EFFECTS OF PROTEIN-ENERGY MALNUTRITION ON SPONTANEOUS MOTOR RECOVERY AFTER STROKE

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ABSTRACT

The development of comorbidity factors such as malnutrition may compromise functional recovery following stroke. The objectives of this study were to elucidate the effects of post-stroke protein-energy malnutrition (PEM) on infarct size, spontaneous motor recovery, and the acute phase response in the chronic period.

Adult, male (12 week old) Sprague-Dawley rats were trained for at least 14 days in the Montoya staircase. Food intake was monitored daily and body weight was recorded weekly. Just prior to inducing stroke, rats were tested in the cylinder and Montoya staircase to determine baseline values for forelimb use during spontaneous exploration and skilled reaching, respectively. These animals were then subjected to photothrombotic stroke targeted to the motor cortex or sham surgery. Animals were tested in the cylinder on day 4 after surgery, before assignment to either control diet (12.5 % protein) or PEM (0.5 % protein) (n= 6-9/experimental group), and again on days 16 and 29. The staircase was abandoned for post-stroke testing because training criteria were not met. On Day 30, blood, brain, and liver were collected for biochemical or histological analysis.

Feeding the low protein diet resulted in PEM as measured by decreased body weight $p<0.001$), food intake ($p=0.016$), and serum albumin ($p<0.001$) and increased liver lipid ($p<0.001$) and serum A2M ($p=0.001$). Both stroke ($p=0.016$) and PEM ($p=0.001$) elicited increases in the positive acute phase protein, A2M.

The effect of PEM on post-stroke cylinder performance varied by specific endpoint. PEM exacerbated forelimb asymmetry during vertical exploration on Days 16 and 29 when scored by

method 1 ($p \leq 0.024$), and this was not due to a change in infarct size ($p=0.775$). Scoring exploration by method 2 and initiation of exploration by first touch demonstrated similar patterns for preferred limb use after stroke, although these endpoints were not significantly affected by PEM ($p \geq 0.301$). The score for takeoff to initiate exploration was also impaired by stroke ($p<0.001$), but PEM had no influence ($p=0.463$). Termination of exploration (landing) was not influenced by stroke ($p=0.332$), and there was no independent effect of PEM ($p=0.959$).

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This thesis is dedicated to my loving parents,
Dennis and Beverly Matwee.

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LIST OF ABBREVIATIONS

°C	**Degrees Celsius**
µL	**Microliter**
µm	**Micrometer**
A2M	**Alpha-2-macroglobulin**
ADP	**Adenosine diphosphate**
AMPA	**Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**
ANOVA	**Analysis of variance**
ATP	**Adenosine triphosphate**
ATPase	**Adenosine triphosphatase**
BASP-1	**Brain abundant membrane attached signal protein-1**
BDNF	**Brain-derived neurotrophic factor**
Ca	**Calcium**
CA1	**Cornu ammonis 1**
cAMP	**Cyclic adenosine monophosphate**
CIMT	**Constraint induced movement therapy**
cm	**Centimetre**
CON	**Control diet**
dL	**Decilitre**
DPSS	**Diode pumped solid state**
ET	**Endovascular thrombectomy**
FOOD	**Feed Or Ordinary Diet**
g	**Grams**

GAP-43	**Growth associated protein-43**
GFAP	**Glial fibrillary acidic protein**
H	**Hydrogen**
hr	**Hour**
IL1-β	**Interleukin 1-β**
ISCH	**Ischemia**
K	**Potassium**
kg	**Kilograms**
L	**Litres**
M	**Molar concentration**
M1	**Microglial classical activation state**
M2	**Microglial alternative activation state**
MARCKS	**Myristoylated alanine rich protein kinase C substrate**
MCA	**Middle cerebral artery**
MCAo	**Middle cerebral artery occlusion**
mg	**Milligrams**
mm	**Millimetres**
mM	**Millimolar**
mW	**Milliwatts**
Na	**Sodium**
NFκβ	**Nuclear factor kappa beta**
nM	**Nanomolar**
NMDA	**N-methyl-D-aspartate receptor**

PEM	**Protein energy malnutrition**
rtPA	**Recombinant tissue plasminogen activator**
SEM	**Standard error of the mean**
SHAM	**Sham surgery**
SNAP-25	**Synaptosomal associated protein-25**
STAIR	**Stroke therapy academic industry roundtable**
TNF-α	**Tumour necrosis factor alpha**
tCDS	**Transcranial direct current stimulation**
trkB	**Tropomysin related kinase B**

CHAPTER 1: INTRODUCTION

1.1 Rationale

Stroke is the third leading cause of death in Canada, accounting for 5.3% of deaths nation-wide (Statistics Canada 2012). Individuals who survive a stroke often bear some form of impairment or disability. A study assessing the impact of disability status on ischemic stroke costs in Canada found that 48.7% of patients were disabled at the time of hospital discharge (Mittmann et al. 2012). The level of independence and quality of life for these individuals depends largely on the extent of recovery of cognitive, communication and motor skills. The pre-existence or later development of medical comorbidity factors such protein-energy malnutrition (PEM) may dramatically compromise functional recovery.

The search for a pharmaceutical treatment for stroke has been met with limited success. The only drug that is currently approved for the treatment of acute ischemic stroke is recombinant tissue plasminogen activator (rtPA) (Meyers et al. 2011). Unfortunately, there are several contraindications for the use of rtPA which exclude a large number of patients from treatment (Novakovic et al. 2009). Furthermore, this thrombolytic agent has an extremely narrow therapeutic window of 4.5 hours (Lansberg et al. 2009). Alternatively, recent advances in technology have resulted in the development of a procedure referred to as endovascular or mechanical thrombectomy (ET), which has been shown to improve functional outcome and decrease mortality rates for some ischemic stroke patients (Goyal et al. 2015).

The complex pathways involved in the ischemic cascade responsible for neuronal cell death offer one important explanation for the continued failure of drug therapies targeting stroke (Novakovic et al. 2009). Although a given pharmaceutical agent may target one pathway, there

are several others through which neuronal damage may occur. Thus, it is recommended that current research approaches focus on combination therapies that target multiple pathways in the ischemic neuronal death cascade as well as placing more emphasis on rehabilitation strategies that target recovery mechanisms. In order to facilitate the translation of these therapies into clinical practice, investigators must also consider comorbidity factors that may influence recovery after stroke (Fisher et al. 2009). This thesis research evaluates the influence of suboptimal nutrition status developing after stroke.

Patients who survive stroke often exhibit some level of functional recovery due to neuroplasticity. Brain plasticity refers to the brain's inherent ability to alter neural pathways in response to injury or experience. These changes may be brought about by the use of pre-existing neuronal connections, axonal sprouting, dendritic growth, the development of dendritic spines or through the growth of additional neurons (Murphy and Corbett 2009; Carmichael 2010). Protein-energy malnutrition may detrimentally affect these repair processes, thereby limiting functional recovery.

PEM that develops after stroke is most likely to be the result of a physical or mental impairment that hinders the ability of the patient to eat. For instance, difficult or painful swallowing may prevent adequate nutritional intake in some stroke patients (Finestone et al. 1995; Smithard et al. 1996). In addition, the changes induced by the stroke may result in depression (Andersen et al. 1994) which in turn may affect food intake (Yang et al. 2009; Morley 2012). A review conducted by Foley et al. (2009) found an increased prevalence of malnutrition at time periods greater than one week after stroke (22 – 35%) compared to studies which focused on the acute period after stroke (8 – 16%). These findings imply that at some point during the post-ischemic period, the nutritional status of a significant proportion of patients deteriorates.

Several previous clinical studies have found malnutrition to be correlated with poor functional outcome in stroke patients (Feed Or Ordinary Diet Collaboration 2003; Yoo et al. 2008; Nip et al. 2011). Although clinical studies are essential for offering insight into factors that may affect stroke recovery, it is not always possible to ascertain a causal link due to confounding variables. The use of an animal model of stroke to study comorbidity factors such as PEM serves to eliminate these confounding variables and thus can provide conclusive data on the effects of malnutrition on recovery after stroke.

Nutritional status may impact stroke recovery in numerous ways. Protein-energy malnutrition pre-existing at the time of brain ischemia left untreated has been shown to modify tropomyosin-related kinase B (trkB) and growth associated protein-43 (GAP-43), proteins that are involved in post-ischemic repair mechanisms (Prosser-Loose et al. 2010). However, this research focused on pre-existing PEM and thus may not accurately reflect what occurs when PEM develops after stroke. A more recent study that examined the effects of post-stroke PEM on markers of neuroplasticity after global ischemia demonstrated that post-ischemic PEM decreased growth associated protein-43, synaptophysin, and synaptosomal-associated protein-25 (SNAP-25) immunoflourescence (Smith et al. 2014). A further limitation, however, is that both of these experiments were conducted using a global brain ischemia model. These findings may not be an accurate reflection of what occurs after focal ischemia, which mimics the more common form of ischemic stroke.

Protein-energy malnutrition may also affect stroke recovery through exacerbation of the brain's inflammatory response to ischemia. Inflammation plays a role in both brain injury and repair processes (Lo 2008; Kriz and Lalancette-Hebert 2009). Thus, an alteration in brain inflammation may hinder neuronal repair and/or exacerbate the initial damage caused by stroke.

Previous studies suggest that PEM could increase brain inflammation (Bobyn et al. 2005; Ji et al. 2008), which in turn could influence injury and repair after stroke (Kriz and Lalancette-Hebert 2009).

Protein-energy malnutrition can independently cause an acute phase response (Alaverdashvili et al. 2015a; Andrade Ramos 2015), which may adversely influence stroke outcome (Dziedzic 2008). Although they are nonspecific indicators, acute phase proteins are often used as markers of systemic inflammation (Gabay and Kushner 1999). A recent study conducted in the Paterson laboratory (Andrade Ramos 2015) found that when 16 week old adult rats were subjected to PEM induced by feeding a 0.5 % protein diet for 30 days, serum concentrations of the positive acute phase proteins alpha-1-acid glycoprotein and alpha-2-macroglobulin (A2M) increased, while serum albumin, a negative acute phase protein, decreased. However, the effect of PEM on inducing an acute phase reaction may differ after stroke, and thus this question was examined in this thesis research.

1.2 Research Objectives

1. Determine the long-term (29d) effects of post-ischemic PEM on motor recovery in the rat photothrombotic stroke model, as assessed by the Montoya staircase and cylinder tests.
2. Assess the impact of post-ischemic PEM on infarct volume caused by photothrombotic stroke.
3. Evaluate the effect of post-stroke PEM on the acute phase response in the chronic period.

1.3 Hypothesis

Protein-energy malnutrition developing after stroke will impair spontaneous motor recovery without altering infarct volume. Post-stroke PEM will be associated with an acute phase response.

CHAPTER 2: LITERATURE REVIEW

2.1 Stroke

Stroke is the third leading cause of death in Canada, accounting for 5.3% of deaths nation-wide (Statistics Canada 2012). Individuals that survive a stroke often bear some form of impairment or disability. A study assessing the impact of disability status on ischemic stroke costs in Canada found that 48.7% of patients were disabled at the time of hospital discharge (Mittmann et al. 2012). In 2013, it was estimated that approximately 405, 000 stroke survivors were living with the effects of stroke (Krueger et al. 2015). The level of independence and quality of life for these individuals depends largely on the extent of recovery of cognitive, communication and motor skills. The pre-existence or later development of medical comorbidity factors such as diabetes, hypertension, hypercholesterolemia and protein-energy malnutrition (PEM) (Feed Or Ordinary Diet Collaboration 2003; Mozaffarian et al. 2015) may dramatically compromise functional recovery. This thesis research focused on elucidating the effects of protein-energy malnutrition developing after stroke on spontaneous motor recovery.

2.1.1 Ischemic Stroke

Roughly 87% of strokes are ischemic stroke, which is modelled in the thesis research; the remaining minority are hemorrhagic (Mozaffarian et al. 2015). Ischemic stroke is caused by the disruption of blood circulation in the brain as the result of a thrombus or an arterial embolism (Sacco et al. 2013). Thrombotic stroke occurs when a blood clot forms directly in an artery supplying the brain; embolic stroke occurs when a blood clot becomes dislodged from elsewhere in the circulatory system and travels to occlude specific cerebral vessels.

Hemorrhagic stroke is characterized by uncontrolled bleeding in the brain. There are two categories of primary hemorrhagic stroke: intracerebral hemorrhage and subarachnoid hemorrhage (Sacco et al. 2013). Intracerebral hemorrhage is the result of a ruptured artery within the brain. Subarachnoid hemorrhage occurs when a blood vessel ruptures, resulting in blood collecting between the arachnoid membrane and the pia surrounding the brain (Sacco et al. 2013). Intracerebral hemorrhage accounts for 10% of all strokes; the remaining 3% are subarachnoid hemorrhages (Sacco et al. 2013).

Throughout the remainder of the thesis, the term "stroke" will be used to refer to ischemic stroke.

2.1.2 Mechanisms of Stroke-Induced Brain Injury

The infarct produced by an ischemic stroke is surrounded by an area of tissue referred to as the penumbra (Sacco et al. 2013). The infarct is composed of tissue that is damaged beyond repair; this tissue will die. By contrast, the penumbra often exhibits reduced blood flow but possesses the potential for recovery (Furlan et al. 1996). It is this salvageable area that is the target of potential stroke treatments (Furlan et al. 1996).

The mechanisms through which ischemic brain damage manifests have been well documented in experimental literature (Ginsberg 2009). The complex sequence of events that occurs following a reduction in cerebral blood flow is referred to as the ischemic cascade. Adenosine triphosphate (ATP) depletion, increased intracellular calcium, glutamate excitotoxicity, membrane depolarization, production of free radicals, blood brain barrier disturbance and inflammation are among the intricate biochemical changes involved that contribute to stroke pathology (Durukan and Tatlisumak 2007).

Ischemic cell death occurs via two main pathways: necrosis and apoptosis. Necrosis is more common within the core of the infarct, with apoptosis becoming progressively more dominant with increasing distance from the core (Smith 2004). Necrosis is characterized by a loss of ionic regulation which subsequently results in cell swelling and membrane degradation (Fink and Cookson 2005). Conversely, the apoptotic process is characterized by decreased cell volume, maintenance of organelle structure, and the reduction and breakdown of the cytoplasm and nucleus (Fink and Cookson 2005). Cells in the penumbra may succumb to death by either of these pathways (Smith 2004).

Brain tissue lacks the ability to store energy and is therefore dependent on a continuous influx of glucose and oxygen in order to produce ATP (Dirnagl et al. 1999). A reduction in cerebral blood flow depletes available oxygen and glucose resulting in the formation of lactate via anaerobic glycolysis (Xing et al. 2012). The ensuing ATP deficiency causes the reverse operation of sodium/calcium transporters and the failure of Na^+/K^+-ATPase (adenosine triphosphatase) and Ca^{2+}/H^+-ATPase pumps (Phan et al. 2002). The failure of these energy dependent transporter channels results in the elevation of intracellular sodium, calcium and chloride ions in tandem with extracellular potassium levels (Phan et al. 2002). The disruption of these ions causes a loss of resting membrane potential and cytotoxic edema (Dirnagl et al. 1999). Once the membranes of neurons and glia become depolarized, excitotoxic neurotransmitters such as glutamate are released from presynaptic neurons. The over-activation of glutamate receptors (N-methyl-D-aspartate receptor (NMDA-), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA-) and metabotropic glutamate receptors) results in further influx of calcium (Phan et al. 2002), which activates phopholipases and proteases that degrade membranes and proteins (Lipton 1999). Glutamate receptor activation also promotes the influx

of sodium and water into the cell, thereby exacerbating cell swelling and edema (Lipton 1999). Elevated calcium, sodium and adenosine diphosphate (ADP) stimulate mitochondrial production of reactive oxygen species (Xing et al. 2012), which in turn trigger various neuronal death mechanisms (including mitochondrial transition pore formation) (Kroemer and Reed 2000) resulting in necrosis, apoptosis and autophagy.

Brain ischemia triggers inflammatory mechanisms that are inherent to the brain and blood. When neuronal integrity is compromised, ATP is released into the extracellular matrix resulting in microglial activation (Zhao et al. 2016). Microglial activation within the penumbra activates matrix metalloproteinases (Amantea et al. 2015). The activation of metalloproteinases causes disruption of the blood-brain barrier by altering microvascular endothelial function, thereby leaving the brain susceptible to peripheral inflammatory reactions (Cunningham et al. 2005). Furthermore, the disruption of the blood-brain barrier in combination with the upregulation of inflammatory mediators by microglia - such as tumor necrosis factor α (TNF-α) and interleukin 1 β (IL1-β) - encourages the infiltration of peripheral inflammatory cells (leukocytes) into the brain during reperfusion (Chen et al. 2014).

Although microglia are responsible for releasing pro-inflammatory mediators, they also play a beneficial role within the brain after stroke. Activated microglia can switch between 2 activation states: M1 and M2 (Chen et al. 2014). The M1 or classical activation state is pro-inflammatory, whereas the M2 or alternative activation state is anti-inflammatory (Chen et al. 2014). Alternatively activated microglia are responsible for clearing away cellular debris and releasing anti-inflammatory cytokines, thereby alleviating the effects of inflammation and promoting the tissue repair process (Chen et al. 2014).

