$). Tukey's HSD post-hoc test showed significant differences between the 0.5% and 1% protein groups ($p < 0.001$), as well as between the 0.5% protein and control group ($p < 0.001$), and between 1% protein and the control group ($p = 0.031$).

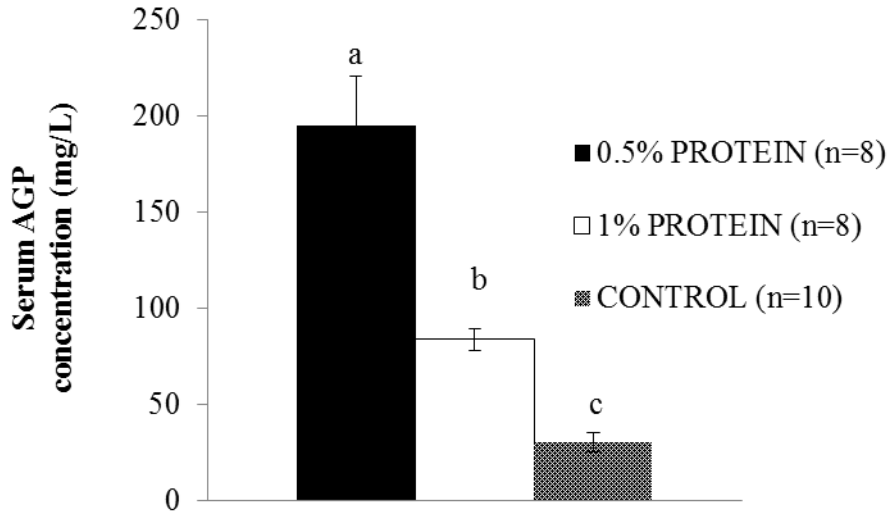


Figure 3.5: Low protein diets elevated mean (± SEM) serum alpha-1-acid glycoprotein concentration (one-way ANOVA, $F(2,23) = 35.84$, $p < 0.001$), and 0.5% protein had the largest effect (0.5% = 195.5 ± 25 mg/L; 1% = 83.9 ± 6 mg/L; Control = 30.2 ± 5 mg/L). Experimental groups not sharing a common superscript letter were significantly different by Tukey's HSD ($p < 0.001$ level).

3.3.1.6 Serum Alpha-2 Macroglobulin

The influence of experimental diet on serum A2M concentration is shown in Figure 3.6. Dietary protein had a statistically significant effect ($F(2,23) = 74.04$, $p < 0.001$). Both low dietary protein groups had significantly increased serum A2M concentration when compared to the control group ($p < 0.001$), and the 0.5% protein diet caused a greater increase than the 1% protein diet ($p < 0.001$).

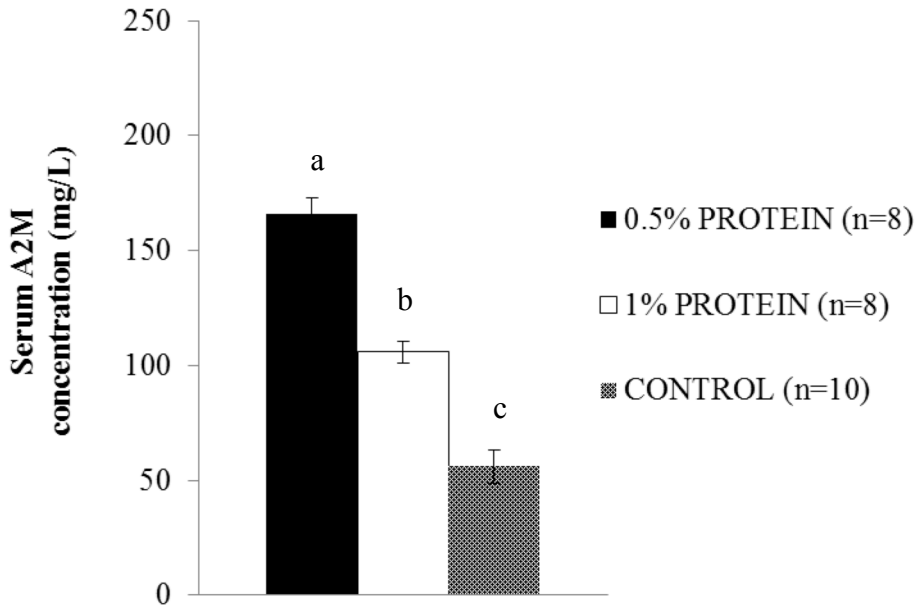


Figure 3.6: A diet containing 0.5% protein elevated mean (± SEM) serum A2M concentration to a greater extent than a 1% protein diet (0.5% = 166.2 ± 6.7 mg/L; 1% = 105.7 ± 4.6 mg/L; Control = 55.7 ± 7.3 mg/L). One way ANOVA showed a significant difference among groups ($F(2,23) = 74.04$, $p < 0.001$). Experimental groups not sharing a common superscript letter were significantly different by Tukey's HSD ($p < 0.001$).

3.3.2 Motor Skills Assessment

3.3.2.1 Montoya Staircase Test

The influence of experimental diet on performance in the Montoya staircase test, expressed as a percent of baseline performance, is shown in Figure 3.7. Three rats did not meet criteria set for the Montoya staircase by the end of the training period and thus were not included in the testing. Mixed-designs ANOVA demonstrated no significant differences among groups over time ($F(4,40) = 1.37$, $p = 0.26$). Group and diet main-effects were not statistically significant ($p > 0.05$).

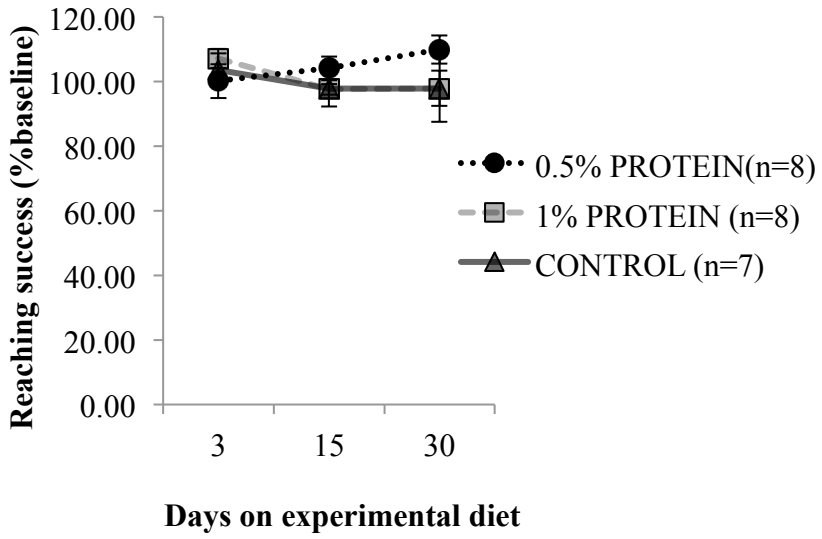


Figure 3.7: Low protein diets did not alter performance in the Montoya staircase after 3, 15, or 30 days of exposure to experimental diet. Data are expressed as mean (± SEM) performance as a % of baseline performance before assignment to experimental diet. No group x time interaction was observed ($F(4,40) = 1.37$, $p = 0.26$). The effect of time was not significant ($p = 0.59$), nor was the effect of dietary protein ($p = 0.72$).

3.3.2.2 Motivation to Reach for Sugar Pellets in the Montoya Staircase

The results in Figure 3.8 show the results of the motivation task expressed as the absolute time to eat 10 sugar pellets. All groups took less time to eat the 10 sugar pellets on repeated testing (Mixed-designs ANOVA, $F(2,46) = 11.52$, $p = 0.0004$). There was no main-effect of experimental diet ($p = 0.19$) or interaction of diet and time ($p = 0.22$).

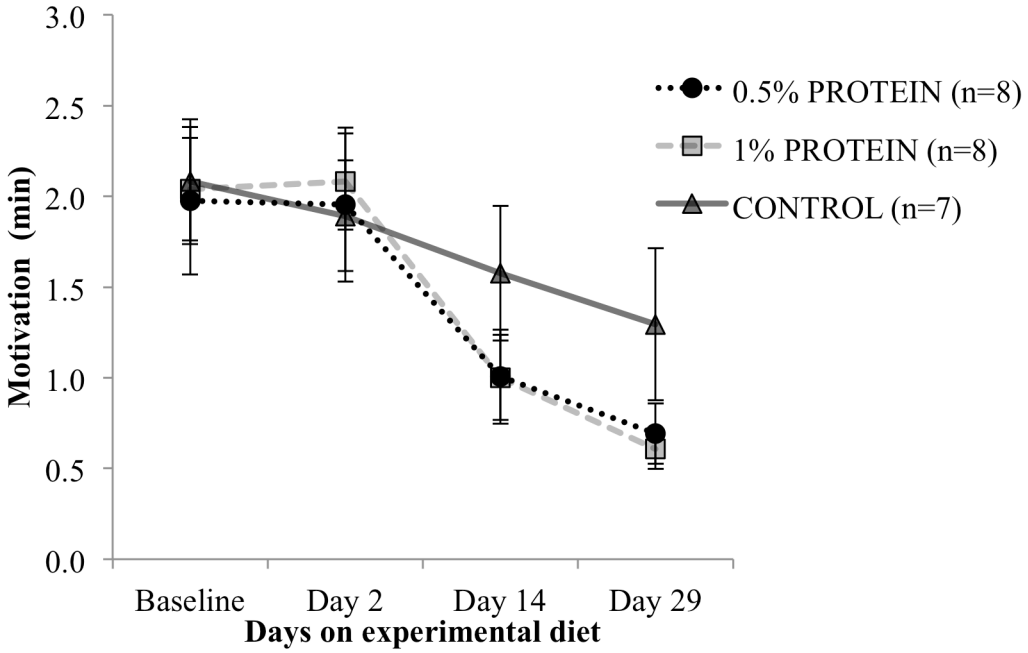


Figure 3.8: All experimental groups ate the sugar pellets more quickly with increased exposure to them (time), but this was not significantly altered by experimental diet. The results of the motivation task are shown as the mean (± SEM) time in minutes to eat 10 sugar pellets. Rats performed differently at each time point (Mixed-designs ANOVA, $F(2,46) = 11.52$, $p = 0.0004$), but showed no main-effect of experimental diet ($p = 0.19$) or interaction of diet and time ($p = 0.22$).

3.4 Discussion

The primary goal of this study was to develop an experimental model of PEM that would serve as a clinically relevant mimick of the malnutrition that arises after stroke. The features of the post-stroke protein-energy malnourished patient important to imitate in the rat included the diminished food intake and the mild to moderate acute drop in body weight (less than 10% in a month (Jönsson, Lindgren et al. 2008, Finestone, Greene-Finestone et al. 2003)), which also affects the biochemical markers of malnutrition. All the mentioned characteristics should be diagnosed with the best methods available for rats, which frequently are not the same as those used in humans. Besides weight loss, the focus for nutritional diagnosis in human studies is usually serum albumin. Rarely do the studies consider the potential effects of inflammation on the expression of this marker, and thus the acute-phase proteins measured in our study are not found in human studies of PEM following stroke. They are, however, recognized as

good markers of the acute-phase response in humans (Kaysen, Dubin et al. 2000), as well as in rats (Lyoumi, Tamion et al. 1998). The analysis of liver lipid content, common in rodent studies (Bobyn, Corbett et al. 2005, Lamri, Meghelli-Bouchenak et al. 1995, Kwon, Kang et al. 2012), is also not commonly estimated in human studies even though PEM in humans also causes fat infiltration into the liver (Garrow, Fletcher et al. 1965, Waterlow 1975, Fong, Nehra et al. 2000).