Astrocytes also become activated in response to brain ischemia. Astrocytes respond to stroke by upregulating glial fibrillary acidic protein (GFAP), an intermediate filament protein (Sofroniew and Vinters 2010; Choudhury and Ding 2016). The upregulation of this protein expands the cytoskeleton, thereby resulting in cellular hypertrophy and a consequent increase in the interlocking of astrocytic processes. The interlocking of these processes forms what is referred to as the glial scar, which surrounds the tissue damaged beyond repair after stroke (Sofroniew and Vinters 2010).

2.1.3 Stroke Neuroprotective Treatment Approaches

Recent advances in technology have led to the development of a procedure referred to as endovascular or mechanical thrombectomy (ET), which has been shown to improve functional outcome and decrease mortality rates for some ischemic stroke patients (Goyal et al. 2015). Endovascular thrombectomy involves the insertion of a thin tube (guide catheter) into an artery in the patient's groin (Rohde et al. 2012). The catheter is then guided via the use of X-ray imaging into the brain. A catheter of smaller diameter (micro-catheter) is used to traverse the thrombus and gain distal access (Rohde et al. 2012). A retrievable stent (stentriever) is then deployed within the thrombus by pulling the micro-catheter back over the expandable stent (Rohde et al. 2012). The stent expands as the catheter is withdrawn, trapping the thrombus between the device and the wall of the blood vessel, thereby restoring blood flow (Rohde et al. 2012). The device is then withdrawn with the micro-catheter under continuous aspiration into the larger catheter(s) (Rohde et al. 2012).

Regrettably, the search for a pharmaceutical treatment for stroke has been met with limited success. The only drug that is currently approved for treatment of acute ischemic stroke is

recombinant tissue plasminogen activator (rtPA) (Meyers et al. 2011). Unfortunately, there are several contraindications for the use of rtPA which exclude a large number of patients from treatment (Novakovic et al. 2009). Furthermore, this thrombolytic agent has an extremely narrow therapeutic window of 4.5 hours (Lansberg et al. 2009).

The complex pathways involved in the ischemic cascade responsible for neuronal cell death offer one important explanation for the continued failure of drug therapies targeting stroke (Novakovic et al. 2009). Although a given pharmaceutical agent may target one pathway, there are several others through which neuronal damage may occur. Thus, it is recommended that current research approaches focus on combination therapies that target multiple pathways in the ischemic neuronal death cascade. A second recommendation is that more emphasis should be placed on developing more effective rehabilitation strategies that target recovery mechanisms. In addition, in order to facilitate the translation of these therapies into clinical practice, investigators must consider comorbidity factors that may influence recovery after stroke (Fisher et al. 2009). This thesis research evaluates the influence of suboptimal nutrition status developing after stroke in order to investigate the effects of this co-morbidity factor.

2.1.4 Post-Stroke Brain Plasticity, Repair and Compensatory Mechanisms

Patients who survive stroke often exhibit some level of functional recovery due to neuroplasticity. Brain plasticity refers to the brain's inherent ability to alter neural pathways in response to injury or experience. These changes may be brought about by the use of pre-existing neuronal connections, axonal sprouting, dendritic growth, the development of dendritic spines or through the growth of additional neurons (Murphy and Corbett 2009; Carmichael 2010).

After stroke, axonal sprouting and the subsequent formation of novel neural circuits are triggered by rhythmic, synchronous neuronal activity (Carmichael and Chesselet 2002). There is an approximately 2-3 week time period during which post-stroke conditions within the central nervous system are conducive to axonal sprouting. During this period, neuronal growth promoting genes are induced, proteins that are inhibitory to growth are removed and growth inhibitory genes have yet to be activated (Carmichael 2006). Stroke has been shown to induce genes that encode the growth cone lipid raft proteins GAP43, BASP-1 (brain abundant membrane attached signal protein-1) and MARCKS (myristoylated alanine rich protein kinase C substrate) (Li and Carmichael 2006). These genes are induced at 3 days post-ischemia and persist throughout the sprouting response (Carmichael 2006). In the healthy adult brain, axonal sprouting is inhibited by 3 classes of proteins: myelin associated proteins, extracellular matrix proteins and developmentally associated growth cone inhibitors (Carmichael 2006). Extracellular matrix proteins known as chondroitin sulfate proteoglycans form perineuronal nets that persist throughout the central nervous system (Karetko-Sysa et al. 2011). It has been suggested that these nets are responsible for preventing the formation of novel synapses and stabilizing existing synapses (Karetko-Sysa et al. 2011). After ischemic insult, perineuronal nets are reduced, inhibitory proteins are temporarily decreased and growth proteins are upregulated in a growth permissive area surrounding the glial scar (Carmichael 2006). It is within this growth permissive zone where axonal sprouting occurs.

Both pre-synaptic (axonal fibres and terminals) (Carmichael 2006) and post-synaptic (dendrites and dendritic spines) (Wang et al. 2016) neuronal remodeling occurs within the peri-infarct cortex in the weeks following stroke. Dendritic spine formation and retraction occurs within the regions of axonal sprouting in the cortical area surrounding the infarct (Carmichael et

al. 2016). This ongoing remodelling peaks at two weeks after the ischemic insult and is most prominent within 200 μm of the core of the infarct (Brown et al. 2010). Post-stroke synaptogenesis may thus occur between dendrites and spared or sprouting neurons (Carmichael et al. 2016).

The regulation of cell death and survival is an important process in the formation of functional neuronal circuits after stroke (Tovar-y-Romo et al. 2016). Cells that contribute to signal transmission within a circuit survive; those that fail to contribute useful connections are pruned from the system (Tovar-y-Romo et al. 2016). Tropomyosin receptor kinase (Trk) activation is moderated by neurotrophins such as brain derived neurotrophic factor (BDNF), which influence several key mediators of cell survival and synaptic plasticity. Ligand binding of a Trk receptor results in the activation of various signalling cascades, which have been implicated in the interception of nuclear and mitochondrial death cascades via the expression of anti-apoptotic proteins and the promotion of synaptic transmission (Lu et al. 2005). Brain derived neurotrophic factor (BDNF) also affects neuronal proliferation, survival and differentiation through binding of its specific tyrosine kinase receptor (Ploughman et al. 2009). Motor learning has been shown to elevate cortical concentrations of BDNF (Klintsova et al. 2004), which may facilitate motor map reorganization, synaptogenesis, enhance dendritic spine formation and dendritic branching after ischemic insult (Biernaskie and Corbett 2001; Monfils et al. 2005). In addition, BDNF signalling also controls GAP-43 activation (Benowitz and Routtenberg 1997), which is integral to the formation of axonal growth cones.

An additional means of repairing damage within the brain is via the generation of new cells. Neural stem cells may be recruited from the subventricular zone to replace damaged neurons (Marlier et al. 2015; Carmichael 2016). These progenitor cells migrate to the damaged

areas of the brain and may survive to become mature, functioning neurons (Arvidsson et al. 2002; Marlier et al. 2015). The survival of these newborn neurons is guided by neurotrophic signalling (trophic support, or lack thereof) and the neuron's subsequent synaptic involvement within a circuit (Hennigan et al. 2007).

Subcortical stroke initially results in bilateral cortical activation in response to sensory or motor stimulation of the affected limb (Carmichael 2006). Over time, this bilateral activation becomes increasingly restricted as the patient recovers. Motor map representation of the affected limb is thereby expanded and reorganized to accommodate functional recovery (Carmichael 2006).

The alteration of diffuse neural circuitry in combination with a natural synaptic redundancy within the brain may facilitate recovery after ischemic insult (Murphy and Corbett 2009). Although the majority of sensorimotor pathways are controlled by the hemisphere opposite the effector, ipsilateral pathways also exist in the healthy brain (Gonzalez et al. 2004; Biernaskie et al. 2005). Thus, the brain may compensate for neuronal loss by recruiting circuits in the contralesional hemisphere to assist in the execution of tasks previously performed by neurons in the damaged hemisphere (Biernaskie et al. 2005; Wahl et al. 2014). However, evidence for a functional contribution of the contralesional hemisphere in sensorimotor recovery is limited (Cramer 2008).

The size of the infarct may influence how the brain re-organizes synaptic connections after stroke (Biernaskie et al. 2005; Carmichael 2016). Large areas of damage may force the brain to rely more heavily on the contralesional hemisphere for recovery (Cramer 2008; Carmichael 2016). Minor areas of ischemic damage may be more efficiently repaired by utilizing recoverable

connections within the penumbra (Biernaskie et al. 2005; Carmichael 2016). Normal patterns of lateralization are associated with better functional recovery (Cramer 2008) . Therefore, abnormal patterns of lateralization may be a reflection of both the magnitude of the insult (Cramer 2008) and the brain's capacity for repairing severely damaged tissue (Biernaskie et al. 2005).

Improvements in motor function are not always indicative of true recovery. Laboratory animals frequently use altered posture or behavioural approaches to improve performance (Murphy and Corbett 2009). This phenomenon, referred to as compensation, is also relevant to clinical settings (Corti et al. 2012). Distinguishing between true recovery and compensation is difficult and time consuming (Murphy and Corbett 2009). Thus the term recovery often encompasses both true recovery and compensation.

2.1.5 Rehabilitation Approaches to Enhance Post-Stroke Plasticity

Developing and testing novel rehabilitation strategies to enhance post-stroke recovery is currently an area of intensive research investigation. Motor recovery after stroke relies on processes of learning and plasticity; therefore, rehabilitation approaches that promote plasticity may improve motor outcome (Allman et al. 2016). Of the many rehabilitation regimens being tested, two examples are presented here.

The effectiveness of most current therapeutic interventions targeting motor recovery is based on task and context specific motor learning and repetition (Veerbeek et al. 2014). A study conducted by Wang et al. (2016) demonstrated that rehabilitation which targets the affected limb promotes structural adaptations in the ipsilesional cortex, thereby facilitating functional recovery. Constraint induced movement therapy (CIMT) is one such therapeutic approach that is based on these principles. This rehabilitation method involves repetitive task practice in combination with

restraint of the unaffected limb in order to force the patient to rely on the affected limb to complete the task (Kwakkel et al. 2015).

Another therapeutic approach that has been investigated in the literature is anodal transcranial direct current stimulation (tCDS) in combination with motor training. Anodal tCDS applied to the motor cortex has been shown to alter membrane polarization, augment excitability (Nitsche and Paulus 2000) and reduce neuronal inhibition (Stagg et al. 2009). This alteration in excitability in combination with motor rehabilitation exercises may assist neurons in forming novel firing patterns associated with a given motor function, thereby facilitating plasticity and motor learning (Allman et al. 2016).

2.2 Protein-Energy Malnutrition

2.2.1 Prevalence & Causes of PEM after Stroke

Protein-energy malnutrition (PEM) in elderly individuals can be the result of various conditions. For instance, anorexia may occur as the result of a decrease in the ability to taste and smell food, early satiation signals, depression, polypharmacy or therapeutic diet (Morley 2012). Several of these factors may coexist in an individual at any given point in time, such as in a subset of elderly patients that present with PEM at the time of stroke (Bouziana and Tziomalos 2011).

However, the PEM that develops after stroke is the subject of this thesis research. In this case, PEM is most likely to be the result of a physical or mental impairment that hinders the ability of the patient to eat. For instance, difficult or painful swallowing may prevent adequate nutritional intake in some stroke patients (Finestone et al. 1995; Smithard et al. 1996). In

addition, the changes induced by the stroke may result in depression (Andersen et al. 1994) which in turn may affect food intake (Yang et al. 2009; Morley 2012).

From a historical perspective, malnutrition in stroke patients is a concern that has been largely ignored for the past 20 years given the persistence of the issue. A study conducted on stroke patients admitted to a hospital in England between 1994 and 1995 found that nutritional status had a tendency to deteriorate, particularly for those patients that remained in the hospital over the 4 week study period (Gariballa et al. 1998). Yoo et al. (2008) came to similar conclusions whereby 12.2% of patients were undernourished on admission, with the prevalence of malnutrition rising to 19.8% at 1 week. A review conducted by Foley et al. that included studies published between 1988 and 2008 found that estimates of PEM in stroke patients ranged from 6.1% - 62% (Foley et al. 2009). However, a major criticism of this review is the lack of segregation according to time of assessment relative to when the stroke occurred. The majority of the studies presented in the Foley et al. review found an increased prevalence of malnutrition at time periods greater than one week (22 – 35%) compared to studies that focused on the acute period after stroke (8 – 16%) (Foley et al. 2009). A more recent study by Kim et al. (2015) found that 25% of study participants experienced weight loss during the first 9 days post-stroke. Another study that investigated the long-term nutritional status in stroke patients found that almost half (47.9%) of the participants were afflicted by malnutrition at time periods greater than 1 year after the ischemic event (Paquereau et al. 2014). Taken together, these findings imply that the issue of malnutrition after stroke has yet to be addressed following nearly 3 decades of research. Furthermore, these studies also suggest that at some point during the post-ischemic period, the nutritional status of a significant proportion of patients deteriorates.

The results of a Canada-wide survey of registered dietitians emphasized the importance of utilizing standardized and validated approaches to nutritional assessment within the stroke patient population (Peters et al. 2015). The survey included 95 dietitians from acute care facilities across the country. The study found that the majority of facilities used screening and assessment approaches that have not been previously validated (Peters et al. 2015). Validated screening methods were used by 34% of respondents in the study; however, only one-third of this group used the original, validated form of the tool while the remaining two-thirds used modified versions of these validated tools (Peters et al. 2015). The majority of facilities (66%) used other means to evaluate nutritional status. In cases where standardized and validated screening methods were not employed, the survey found that a wide variety of individual indicators with variable cut-off points were used to identify malnourished patients (Peters et al. 2015). The utilization of unverified assessment tools increases the likelihood of misdiagnosing PEM, which may result in the underestimation of malnutrition rates within the vulnerable stroke patient population.

2.2.2 Effects of PEM Developing after Stroke on Functional Recovery

A recent study conducted by Nii et al. (2016) concluded that both energy intake at admission to a rehabilitation facility and improvement in nutritional status at discharge were associated with the recovery in activities of daily living in stroke patients. However, this study lists several limitations including a small sample size, possible selection bias for inclusion in the study and a lack of control for stroke severity or type of rehabilitation. Conversely, the Feed or Ordinary Diet (FOOD) trial found that nutritional supplementation after stroke did not improve stroke outcome (Dennis et al. 2005). Although this study involved a large number of patients, it has been subjected to a number of criticisms (Rabadi et al. 2008). The majority of patients (92%)

included in the FOOD trial were adequately nourished prior to enrollment in the study and were therefore unlikely to benefit from nutritional support (Rabadi et al. 2008). In addition, the parameters that were used to define malnutrition in this study were inadequate, which may have contributed to the misclassification of adequately nourished participants as malnourished. Furthermore, stroke severity was not controlled for by a validated measure and patients with swallowing difficulties were excluded from the trial (Rabadi et al. 2008).

A study conducted by Rhabadi et al. (2008) compared the effects of standard nutritional supplementation (127 calories, 5 g of protein) to intensive nutritional supplementation (240 calories, 11 g of protein) in malnourished patients admitted to a rehabilitation unit 2 weeks after stroke. Patients who received intensive nutritional support achieved greater functional independence compared to those assigned to the standard nutritional supplementation group (Rabadi et al. 2008). The findings of this study are a stark contrast to the conclusions drawn by the FOOD trial (Dennis et al. 2005). These conflicting results are likely attributable to 3 key differences: 1) The inclusion criteria for the Rhabadi et al. (2008) study in which only patients that exhibited significant weight loss (2.5%) within 2 weeks post-stroke were included in the study relative to all eligible patients that were able to swallow in the FOOD trial (Dennis et al. 2005). 2) The time period post-stroke when nutritional intervention was received: 2 weeks versus a span with a median of 5 days and an interquartile range of 3 to 9 days. 3) The Rhabadi et al. (2008) study included dysphagic patients whereas the FOOD trial (Dennis et al. 2005) excluded patients with eating difficulties. The exclusion of dysphagic patients would have exempted those patients that may have benefitted the most from nutritional intervention. Some limitations of the Rhabadi study include a small sample size and a lack of total nutritional intake information.

A study conducted by Ha et al. (2010) demonstrated that poor nutritional status after stroke has a detrimental influence on quality of life and muscle strength. Several previous clinical studies also found malnutrition to be correlated with poor functional outcome (Feed Or Ordinary Diet Collaboration 2003; Yoo et al. 2008; Nip et al. 2011) and decreased survival rates in stroke patients (Davis et al. 2004). The clinical effects of malnutrition are related to the duration and severity of nutritional deficiency, and thus early detection is paramount for limiting detrimental effects on patients coping with other pathological conditions.

Unfortunately, many of the aforementioned clinical studies are confounded by small sample sizes and a lack of standardization with regard to nutritional assessment, particularly where multiple care centers are involved. It is also possible that these studies underestimate the prevalence of malnutrition in patients when simplified classification systems are utilized. Although clinical studies are essential for offering insight into factors that may affect stroke recovery, it is not always possible to ascertain a causal link due to confounding variables. Although it too has limitations, the use of an animal model of stroke to study comorbidity factors such as PEM serves to eliminate these confounding variables and thus can provide conclusive data on the effects of malnutrition on functional outcome after stroke. In addition, animal models are essential to examine how functional outcome is affected by biochemical changes within the brain.

2.2.3 Proposed Mechanisms by which PEM can Influence Post-Stroke Recovery

Nutritional status may impact stroke recovery in numerous ways. Protein-energy malnutrition pre-existing at the time of brain ischemia and left untreated has been shown to modify tropomyosin-related kinase B (trkB) and growth associated protein-43 (GAP-43),

proteins that are involved in post-ischemic repair mechanisms (Prosser-Loose et al. 2010). However, this research focused on pre-existing PEM and thus may not accurately reflect what occurs when PEM develops after stroke. In addition, these experiments were conducted using a global brain ischemia model that causes a reduction in blood flow to the entire brain such as can occur as a result of cardiac arrest (Khodanovich and Kisel 2015). The CA1 (Cornu ammonis 1) region of the hippocampus is the most vulnerable in this model (Harukuni and Bhardwaj 2006), and the main deficits are in cognition. Therefore, these findings may not be an accurate reflection of the mechanisms involved in focal ischemia targeted to the motor cortex.

A more recent study that examined the effects of post-stroke PEM on markers of neuroplasticity after global ischemia demonstrated that post-ischemic PEM decreased growth associated protein-43, synaptophysin and synaptosomal-associated protein-25 immunofluorescence (Smith et al. 2014). These proteins are indicators of active axonal growth cones and functional synaptic connections, respectively (Smith et al. 2014). Thus, PEM may interfere with post-ischemic neuroplastic repair mechanisms (Smith et al. 2014). However, study limitations include the use of a global model of ischemia and the fact that the functional impact of these biochemical changes was not evaluated.

Protein-energy malnutrition may also affect stroke recovery by exacerbating the brain's inflammatory response to ischemia (Bobyn et al. 2005; Ji et al. 2008). Inflammation plays a role in both brain injury and repair processes after stroke (Lo 2008; Kriz and Lalancette-Hebert 2009). Thus, increased or decreased brain inflammation could hinder neuronal repair and/or exacerbate the initial damage caused by stroke. Previous studies suggest that PEM could influence brain inflammation caused by brain ischemia (Bobyn et al. 2005; Ji et al. 2008); this in turn could influence injury and repair after stroke (Kriz and Lalancette-Hebert 2009). However,

it is important to note that increased brain inflammation has not been reliably found with either pre-existing (Smith et al. 2011) or post-ischemic (Smith et al. 2014) PEM when other models of brain ischemia have been employed. As above, all of this evidence is restricted to models of global brain ischemia.

Altered muscle structure and function is a third potential contributor to PEM-induced hindrance of post-stroke recovery, as has been demonstrated in a clinical study by Ha et al. (2010). Alaverdashvili et al. (2015a) reported in adult rats that protein-energy malnutrition causes forelimb muscle dysfunction and reduces the diameter of muscles containing more fast-twitch fibers. Other research has demonstrated that a diet low in protein causes a reduction in size of both fast and slow twitch muscle fibers (Prescod et al. 2011). Indeed, the contribution of inadequate protein intake to muscle wasting and reduced strength is a widely documented phenomenon (Norman et al. 2011). It has been suggested that animals may compensate for muscle weakness induced by PEM during behavioural testing by employing body positions that engage more slow-twitch muscle fibers (Alaverdashvili et al. 2015a), which have a lower metabolic demand (Delp and Duan 1996). Compensation after sensorimotor insult such as stroke is also a well-documented occurrence (Murphy and Corbett 2009; Alaverdashvili and Whishaw 2013). Thus, PEM may present additional physical hurdles to the recovery of normal motor function after stroke. However, the assessment of muscle morphology is beyond the scope of the present study.

A fourth mechanism by which PEM could hinder recovery after stroke is by inducing an acute phase response (Smith et al. 2013; Smith et al. 2014). Protein-energy malnutrition can independently cause an acute phase response (Alaverdashvili et al. 2015a; Andrade Ramos 2015), which may adversely influence stroke outcome (Dziedzic 2008). A small acute phase

response is induced by stroke; however, this response varies among patients and is influenced by stroke severity (Dziedzic 2015). More pronounced acute phase reactions are associated with poorer stroke outcome (den Hertog et al. 2009; Nezu et al. 2013; Dziedzic 2015). Although we hypothesize that the acute phase response induced by protein-energy malnutrition is indicative of systemic inflammation, it is acknowledged that acute phase proteins are nonspecific indicators (Gabay and Kushner 1999; Omran and Morley 2000b), and thus the underlying mechanism is not clear.

A recent study conducted in the Paterson laboratory (Andrade Ramos 2015) found that when 16 week old adult rats were subjected to PEM induced by feeding a 0.5 % protein diet for 30 days, serum concentrations of the positive acute phase proteins alpha-1-acid glycoprotein and alpha-2-macroglobulin increased, while serum albumin, a negative acute phase protein, decreased. However, the effect of PEM on inducing an acute phase reaction may differ after stroke, and this question was examined in this thesis research.

2.3 Selection of Experimental Stroke Model

2.3.1 STAIR Report Guidelines and Models of Stroke

The Stroke Therapy Academic Industry Roundtable (STAIR) reports outline several standards for the investigation of stroke using animal models. These standards are intended as a methodological guide to maximize the efficiency and success of research targeting stroke therapies and improve translation of preclinical findings to the clinic. The report, initially published in 1999 and later updated in 2009, emphasizes the necessity of multiple outcome measures with the absolute minimum of functional response and infarct volume assessment. Additional measures that may add valuable information to an investigation include

immunohistochemical analysis and neuropathology. The authors suggest the use of both acute and long term monitoring, since the beneficial effects of a given treatment may vanish over time (Stroke Therapy Academic Industry Roundtable 1999; Fisher et al. 2009). The key physiological variables recommended for monitoring during the surgeries required to induce stroke include brain temperature, blood pressure and blood gases (Stroke Therapy Academic Industry Roundtable 1999; Fisher et al. 2009), since variation in these has been shown to induce inconsistency in infarct volume (Stroke Therapy Academic Industry Roundtable 1999). The updated report also suggests that stroke therapies be evaluated in the context of sex, aging and comorbidity factors that may be associated with the aging process. Recovery endpoints, biological markers and animal age should be a reflection of the target human population that the research is to be translated for (Fisher et al. 2009).

2.3.2 Preclinical Models of Focal and Global Ischemia

Human stroke exhibits a pattern of localization to neuronal circuits that control a given function, thereby resulting in behavioural impairments (Carmichael 2005). These infarcts are limited to 4.5 – 14% of the volume of a given hemisphere (Carmichael 2005). Thus, an ideal stroke model will allow for the investigator to define the size and location of the infarct.

There are various methods available to model stroke in rodents. Each model has advantages and disadvantages, and thus the best model depends upon the individual research question. There are two major categories of brain ischemia models: global and focal. Global brain ischemia models such as two-vessel occlusion (carotid artery occlusion and hypotension) mimic brain injury associated with cardiac arrest (Small and Buchan 2000). These models affect the brain as a whole.

Focal brain ischemia models that target specific brain regions are intended to mimic human thromboembolic stroke. There are two categories of focal ischemia models that are distinguished by whether they produce transient or permanent occlusion (Sicard and Fisher 2009). Most of these models involve some variation on occlusion of the middle cerebral artery (MCAo) as described by Tamura et al. (1981). Transient middle cerebral artery occlusion can be produced through the use of microvascular clips or the injection of endothelin-1 to induce vasospasms (Murphy and Corbett 2009; Kumar et al. 2016). Permanent middle cerebral artery occlusion may be achieved through the use of cauterization (Murphy and Corbett 2009). A common drawback to the aforementioned MCAo methods is that they all necessitate the use of invasive surgical techniques. Some protocols involve a craniectomy, which induces skull trauma and results in changes in brain temperature and pressure (Braeuninger and Kleinschnitz 2009). The function of the blood brain barrier may also be affected (Tamura et al. 1981).

A technique known as the intraluminal suture approach can also be used to induce MCAo in rats. This technique requires the insertion of a suture into the internal carotid artery and subsequent intracranial advancement to block the MCA (Murphy and Corbett 2009; Khodanovich and Kisel 2015; Kumar et al. 2016). The suture may then be left in place or removed, depending on whether permanent or transient ischemia is desired. Although this method has the advantage of producing a well-defined penumbra, it also carries the risk of subarachnoid haemorrhage (Sicard and Fisher 2009; Khodanovich and Kisel 2015; Kumar et al. 2016). Furthermore, lesion size may be affected by several variables including suture length, diameter and coating (Belayev et al. 1996; Hata et al. 1998; Shah et al. 2006; Kumar et al. 2016). Additionally, the surgical procedure that is required by the intraluminal suture model may

damage muscles involved in mastication and swallowing, resulting in decreased food intake and declining weight (Bederson et al. 1986).

An alternative to the suture model is the thromboembolic model of focal ischemia. This permanent occlusion model involves the introduction of a blood clot into the internal carotid artery (Sicard and Fisher 2009; Khodanovich and Kisel 2015; Kumar et al. 2016). The blood clot then becomes fixed in the MCA. Although this model is highly analogous to human stroke, the level of variability in infarct volume and a high mortality rate (Murphy and Corbett 2009; Khodanovich and Kisel 2015; Kumar et al. 2016) rendered this technique undesirable for use in the present study. The photothrombotic model of focal ischemia was chosen for use in the present study primarily because it is reproducible and the least surgically invasive technique.

2.3.3 Photothrombotic Model of Focal Ischemia

The photochemical model of focal ischemia, as developed by Watson et al. (1985), produces an infarct via the illumination of Rose Bengal dye. The dye is injected through a catheter into the tail vein for systemic circulation and a laser of specific wavelength is focused onto the desired brain region after thinning of the skull. Upon contact with the light, the dye releases energy to oxygen molecules which generates reactive oxygen species. These highly reactive oxygen products react with endothelial cell membranes which results in platelet adhesion and aggregation and the subsequent formation of thrombi in various micro vessels within the brain parenchyma (Watson et al. 1985; Carmichael 2005). This is a less invasive procedure, as no craniotomy is required. The main advantage for the thesis study is that minimizing surgical stress limits the neuroendocrine response elicited by invasive surgery (Roberts 1995; Twyman 1997). This is important, since the latter is minimal after clinical stroke

(Syrjanen et al. 1989; Finestone et al. 2003). Thus, the influence of increased gluconeogenesis and skeletal muscle breakdown, which has marked effects on protein-energy status, can be reduced through use of the proposed model. The use of a surgical sham further controls for this potential confounding variable.

The photochemical model of focal ischemia allows for highly reproducible infarcts that can be placed as desired by the investigator (Watson et al. 1985). Additional refinements to the laser system made by our lab have been shown to further decrease variability and increase reproducibility in infarct size (Alaverdashvili et al. 2015b). The addition of a polarizer or variable neutral density filter to the laser apparatus allows for control over the intensity of the laser beam. Control over light intensity is imperative, since the photochemical rate of reaction that results in formation of the infarct is proportional to beam intensity (Alaverdashvili et al. 2015b). Beam stability is monitored throughout the irradiation period to ensure that intensity remains consistent by splitting the beam and measuring power output using a photodiode sensor (Alaverdashvili et al. 2015b).

The thesis study relied on targeting the region of the motor cortex that corresponds to forepaw function. This technique is a widely used and well validated approach for modelling stroke (Shanina et al. 2006; Moon et al. 2009; Jablonka et al. 2010). One notable disadvantage of this model is a limited penumbra (Carmichael 2005). An additional criticism of this approach is the production of extracellular (vasogenic) edema in combination with intracellular (cytotoxic) edema (Carmichael 2005; Kumar et al. 2016). By contrast, human stroke develops with cytotoxic edema characterized by decreased water diffusion (Carmichael 2005). Consequently, some investigators consider the pathophysiology of photothrombosis to bear more resemblance to traumatic brain injury than to focal ischemia (Carmichael 2005).

2.4 Functional Assessment in Focal Ischemia Models

2.4.1 Overview

Histological endpoints are essential to evaluate outcome in animal stroke models. For focal ischemia models, infarct volume is measured (MacLellan et al. 2013; Alaverdashvili et al. 2015b). However, conclusions that rely exclusively on evaluation of neuron death can provide an inaccurate assessment of neuronal viability (Corbett and Nurse 1998). Moreover, although neurons may appear structurally sound, these cells can be functionally abnormal (Corbett and Nurse 1998). The most relevant clinical endpoint for stroke patients is functional outcome. By incorporating functional analysis into the study design, the full impact of treatment or comorbidity factors (e.g. PEM) on stroke outcome may be determined.

There are several tests that are commonly utilized to assess motor outcome after cortical stroke. Among these tests are the Montoya Staircase (MacLellan et al. 2013), cylinder task (Kirkland et al. 2012), ladder task (Mestriner et al. 2013), beam walking task (Clark et al. 2008), and single pellet reach (Moon et al. 2009). The tests that were selected for this thesis research were the cylinder test and the Montoya staircase task. Both of these behavioural assessment techniques have been shown to be sensitive measures of function after focal damage to the motor cortex.

2.4.2 Cylinder Test

The cylinder test is used to appraise the development of asymmetrical reliance on a given forelimb when the animal is placed in a Plexiglas cylinder and rears up to engage in spontaneous exploration (Schallert et al. 1997). Rats are filmed from below the cylinder. The number of wall contacts with each forelimb is recorded for detailed analysis. Although scoring is somewhat

subjective, the system is relatively simple and easy to utilize (Schallert and Woodlee 2004). In addition, the cylinder test does not require a food reward to encourage the rats to perform, which is particularly advantageous for nutritional studies where nutrient intake must be tightly controlled. A third advantage of the cylinder task is that limb use scores are relatively unaffected by practice effects as the result of repeat testing or by compensatory strategies frequently employed by animals after sensory-motor injury (Schallert and Woodlee 2004). For instance, animals exposed to the single pellet reaching task after stroke may make additional attempts or adjust their posture or movements in order to achieve success in the task (Alaverdashvili and Whishaw 2013).

2.4.2.1 Approaches to Scoring Performance in the Cylinder

The most common approach to evaluating spontaneous limb use in the cylinder apparatus is to record the number of independent, bilateral and simultaneous limb placements on the wall during exploration (Gonzalez et al. 2004; Li et al. 2004; Clark et al. 2008; MacLellan et al. 2013; Andrade Ramos 2015). Affected limb use (or use of the limb of interest) is calculated as a percentage of the total number of wall placements (Schallert et al. 2002). Additional measures of limb use asymmetry that can be obtained from the cylinder apparatus include paw use for initiation (MacLellan et al. 2002; Field et al. 2006) and termination (Schallert et al. 2000) of vertical bouts (take-off and landing) and first touch after initiation of exploration.

Evaluating several endpoints in the cylinder apparatus may increase the chances of detecting different rates of recovery, thereby increasing sensitivity (Schallert and Tillerson 2000). One challenge to this approach is the limited number of movements that can be scored in the task. In order to ensure a sufficient number of wall contacts, Schallert et al. recommend

filming each animal for a minimum of 20 touches (Schallert et al. 2002). When evaluating paw use during landing, Schallert suggests recording 20 wall contacts and at least 10 landing movements (Schallert and Tillerson 2000). The evaluation of these endpoints, which are often overlooked (Li et al. 2004; Bretzner et al. 2008; MacLellan et al. 2013; Andrade Ramos 2015), has been facilitated in the thesis study by filming for a minimum of 5 minutes and 20 contacts.

2.4.3 Montoya Staircase

The parallels between human upper extremity and rat forelimb movements during reaching tasks (Whishaw et al. 2002; Whishaw et al. 2008) offer a viable means of studying conditions that affect motor impairment as a result of stroke. There are various tests available that may be used to evaluate post-stroke changes in the skilled reaching and grasping ability of laboratory rats. These tests include the Montoya Staircase (Montoya et al. 1991), single pellet reach (Whishaw et al. 1991) and tray task (Whishaw et al. 1986). All of the aforementioned assessment tools have been demonstrated to be sensitive measures of functional outcome in rat focal ischemia models (Gharbawie et al. 2005; Shanina et al. 2006; Moon et al. 2009).

In the staircase task, rats are trained to reach for sucrose pellets placed on 7 steps located on either side of an elevated central platform. By contrast, the single pellet reach and tray tasks require the rat to reach through vertical openings to obtain food from a table or shallow trough respectively. One advantage of the staircase test over the single pellet reach and tray tasks is the ease with which the rat may be constrained to the limb of interest. In addition, the staircase test may be readily administered to several rats at once, allowing for rapid data collection.

A drawback of the Montoya Staircase apparatus is that it is less conducive to qualitative analysis of skilled reaching. The benefit of qualitative analysis is that it provides information on

the use of compensatory strategies during a given reaching task (Whishaw et al. 1991). Both the tray task and the single pellet reach task readily allow for endpoint (quantitative) and qualitative assessment. However, the latter requires the utilization of complex, subjective, and time consuming movement notation techniques. A modified version of the staircase, in which the pellets are colour coded to correspond to a specific step was proposed by Kloth et al. (2006). The use of colour coded pellets may help provide some insight into the degree of deterioration of grasping ability in ischemic rats without necessitating complex movement analysis.