Our results showed that both low protein diets caused weight loss, and the range of all individual rats in both groups was 1.7- 4.9% after seven days, which is characteristic of the desired acute PEM state (Jönsson, Lindgren et al. 2008). After 2 weeks being fed protein deficient diets, rats had lost 2.5-7% of their initial body weight. The literature regarding nutritional status of stroke patients shows a similar pattern of weight loss. Gariballa et al. (1998) showed a 2.8% decrease in body weight 2 weeks after stroke. Unosson et al. (1994) reported a drop in weight of 2.3% at 2 weeks after stroke. After 4 weeks on 0.5% protein diet, our rats showed a 10.7% decrease in body weight from the beginning of the study, whereas the 1% protein diet caused a 5.8% weight loss. Finestone et al. (1995) reported that after 4 weeks, 14% of stroke patients had lost 5-15% of their usual weight (collected as weight before stroke, reported by patient or family member). The authors classify this weight loss as a sign of mild malnutrition, which is the severity we aimed to achieve with our study. Although similar, it is important to acknowledge the limitations of the comparisons. The Finestone et al. (1995) study reported weight, which, especially when dealing with elderly patients, could show biased results. Also, the adult rats used in our study are past their rapid growth slope (Duffy, Lewis et al. 2002), which has advantages over studying adolescent rats. However, the comparison with the human situation still has limitations, since the rat continues to grow until 96 weeks, although at a much slower rate (Levin, Keesey 1998, Duffy, Lewis et al. 2002).

Over the 31 days on diets, protein deprived rats showed diminished food intake. The 1% protein group consumed 15.9% less food than the control animals, whereas the 0.5% protein group had a more dramatic decrease in food consumption of 20.6%. This diminished food intake caused the energy deficit, which combined with the protein deficiency of the diets caused the desired PEM. One study after stroke showed a similar decrease in food intake of 15.9% in an orally fed group over 21 days (Foley, Finestone et al. 2006). However, an unusual pattern was observed in food intake in the current study. First, food intake on the first 2 days after assignment was peculiarly high, which is likely due to the previous food restriction required for

training in the Montoya staircase. The hypothesis is that after being restricted for 15 days, animals were trying to compensate and eat more, after which food intake appeared to stabilize on the third day. The protein deprived groups, because of the introduction to the new diets, ate less than the control group. Thus, these first few days of data are less reliable data for documenting how the low protein diet affects food intake and how soon the low protein diets cause the voluntary reduction in food intake. These results highlight a limitation to our experimental design, as a period of free access to control diet (i.e., wash-out period) should have been provided before assigning the animals to their experimental diet groups. Had this been done, it is quite possible that the food intake of the three experimental groups would appear similar on days 1-3. Another limitation of the study is that the acute nature of the PEM models could be assessed only on the basis of daily food intake and body weight measured 2 times per week. All other diagnostic tools were only assessed after 31 days. It is suggested that future studies assess serum acute-phase proteins and liver lipid content at earlier time points to allow for a more complete diagnosis of the type of PEM and how it evolves.

The biochemical data support the conclusion that both deficient diets induce PEM. However, the diet containing 0.5% protein is the one that is best suited to produce mild to moderate acute PEM in adult rats. Both protein deficient diets caused liver lipid content to be increased, but the severity of the protein depletion did not affect liver lipid accumulation. Our animal study provided animals with a balanced diet prior to assignment into deficient diets. In the clinical reality, conversely, many people are consuming the typical Western diet, high in saturated fats and sugars, prior to having a stroke. Future studies could be designed to feed Western diets prior to stroke and the development of PEM. As a Western diet also triggers the development of fatty liver (Zivkovic, German et al. 2007), the effects of post-stroke PEM may be further magnified by the previous dietary history.

Another objective of this study was to assess the degree of acute-phase response induced by the models of PEM. A decrease in serum albumin occurs with low dietary amino acid availability (Qu, Ling et al. 1996) such as would occur with the 0.5% and 1% protein diets. However, the increase in the serum concentration of the positive acute-phase proteins, AGP and A2M, suggest that at least some of the decline in serum albumin is due to inflammation. These results extend previous findings reported in adolescent rats fed low protein diets (Lyoumi et al. 1998, Smith, Andrade Ramos et al. 2013) to less severe PEM in more slowly growing adult rats.

In our study, rats fed 0.5% protein diet showed a greater acute phase response on the basis of higher concentrations of both positive acute phase markers; this was not the case with the negative acute phase reactant, albumin. The acute-phase response in malnourished stroke patients would be important to characterize in future studies, as it has not yet been described. Overall, the analyses of inflammatory markers in this study strengthen the previous idea that PEM can independently cause an acute-phase response (Ling, Smith et al. 2004), since there was no other stimulus present.

Since our dietary prototype did not influence performance on the Montoya staircase test, this behavioural protocol can be reliably used in future studies with the goal of studying whether nutritional status influences post-stroke deficits. The effects of PEM on motor skills have previously only been studied during the developmental period. One rat study previously showed that maternal malnutrition resulted in a delay in reflex maturation and locomotor activity evolution in pups in the postnatal period (Barros, Manhães-De-Castro et al. 2006). However, these investigators did not use purified diets, the low-protein diet was manipulated with use of food ingredients, and unmatched rat chow was fed to control rats. This approach cannot guarantee isocaloric diets between PEM and control groups or diets matched for micronutrients, and thus the effects cannot be attributed to PEM with certainty. A more recent study on mice confirmed that animals born to protein-energy malnourished mothers have delayed motor development, and in this case the nutrition protocol was well designed, with purified, well-matched diets (Ranade, Sarfaraz Nawaz et al. 2012). One human study correlated PEM in early infancy with poorer motor abilities in adolescence (Hoorweg, Stanfield 1976). Another study established that motor scores were significantly lower in malnourished children than in normal counterparts, especially in chronically malnourished children (Reyes, Valdecanas et al. 1990). Although these studies relate malnutrition with poor motor function, this has not previously been studied in the adult. To my knowledge, this is the first study to assess the relationship between PEM in adult rats and skilled motor abilities. One study limitation that must be noted, however, is that motor performance was assessed by two measurements performed on the test day. This did not follow the recommendation to test on a minimum of two independent days for preclinical stroke models, which is intended to capture intra-animal variability in performance (reviewed in Kleim, Boychuk et al. 2007). This may have reduced the sensitivity for detecting small differences between the experimental groups. However, in stroke studies, a careful design should

also consider that multiple testing can also act as rehabilitation in addition to outcome assessment.

This study also demonstrated that evolving PEM did not alter the rats' motivation to obtain the sugar pellets, making it a valid test for studying the effects of PEM in the post-stroke period. Altered motivation would have compromised the use of the Montoya staircase to test motor skill as intended. Interestingly, the malnourished animals followed the same pattern as control animals, and increased the speed at which they ate the sugar pellets each time they were tested. This is likely explained by the need, on the first testing day to take longer to acclimatize to the cage; with increasing exposure over time, the rats did not need to perform cage recognition before going after the pellets. Thus, the acclimatization factor reduces time to find pellets in subsequent trials. However, some observations suggest that this task could be refined to be a better test of motivation. For example, random placement of pellets in the cage has an effect on how fast rats are able to eat the sugar treats. For instance, if most pellets fall into a corner and the animal does not need to explore the cage to find pellets, this is another explanation, in addition to motivation, for an increase in speed. For further studies, consistently placing both the pellets and the rat in pre-selected spots in close proximity would possibly render more accurate results. Also, an automated activity chamber could be used for tracking locomotor activity to determine whether there are any differences among experimental groups. A period of exposure to an empty cage prior to the addition of sugar pellets would also allow familiarity with that environment prior to testing so that this aspect would not confound the test of motivation to eat the sugar pellets. Finally, although the low-protein diets did not significantly affect rat motivation to eat sugar pellets, it is noteworthy that a trend towards malnourished animals being more motivated appeared to be developing. Thus, it is recommended that future studies of malnourished rats always include a test of motivation, with the suggested adaptations, particularly if the test period exceeds 30 days.

In summary, this study showed that feeding a 0.5% protein diet for 31 days produces an acute, moderate PEM with some similar features to what is observed in human patients after stroke. This model is suitable for use in future experimental stroke studies because it does not affect motor ability in the Montoya staircase test and does not change motivation to reach for sugar pellets.

CHAPTER 4
ESTABLISHING THE PHOTOTHROMBOTIC FOCAL ISCHEMIA MODEL

4.1 Introduction

The PEM that develops after stroke may worsen recovery (Martineau, Bauer et al. 2005, Yoo, Kim et al. 2008). Experimental models of global brain ischemia have shown that PEM can have effects on both the brain cell death cascade (Bobyn, Corbett et al. 2005) and plasticity mechanisms that aid brain repair (Prosser-Loose, Verge et al. 2010). However, these studies have addressed the effects of PEM present at the time of brain ischemia, which is a problem affecting around 16% of patients (Gariballa, Parker et al. 1998a, Gariballa, Parker et al. 1998b). While important, PEM developing after brain ischemia affects a much larger segment (35-49%) of patients (Shen, Chen et al. 2011, Chai, Chu et al. 2008, Poels, Brinkman-Zijlker et al. 2006). Also, the effects have never been studied in a focal ischemia model, which is a better mimick of stroke. To address both the functional and biochemical effects of post-stroke PEM, a reliable experimental stroke model is needed to combine with the PEM model developed and described in Chapter 3.

The photothrombotic stroke model is a good model choice, because it mimicks active clot formation in human stroke (Watson, Dietrich et al. 1985). Also, it does not require as invasive surgery, such as a craniotomy, as some other accepted models. When studying malnutrition effects, it is particularly important that the stroke model not require extensive surgery, since the latter has dramatic effects on nutritional status and could confound the study of stroke. The metabolic response to surgery increases mobilization of glucose and fat, increases lipolysis and gluconeogenesis, decreases utilization of insulin, and generates hyperglycemia (Cuthbertson, Fell et al. 1972). Major surgery thus increases protein and energy requirements dramatically (Hill, Douglas et al. 1993), and this is not a feature observed in stroke patients (Finestone, Greene-Finestone et al. 2003). Thus, choosing a model that minimizes these effects is important. Another advantage of the photothrombotic stroke model is that it can be readily applied to both adult and aged rats, which best mimicks the majority of stroke cases (Brown, Marlowe et al. 2003, Badan, Buchhold et al. 2003). Nevertheless, there are also recognized limitations of this model, the main one being vasogenic edema, which is atypical of human stroke (Carmichael 2005). This can be overcome with long term endpoints that allow for

resolution of edema. Another limitation for neuroprotection studies (such as drug candidate studies) is the lack of reperfusion and consequently a penumbra (Carmichael 2005). However, this does not present limitations for the intended post-stroke PEM studies that are hypothesized to exert effects through plasticity mechanisms. Modifications to the method have also been proposed to overcome this limitation to produce a penumbra-like region (Hilger, Blunk et al. 2004).

The photothrombotic stroke model was created by Watson and colleagues (1985). A thrombus was produced via a photochemical method to occlude the cerebral microvasculature (Watson, Dietrich et al. 1985). They injected a photosensitive dye and irradiated the exposed skull with a green light from a filtered arc lamp. Platelet aggregates were observed in the vascular endothelium, resulting in occlusion. However, Rosenblum and el-Sabban (1977) were the ones who originally pioneered the idea of photo-altering dye for thrombus formation. They used sodium fluorescein dye and blue light in mice to occlude pial vessels. Since the dye was capable of absorbing light, it was thought to produce damage to the endothelium because of heat transmission and to cause platelet aggregation (Rosenblum, el-Sabban 1977). However, the dye reemitted the absorbed light by fluorescence and was not very efficient. The use of other dyes was shown to be more efficient, rose Bengal being one of them (reviewed in Watson 1998). Disodium tetrachlorotetraiodofluorescein, known as rose Bengal, is very efficient in injuring the endothelium. The energy absorbed by the dye when irradiated is transferred to the heavy atom of iodine in the structure. Because of this light-iodine interaction, oxygen becomes excited (singlet state), and singlet oxygen directly interacts with endothelial lipids and proteins, and causes peroxidation. The result is a damaged endothelium that attracts and causes platelets to aggregate (Watson 1998). However, the triplet state of the photosensitized dye acts as a free radical and can lead to other damaging reactions (Dietrich, Busto et al. 1987). The photothrombotic phenomenon is cumulative and generated by the rate of endothelial damage and the response of the platelets to this damage (Dietrich, Busto et al. 1987).