Rats have been shown to learn to utilize compensatory movements in all three of the aforementioned skilled reaching tasks (Whishaw et al. 1997; Whishaw et al. 2002; Kirkland et al. 2012). In addition, it has been demonstrated that repeated testing in a skilled reach training apparatus promotes neural recovery (Wurm et al. 2007; Kim et al. 2012). Both compensation and recovery as a result of rehabilitative training occur in clinical stroke patients (Corti et al. 2012). Each of the aforementioned phenomena confound the effect of spontaneous motor recovery, which is examined in the thesis study. In order to minimize the effects of induced rehabilitation and the acquisition of compensatory strategies, particularly with regard to the staircase apparatus, the number of testing sessions in the post-ischemic period was limited.

The thesis research relied on validated techniques described in Sections 2.3 and 2.4 to address the hypothesis that protein-energy malnutrition developing after stroke would impair spontaneous motor recovery without altering infarct volume, and that this would be associated with an acute phase response. The rat photothrombotic model of focal ischemia was utilized to investigate the effect of protein-energy malnutrition developing after stroke on spontaneous motor recovery. Infarct size was measured and the Montoya staircase and cylinder tasks were chosen to evaluate the influence of protein-energy malnutrition on forelimb use asymmetries that

developed in response to stroke. Nutritional status was assessed by a combination of food intake, body weight, serum albumin concentration and the degree of liver steatosis. The acute phase response in the chronic period was monitored by serum albumin and alpha-2-macroglobulin concentrations.

CHAPTER 3: MATERIALS & METHODS

This experiment was approved by the University of Saskatchewan's Animal Research Ethics Board and adheres to the Canadian Council on Animal Care guidelines for humane animal use.

3.1 Experimental Design

Adult, male (12 week old) Sprague-Dawley rats, (n=30) were caged in pairs in a facility maintained at 22°C with a 12 hour light/dark cycle. Animals were acclimatized to the facility for 10 days before introduction to behavioural training in the Montoya staircase. Beginning on the 3rd day after arrival, the animals were handled for a minimum of 5 minutes each day until introduction to the Montoya staircase apparatus. Rats were placed on experimental purified control diet on the 4th day of handling. The control diet (Alaverdashvili et al. 2015a) consisted of a modified version of the American Institute of Nutrition -93M diet (Reeves et al. 1993). The diet was tertiary-butylhydroquinone free and contained 12.5% protein. Banana flavoured sucrose pellets were introduced into the cages of the animals 5 days prior to training in the Montoya staircase. Animals were trained for a minimum of 14 days with 3 additional days of training where needed to meet the minimum training criteria. The pre-stroke baseline performance in the Montoya staircase was calculated using data from the last two days of training in the staircase. Baseline cylinder testing occurred on the final day of staircase testing after the last staircase trial.

Animals were then assigned to undergo either sham surgery or focal ischemia (photothrombotic stroke). On Days 3 and 4 after surgery, animals that showed promise of training in the Montoya staircase were tested to determine the post-stroke deficit. On Day 4, the cylinder test was performed, after which rats were assigned to either a low protein or adequate

protein (control) diet. Montoya staircase testing was conducted again on Days 15, 16, 28 and 29 with cylinder testing on Days 16 and 29.

Body weight was monitored on a weekly basis throughout the experiment with the exception of more frequent measurement during the brief period immediately after surgery. Food intake was recorded daily.

On Day 30, animals were humanely euthanized via isoflurane overdose. Blood and liver were preserved in order to assess acute phase response and protein-energy status. Brains were collected for infarct volume assessment. The details of harvesting tissue and serum are found below.

3.1.1 Motor Task Training and Pre-Stroke Baseline Measurements

3.1.1.1 Montoya Staircase

The Montoya staircase was used to assess the skilled reaching deficit after cortical stroke and the potential for recovery. The standard and intended methodology for the Montoya staircase is as follows: Each staircase box is constructed in such a manner that the animal can only reach for pellets in the right or left staircase with the ipsilateral paw. Each side consists of 7 steps, each containing a well that holds 3 sucrose pellets (45 mg, Bio-Serv). Rats are trained twice daily for 14 consecutive days in the staircase apparatus for a total of 28 trials (Ploughman et al. 2009; MacLellan et al. 2013). Trials run on the same day are separated by a minimum of 2 hours. Additional trials are recorded for animals that require further training to meet minimum inclusion criteria. Each animal is placed in the box for a period of 15 minutes, after which the animal is removed and the total number of pellets eaten, dropped and remaining on the steps is recorded. Rats are excluded from post-stroke staircase analysis if they cannot obtain an average of at least

12/21 pellets with a standard deviation less than ± 2 on the last 8 training trials (Ploughman et al. 2009; MacLellan et al. 2013). The baseline (pre-stroke) value for each animal is obtained for later comparison with post-surgery performance in order to evaluate the effect of stroke on skilled reaching and grasping ability. Baseline values are calculated using an average of the last 4 training trials (2 days).

The Montoya staircase baseline data were used to determine the preferred paw of each rat (when possible) and thus guide infarct placement during the induction of stroke. The infarct is then positioned in the forelimb motor cortex contralateral to the preferred paw. The side on which the rat consistently collects more pellets is considered the preferred side. For example, strong right paw preference is defined by the rat collecting at least 5 more pellets with the right paw (relative to the left); this is calculated as a mean over the last 8 trials. When training resulted in inadequate staircase performance (and thus preferred paw use could not be determined), the targeted hemisphere was chosen randomly.

Unfortunately, some major challenges arose during the Montoya staircase training. Rats were introduced to the Montoya Staircase on the 10^{th} day after arrival at the facility without any prior food restriction. It is common practice to employ a food restriction throughout the training period to motivate the rats to learn to reach for the sugar pellets (Sacrey et al. 2009; MacLellan et al. 2013). However, since the major goal of this experiment was to determine the effect of a nutritional intervention (post-stroke PEM), I proposed to omit the food restriction to eliminate the potential confounding influence of food restriction. The results of two previous pilot studies performed on 6 rats had supported the idea that the rats would learn this task without food restriction. Contrary to these findings, it became apparent after 7 days of training in the current study that the rats were not learning the task as expected. As time progressed, a large portion of

the rats failed to demonstrate sufficient training progress in the pellet retrieval task. Thus, on Day 9 of training, a 15% food restriction was implemented for all animals. Rats were returned to ad libitum diet access 2 days prior to surgery.

This attempt to improve performance by introducing food restriction at a late stage was unsuccessful, and the majority of the animals (21/30) failed to meet minimum pellet retrieval criteria upon the completion of 16 days of training. Only 9 rats achieved at least the minimum standard of 12/21 pellets ± 2 standard deviations. One trained rat was excluded due to a failed surgery.

3.1.1.2 Cylinder

Baseline (pre-stroke) measurements for the cylinder test were conducted on the last day of staircase training prior to stroke. The cylinder test was utilized to assess motor recovery during spontaneous exploration following stroke. Animals were placed in a vertical Plexiglas® cylinder (41 cm high and 20 cm in diameter) and filmed for a period of time that met 2 criteria; the filming was conducted for a minimum of 5 minutes and until each rat made at least 20 wall contacts (Ploughman et al. 2009; MacLellan et al. 2013). Rats were filmed from below by aiming a camera at a mirror positioned at a 45° angle beneath a transparent surface on which the cylinder rested. The video file was then scored for forelimb use during initiation of exploratory behaviour, exploration and termination of exploratory behaviour (Schallert and Tillerson 2000). Each behaviour is defined in the relevant section below. All endpoints were evaluated through to completion of the data collected.

3.1.1.2.1 Initiation of Exploration: First Touch and Takeoff

Initiation of exploratory behaviour was quantified by two methods: tracking the forepaw used to push off from the cylinder floor (takeoff) and the forepaw used to make initial contact with the cylinder wall (first touch). Takeoff was defined as the movement used by the rat to initiate exploration by lifting its upper body off the cylinder floor prior to a bout of exploration. The last forepaw in contact with the cylinder floor was considered the limb used for takeoff. Bilateral use was scored if both forelimbs left the cylinder floor simultaneously. First touch was defined as the initial wall contact made after takeoff during each bout of exploration. Bilateral use was scored only if both forelimbs were used to contact the cylinder wall simultaneously.

3.1.1.2.2 Exploratory Behaviour

A bout of exploration is the period of exploration that occurs between the time when the rat's forepaws leave the cylinder floor and when the rat returns both forepaws to the cylinder floor. During the time spent in the cylinder, any given animal will undergo several bouts of exploration with and without making wall contact. During a bout of exploration, a rat may touch the cylinder with either the left or right forelimb alone or both forelimbs simultaneously during weight shifting movements. In addition, the animal may contact the wall with one forelimb and then the other in rapid succession while maintaining wall contact with one forepaw at all times. This movement is referred to as wall 'stepping' as described by Schallert et al. (Schallert et al. 2000; Schallert and Woodlee 2004). For instance, if a rat were to place its left forepaw on the cylinder wall and subsequently place its right forepaw on the wall before lifting the left forepaw and replacing the left forepaw on the cylinder wall in a different position, this action would be considered wall stepping. The rat may continue to 'step' by then lifting its right forepaw (without removing the left) and replacing the right forepaw on the cylinder wall in a new position. A stepping sequence is terminated when the animal removes both forepaws simultaneously from

the cylinder wall. Forelimb use during exploratory behaviour was tallied using two different scoring systems, hereby referred to as scoring methods one and two.

3.1.1.2.2.1 Exploratory Behaviour: Scoring Method 1

The scoring system used in method 1 was defined as a movement score that equalizes right and left forelimb use since one forelimb is considered to be used for postural support during a stepping sequence. Placement of the left forepaw on the cylinder wall was scored as a single contact by the left forelimb. Removal of the left forepaw from the cylinder wall followed by placement of the right paw on the cylinder wall was scored as a single contact by the right forelimb. If the animal made wall contact with one forepaw and subsequently placed the other forepaw on the cylinder wall without removing the first limb, this action was scored as one independent wall contact and one bilateral wall contact. Any subsequent stepping was scored as bilateral use until both paws were removed from the wall. Wall contact made by using both limbs simultaneously was also scored as bilateral use.

3.1.1.2.2.2 Exploratory Behaviour: Scoring Method 2

Scoring method 2 was defined as a movement score in which right and left forepaw use during a sequence of forepaw placements is scored as independent use. Bilateral contacts with the cylinder wall were only scored as bilateral use if the animal made wall contact using both limbs simultaneously. All other contacts were scored as independent forelimb use. Scoring method 2 places emphasis on overall limb use and does not account for use of the opposing forelimb for postural support during stepping movements. Wall stepping was broken down into the number of contacts made by each forelimb, regardless of the position of the other limb. For example, if the rat contacted the cylinder wall with the right forepaw and proceeded to place the

left forepaw on the wall without removing the right forepaw, this movement was scored as one contact by the right forelimb and one contact by the left forelimb. If the rat then removed the right forepaw and replaced the right forepaw on the cylinder wall without removing the left forepaw, this action was still scored as use of the right forelimb. If the animal placed both forelimbs on the cylinder wall at the same time, this action was scored as bilateral use.

3.1.1.2.3 Termination of Exploration: Landing

Landing was defined as the movement used by the rat to return its upper body to the cylinder floor after a bout of exploration. The first forepaw in contact with the cylinder floor was considered the limb used for landing. Bilateral use was scored if both forelimbs were used to contact the cylinder floor simultaneously after a bout of exploration.

3.1.1.2.4 Total Number of Touches

The total number of touches with any forepaw for each activity and test day was also evaluated to ensure that diet did not alter activity in the cylinder apparatus, since this could be a confounding factor.

3.2 Induction of Photothrombotic Stroke or Sham Surgery

Following the behavioural training period, rats were assigned to receive either photothrombotic cortical stroke (ISCH) (Alaverdashvili et al. 2015b) or sham surgery (SHAM). The rats, now between 16 and 17 weeks of age, were anesthetized with 4.0 to 4.5% isoflurane in oxygen and stabilized at 1.5-2.0% isoflurane mixed with 40% oxygen and 60% nitrous oxide gas. Once anesthetized, the incision area was shaved and cleaned of stray fur. The rat was then mounted in a stereotaxic frame on top of a homeothermic blanket system with feedback via a

rectal probe that served to maintain the animal's temperature close to 37 degrees Celsius. The incision site was cleansed with chlorahexadine soap followed by 70% ethanol. Aseptic surgical technique was employed thereafter for the remainder of the procedure. Respiration rate, oxygen saturation, and pulse were monitored throughout the surgical period and recorded at 10 minute intervals using the MouseOx Plus (STARR Life Sciences). A 1-2 cm midline incision was made beginning between the eyes and extending back to the ears. The tissue was separated to offer a clear view of the skull. As described above, the infarct was positioned in the forelimb motor cortex contralateral to the preferred paw as discerned during training for the staircase reaching task where possible. In instances where forelimb preference could not be determined as a result of inadequate pellet retrieval success in the staircase apparatus, the infarct was randomly placed in either the right or left hemisphere. The skull was thinned with a dental drill directly over the area of the motor cortex that controls forelimb function (+2 mm to -1 mm anterior-posterior from Bregma and +/- 2 mm to +/- 5 mm lateral of midline). The photothrombotic dye Rose Bengal (10 mg/kg) was drawn up into a syringe wrapped in aluminum foil to prevent photodegradation. The Rose Bengal was then injected intravenously through the tail via a catheter over a period of 45 seconds followed by a saline injection over an equivalent period. A pre-warmed Diode Pumped Solid State (DPSS) laser emitting 532 nm (green) light with a 4.0 mm diameter beam, delivering a power density between 280 and 300 mW/cm^2 was then aimed for 10 minutes over the area of the previously thinned skull. Power density was controlled by a rotatable polarizer. After removal of the laser, the incision was sutured and 2 mg/kg of bupivacaine hydrochloride (Marcaineä, Hospira, Inc.) was infiltrated at the incision site. Finally, an injection of sterile saline (10 mg/kg/hr) was administered to replace fluid lost during surgery. Sham animals underwent the same procedure without exposure to the laser beam.

Each animal was then moved to a recovery cage until he regained mobility. Half of the cage was placed on a warm, water-filled heating blanket. Once recovered, the animal was free to choose to remain in the heated area of the cage or move to a cooler area. Animals underwent close post-surgical monitoring for 3 days.

3.3 Assignment to Post-Stroke Experimental Diet

After surgery, rats continued on the purified control diet until Day 4. This design was intended to mimic the clinical scenario in which people develop PEM following a stroke. Since the goal of this study was to examine the effects of PEM developing after stroke on brain plasticity, the delay in diet assignment also reduces the chance of a dietary effect on the ischemic neuronal death cascade and infarct volume. Delaying diet assignment also allows for the assessment of the independent effect of stroke (prior to any dietary influence) on Days 3 and 4 as compared to sham animals. On Day 4, after behavioural testing, half of the stroke animals and half of the sham animals were randomly assigned to 1 of 2 diet groups, control diet (CON) or low protein diet (PEM). The basal diet composition (Alaverdashvili et al. 2015a) is a modified version of the American Institute of Nutrition 93-M diet (Reeves et al. 1993). The control diet consisted of 12.5% protein, which is sufficient to meet dietary requirements for adult rats (Reeves et al. 1993). The group assigned to PEM was given an isocaloric diet identical to the control diet with the exception of lower protein content (0.5%), in which the protein was replaced by carbohydrate (Alaverdashvili et al. 2015a). In 16 week old (adult) rats, a 0.5% protein diet results in mixed PEM due to a voluntary reduction in caloric intake (Andrade Ramos 2015). Feeding a 0.5% protein diet to 16 week old, male Sprague-Dawley rats has been shown in our lab to result in PEM as demonstrated by a reduction in food intake and 10.6% decrease in body weight over 4 weeks; as compared to the control group, the low protein diet caused a

22.2% decrease in serum albumin and a ~6 fold increase in liver lipid content (Andrade Ramos 2015). These measures will also be employed in the present study to assess protein-energy status.

The following experimental groups thus resulted from the assignment to the aforementioned experimental diets: CON-SHAM (n=6), PEM-SHAM (n=6), CON-ISCH (n=9), PEM-ISCH (n=7).

3.4 Post-Stroke Functional Assessment

Cylinder testing was conducted as described above on post-stroke Days 4, 16 and 29. For each endpoint, the percent of preferred or affected forelimb use was calculated using the following formulae:

For ischemic rats, affected limb use was calculated as [(forelimb contralateral to infarct + ½ bilateral contacts)/total contacts]x100%.(Schallert 2006)

For sham rats, percent preferred limb use was calculated as [(preferred forelimb + ½ bilateral contacts)/total contacts]x100%.(Schallert 2006)

Although the majority of the rats did not meet training criteria for the Montoya staircase, any rats that showed some interest in retrieving pellets located beyond the top 2 steps (19/30 rats) (CON-SHAM n=3, CON-ISCH n=8, PEM-SHAM n=5, PEM-ISCH n=3) were evaluated in the staircase apparatus post-surgically. Using the methods described above, rats were tested twice per day in the Montoya Staircase on Days 3, 4, 15, 16, 28 and 29 after surgery in order to assess skilled reaching after stroke. The results of the 4 trials from each pair of consecutive day testing were averaged. On the day prior to testing, sucrose pellets were offered to the rats in their home cages to re-familiarize the animals with the taste. To motivate the rats to reach, food was

removed from the cage 12 hours prior to testing and was returned to the cage once testing was complete. Post-stroke reaching data were calculated as a percentage compared to baseline data obtained during the training period prior to stroke.