Consistency in any experimental stroke model is essential to allow for the study of cause and effect (Carmichael 2005). Thus, in establishing this model in our laboratory, reliability needed to be assessed. Since infarct location and extent influence post-stroke function and brain reorganization, histological methods are used to assess infarct volume, ensure consistent infarct location, and interpret the accompanying functional measurements (Carmichael 2005). The final

volume of infarct is determined by the: [1] type and concentration of the dye; [2] type and intensity of irradiation source used; [3] irradiation time; and [4] resolution of edema (Watson, Prado 2009). In the present study, it was hypothesized that laser irradiation at 532 nm (25 mW power in a 5 mm diameter) targeted to the forepaw area of the cortex for 10 minutes combined with 30 mg/kg body weight of rose Bengal injection would result in medium size infarcts associated with motor alterations of the targeted forelimb. The parameters used in this methodology are known to occlude 10 to 40 μm diameter cortical vessels (Watson, Prado 2009). A major objective was to determine the consistency of the model by measurements of infarct volume.

The motor cortex is a common site of human stroke, which often results in limb impairments or paralysis (Fridman, Hanakawa et al. 2004, Chollet, Dipiero et al. 1991, Liepert, Storch et al. 2000). In the photothrombotic model, a reproducible infarct in the motor cortex associated with a functional domain (such as forepaw use) can be produced (Watson, Dietrich et al. 1985) and generate alterations in sensorimotor function (Moon, Alaverdashvili et al. 2009, Alaverdashvili, Moon et al. 2008, Maxwell, Dyck 2005, Brown, Aminoltejari et al. 2009, Markgraf, Green et al. 1994, Markgraf, Kraydieh et al. 1993). Forepaw impairments resemble human limb disabilities, and thus are useful for the study of motor recovery. Photothrombotic stroke in the forelimb area of the motor cortex should result in defined functional deficits, which are essential to characterize in experimental stroke models to allow for understanding of behavioral alterations and neuroplasticity. The Montoya staircase and cylinder test are well-validated for experimental stroke assessment (Jackson 2009). The Montoya staircase is a food rewarded test that can quantitatively assess the ability of a trained rat to reach. After focal ischemia, animals are expected to have an impaired capacity to retrieve sugar pellets and to show some recovery as the post-stroke period progresses (Montoya, Campbell-Hope et al. 1991). Rats also show alterations in their ability to support themselves with the affected paw after stroke. The cylinder test, which is a non-baited assessment, allows for the observation of paw preference and postural support of the rat placed inside a cylinder (Gharbawie, Whishaw et al. 2004). Functional post-stroke assessment is a critical supplement to histological findings, because often infarct size does not predict the degree of disability. Also, whereas histology is limited to one terminal time point, behavior can be measured at multiple times to show the rate of recovery (Lemon, Griffiths 2005, Hossmann 2006). Thus, the second objective in this study was to evaluate the consistency

of the motor alterations after stroke by behavioural assessments.

Another important aim of a newly developed laboratory stroke model is incomplete functional recovery, since that is what most often occurs in the clinical reality. Stroke has a high disability rate among survivors (Go, Mozaffarian et al. 2013). Since 76% of stroke patients survive, the support to disabled individuals represents more than 56% of the annual cost of stroke (Statistics Canada 2011). Stroke has a major impact on quality of life. For example, one study showed that only 36.8% of stroke survivors report themselves as being in good health (Centre for Chronic Disease Prevention and Control, Health Canada Canadian Cardiovascular Society Heart and Stroke Foundation of Canada 2003). In 2000, a high percentage of individuals (77.2%) who had a stroke reported having activity restrictions and needing help with activities of daily living (Centre for Chronic Disease Prevention and Control, Health Canada Canadian Cardiovascular Society Heart and Stroke Foundation of Canada 2003). The degrees of impairment and disability depend on the type and severity of stroke, but also on the location of the infarct (de Haan, Limburg et al. 1995). Thus, while stroke patients can exhibit some functional recovery after the initial degree of disability, recovery is incomplete for most patients. Rat stroke models also demonstrate spontaneous recovery (reviewed in Murphy, Corbett 2009), but the extent varies among different models and with varying methodological refinements. In order to mimick the clinical reality, the third study objective was to investigate whether the model developed would meet the aim of incomplete functional recovery after 30 days.

4.2 Materials and Methods

4.2.1 Experimental Design

Adult, male, Sprague-Dawley rats, 13 weeks old (N=18) were caged in groups of 2 or 3. Rats were acclimatized to the facility for 3 days with *ad libitum* access to control diet (see below). On the fourth day, animals were removed from the colony to the behavioral room where they remained for 30 minutes in their cages, before being handled for 10 minutes each. Daily handling continued for 1 week. During this time, animals were weighed daily to allow them to acclimate to the procedure. Food intake was measured, during the first week to permit calculation of the food restriction to be imposed during the training period. Over the next 15 days, rats were exposed to behavioural training in the Montoya staircase task 2 times/day. They were then randomized to either sham surgery (SHAM) or focal ischemia (ISCHEMIA). The

sample size was 15 rats in the ISCHEMIA group and 3 animals in SHAM group. The rationale for the small sample size in the SHAM group was that their main purpose was to provide normal brain specimens for histological comparison.

To assess pre-stroke baseline (day before surgery) and post-stroke sensorimotor function, two validated functional tests were used, the cylinder test (on days 3 and 30 post-surgery) and the Montoya staircase test (on days 3, 15 and 30 post-surgery). Rats were humanely euthanized on day 31, with the exception of 2 animals euthanized on day 30 due to ethical reasons related to tail necrosis. Brains were then collected for histological assessment of infarct volume. The details of each step in the experimental design are provided below. 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.

4.2.2 Diet

The control diet used in this experiment was identical to that used in the first study and described in Chapter 3 (Table 3.1). Body weights were obtained on a weekly basis, except during the Montoya staircase training period and for 4 days following the surgery, when body weight was measured daily.

4.2.3 Montoya Staircase Training and Pre-Stroke Performance Assessment

Rats were trained in the Montoya staircase task as described in Chapter 3. All rats were food restricted by 15% during the first week of training and 10% during the second week of training to provide sufficient motivation to reach for the banana flavored sucrose pellets (TestDiet, Richmond, IN). During the training period, rats were left in the chamber for 15 minutes and the total number of pellets eaten, dropped and remaining on the steps was recorded. This procedure was repeated after 2 hours, to allow for 2 training sessions per day.

Baseline (pre-stroke) performance testing in the Montoya staircase was done on the night before surgery at the end of the training period, under the same conditions used for training days. Animals were tested twice for 15 minutes each, with a 2 hour interval. Rats that were able to eat $\geq$ 12/21 pellets and a standard deviation of $\leq$ 2 over a period of 8 trials were included in the post-stroke Montoya staircase testing. Those rats that did not meet this criteria (n = 2) were still assigned to surgical groups and used for all other assessments.

4.2.4 Cylinder Task: Pre-Stroke Performance Assessment

This test assesses spontaneous forelimb use (asymmetry) during exploration. The pre-stroke baseline test took place on the day prior to surgery. This test also served to determine preferred paw, so that the infarct could be placed in the hemisphere contralateral to this paw and thus affect function of this paw. Animals were placed into a Plexiglass cylinder (20 cm diameter) situated on a glass tabletop and were videotaped from below. Rats were left in the cylinder for 5 minutes and a minimum of 20 independent wall contacts. Since 3 animals did not have the minimum required number of touches in 5 minutes, each remained in the cylinder for an additional 7 minutes. The videotapes were later viewed by a blinded observer to count the number of forelimb wall contacts (single and bilateral) used for postural support. Forelimb use preference was calculated as follows:

- For ISCHEMIA rats, percent affected limb use was calculated using the following formula: [(contralateral forelimb contacts + 1/2 bilateral contacts) / (total contacts)] x 100% (Woodle, Asseo-Garcia et al. 2005).
- For SHAM rats, percent preferred limb use was calculated as: [(preferred forelimb contacts + 1/2 bilateral contacts) / (total contacts)] x 100% (Woodle, Asseo-Garcia et al. 2005).

4.2.5 Surgical Induction of Stroke

For photothrombotic stroke, the method was modified from that described by Watson and colleagues (Watson, Dietrich et al. 1985) and performed under sterile conditions. Rats, now 16 weeks old, were anaesthetised with 4% isoflurane in an induction chamber. Animals were transferred to the surgical area after their heads were shaved. Once placed in the stereotactic device, the incision site on the skull was cleaned 3 times with chlorhexidine soap followed by 70% ethanol and a 1-2 cm midline incision was made through the scalp from between the eyes to between the ears. The skin was held open with bulldog clamps to keep the skull exposed. The hemisphere contralateral to the preferred side was used for placement of a unilateral lesion.

The region of interest was defined using the following stereotaxic coordinates: +2 mm to -1 mm anterior-posterior from Bregma and from +2 mm to +5 mm lateral of midline, chosen to target the area of forepaw sensorimotor cortex (Paxinos, Watson 1998). Using a

stereotaxic frame, this region was demarcated and the overlying skull was thinned with a bone drill until brain surface blood vessels were visualized, when the skull was dry, under a dissecting microscope at x16 magnification. The area surrounding the thinned box was colored with a black sharpie marker to avoid laser refraction. The rat tail was then cleaned with warm water and chlorhexidine soap, and a tourniquet was applied. A catheter (BD Insyte IV catheter, 24GA 0.75IN) was inserted into the lateral tail vein, the tourniquet and needle were removed, and the syringe containing the 30 mg/kg body weight dose of rose Bengal (using a stock concentration of 100 mg/ml) was attached to the catheter. The syringe was kept covered in tin foil to avoid light degradation. At this point, a 532 nm green laser (Laserglow Technology) delivering 25mW in a 5 mm diameter was moved into position above the thinned area of the skull. The timer was started just as the rose Bengal infusion began and was set to maintain illumination for 10 minutes, allowing for photoactivation of the dye. Rose Bengal dye was injected over 45 seconds into the tail vein, followed by sterile saline injection (over 45 seconds) to clear the catheter. The homoeothermic blanket system was set to maintain rectal temperature controlled to 37 ± 0.2 C°. Following photostimulation, the tail vein catheter was removed. The head incision was sutured and local anesthetic (bupivicane, 2mg/kg) in physiological saline was injected in the surrounding area. For fluid replacement, a subcutaneous injection of sterile physiological saline (0.9%) was administered into the scruff of the neck. Sham animals were treated identically (including time under anaesthesia) except that the laser was not turned on. Physiological variables monitored included: [1] Pulse, measured by the multiparameter monitor (Lifewindow 6000V), [2] oxygen saturation, measured by pulse oximetry in the multiparamenter monitor (Lifewindow 6000V), [3] respiration rate (breaths/minute), measured by counting the number of breaths in 15 seconds and multiplying by 4, and [4] rectal temperature, measured by rectal probe.

During the post-surgical period, rats were monitored twice daily for the first 4 days, and then once a day for the remainder of the study.