3.5 Tissue Collection and Histology

On Day 30, rats were anesthetized and a 3 ml blood sample was collected from each animal via cardiac puncture for serum protein analysis. Blood samples were split between 2 tubes and allowed to coagulate at room temperature for 45 to 90 minutes before centrifugation in a Beckman Coulter Allegra X-12R centrifuge for 10 minutes at 1500 x g (4°C). Serum was extracted via pipette and divided equally among 6 sample vials, which were then stored at -80 degrees Celsius for future processing.

After blood sampling, rats were perfused transcardially with 0.9% saline, and liver samples were collected and flash frozen via submersion in liquid nitrogen. The rats were then perfused transcardially with 4% paraformaldehyde. The animals were then decapitated, and the brains removed and placed in a fresh 4% paraformaldehyde solution overnight. The brains were then placed in a 10% sucrose solution made in 0.1M phosphate buffer. Brains were then transferred to 20% and finally 30% sucrose solutions after they sank to the bottom of the containers. Brains were then frozen in optimal cutting temperature compound prior to sectioning. Sections of 40 µm thickness were captured using a cryostat (AO Scientific Instruments HistoStat Microtome) starting just prior to the infarct and ending just after the injured area. Serial sectioned sets of every 3rd section were preserved for the estimation of infarct volume and histopathological assessment after cresyl violet staining. ImageJ software (National Institutes of Health) was used to assist in the estimation of infarct volume. Initially, it was planned to trace the area of normal

tissue in each hemisphere and measure it with ImageJ in order to calculate the volume of lost tissue according to a method previously described by Clark et al. (2008) using the following formulae:

Volume of tissue lost = remaining volume of normal hemisphere – remaining volume of injured hemisphere.

Volume of a hemisphere = average (area of complete coronal section of hemisphere – area of ventricle – area of damage) x interval between sections x number of sections

As a result of artifacts introduced during the freezing, sectioning and staining of the brains, it was determined that the Clark et al. (2008) method for infarct volume assessment would not be an accurate approach to analyzing infarct volume in this experiment. A modified approach was taken in which the size of the infarct was determined by outlining the area of damage in each section and measured using ImageJ. When sections were missing, total infarct volume was determined by summing the area of damage in each section multiplied by the distance between sections. When all sections were available, the area of damage was averaged and multiplied by the distance between sections and the number of sections as follows:

Total infarct volume = average area of damage x interval between sections x number of sections.

3.6 Serum Albumin Concentration

Serum samples were removed from storage at -80° C to -20°C approximately 24 hours prior to processing. On the day of analysis, the samples were thawed on ice at room temperature. Albumin standard stock solution (10 g/dL) was prepared by dissolving 100 g of Bovine Serum

Albumin Fraction V in 1L deionized distilled water. The albumin stock solution was stored in aliquots at -80°C for future use. Bromocresol Green stock solution (0.60 mM) was prepared by dissolving 419 mg of Bromocresol Green in 10 ml of 0.1N sodium hydroxide. The solution was then diluted to 1L with deionized distilled water. Succinate buffer (0.1M) was prepared by combining 11.9 g of succinic acid in 800 ml of deionized distilled water. The pH was then adjusted to 4.0 with sodium hydroxide and the volume was subsequently brought up to 1.0 liter with deionized distilled water. The Bromocresol Green working solution was prepared by combining 3 parts 0.1M succinate buffer with 1 part stock Bromocresol Green solution and 4 g/L of 30% Brij-35. The pH was then adjusted to 4.2 ± 0.05. Six standard albumin concentrations were prepared: 2, 3, 4, 5, 6 and 7 g/dL in addition to a blank.

A 25µl aliquot of each standard, blank and serum sample was then pipetted into pre-labeled, 10 ml Eppendorf tubes. Five ml of Bromocresol Green working solution was added and the contents were mixed well. After a 30 minute incubation at room temperature, 1 ml aliquots were transferred into 1.5 ml disposable cuvettes in triplicate. The cuvettes were then read using a spectrophotometer (SpectraMax®M5, Molecular Devices Inc.) set to 628 nm. The data were recorded using SoftMax Pro 6.4. The absorbance of the blank solution was subtracted from that of the standards and serum samples, and the standard curve was plotted as absorbance vs. concentration. Linear regression was then used to calculate the serum albumin concentration of the samples.

3.7 Serum Alpha-2-Macroglobulin Concentration

Serum samples were allowed to thaw on ice at room temperature. The protocol provided in the ELISA kit manufactured by Immunology Consultants Laboratory Inc. was followed as

described here in brief: Reagents were brought to room temperature and diluted to appropriate working concentrations. Serum samples were diluted in triplicate to 1/500 by first transferring 5 µl of each sample into Eppendorf tubes and diluting with 495 µl of diluent. These 1/100 samples were then further diluted by mixing 40 µl of the aforementioned dilution with 160 µl of diluent to produce a 1/500 dilution of the sample. The diluted serum was pipetted into anti-A2M antibody coated wells. The wells were then covered and incubated for 60 minutes. The contents of the wells were subsequently washed 4 times to remove unbound proteins. Anti-A2M antibodies conjugated with horseradish peroxidase were added and allowed to incubate for 10 minutes to form a complex with the previously bound A2M. After 4 washes, a chromogenic substrate containing 3,3′,5,5′- tetramethylbenzidine and hydrogen peroxide was added and incubated for 10 minutes in the dark. A stop solution consisting of 0.3M sulfuric acid was added and the absorbance of the wells was read at 450 nm. Since the quantity of bound enzyme is directly related to the concentration of A2M in the sample, the concentration of A2M was estimated from absorbance of the sample by applying a quadratic equation. The concentrations of A2M were thus interpolated from the standard curve and corrected for the dilution factor. All standards were run in duplicates and all samples in triplicates.

3.8 Liver Lipid Concentration

Liver samples were removed from storage at -80° C to -20°C on the day prior to processing. On the day of analysis, the samples were thawed at room temperature for 1 hour. Two ~500 mg pieces of liver taken from each sample were deposited into separate pre-labeled, 10 mL, additive-free vacutainer tubes. Each replicate was then homogenized with 1 mL of 0.15M sodium chloride using a Polytron homogenizer on setting 7 for approximately 30 seconds. The blades were then rinsed with an additional 1 mL of sodium chloride into the vacutainer tube.

A graduated cylinder was used to add 5 mL of 2:1 chloroform:methanol to each tube in a fume hood. The tubes were then capped and centrifuged at 2175 x g for 10 minutes in a Beckman Centrifuge Allegra 25 using a TS-5.1 rotor. The upper methanol/water layer in each tube was then carefully pipetted off into a waste container. The middle layer, the homogenized liver pellet, was moved to the side of the tube and 1 g of anhydrous sodium sulphate was added to each sample. The tubes were then capped with stoppers and shaken until the liver pellet was broken down into a grain-like consistency. The contents of the vacutainer tubes were then filtered through #1 Whatman filter papers dampened with chloroform into small, glass test tubes that had been previously labeled and weighed. The empty vacutainer tubes were rinsed 3 times with approximately 0.5 mL of chloroform into the filter paper. An additional 0.5 mL of chloroform was used to wash each sample. Once the filter paper appeared dry, the test tubes were transferred to a vacuum centrifuge with the water bath temperature set at 30°C and the pressure set at 100 millibars for approximately 2 hours. The tubes were then removed from the centrifuge and allowed to cool to room temperature before they were re-weighed. Liver lipid was determined by subtracting the weight of the test tube from the combined weight of the test tube and final sample weight. Percent liver lipid was then determined by the following formula:

Liver Lipid = Final Sample Weight / Liver Wet Weight x 100%

3.9 Statistical Analysis

Post-surgical body weight and food intake prior to diet assignment (on Days 1-4) was evaluated by independent t-test. Food intake was analyzed on a cage basis. Due to the small sample size, food intake after day 4 was analyzed on each day by 2-factor ANOVA until a sustained effect of diet was identified. Body weight after diet assignment was analyzed by 3-

factor, mixed design, repeated measures ANOVA, with diet and surgery as between-subject variables and time as a within-subject variable. Independent samples t-tests were used to determine the first day on which body weight differences became statistically significant.

All cylinder behavioural endpoints were analyzed by 3-factor mixed design repeated measures ANOVA, with diet and surgery as between-subject variables and time as a within-subject variable.

First, to establish that stroke induced a functional deficit and that there was no inherent bias between surgical groups prior to dietary assignment (on Day 4), the Baseline and Day 4 data were analyzed. When there was a significant time x surgery interaction, the diet groups were combined, and T-tests were performed at each time point in order to determine if stroke affected the behavioural endpoint on Day 4.

Secondly, to determine if PEM affected the stroke-induced functional deficits, the data from Days 4, 16 and 29 were then analyzed by a second 3-factor, mixed design, repeated measures ANOVA. Independent t-tests were used when appropriate to determine on what day differences in behaviour occurred between groups. For the exploratory behaviour category only (both scoring methods 1 and 2), the 3-factor mixed design repeated measures ANOVA was followed up by splitting the SHAM and ISCH groups for separate analysis by t-tests on Day 16 and Day 29.

In addition to the key endpoints that assessed forelimb use asymmetry, each cylinder endpoint was analyzed for the total number of contacts (or number of movements as described) as above to rule out the possibility that diet influenced overall activity, since this could confound the interpretation of the results.

Physiological parameters from the surgical period, serum A2M and serum albumin concentrations, and liver lipid content were analyzed with 2-factor analysis of variance (ANOVA), with diet and surgery as independent variables. This was followed by post-hoc analysis (Tukey's test) if applicable. Infarct volume was evaluated using an independent t-test.

In order to determine if motor deficits in the cylinder task identified by method 1 on post-stroke day 4 were related to the extent of brain damage (total infarct volume), the percent change in performance on post-stroke day 4, relative to pre-stroke baseline, was calculated and correlated with the volume of damage in the two ISCH groups using Pearson's correlation analysis.

Statistical analysis was conducted using SPSS 18.0 for Windows. Differences were considered statistically significant at $p<0.05$. Data are shown as mean ± SEM.

CHAPTER 4: RESULTS

4.1 Excluded Animals

Two animals were excluded from the experiment prior to completion, one due to a failed tail vein injection during surgery, and an unexplained potentially confounding pathological condition (dermatitis) in the other. Two additional animals were excluded due to failed surgical techniques used to induce brain ischemia. Although these animals were excluded from all other endpoints, they could not be excluded from food intake analysis, as these data were collected on a cage basis and animals were caged in pairs. The final number of individual animals in each group after exclusions was as follows: CON-SHAM (n=6), PEM-SHAM (n=6), CON-ISCH (n=8), PEM-ISCH (n=6).

4.2 Physiological Monitoring During Surgery

Table 4.1 shows the physiological parameters measured during the surgery. Data were analyzed by the 4-group assignment to ensure that there was no inherent physiological bias prior to diet assignment on day 4. Mean oxygen saturation by experimental group ranged from 97 to 99% across all 4 groups. Although there was a significant diet x surgery effect (2-factor ANOVA, $p=0.014$), post-hoc analysis revealed no significant differences in oxygen saturation levels among the 4 groups (Tukey's test, $p \geq 0.063$).

Pulse showed greater inter-animal variability with average pulse rate by group ranging between 284 and 304 beats per minute. There were no diet ($p=0.462$), surgery ($p=0.402$) or diet x surgery ($p=0.070$) effects on pulse rate.

Body temperature was tightly controlled during surgical procedures. Statistical analysis found no effect of diet group ($p=0.966$) and no diet x surgery interaction ($p=0.992$); however, an independent effect of surgery was detected ($p=0.037$).

Table 4.1: Physiological parameters measured during surgery.

Experimental Group	Oxygen Saturation	Pulse	Temperature
PEM-ISCH*	97 ± 0.4	284 ± 5.5	36.9 ± 0.1
PEM-SHAM‡	99 ± 0.2	304 ± 3.8	36.8 ± 0.1
CON-ISCH†	98 ± 0.2	304 ± 3.8	36.9 ± 0.1
CON-SHAM§	97 ± 0.3	296 ± 3.8	36.8 ± 0.1

* n=6 ‡ n=6 † n=8 § n=6 (Mean ± SEM)

4.3 Food Intake

Figure 4.1 depicts post-surgical food intake, with experimental diet (control diet or low protein diet) assignment occurring on Day 4. Prior to Day 4, all animals received control diet. The data are presented as the mean (±SEM) food intake/rat/day; however, food intake was collected and analyzed on a cage basis (3-5 cages) with 1 to 2 rats housed per cage. Although not statistically analyzed by repeated measures ANOVA due to insufficient sample size, food intake showed an apparent decrease in all experimental groups during the first few days of post-surgical recovery, relative to pre-surgical food intake (Figure 4.1). Food intake was not different between SHAM and ISCH groups prior to diet assignment on Day 4 (t-test, $p \geq 0.447$).

After diet assignment, the first sustained independent effect of diet on food intake emerged on Day 13, where the low protein diet decreased food intake compared to control diet fed groups (2-factor ANOVA, $p=0.016$). Surgery had no influence on food intake ($p=0.668$), nor was there a diet x surgery interaction ($p=0.839$). A transient independent depressing effect of the low protein diet first occurred on Day 11(diet - $p=0.027$; surgery – $p=0.990$; diet x surgery interaction – $p=0.050$); however, this was not significant on Day 12 (diet – $p=0.178$; surgery – $p=0.690$; diet x surgery interaction – $p=0.969$). The dip in food intake on Days 14, 17, 29 and 30 that is apparent across all experimental groups is likely due to overnight fasting required for Montoya staircase testing.

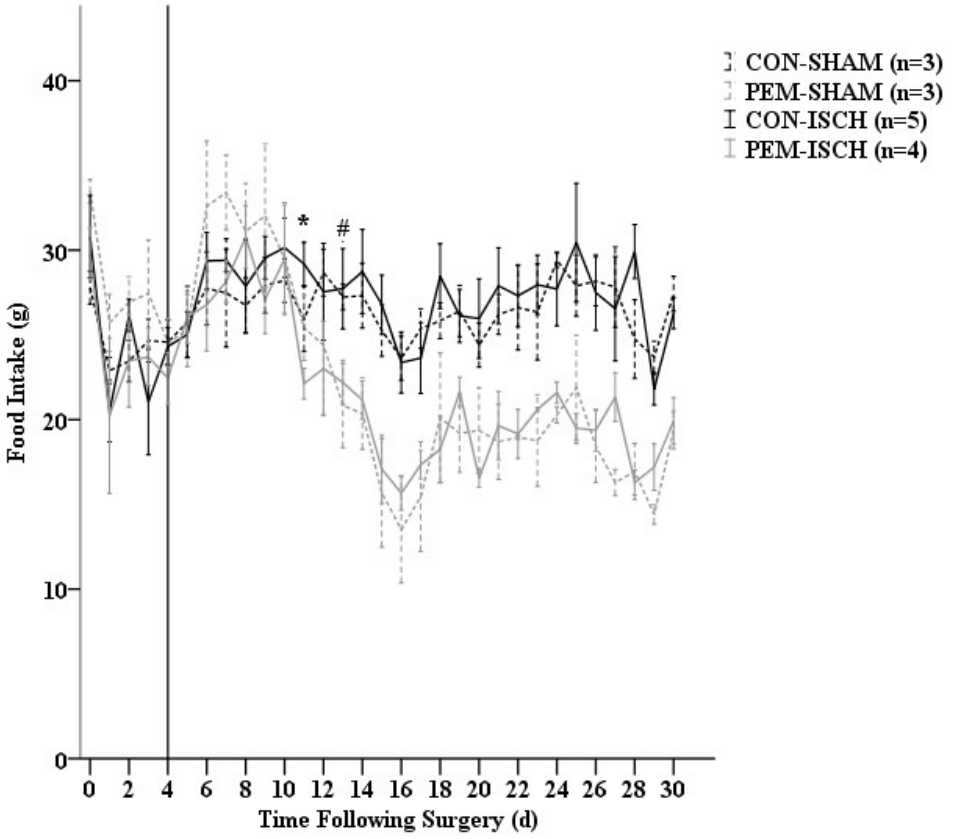


Figure 4.1: Mean (±SEM) food intake. The low protein diet introduced on Day 4 caused a sustained decrease in food intake by Day 13 post-surgery, resulting in protein-energy malnutrition. Data collected on a cage basis (3-5 cages [1-2 rats/cage]). Day 0 = surgery day. Vertical line = day of diet assignment. *Indicates the first day that food intake was decreased in the PEM groups compared to the CON groups (2-Factor ANOVA, p= 0.027). # Indicates the first day that the independent effect of diet was sustained (2-factor ANOVA; p=0.016).

4.4 Body Weight

Figure 4.2 presents the mean (± SEM) body weight throughout the post-surgical period. Body weight was not significantly different between sham and ischemic groups prior to dietary assignment on Day 4 (t-test, $p \geq 0.406$). Analysis of body weight between Day 4 and the end of the experiment by 3-factor, repeated measures ANOVA demonstrated a significant time x diet interaction (within-subjects effects, $p < 0.001$). There were no significant time x surgery or time x diet x surgery within-subjects effects ($p \geq 0.183$). The low-protein diet decreased body weight compared to CON groups by Day 14 (t-test, $p < 0.001$). Control fed animals gained an average of 22% of their pre-surgical body weight over the 30 day test period. By comparison, protein-energy malnourished animals lost an average of 9% of their body weight.

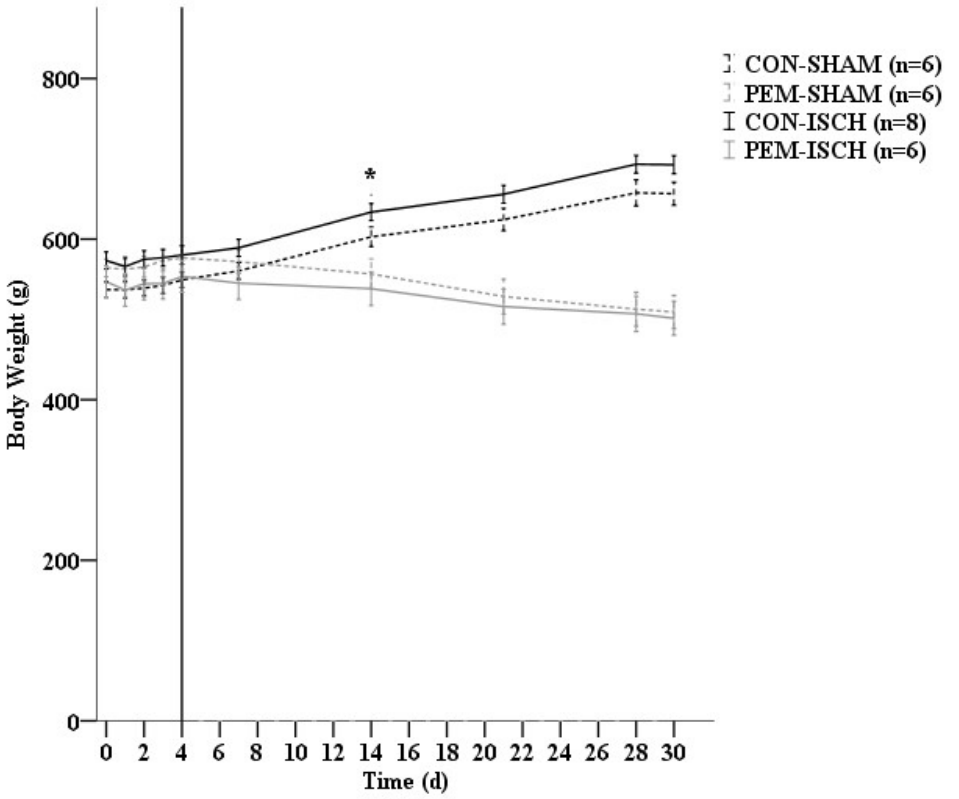


Figure 4.2: Mean (± SEM) body weight. The low-protein diet started on day 4 decreased body weight by day 14 post-surgery. Day 0 = surgery day. Vertical line = day of diet assignment. Three-factor repeated measures ANOVA demonstrated an interaction between time and diet ($p < 0.001$; within-subjects effects). *Indicates the first day for an independent effect of diet on body weight (t-test, $p < 0.001$).

4.5 Serum Albumin Concentration

Rats in the low protein diet group that developed PEM experienced a 20% decrease in serum albumin concentrations compared to control diet groups by Day 30 (Figure 4.3). The low-protein diet significantly decreased serum albumin concentration independently by Day 30 relative to animals fed the control diet (2-factor ANOVA, $p<0.001$). There was no significant effect of surgery ($p=0.837$) and no diet x surgery interaction ($p=0.096$).

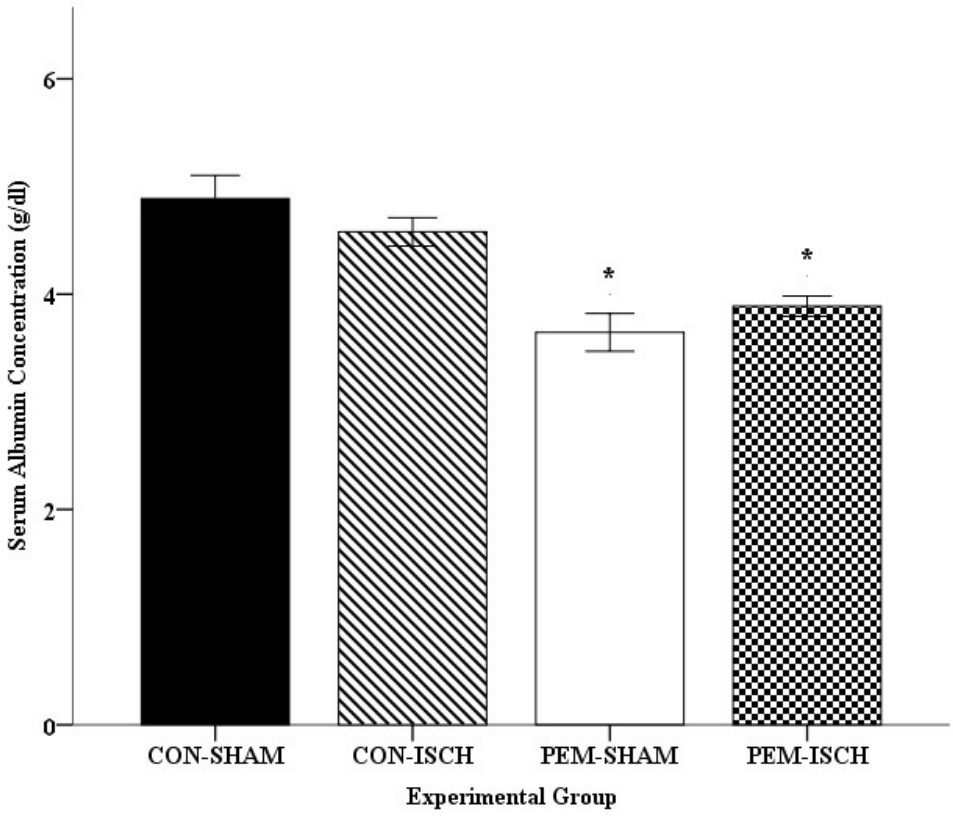


Figure 4.3: Mean (± SEM) serum albumin concentration was decreased by protein-energy malnutrition on Day 30. *Analysis by 2-factor ANOVA revealed an independent effect of diet ($p<0.001$). CON-SHAM: n= 6, CON-ISCH: n = 8, PEM-SHAM: n = 6, PEM-ISCH: n = 6.

4.6 Serum Alpha-2-Macroglobulin Concentration

Analysis of Day 30 serum alpha-2-macroglobin concentration by 2-factor ANOVA (Figure 4.4) indicated that there were independent effects of diet (p=0.001) and surgery (p=0.016); the diet x surgery interaction was not significant (p=0.081). Ischemia increased serum alpha-2-macroglobulin levels compared to SHAM surgery, and PEM increased serum alpha-2-macroglobulin relative to CON diet.

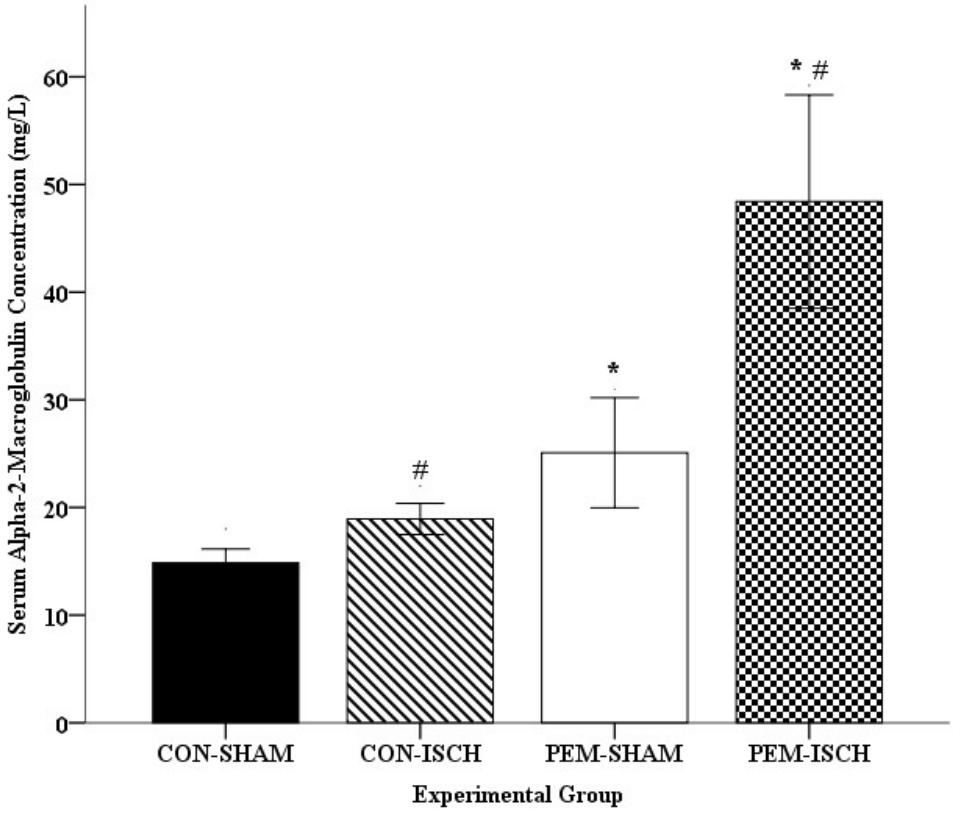


Figure 4.4: Mean (± SEM) serum alpha-2-macroglobulin concentration was increased by both protein-energy malnutrition and stroke on Day 30. Analysis by 2-factor ANOVA revealed independent effects of (*) diet (p=0.001) and (#) surgery (p=0.016). CON-SHAM: n= 6, CON-ISCH: n = 8, PEM-SHAM: n = 6, PEM-ISCH: n = 6.

4.7 Liver Lipid Concentration

Liver lipid concentrations in rats fed the low protein diet resulting in PEM were elevated relative to animals fed a nutritionally adequate diet (CON) on Day 30 (Figure 4.5). Analysis by 2-factor ANOVA determined that there was an independent effect of diet ($p<0.001$). There was no effect of surgery ($p=0.348$) or interaction between diet and surgery ($p=0.965$).

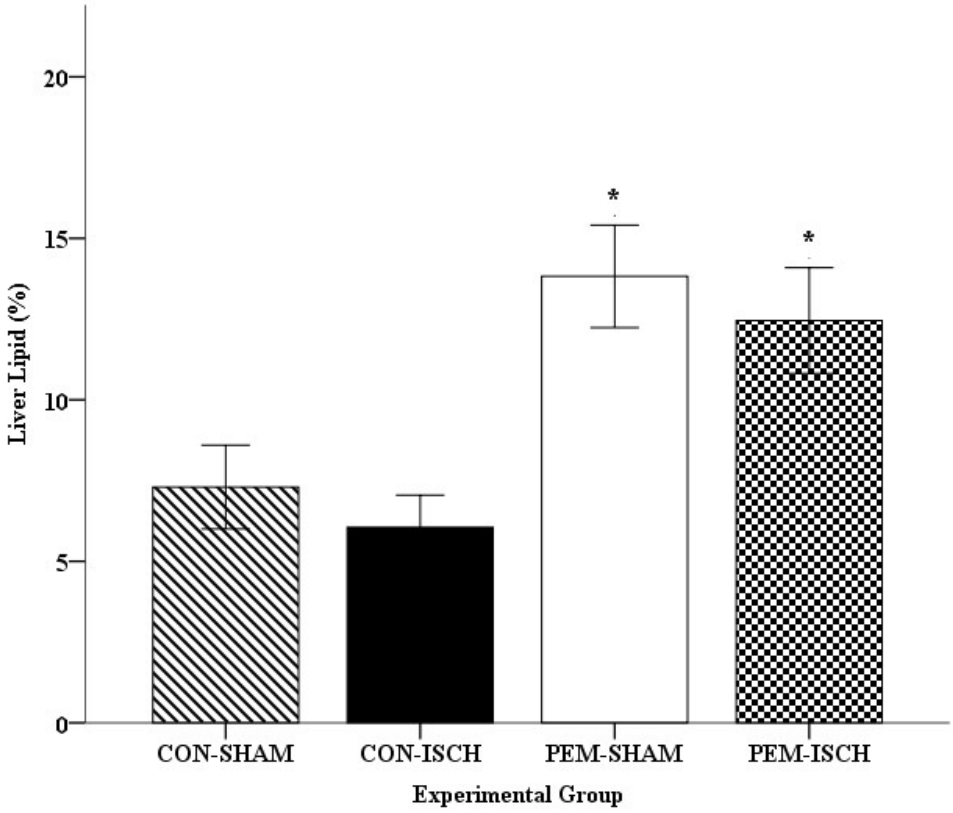


Figure 4.5: Mean (± SEM) liver lipid concentration (% wet weight) was increased by protein-energy malnutrition on Day 30. *An independent effect of diet on liver lipid concentration was found on Day 30 (2-factor ANOVA, $p<0.001$). CON-SHAM: n = 6, CON-ISCH: n = 8, PEM-SHAM: n = 6, PEM-ISCH: n = 6.

4.8 Infarct Volume

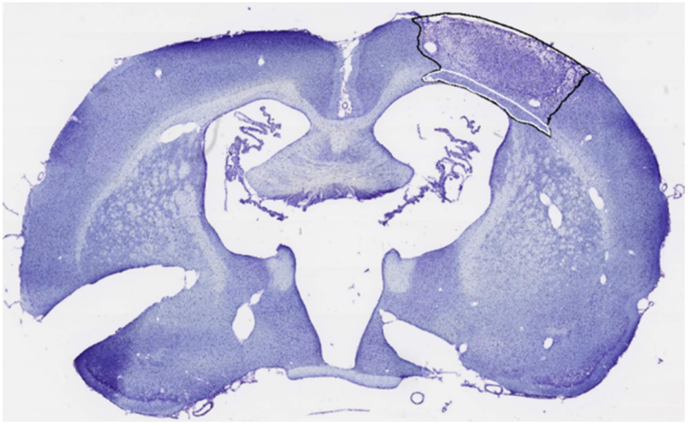
Table 4.2 compares infarct volume between PEM-ISCH and CON-ISCH groups. No difference was found in total infarct volume (cortex and corpus callosum) between groups (independent samples Student's t-test, p=0.775). There was also no difference between the groups in the volume of damage to the corpus callosum alone (independent samples Student's t-test, p=0.423). Figures 4.6a and 4.6b depict representative photographs from the core of the infarct of the brains with the largest and smallest infarcts, respectively. These photographs demonstrate the range of damage to the corpus callosum in the thesis study. Figure 4.6c is representative of the extent of damage to the corpus callosum in the majority of animals in the experiment (n=12).

Table 4.2: Neither total infarct volume nor damage to corpus callosum was exacerbated by protein-energy malnutrition.

Experimental Group	Total Infarct Volume (mm^3)	Corpus Callosum Damage (mm^3)
PEM-ISCH*	7.4 ± 0.9	0.23 ± 0.13
CON-ISCH‡	7.9 ± 1.0	0.35 ± 0.34

* n= 6 ‡ n= 8. Data are shown as mean ±SEM. Total infarct volume (cortex and corpus callosum) and corpus callosum damage were not influenced by PEM (t-test; p=0.775 and p=0.423, respectively).

(a)



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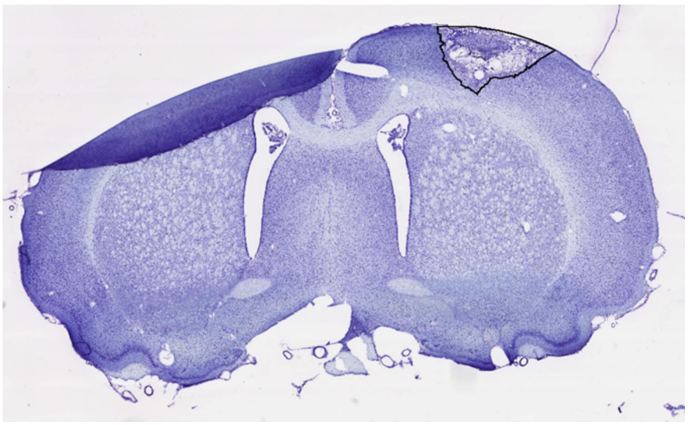

(c)

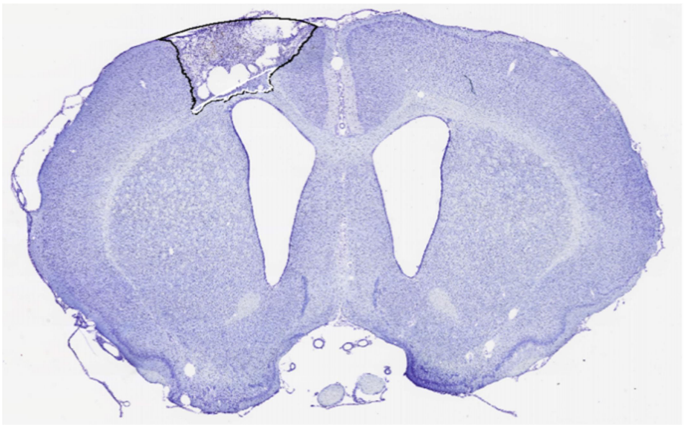

Figure 4.6: Representative photographs (20x magnification) of coronal sections taken at the core of the infarct for 3 animals. The black outline demarcates the infarct. (a) A photograph of a slice from a brain with extensive damage to the corpus callosum (white outline) (Bregma -0.24). (b) A photograph of a slice from a brain with no damage to the corpus callosum (Bregma +0.72). (c) A photograph that is representative of the most typical extent of damage to the corpus callosum (white outline) (Bregma +0.84).