4.2.6 Post-Stroke Functional Assessments: Montoya Staircase and Cylinder Testing

For the testing period after the focal ischemia (on days 3, 15 and 30 post-stroke), animals were tested in the Montoya staircase 2 times/day for 15 minutes each, with an interval of at least 2 hours in between trials. The total number of pellets eaten, dropped and remaining on the steps was counted for analysis. The mean of the two tests was used for analysis. Reaching

success was expressed as a percent of baseline performance and calculated as:

- For ISCHEMIA rats: [(number of pellets retrieved and eaten with the affected paw on testing day)/ (number of pellets retrieved and eaten with the affected paw at pre-stroke baseline)] x 100%.
- For SHAM rats: [(number of pellets retrieved and eaten with the preferred paw on testing day)/ (number of pellets retrieved and eaten with the preferred paw at pre-surgery baseline)] x 100%.

Cylinder testing took place on days 3 and 30. Animals were video recorded in the cylinder for 5 minutes, with the exception of the 3 animals who did not reach criteria at baseline; each of these rats remained in the cylinder for a total of 12 minutes. The percent affected/ preferred limb use was calculated as described above in Section 4.2.4.

4.2.7 Histological Assessment of Infarct Size

Rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The heads were placed in a 4% paraformaldehyde solution for 24 hours. Brains were then dissected, postfixed in 4% paraformaldehyde overnight and then transferred to another jar containing 20% sucrose in phosphate buffered saline(PBS) for 3 days. Subsequently, brains were placed in OCT (Optimal Cutting Temperature) compound for 30 minutes, after which they were frozen using dry-ice cooled isopentane in a mold containing the OCT compound. Samples were stored at -80°C until sectioning. Coronal brain sections (40 μm thick) extending throughout the infarct were cut in a cryostat. Every third section was collected for cresyl violet staining.

The brain sections were inspected under a light microscope (Nikon Eclipse 50i). Digital images were captured using the DS-Fi1 camera attached to the microscope (Nikon Eclipse 50i) on 10x magnification. Each section was assigned the coordinate from Bregma in accordance with a rat brain atlas (Paxinos, Watson 1998). If two hemispheres in a given section were asymmetrical (at different coordinates due to sectioning artifact or stroke-associated displacement), a coordinate was defined for each hemisphere. Since a considerable number of sections were of poor histological quality, each brain section was classified as “usable” or “unusable” based on staining and sectioning quality. If the region of infarction was obscured or damaged, it was discarded from further analysis. The minimum number of sections used to make

a volume estimate was 12.

For each section judged to be of adequate quality, the dorsal border of the infarct was traced using a template of contralateral (healthy) hemisphere (a mirror image). The border of the infarction was then traced, and this included both necrotic tissue and the surrounding reactive gliosis. The area of infarct (mm^2) was measured using the Image/J software from National Institutes of Health (NIH) (http://rsb.info.nih.gov/ij/download.html). The area was measured 3 times, and the resulting average "infarction area" for each section was used for a volume calculation. The distance between "usable" sections was calculated and was referred to as the "corrected between section interval" considering the number of "unusable", rejected brain sections. The volume of brain infarct for each rat was calculated using the following equation:

$$\text{Infarct volume } (mm^3)_{\text{ each animal}} = \sum [\text{Infarct area of each section } (mm^2) \times \text{Corrected between section interval } (mm)]$$

4.2.8 Statistical Analysis

Statistical analysis was performed using SPSS 18. Behavioral endpoints were treated as interval data and tested for normality. Repeated measures ANOVA was used to analyse ISCHEMIA group on: reaching success on Montoya staircase over time (days 3, 15 and 30), affected limb use for support in the cylinder test over time (days 3 and 30), and pattern of limb use in the cylinder over time (days 3 and 30). The large difference in group size between the ISCHEMIA and SHAM groups would not allow for an unbiased comparison and thus, the SHAM group was not included in the analysis. Post hoc analysis, where applicable, was done using paired T-tests.

To establish if motor function was related to the extent of cortical damage, the correlation between infarct volume and motor deficit in the Montoya staircase and cylinder test, at each time point (days 3, 15 and 30 for Montoya staircase and days 3 and 30 for cylinder) was determined by Pearson's correlation.

A multiple regression analysis was performed to examine the effects of tail necrosis on behavioural endpoints (Montoya staircase performance on days 3, 15 and 30, and cylinder performance on days 3 and 30) and infarct volume.

Statistical significance for all analyses was set at $p < 0.05$.

4.3 Results

4.3.1 Pre and Post-Surgical Outcomes

Physiological variables monitored during surgery are summarized in Table 4.1. The mean ($\pm$ SEM) presented for the group was calculated from the mean values over the surgical period for each animal. Ranges show the lowest and highest values obtained for the group and thus demonstrate inter-animal variability. Both oxygen saturation and temperature were kept within a tight range. The range in oxygen saturation was 98 –100% in both groups. Mean (± SEM) temperature was 36.8 ± 0.1°C and 36.8 ± 0.3°C for ISCHEMIA and SHAM groups, respectively. Pulse rate had wider inter-animal variability, and thus the mean (± SEM) pulse rate was 301.4 ± 4.9 beats per minute in the ISCHEMIA group, and 308 ± 8.8 beats per minute in the SHAM group. Over the dye injection period, 6 animals (5 ISCHEMIA and 1 SHAM) showed a brief ~ 60% decrease in pulse rate, which returned to normal in $\leq$ 40 seconds. Another set of 4 animals (2 ISCHEMIA and 2 SHAM) lost pulse for about 20 seconds and returned to normal after that.

After surgery, ISCHEMIA rats lost an average (± SEM) of 0.9 ± 0.1% of their pre-surgery weight, whereas SHAM animals had a mean ± SEM drop of 0.8 ± 0.1% relative to their pre-surgery weight. All animals had started regaining weight by 1 day after surgery, and all had returned to pre-surgery weight by day 4 post-surgery. Body weight data are shown in Figure 4.1. This figure also illustrates body weight during the pre-surgical period when rats were chronically food-restricted for training in the Montoya staircase.

Tail problems related to the rose Bengal administration developed in 6 rats during the 31 day post-surgical period. The tails turned pink or purple and, over time, showed signs of necrosis. Working in close consultation with the Veterinarian, criteria were developed to humanely euthanize any rat showing sign of distress, pain, or tail infection. All rats that showed tail problems were monitored twice a day for the entire study period. Four rats were kept until the end of the study, whereas 2 rats had to be euthanized one day prior to the end of the study.

Table 4.1: Summary of physiological variables monitored during surgery.

Group	Oxygen Saturation (%)		Pulse Rate (beats per minute)		Temperature (°C)	
	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
ISCHEMIA (n=15)	99.4 ± 0.1	98 - 100	301.4 ± 4.9	256 - 348	36.8 ± 0.1	36.2 – 37.7
SHAM (n=3)	99.3 ± 0.3	98 - 100	308.8 ± 8.8	287 - 335	36.8 ± 0.3	36.6 – 37.9

Means are calculated as the average of means for each rat over the surgical period. Ranges display the lowest and highest values in the group, to show inter-animal variability.

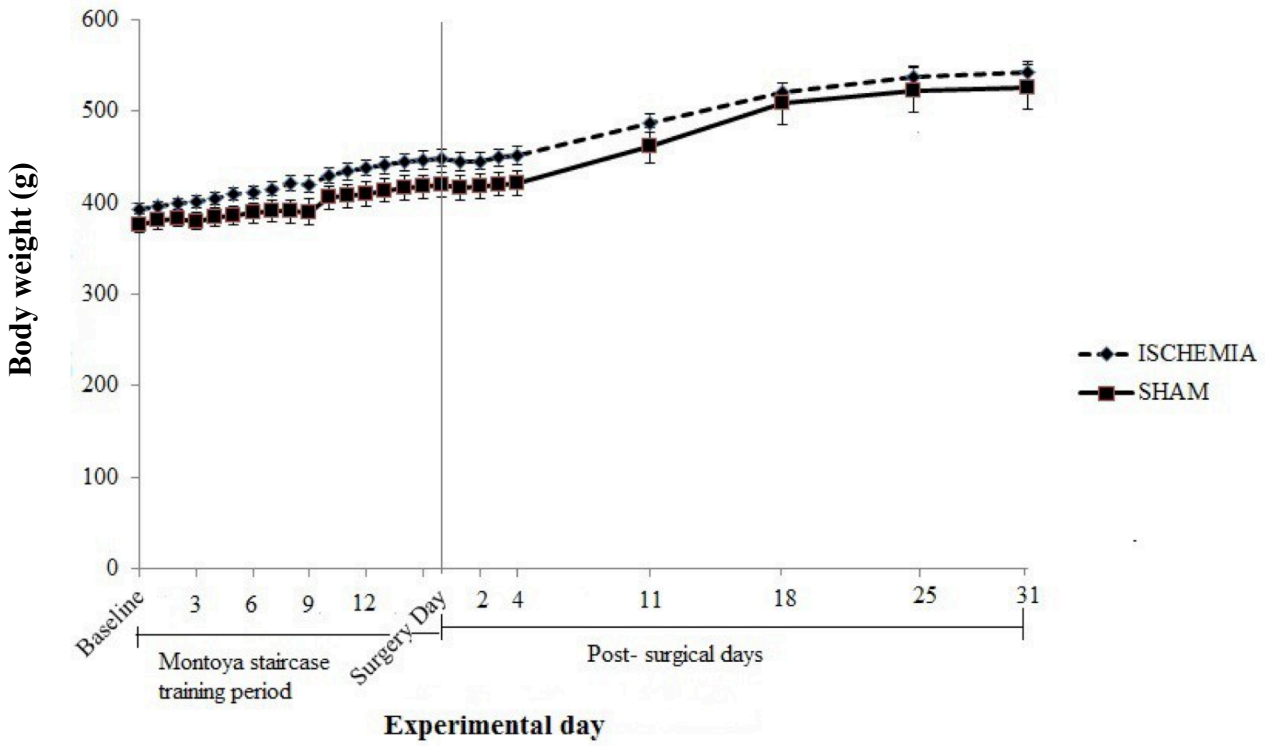


Figure 4.1: Body weight (g) during the experimental period. Values are shown as mean ± SEM for ISCHEMIA (n= 15) and SHAM (n=3) groups.

4.3.2 Histological Outcomes

Control (Sham) rats demonstrated no histological evidence of cortical damage. An infarct was present in 87% of rats exposed to photothrombotic stroke. The infarct volume varied from 5.7 mm^3 to 12.8 mm^3 in those 13/15 rats exposed to photothrombotic stroke. The 2 animals without visible infarcts experienced problems with rose Bengal dye injection. In both cases, the needle was inserted multiple times before the catheter could be positioned in the tail vein. Resistance while injecting rose Bengal was also reported, suggesting avascular leakage of the dye. The variation in infarct size is shown in Figure 4.2.

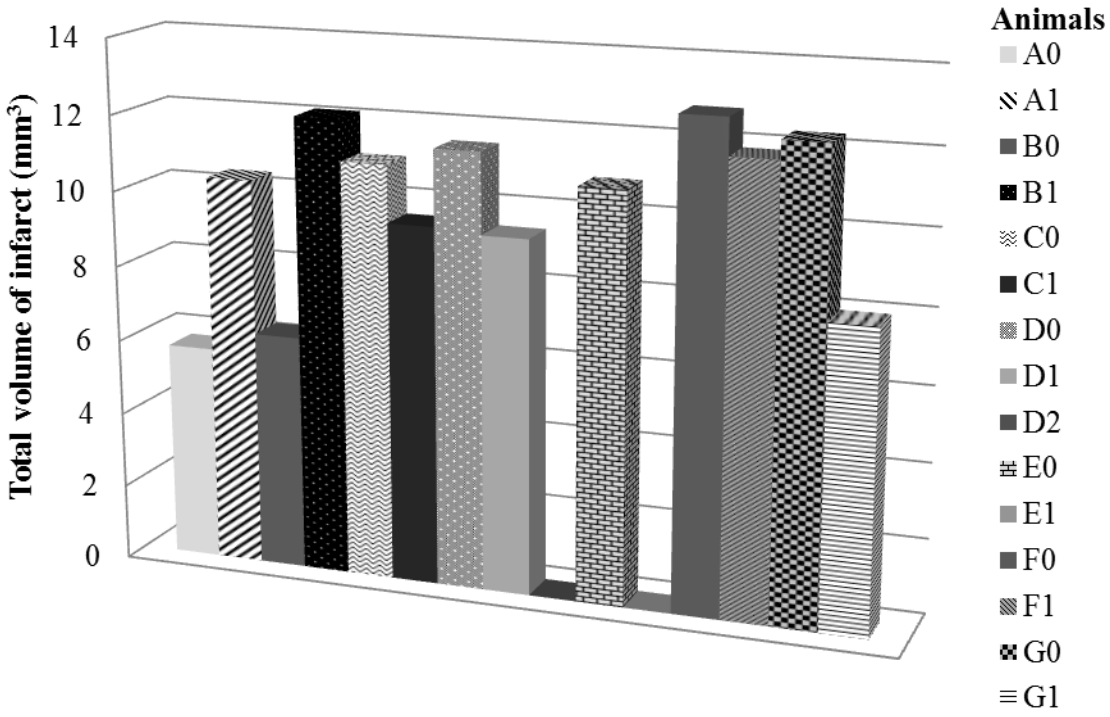


Figure 4.2: Variation in total volume of infarct (mm^3) among rats exposed to photothrombotic stroke (n=15). Two rats had no visible infarct. The infarcts of all other animals ranged from 5.7 mm^3 to 12.8 mm^3. For labelling, animals with the same letter were caged together.

Figure 4.3 shows a photograph of a coronal section, at 10x magnification, taken through the core of the lesion (~0.5 mm from Bregma) for 2 rats from the ISCHEMIA group. These sections demonstrate two differences commonly observed during the preparation of brain sections: (1) the shape of the lesion is different; and (2) the section on the left side shows that the

infarct (necrotic tissue) was lost during sectioning, while the infarct on the right side is intact. Inspection of coronal sections under the microscope at higher magnification (100x) revealed the necrotic area of infarct with an adjacent area of marked reactive gliosis. For most animals, the infarction extended through all cortical layers but did not affect subcortical structures. However, some animals showed damage to the underlying corpus callosum.

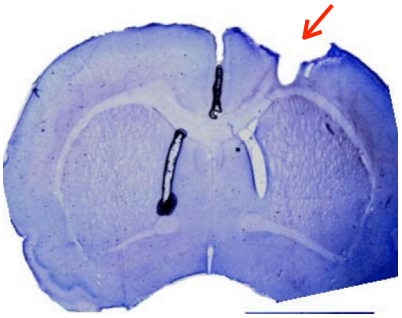
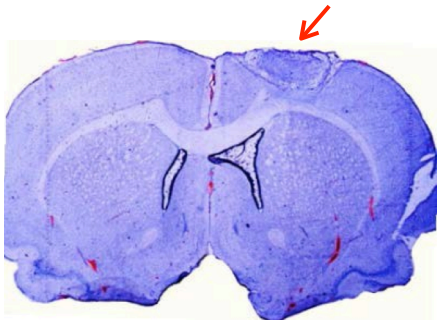
(a)  (b) 

Figure 4.3: Photographs (10x magnification) of coronal sections taken at the core of the infarct for 2 animals, located at ~0.5mm from Bregma. (a) This photograph is representative of cases in which the necrotic tissue was lost during sectioning; (b) This is an example of a coronal section with intact infarcted tissue. Each section represents one of many different lesion shapes observed. The region of infarct is shown by the arrow.

Figure 4.4 shows the variability in the locations of the infarcts in the brain cortex from the 13 rats that had a visible infarct. Most animals had infarcts positioned less rostral and lateral than was targeted with the photothrombotic stroke planned.

The infarct distribution and position in selected brain coordinates are illustrated in Figure 4.5 for the smallest (5.7 mm^3) and largest (12.8 mm^3) infarcts obtained. Both representations depict infarcts located in the forepaw area of sensorimotor cortex.

Lesion volume (mm^3) and location (coordinates of infarct in mm) are provided in Table 4.2 for direct comparison for each ISCHEMIA rat.

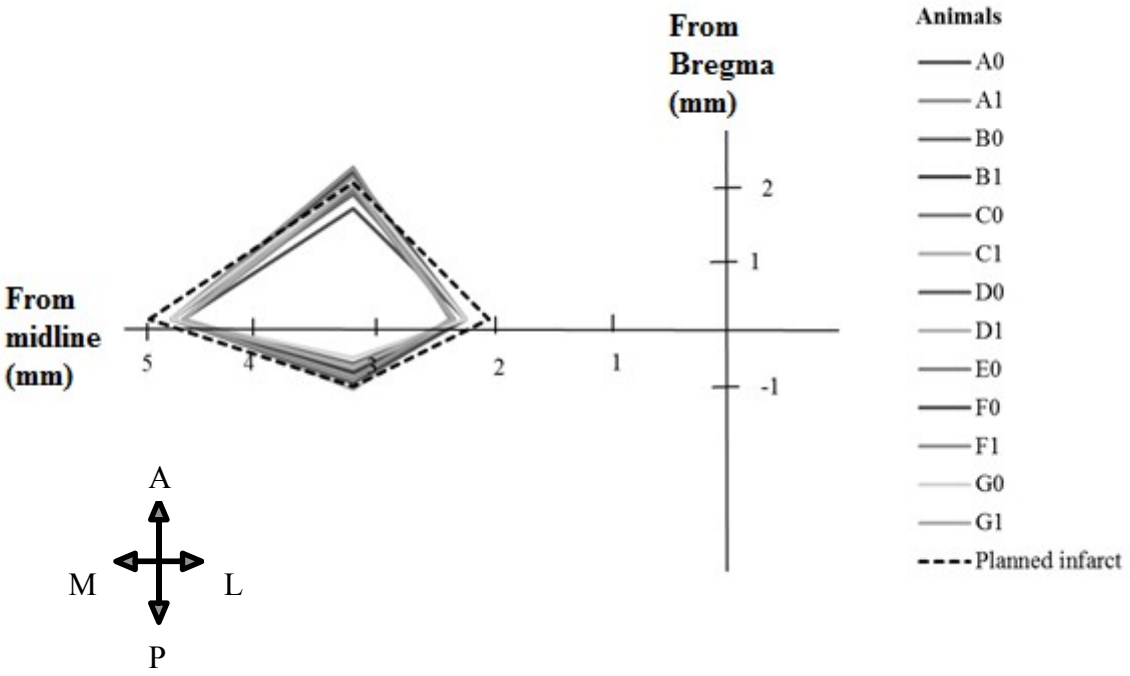


Figure 4.4: Representation of the location of the infarct in the brain cortex of those ISCHEMIA rats with an observable infarct (n=13) in comparison to the intended placement (+2 mm to -1 mm anterior-posterior from Bregma and from +2 mm to +5 mm lateral of midline). Most infarcts are positioned less rostral than planned. None of the ISCHEMIA animals showed the lateralization proposed. For labelling, animals with the same letter were caged together.

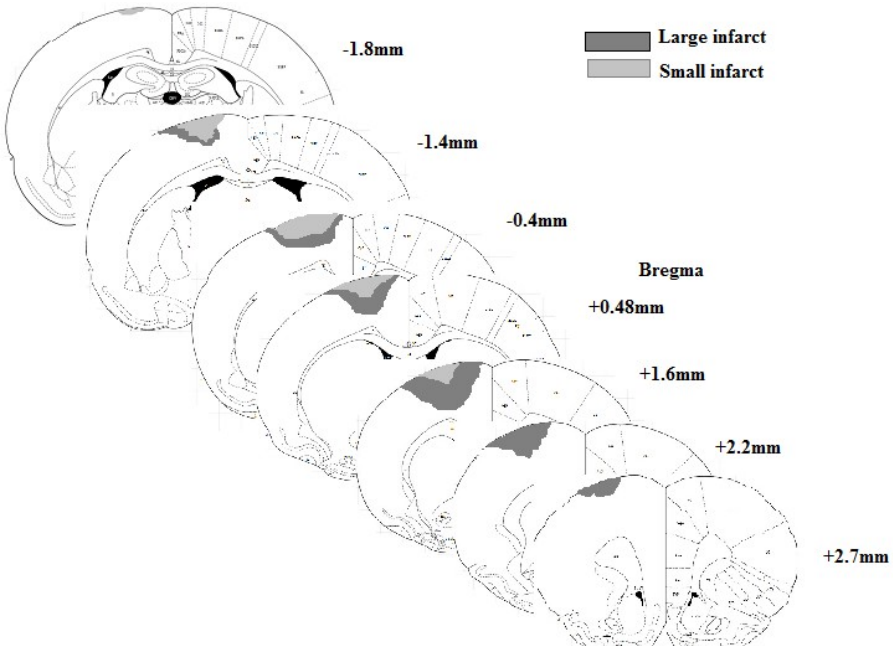


Figure 4.5: Area and regional distribution of two sample brains with the largest (12.8 mm^3) and smallest (5.7 mm^3) infarcts are represented in coronal diagrams. The lesions were localized in the sensorimotor cortex, and targeted the area of forepaw.

Table 4.2: Summary of lesion location and size for ISCHEMIA animals.

	Coordinate of Lesion Relative to Bregma (mm)		Coordinate at Core of Lesion (mm)		Total Volume (mm^3)
Animal	**Anterior**	**Posterior**	**Medial**	**Lateral**	
A0	0.88	-1.6	1	3.5	5.7
A1	2.2	-1.4	0.5	4	10.4
B0	1.5	-2.04	1	4	6.3
B1	2.2	-1.7	0.5	4	12.1
C0	1.7	-1.8	0.5	3.5	11.0
C1	2.3	-0.9	0.5	3.5	9.5
D0	2.1	-1.8	0.5	4	11.5
D1	2.08	-1.88	1	4	9.4
D2	No infarct.				0
E0	2.2	-1.3	1	4	10.8
E1	No infarct.				0
F0	2.5	-1.16	1	4	12.8
F1	2.7	-1.3	0.3	4	11.8
G0	2.08	-2.3	1	4	12.3
G1	1.88	-1.8	0.5	3.5	7.9
Mean	2.02	-1.61	0.72	3.85	8.8
SEM	0.13	0.11	0.08	0.07	1.2

For labelling, animals with the same letter were caged together.

4.3.3 Behavioral Outcomes

Montoya staircase tests confirmed that ISCHEMIC animals show a decline in reaching success after stroke, which then improves over time. Figure 4.6 shows that on day 3 after surgery, animals' ability to retrieve and eat sugar pellets had declined to a mean (± SEM) of 34.3 ± 7.3% of their baseline performance. After that, function started improving, and by day 15, the rats showed mean (± SEM) reaching ability of 60.5 ± 4.3% of their baseline performance. Mean (± SEM) reaching ability after 30 days was 65.5 ± 5.0% of baseline performance. Repeated measures ANOVA confirmed that there was a significant difference in reaching ability over time ($F(2,24) = 14.9$, $p < 0.001$), and post-hoc testing showed that performance on days 15 and 30 were not statistically different ($t(12) = -0.96$, $p = 0.36$). SHAM animals are plotted for illustration of normal function and were not used in statistical analysis due to small sample size.