Table 4.3: Summary of infarct location in rats exposed to photothrombotic stroke.

		*Coordinate of Lesion Relative to Bregma (mm)		‡Coordinate at Core of Lesion (mm)		Total Infarct Volume (mm^3)
Animal ID	Experimental Group	Anterior	Posterior	Medial	Lateral	
Cage 1, Rat 0	PEM-ISCH	2.76	-1.56	1.2	4.2	8.3
Cage 1, Rat 1	PEM-ISCH	1.8	-1.56	2	4.6	5.6
Cage 2, Rat 1	PEM-ISCH	2.94	-0.54	1.9	4.8	6.9
Cage 4, Rat 0	CON-STROKE	1.56	-1.32	1.1	4.4	6.6
Cage 4, Rat 1	CON-STROKE	1.44	-1.08	1.5	4.3	5.7
Cage 7, Rat 1	PEM-ISCH	2.04	-1.2	1.9	4.3	5.0
Cage 8, Rat 0	PEM-ISCH	1.8	-1.32	1.9	4.9	7.4
Cage 8, Rat 1	PEM-ISCH	2.76	-1.56	0.9	5.0	11.5
Cage 10, Rat 0	CON-STROKE	2.52	-0.6	1.4	5.1	9.3
Cage 11, Rat 0	CON-STROKE	0.84	-1.68	1.2	4.2	6.6
Cage 11, Rat 1	CON-STROKE	2.16	-0.72	1.8	4.6	3.4
Cage 13, Rat 0	CON-STROKE	1.68	-1.92	1.7	5.8	12.6
Cage 13, Rat 1	CON-STROKE	1.44	-1.68	0	3.9	10.1
Cage 14, Rat 0	CON-STROKE	2.52	-0.72	0.4	4.2	8.5

*Targeted coordinates were +2 mm to -1 mm anterior-posterior from Bregma ‡ Targeted coordinates were +/- 2 mm to +/-5 mm lateral of midline

4.9 Assessment of Motor Skills

4.9.1 Cylinder Test

4.9.1.1 Exploratory Behaviour

4.9.1.1.1 Exploratory Behaviour: Scoring Method 1

Figure 4.7 demonstrates that affected paw use during exploration is reduced in animals after stroke, and this deficit does not recover in protein-energy malnourished animals. Three-factor repeated measures ANOVA of baseline and Day 4 data indicated a significant time x surgery interaction ($p<0.001$, within-subjects analysis) in addition to an independent effect of surgery ($p=0.014$, between-subjects effect). No time x diet or time x diet x surgery interaction effects were found (within-subjects effects, $p\geq0.313$). For between-subjects effects, there was an independent effect of surgery ($p=0.014$), but no diet or diet x surgery interaction effects ($p\geq0.216$). There was no difference in performance between ISCH and SHAM groups at baseline (independent sample Student's t-test, $p = 0.338$). Ischemia significantly reduced use of the affected forelimb for postural support on Day 4 compared to SHAM (independent sample Student's t-test, $p<0.001$).

Analysis of the data from Days 4, 16, and 29 via 3-factor ANOVA revealed a significant time x diet x surgery interaction ($p=0.045$, within-subjects). Between-subjects analysis yielded a significant diet x surgery interaction ($p=0.013$).

When the two ISCH groups were separated from the two SHAM groups for separate analysis of each on Days 16 and 29, affected forepaw use was significantly lower in the PEM-ISCH group than the CON-ISCH group on both Days 16 (independent sample Student's t-test,

p=0.024) and 29 (independent sample Student's t-test, p=0.013). There was no significant differences in preferred paw use between PEM-SHAM and CON-SHAM groups on Day 16 (independent sample Student's t-test, p=0.257) or Day 29 (independent sample Student's t-test, p=0.264).

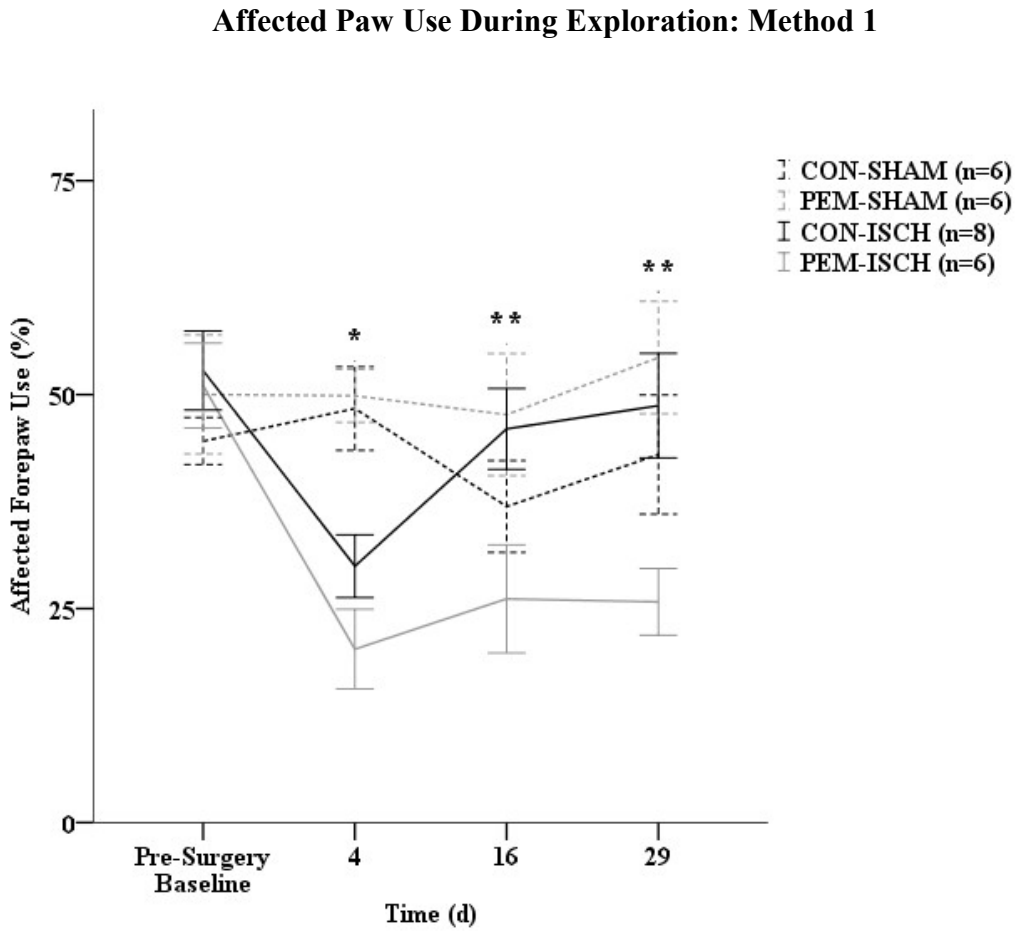

Figure 4.7: Mean (±SEM) percent affected paw use during exploration as assessed by scoring method 1. The dietary regimen was started on day 4 after behavioural testing. *Affected forepaw use was independently decreased by ISCH on Day 4 as determined by 3-factor repeated measures ANOVA (p<0.001) and independent sample Student's t-test (p<0.001). **Affected paw use was significantly lower in the PEM-ISCH group than the CON-ISCH group on Days 16 (independent sample Student's t-test, p=0.024) and 29 (independent sample Student's t-test, p=0.013).

4.9.1.1.2 Exploratory Behaviour: Scoring Method 2

Similar to Figure 4.7, Figure 4.8 demonstrates that affected paw use during exploration is reduced in animals after stroke and this deficit does not recover if the animals are protein-energy malnourished. Analysis of Baseline and Day 4 data by 3-factor, repeated measures ANOVA revealed a significant time x surgery interaction ($p<0.001$, within-subjects). There were no significant time x diet or time x diet x surgery effects ($p\geq0.213$, within-subjects effects). Between-subjects analysis showed a significant surgery effect ($p=0.024$), whereas there were no significant diet or diet x surgery effects ($p\geq0.134$). An independent sample Student's t-test determined that there was no difference ($p=0.161$) between ISCH and SHAM groups at baseline, indicating that there was no functional bias prior to surgical assignment. On Day 4 after surgery, ISCH significantly decreased affected paw use as compared to SHAM ($p<0.001$). Note that this test was conducted just prior to diet assignment.

Repeated measures ANOVA of data from Days 4, 16, and 29 resulted in a significant time x surgery interaction ($p=0.004$, within-subjects) with no time x diet ($p=0.936$) or time x diet x surgery ($p= 0.122$) interaction. Between-subjects analysis revealed a significant diet x surgery effect ($p=0.009$). When the two ISCH groups were separated from the two SHAM groups for separate analysis of each on Days 16 and 29, affected forepaw use was decreased in PEM-ISCH rats as compared to CON-ISCH on both Day 16 (independent Student's t-test, $p=0.021$) and Day 29 (t-test, $p=0.018$). Diet did not alter preferred paw use in CON-SHAM relative to PEM-SHAM animals on Day 16 (t-test, $p=0.225$) or Day 29 (t-test, $p=0.230$).

It is noted that since the 3-factor, repeated measures ANOVA did not reveal a significant time x diet x surgery interaction, splitting the SHAM and ISCH groups for analysis by t-tests on

Day 16 and Day 29 is a liberal approach that is less justified than for the Scoring Method 1 data above. However, since the results of scoring method 1 and 2 are highly related measures that exhibited a similar behavioural pattern, the data were treated with the same statistical approach.

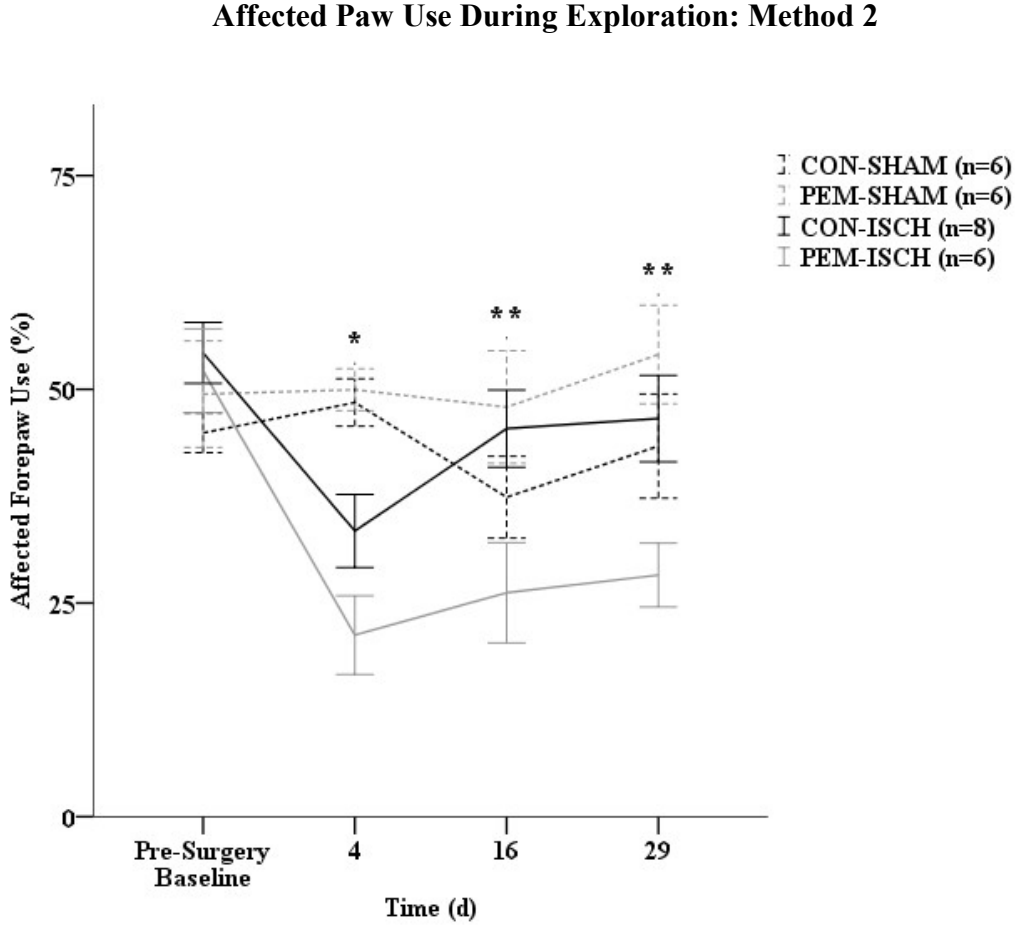

Figure 4.8: Mean (±SEM) percent affected paw use during exploration as assessed by scoring method 2. The dietary regimen was started on day 4 after behavioural testing. *Affected forepaw use was independently decreased by ISCH on Day 4 (3-factor repeated measures ANOVA, $p=0.024$; independent sample Student's t-test, $p<0.001$). ** Affected paw use was significantly lower in the PEM-ISCH group compared to the CON-ISCH group on Days 16 (independent Student's t-test, $p=0.021$) and 29 (independent Student's t-test, $p=0.018$)

4.9.1.1.3 Initiation of Exploration Behaviour: First Touch

Figure 4.9 demonstrates that affected paw use during first touch is reduced in animals after stroke. Analysis by 3-factor, repeated measures ANOVA of Baseline and Day 4 data demonstrated a significant time x surgery interaction ($p=0.001$, within-subjects). There were no significant time x diet or time x diet x surgery interactions within-subjects effects ($p\geq0.314$). There were also no significant between-subjects effects ($p\geq0.140$). Post-hoc analysis determined that ISCH decreased affected forepaw use on Day 4 relative to SHAM (independent samples Student's t-test, $p=0.003$), but not at baseline ($p=0.456$).

Analysis of the data from Days 4, 16, and 29 by 3-factor repeated measures ANOVA determined that there was no significant effect of time (within-subjects, $p=0.090$) or time x diet or time x surgery x diet effect (within-subjects, $p\geq0.181$). Although the time x surgery interaction was not statistically significant, a trend was observed (within-subjects, $p=0.066$). For between-subjects analysis, the diet x surgery interaction was statistically significant ($p=0.016$). There was also a significant independent surgical effect (between-subjects, $p=0.043$). The effect of diet was not significant between subjects ($p=0.572$).

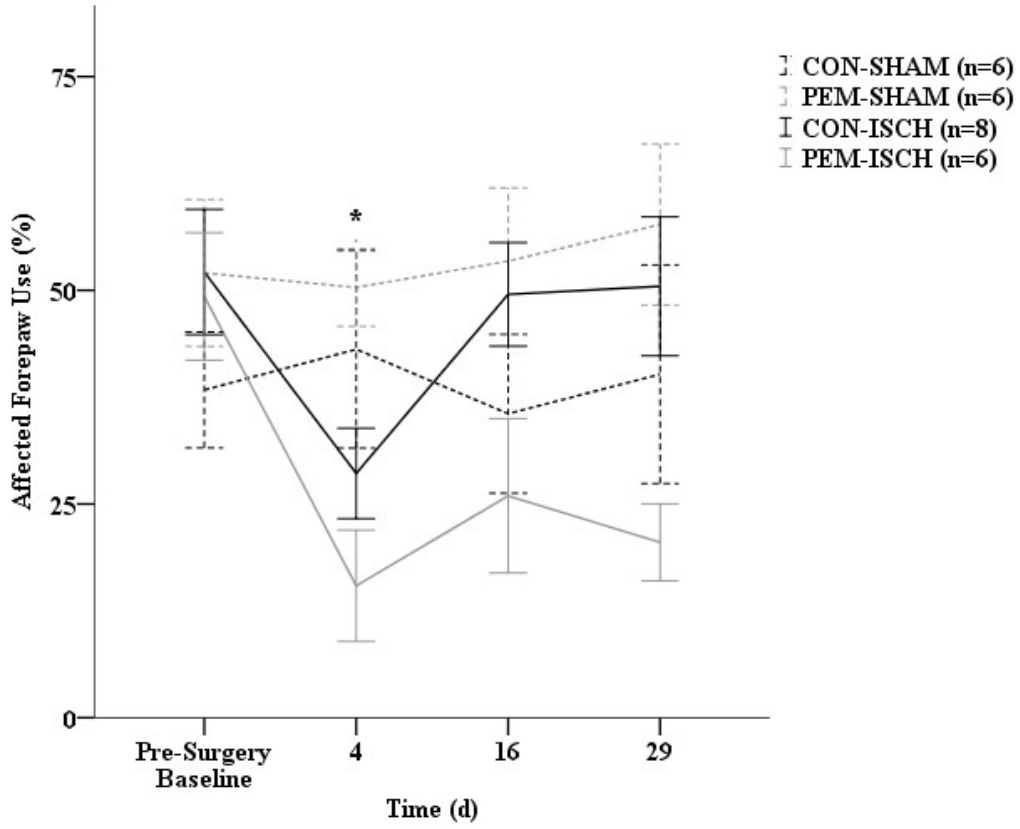

Figure 4.9: Mean (±SEM) percent affected paw use during first touch. The dietary regimen was started on day 4 after behavioural testing. *Analysis of Baseline and Day 4 revealed that affected forepaw use was independently decreased in ISCH animals compared to SHAM animals (independent samples Student's t-test, p=0.003).