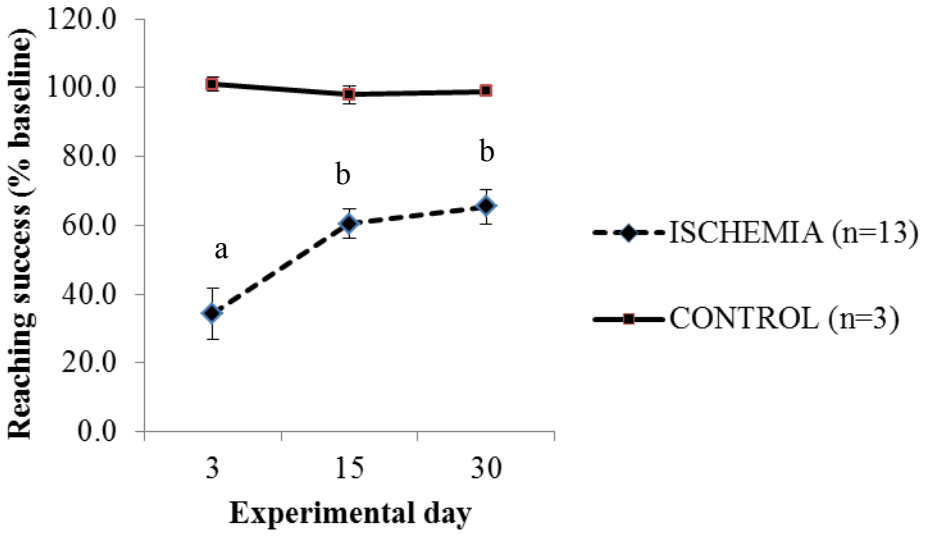


Figure 4.6: Reaching success in the Montoya staircase test was impaired after stroke and improves over time, but there was incomplete recovery by d30. Data are expressed as mean (± SEM) % of baseline performance before stroke (day 3 = 34.3 ± 7.3; day 15 = 60.5 ± 4.3; day 30 = 65.5 ± 5.0). ISCHEMIA rats performed differently over time ($F(2,24) = 14.9$, $p < 0.001$), and there was no significant difference on performance between days 15 and 30 ($t(12) = -0.96$, $p = 0.36$).

Figure 4.7 illustrates preferred/affected forepaw use in the cylinder test. ISCHEMIA rats showed a significant decline in the affected limb use for postural support, and the group mean showed complete recovery by 30 days. There was a statistically significant difference in % affected limb use over time ($F(2,28) = 14.94$, $p < 0.001$). Further post-hoc testing showed that there was a significant improvement in the use of affected paw for postural support from day 3 to day 30 ($t(14) = -2.7$, $p = 0.018$). Baseline values and day 30 values were not statistically different ($t(14) = 2.63$, $p = 0.2$). The mean % affected limb use on day 30 was 42.7%, as compared to the 53.9% use at the baseline assessment. However, when examining individual rat performance, there was considerable variability. Only 2 animals in fact showed complete recovery, and 2 other animals had smaller than 10% deficits at 30d.

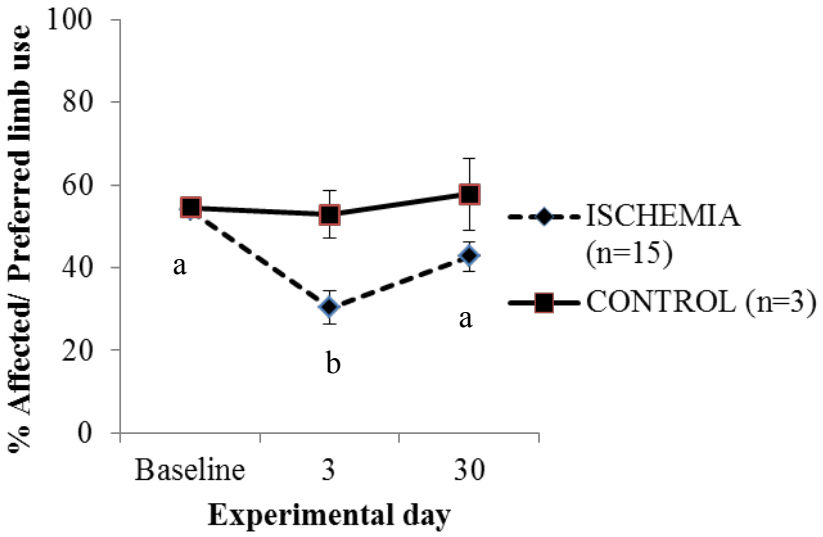


Figure 4.7: ISCHEMIA rats used their affected limb for support in the cylinder significantly less after stroke ($F(2,28) = 14.94$, $p < 0.001$) and improved function significantly over 30 days ($t(14) = -2.7$, $p = 0.018$). Use of the preferred paw after 30 days was not statistically different than that at baseline ($t(14) = 2.63$, $p = 0.2$), showing complete recovery.

Figure 4.8 shows the complete pattern of forepaw use in the cylinder in the ISCHEMIA rats over the 30 days after stroke. ISCHEMIA rats showed significant forelimb asymmetry for postural support, and this asymmetry changed over time (interaction between time and forepaw use) ($F(4,84) = 7.96$, $p < 0.001$). The concomitant use of both paws for support was significantly decreased on repeated testing ($p = 0.001$), and the use of the affected paw and the unaffected paw for unilateral support was approximately equal ($p = 0.76$).

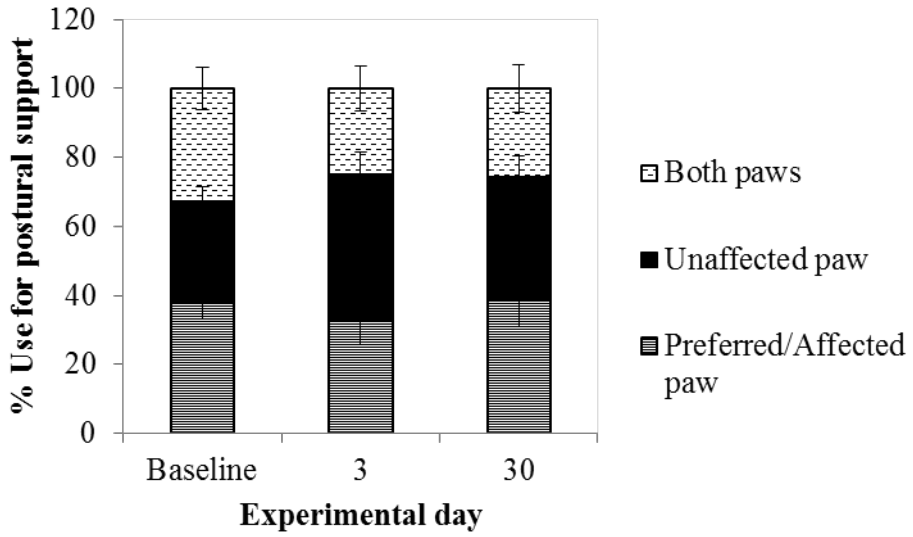


Figure 4.8: Pattern of limb use in the ISCHEMIA group for postural support in the cylinder was significantly different over time ($F(4,84) = 7.96$, $p < 0.01$) (interaction term). The bilateral use of forelimbs was decreased over time ($p = 0.001$), whereas affected and unaffected paws were used unilaterally approximately the same ($p = 0.76$). n=15.

4.3.4 Relationship between Infarct Size and Behavioral Outcomes

To examine if performance in the Montoya staircase was related to the size of the stroke (infarct volume), the deficit in this task was calculated as the percent change in performance between the pre-stroke baseline assessment and the test on post-stroke d 3, 15 and 30. Figure 4.9 shows that there was a significant positive correlation on d3 ($r^2 = 0.47$, $p = 0.006$, Pearson's correlation). Figures 4.10 and 4.11 show that infarct volume and extent of deficit in the task were not significantly correlated on d 15 ($r^2 = 0.14$, $p = 0.20$, Pearson's correlation) or d 30 ($r^2 = 0.19$, $p = 0.14$, Pearson's correlation).

To examine the extent to which performance in the cylinder test was related to the size of the stroke (infarct volume), the deficit in this task was calculated as the percent change in performance in between the pre-stroke baseline assessment and the test on post-stroke days (d 3 and 30). Figures 4.12 and 4.13 demonstrate that there was a significant positive correlation on d3 ($r^2 = 0.62$, $p < 0.001$, Pearson's correlation) but not on d30 ($r^2 = 0.02$, $p = 0.62$, Pearson's correlation).

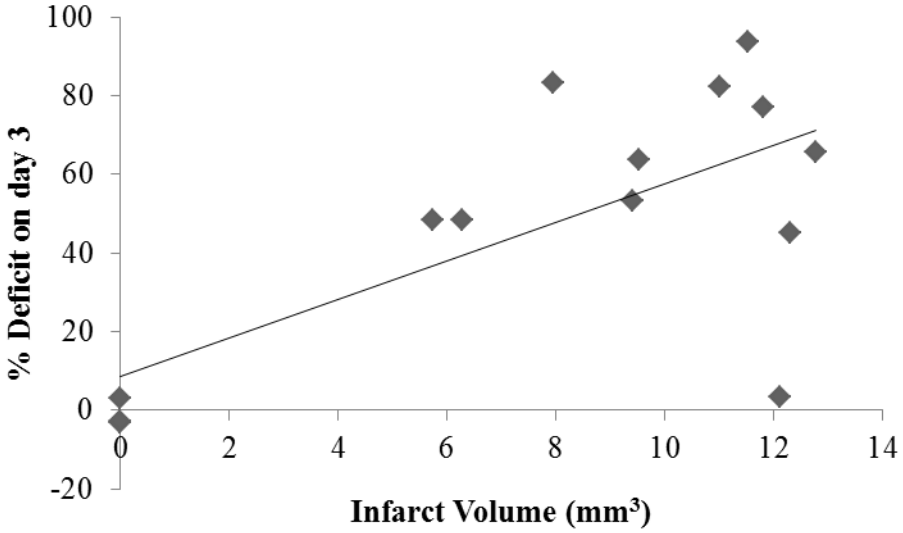


Figure 4.9: The d3 deficit in the Montoya staircase significantly increased with increasing infarct volume (r^2 =0.47, p = 0.006, Pearson's correlation) in rats following photothrombotic stroke. Deficit (%) was calculated as 100- [(pellets eaten on day 3/ pellets eaten at baseline) x 100]. n = 13.

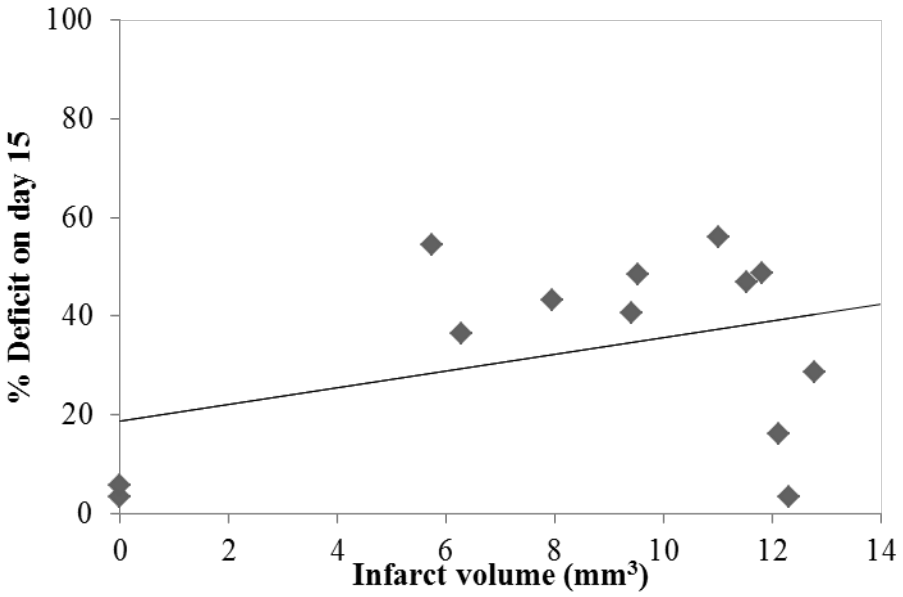


Figure 4.10: The d15 deficit in the Montoya staircase was not significantly correlated with infarct volume (r^2 = 0.14, p = 0.20, Pearson's correlation) in rats following photothrombotic stroke. Deficit was calculated as 100- [(pellets eaten on day 15/ pellets eaten at baseline) x 100]. n = 13.

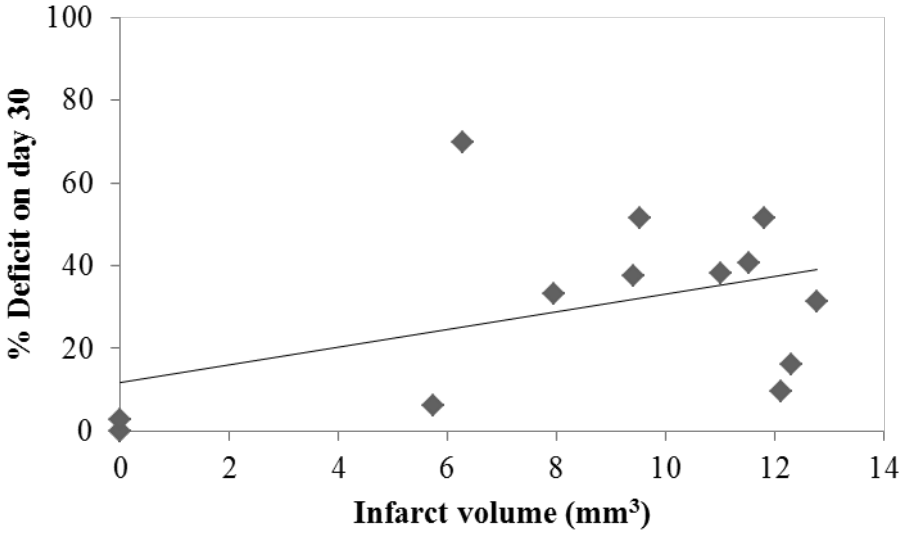


Figure 4.11: The deficit in the Montoya staircase on d 30 after photothrombotic stroke was not significantly correlated with infarct volume (r^2 = 0.19, p = 0.14, Pearson's correlation). Deficit was calculated as 100- [(pellets eaten on day 15/ pellets eaten at baseline) x 100]. n= 13.

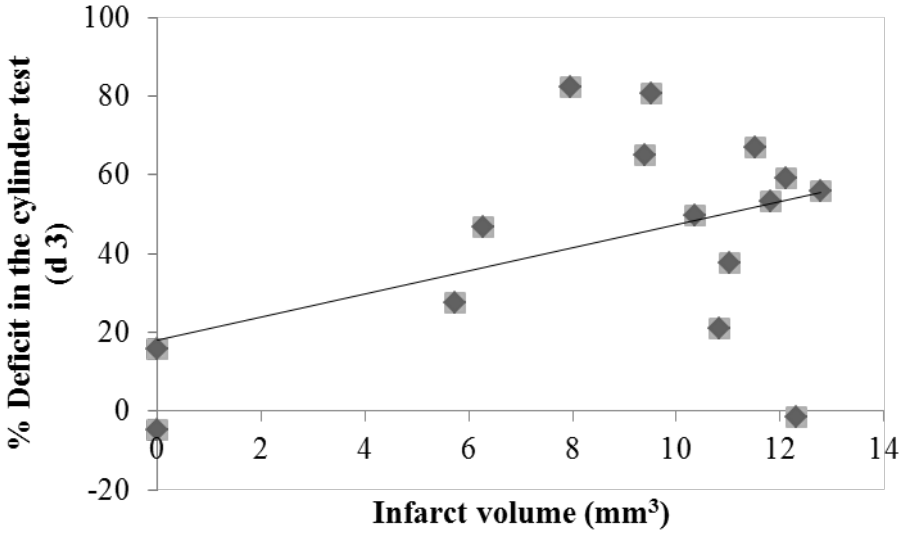


Figure 4.12: The deficit in the cylinder test on d3 after photothrombotic stroke was significantly correlated to infarct volume (r^2 = 0.62, p < 0.001, Pearson's correlation). n = 15.

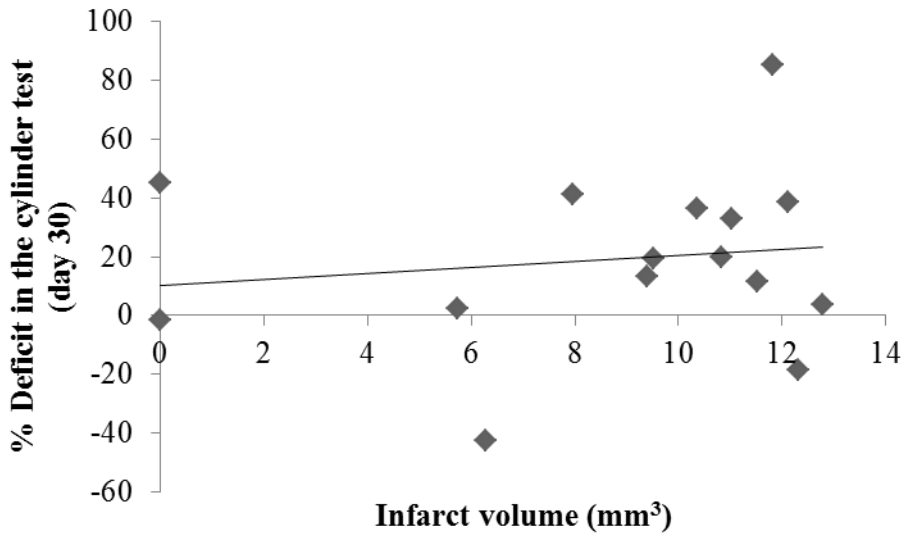


Figure 4.13: The deficit in the cylinder test on d30 after photothrombotic stroke did not show significant correlation with infarct volume ($r^2 = 0.02$, $p = 0.62$, Pearson's correlation). n = 15.

The presence of tail necrosis could influence performance in the behavioural tasks and could also be a marker of incomplete dye infusion (and thus predictive of smaller infarct size). Thus, multiple regression analysis was performed to determine if the presence of tail necrosis affected the dependent variables: d 3, 15 and 30 Montoya staircase performance, d3 and 30 cylinder test performance, and infarct volume. The prediction model indicated that tail necrosis had a significant effect on all variables when pooled together ($F(6, 6) = 7.374$, $p = 0.014$). Partial correlations revealed that tail health problems were negatively correlated with infarct size ($\beta = -0.542$, $p = 0.03$); the presence of tail problems explained approximately 24% of the infarct size variance ($r^2 = 0.24$). Also, tail problems had a positive correlation with Montoya performance on day 30; that is, rats with a tail problem had smaller chronic deficits in the Montoya staircase ($\beta = 0.707$, $r^2 = 0.50$, $p = 0.003$).

4.4 Discussion

The main objective of the present study was to determine the success and reliability of the photothrombotic stroke techniques for inducing cortical stroke. A visible infarct was apparent in 87% of the rats. As intended, the infarct was located in the sensorimotor cortex and removed a small part of the forelimb representation of the neocortex as previously described in published maps (Kleim, Barbay et al. 1998, Donoghue, Wise 1982, Neafsey, Bold et al. 1986).

Although some brains also showed damage to the corpus callosum, other subcortical regions were not damaged, and previous work has shown that this lesion extension could be due to degeneration of efferent fibers from the motor cortex and should not affect function (Karl, Alaverdashvili et al. 2010). The photothrombotic stroke surgery was also successful in that the minimal invasiveness allowed for rapid post-surgical recuperation and minimal distress to the animals. The weight loss after surgery was small and quickly recovered, which reinforces the model as a good one for addressing the interaction between nutritional status and stroke.

However, the extent of variability in the size, shape, and exact placement of the infarct suggests that further technical refinements are needed. To allow for comparison of study variability in infarct volume [mean (± SEM) of 8.8 ± 1.2 mm^3] with other studies reporting different mean infarct volumes, the coefficient of variation was calculated to be 47%. The variability associated with this model is usually reported to be considerably lower than observed in the current study. One study with a mean infarct size similar to ours (12 mm^3) reported a coefficient of variation of 19% (Yao, Sugimori et al. 2003). Another study with similar methods but smaller infarcts (2.84 mm^3), showed a coefficient of variation of 14% (Moon, Alaverdashvili et al. 2009). We also found a large variability in lesion shape, whereas photothrombotic stroke in the cortex usually results in a triangular shaped infarct (Jablonka, Burnat et al. 2010, Moon, Alaverdashvili et al. 2009, Imbrosci, Eysel et al. 2010, Schmidt, Bruehl et al. 2012). There was also some misplacement of the infarct relative to the intended brain coordinates.

One of the variables contributing to the large variability is difficulty in tail vein injection of the rose Bengal dye in some rats. Multiple attempts at correctly positioning the catheter could have caused dye leakage, and the high concentration of dye in the stock solution could have caused direct endothelial damage. The result in these cases was that less than the optimal dye dose reached the targeted area. Increased variability could also be attributed to some variation in skull thinning and quality of skull area demarcation with the drill. Our procedure of illuminating the skull with the laser prior to dye injection may have contributed to variability in infarct shape and volume. It is possible that the cortical vessels were affected by the heat produced by irradiation and that some clotting was initiated before the dye reached the area (Nishimura, Schaffer et al. 2006). This variable, as well as difficulty in thinning the bone in the lateral portion of the skull, may also have contributed to some misplacement of lesions.

This study was also designed to assess the extent and consistency of the motor

alterations caused by stroke. The Montoya staircase results showed that our model of photothrombotic stroke resulted in impairments in skilled reaching for sugar pellets. On day 3 after stroke, mean reaching success with the affected paw was 34.3 % of baseline performance, and this recovered to 60.5% of baseline values by day 15. One study that assessed reaching ability after focal ischemia induced by endothelin-1 reported reaching success of 38.5 % of baseline performance after 15 days (Biernaskie, Corbett 2001). However, the infarcts in this study were larger than ours, the rats were younger, and the stroke affected not only the sensorimotor cortex, but also the lateral portion of the caudate putamen. That explains their deficit on day 15 being similar to our deficit on day 3. Our results also agree with another report which showed that with a lesion similar to our largest lesion (~ 12 mm^2), the reaching ability in the Montoya staircase at 5 days after stroke was approximately 25% of baseline performance (MacLellan, Keough et al. 2011). Our analysis also showed a positive correlation between the presence of tail necrosis and performance on day 30 in the Montoya staircase. This is expected since post-surgical tail problems predict inefficient delivery of rose Bengal and smaller infarcts, and rats with smaller infarcts have a greater tendency to recover. The cylinder test results showed that the affected limb was used for support significantly less after stroke, and the mean deficit on day 3 was 56.3 %. Other studies have shown similar deficits. One group, using the endothelin-1 model of stroke, evaluated affected limb use in the cylinder on day 5 after stroke, and reported a 43.5% deficit (MacLellan, Keough et al. 2011). Another group, using the photothrombotic stroke model, reported a deficit of 52.1% on day 1 after stroke (Shanina, Schallert et al. 2006).

The functional deficit in the cylinder and Montoya staircase tests on day 3 after stroke was correlated with infarct volume measured at 30 days post-stroke. This was expected since a larger infarct to the motor cortex usually produces larger functional abnormalities (Grabowski, Brundin et al. 1993, Baird, Meldrum et al. 2001, Roof, Schielke et al. 2001). This relationship was lost on post-stroke days 15 and 30 on the Montoya staircase test and on day 30 in the cylinder test. These results are in agreement with previous studies demonstrating that the rate of functional recovery depends not only on the extent of infarct, but also on plasticity mechanisms that assist this recovery (Levin, Kleim et al. 2009, Cramer 2008).

An important study finding was that recovery of reaching function in the Montoya staircase was incomplete (65.5% of baseline performance) at 30 days after stroke. The cylinder

results in this respect were particularly influenced by the high stroke model variability. Whereas 27% of rats either completely recovered the use of the affected limb for support or had very small deficits (2 – 3%) by the end of the study, the other 73% recovered to only 67% of pre-stroke baseline performance. It is predicted that technical refinements that further reduce the variability in the photothrombotic stroke model in future should thus result in the desired functional outcome. Although it is difficult to compare our results directly to the clinical reality, the majority of patients who survive ischemic stroke and show motor impairments have incomplete functional recovery that varies from notable to limited; skilled functions are more difficult to recover than support functions (reviewed in Stinear 2010).

There were two methodological limitations of this study that can be improved in future experimental designs. The first was the use of the cylinder test for determination of paw preference and thus infarct placement. Most animals are extremely ambidextrous, and the interchangeable use of the paws is more noticeable in unskilled movements (Whishaw 1992, Pençe 2002). Since the Montoya staircase assesses skilled function, the probability of finding real paw preference would be greater if this test had been used to assign paw preference. A second limitation also noted in the Discussion of Chapter 3 is that Montoya staircase testing on at least two independent days for each time point would have better captured true variability. By testing twice in the same day, the estimate of consistency and extent of recovery in this study was possibly compromised. However, it is also noted that additional testing days increase risk that the assessment could also serve a rehabilitative function (Clarke, Mala et al. 2009).

In summary, this study allowed for the methodological development of the stroke photothrombotic model and two well accepted behavioural protocols (Montoya staircase and cylinder tests) that work in combination with the model. While the basic methods were established for inducing stroke, further refinements are needed to reduce variability in infarct size and functional outcome.

CHAPTER 5
DISCUSSION

5.1 Conclusions

The studies described in this thesis resulted in two rat models required for future study of the PEM that develops after stroke. These models, with some additional refinements, form a good basis for addressing the hypothesis that PEM that develops after stroke will impair motor neuroplasticity. Separately, the two experiments established both a nutritional model to cause the desired PEM in adult rats, and a focal ischemia model to mimick the human stroke patient.

The PEM model development described in Chapter 3 yielded some novel findings. First, a 0.5% protein diet fed for 31 days achieved the desired goal of acute moderate PEM in adult (16 week old) rats. Also, we confirmed previous findings from my laboratory that PEM can independently cause an acute phase response, although there were specific differences when compared against the acute phase reactant response observed in protein-energy malnourished adolescent rats (Smith, Andrade Ramos et al. 2013). The current study also demonstrated the absence of an effect of PEM on skilled reaching ability in the adult rat. Although protein-energy malnutrition is associated with diminished muscle strength in elderly individuals (reviewed in Mithal, Bonjour et al. 2012), there were no previous data on forelimb skilled function. This finding is especially important for future studies of the effects of PEM on this well-established measure of motor deficit caused by experimental stroke. Future stroke studies should also include malnourished sham-operated rats to address the possibility of independent effects of PEM on skilled reaching, as this could arise with longer exposure to PEM.

The study described in Chapter 4 established the initial methodology for the photothrombotic stroke model. Most surgeries were effective in inducing an infarct in the forepaw functional domain of the neocortex. Histological damage was translated to functional outcomes in the sense that animals with infarcted brains showed behavioral abnormalities. Although rats improved their performance over time, the final functional testing after 30 days showed incomplete recovery in 70% of rats. Evidence for the lack of invasiveness with this stroke model was observed in the post-surgical period, during which rats had minimal weight loss and showed no signs of lethargy. However, the extent of variability in the size, shape, and

exact placement of the infarct combined with some cases of tail necrosis indicate that further technical refinements are required.

5.2 Strengths and Limitations

The major strength of the studies performed herein is the development of animal models that will allow for carefully controlled analysis of the effects of post-stroke nutritional status on functional recovery. Clinical studies have not been able to establish cause and effect relationships between PEM and outcome from stroke. Also, a clinical study would not be able to examine exact mechanisms and carefully analyse brain biochemistry, which is possible with animal research (Lieschke, Currie 2007, Graham, McCullough et al. 2004). Moreover, the two studies in this thesis were carefully designed to mimick the human conditions of stroke and malnutrition as observed in patients after stroke. Rat studies permit strict control over diet consumption, lifestyle and body weight status (Lieschke, Currie 2007, Small, Buchan 2000). Performing the two model-building experiments separately also allowed for isolating a number of possible confounding variables and analysing the causes for the outcome measures individually.

Another strong point of the research design was the choice of behavioral tests for both studies. Although time-consuming, rats can be readily trained in the Montoya staircase, and a high proportion are able to reach criteria, a feature noticed by other groups as well (Pagnussat, Michaelsen et al. 2009, Abrous, Dunnett 1994). This is in contrast to another skilled reaching test, the single pellet reach test, in which many rats cannot reach criteria and have to be eliminated from the study (Silasi, Hamilton et al. 2008). Neither protein-energy malnourished nor stroke rendered rats were unable to perform the task, although in the latter case, rats clearly showed the expected disability. The most positive aspect of the behavioral tests chosen is the fact that they assess different characteristics of forepaw function. The Montoya staircase assessed skilled function and is a food rewarded test, which means that the rat is able to obtain a treat (sugar pellet) for performing well (Montoya, Campbell-Hope et al. 1991). The cylinder test measured use of the limb for support and is a non-baited test in that the rat is not rewarded for doing the task (Gharbawie, Whishaw et al. 2004). The lack of food reward is especially important in nutrition studies since it prevents confounding variables such as increased energy intake or alterations in protein/carbohydrate ratio or intake of experimental diet. An asset of the

Montoya staircase is that the function of the two forelimbs can be investigated separately (Grabowski, Brundin et al. 1993), although in the thesis research, the task was used to assess function of only the affected or preferred paw. One limitation of the experimental design is that the cylinder test was not included in the PEM study and thus, we cannot infer about how the evolving malnutrition would affect the use of limbs for postural support. One of the problems of not conducting the cylinder test in the malnutrition study is that there was a decrease in the number of wall touches with increased exposure to the task in the stroke study. Should PEM also affect the number of touches, for example by altering motivation to explore, this would reduce test sensitivity and potentially introduce a confounding variable (Gharbawie, Whishaw et al. 2004).

A major asset of the PEM study was the creation of two different PEM prototypes by manipulating the protein content of the diet. The main limitation was limited sensitivity of the test used to assess whether PEM altered motivation to reach for a sugar pellet. After performing this test, it became evident that it is a relatively crude test subject to several errors, which may account for the large variability observed among animals. When considering within-animal variance, it was noticed that the rats became more comfortable with the task over time, as assessed by less time spent rearing and less urination and defecation in the testing cage. This could be another flaw of this assessment, since the goal was to assess motivation and not acclimatization. It is further noted that the design tested only the effects of evolving malnutrition on motivation to obtain a sugar pellet, and the results could differ with PEM presenting after a stroke. Human studies have shown, for example, that stroke causes altered taste perceptions (Dutta, Josiah et al. 2013).

Another limitation of the malnutrition study concerns the grouping of the animals. Cage mates were not weight-paired, which in some cases rendered a larger, presumably more dominant rat in the same cage with a smaller dominated one. This difference would have been exaggerated during the training period for the Montoya staircase when animals were chronically food-restricted. This resulted in some cases in which one rat ate substantially more than the other, which affected the baseline weight for starting the protein-restricted diets (Baenninger 1970).

The photothrombotic stroke model chosen for the second study had the advantage of being well-established in the literature (Watson, Dietrich et al. 1985, Dietrich, Busto et al. 1987,

Watson 1998, Diederich, Quennet et al. 2012b, Jablonka, Burnat et al. 2010, Sulejczak, Ziemlinska et al. 2007), which gave insight into potential problems before they happened. The most positive aspect of this model, relative to other rat stroke models, is the previously reported lack of surgical invasiveness and the fast post-surgical recovery (Dietrich, Busto et al. 1987), which was confirmed in this study. This feature is very important for future study of post-stroke PEM in order to minimize the confounding influence of the stress response to surgery on nutritional requirements and nutritional status.

This study was a good first step towards establishing the photothrombotic model in our laboratory. Although well-established in the literature, there is no one universally accepted standard for the methodology (Dietrich, Busto et al. 1987, Maxwell, Dyck 2005, Sulejczak, Ziemlinska et al. 2007, Schmidt, Bruehl et al. 2012), which renders each laboratory responsible for establishing and refining its own. In the case of my laboratory, some refinements are still needed to overcome some inconsistencies in infarct volume and placement and functional outcome. The damage caused to the tails of some rats by the rose Bengal injection proved to be an important limitation. However, my laboratory has recently completed a pilot study demonstrating that using both a lower dose of rose Bengal (10-20 mg/kg) and a less concentrated stock solution for injection can induce a reproducible brain infarct while minimizing any side effects related to dye injection.

There are many variables that can influence performance on behavioural tests, and most rely on the individuality of each rat. Although there was a general pattern in behaviour in both the Montoya staircase and the cylinder test after stroke, each rat had an individual pattern of performance and recovery that could not always be accounted for by the size of the brain infarct. Different rat strains are known to behave differently in the Montoya staircase, but in addition, there is variability within strains, especially in the training phase, but also in the maximum level of performance (Nikkhah, Rosenthal et al. 1998). Similarly, we noticed that rats that looked visibly more distressed had fewer wall touches in the cylinder.

5.3 Future Directions

Although the two studies together established essential basic methodology, additional adjustments are necessary before using the models to study the influence of post-stroke PEM. A wash-out period should be added between any behavioural training period that

requires food restriction and stroke surgery and/or randomization into experimental diet groups. This extra step will avoid the early differences in food intake observed in the present study. Also, a pilot study since performed in my laboratory suggests that it will be possible to train rats to reach in the Montoya staircase without food restriction (unpublished findings), which will offer a considerable advantage for future nutrition-related studies. It would also be interesting to examine the effects of a pre-stroke Western diet, which frequently contains a higher content of refined grains and sugar products and excessive saturated and trans fatty acids (Cordain, Eaton et al. 2005). This would be of interest because a high proportion of people are eating foods high in fat and sugars (Sherzai, Heim et al. 2012). After feeding the Western diet for a few weeks, the influence of post-stroke PEM would be an even better mimick of the clinical scenario for many patients. This Western diet on its own can exert detrimental effects on stroke outcome (Langdon, Clarke et al. 2011), so it would be interesting to study the combined effects of pre-stroke Western diet and post-stroke PEM.

Although all behavioral training and testing for the Montoya staircase was done in the active (dark) phase of the cycle, it would be advantageous to complete this testing during the day to increase efficiency and expand flexibility for scheduling available for the testing. Other groups have successfully trained and tested in the light phase (Mestriner, Pagnussat et al. 2011; Auriat, Colbourne 2009); however, there are also reports of lower activity during this period (Stephenson, Lim et al. 2012). A pilot study in my laboratory now suggests that both training and testing can be accomplished during the light phase without increasing the training period excessively (unpublished findings).

The studies described in this thesis with adult rats have provided the first step towards studying older animals. The Stroke Treatment Academic Industry Roundtable (STAIR), which aims to advance the development of acute and restorative stroke therapies, has recommended that after initial evaluations in young, healthy male animals, further preclinical stroke studies should be performed in females, aged animals, and animals with comorbid conditions such as hypertension, diabetes, and hypercholesterolemia (Albers, Goldstein et al. 2011). The future planned studies addressing PEM as a comorbidity factor meets the latter goal, but will focus on nutrition as a commonly overlooked determinant of outcome after stroke. Future studies of PEM should also include both aged and female rats to more completely mimick the clinical reality. However, studies with older animals are expensive, and a surgical

model of stroke may well need further adjustment before application to aged rats that also will require extended care and attention. Studies with female rats require strict monitoring of hormonal fluctuations, which could affect many outcome variables. Such challenges are best addressed after an experimental model of stroke is very well-established and reliable in the adult.

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