4.9.1.1.4 Initiation of Exploration Behaviour: Takeoff

Figure 4.10 demonstrates that affected paw use during takeoff is reduced in animals after stroke. For Baseline and Day 4 data, 3-factor, repeated measures analysis determined a significant time x surgery interaction for within-subjects effects ($p<0.001$); there was no time x diet or time x diet x surgery effects (within-subjects, $p\geq0.375$). Between-subjects analysis showed an independent effect of surgery ($p<0.004$), whereas there were no diet or diet x surgery effects ($p\geq0.441$). Independent sample Student's t-tests determined that there was no bias in paw use at baseline prior to surgical assignment ($p=0.519$) and ISCH decreased affected paw use on Day 4 compared to SHAM surgery ($p<0.001$).

Analysis of Day 4 through 29 data revealed that although time was significant ($p=0.001$), there were no significant effects for time x diet, time x surgery or time x surgery x diet (within-subjects, $p\geq0.128$). For between-subjects analysis, there was an independent effect of surgery ($p<0.001$) with ISCH depressing use of the affected forepaw for takeoff, but no effect of diet or diet x surgery interaction ($p\geq0.463$).

Affected Paw Use During Initiation of Exploration: Takeoff

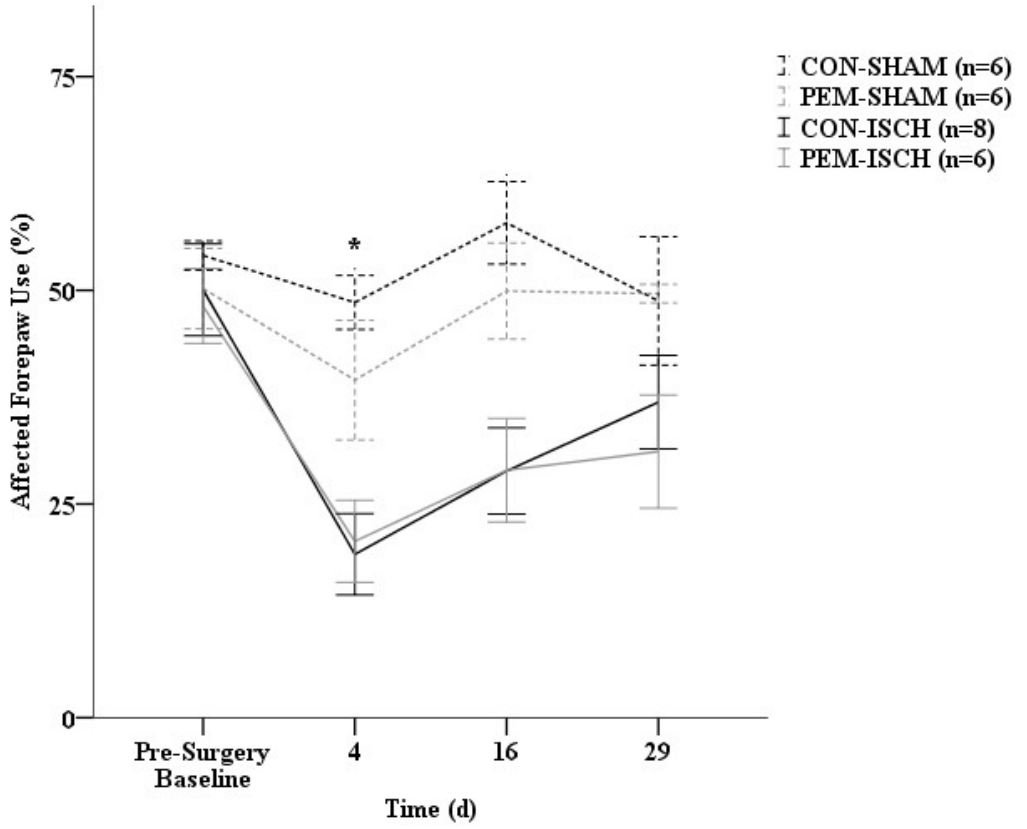

Figure 4.10: Mean (± SEM) percent affected forepaw use during takeoff. The dietary regimen was started on day 4 after behavioural testing. *Analysis of Baseline and Day 4 data indicated that ISCH independently decreased forepaw use during takeoff (3-factor, repeated measures ANOVA, $p=0.004$; independent samples Student's t-test, $p<0.001$). PEM did not alter post-stroke recovery of percent affected paw use during takeoff. An independent effect of ISCH on affected paw use was also found when data was analyzed across Days 4, 16 and 29 (3-factor ANOVA, between-subjects, $p < 0.001$).

4.9.1.1.5 Termination of Exploration: Landing

Figure 4.11 demonstrates that percent paw use during landing was not affected by ischemic stroke. Evaluation of landing data from Baseline and Day 4 (Figure 4.11) revealed a significant time x surgery interaction (3-factor repeated measures ANOVA, within-subjects, $p=0.011$). There were no within-subjects time x diet or time x diet x surgery interactions ($p\geq0.814$). Between-subjects analysis failed to detect any diet, surgery or diet x surgery effects ($p\geq0.325$). Further analysis of affected forepaw use for landing by way of t-tests at Baseline and Day 4 revealed no effect of surgery on preferred paw use on either day ($p\geq0.180$).

Analysis of affected forepaw use for landing on Days 4, 16 and 29 by 3-factor ANOVA revealed no significant within-subjects effects for time x diet x surgery, time x surgery or time x surgery ($p\geq0.228$); however, time was significant ($p<0.001$). There were also no between-subjects diet, surgery, or diet x surgery interaction effects ($p\geq0.225$).

Affected Paw Use During Termination of Exploration: Landing

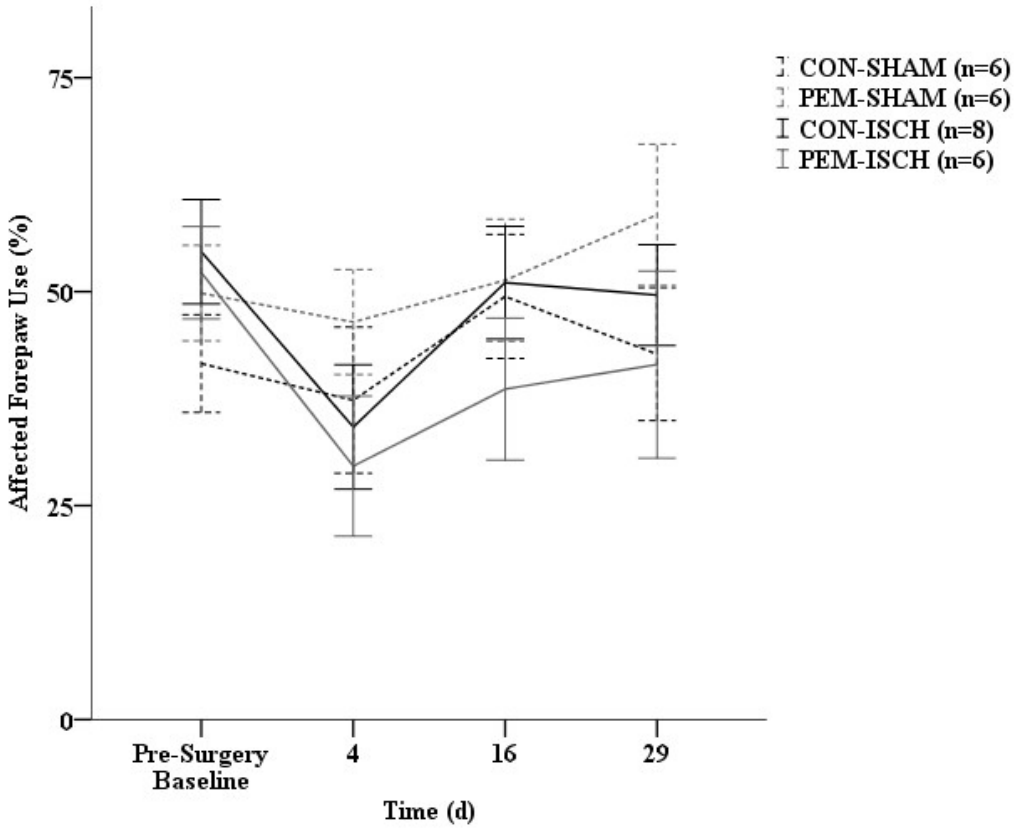

Figure 4.11: Mean (± SEM) percent affected paw use during landing is not sensitive to effects of ISCH or PEM. Three-factor repeated measures ANOVA revealed a significant within-subjects interaction between time and surgery for analysis of Baseline and Day 4 data ($p=0.011$); however, further analysis determined that there was no difference between surgery groups at Baseline or Day 4 (t-test, $p>0.180$). Analysis of Day 4 through 29 by 3-factor ANOVA revealed no significant between-subjects or within-subjects effects ($p>0.225$).

4.9.1.1.6 Exploratory Behaviour: Total Number of Touches

Analysis of the total number of contact (or number of movements) for each cylinder endpoint by 3-factor repeated measures ANOVA at baseline and D4 demonstrated no significant surgery x time, diet x time, or diet x time x surgery effects (within-subjects effects, $p \geq 0.063$) (data not shown). There was an independent effect of time, with the total number of contacts/motions declining over time ($p \leq 0.014$, within-subjects effects). For between-subjects analysis, there was no significant effect on the activity level of animals of diet ($p \geq 0.738$) or surgery ($p \geq 0.059$), nor was there a significant diet x surgery interaction ($p \geq 0.388$).

Analysis of these data for day 4, 16 and 29 found no significant time, diet x time, surgery x time or diet x surgery x time effects ($p \geq 0.068$, within-subjects effects). Between-subjects analysis showed no effect of diet ($p \geq 0.253$, between-subjects effects) and no diet x surgery interaction ($p \geq 0.550$, between-subjects effects). However, there was a significant surgical effect present for exploration scored by either method 1 or 2 ($p = 0.014$, between-subjects effects). No significant surgical effect was found for takeoff, landing or first touch ($p \geq 0.087$).

4.9.1.2 Correlation between Infarct Size and Functional Outcome in the Cylinder

No correlation was found between total infarct volume and deficit in the cylinder task as assessed by scoring method 1 on Day 4 ($r^2 = 0.293$, $p=0.310$, Pearson's correlation). Figure 4.12 depicts graphically the relationship between total infarct volume and the deficit in affected forepaw use on D4 post-stroke. The deficit in this task was calculated as the percent change in performance between pre-stroke baseline assessment and the initial post-stroke test on Day 4.

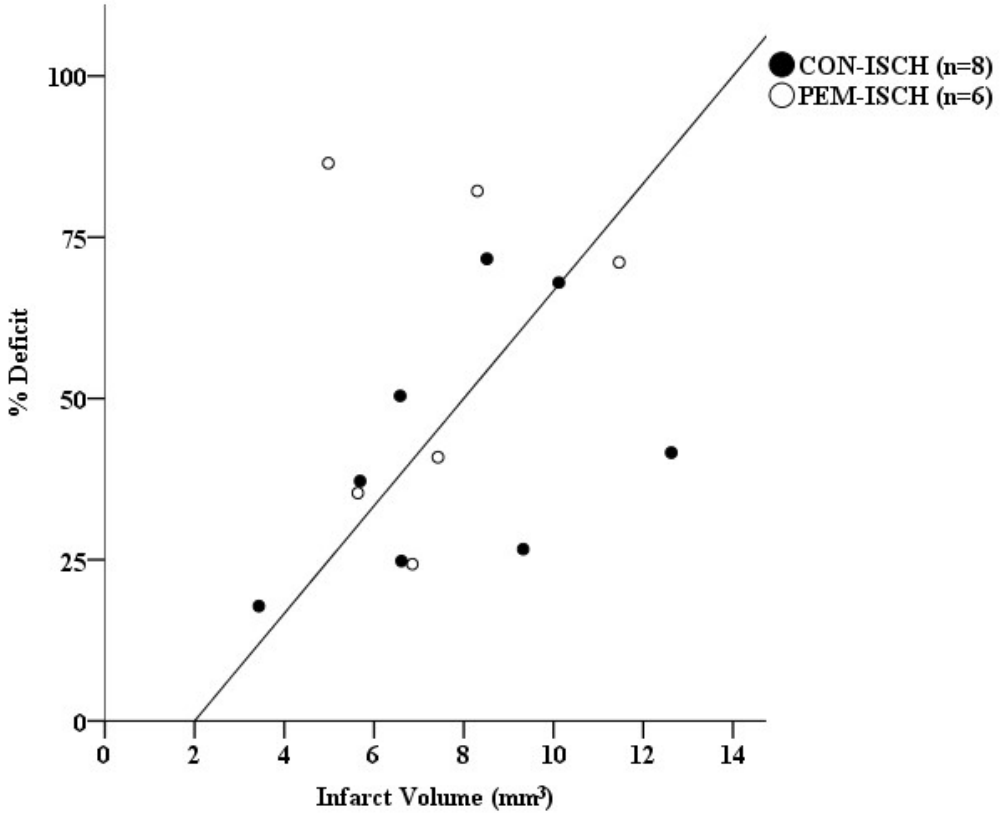

Figure 4.12: The deficit in the cylinder test on D4 after photothrombotic stroke was not significantly correlated to infarct volume ($r^2 = 0.293$, P=0.310, Pearson's correlation).

4.9.2 Montoya Staircase

Since the majority of the rats failed to train to criteria in the staircase apparatus prior to stroke, this endpoint was invalidated as a measure of functional deficit after stroke. The following number of rats met criteria: 2/6 CON-SHAM, 4/10 CON-ISCH, 2/6 PEM-SHAM, and 1/8 PEM-ISCH rats. Note that 2 trained rats were also excluded from the CON-STROKE group due to failed surgery.

Graphs of the staircase test results for individual animals that were tested after surgery are found in Appendix A. The data set is split into sub-groups consisting of rats that failed to meet

pre-stroke baseline criteria and rats that met criteria in the staircase apparatus. The data presented shows high inter-animal variability. Interpretation of the data can be found in Appendix A.

CHAPTER 5: DISCUSSION & CONCLUSION

This thesis research tested the hypothesis that protein-energy malnutrition developing after stroke would impair spontaneous motor recovery without altering infarct volume and that post-stroke PEM would be associated with an acute phase response. Our findings suggest that protein-energy malnutrition developing after stroke has a detrimental impact on gross motor function that is independent of an influence on infarct volume. In addition, PEM appeared to initiate a systemic acute phase response as demonstrated by elevated serum alpha-2-macroglobulin concentration (a positive acute phase protein) and depressed serum albumin levels (a negative acute phase protein); stroke independently increased only serum-alpha-2-macroglobulin (Table 5.1). These findings support the hypothesis and imply that treating malnutrition is necessary in order to enhance recovery after stroke.

Nutritional status is often overlooked when it comes to post-stroke care (Gomes et al. 2016). In a clinical setting, information about the nutritional status of an individual is gathered by measuring body composition and determining if there has been a history of weight loss and inadequate food intake (Omran and Morley 2000a). In addition, nutritional status is assessed through biochemical indices, and serum albumin has traditionally been employed to assess protein status (Omran and Morley 2000b). However, serum albumin concentration has low specificity, since it is influenced by both amino acid supply (nutritional status) and the acute phase reaction (Qu et al. 1996). Thus, it is necessary to also monitor other markers of nutritional status in order to confirm the existence of malnutrition. The degree of liver steatosis may also be used as evidence for protein-energy malnutrition (Edozien and Switzer 1978). Thus, the present study presents food intake, body weight, liver lipid and serum albumin as markers of protein-energy status.

By Day 13 after surgery, food intake in animals fed the low protein diet was depressed by 22% relative to animals fed control diet (Figure 4.1). Body weight was significantly decreased in the low protein diet-fed groups relative to the control diet groups by Day 14 (Figure 4.2). On Day 14, animals fed control diet had gained 11% of their baseline (pre-surgery) body weight. Conversely, animals fed the low protein diet lost 1% of their pre-surgery body weight by Day 14. After 26 days (Day 30) on the low-protein diet, malnourished animals had lost an average of 9% of their pre-surgery body weight. By contrast, the animals on control diet gained 21% of their pre-surgery weight (Figure 4.2). The low protein diet also increased liver lipid concentration by 50% (Figure 4.5) and decreased serum albumin concentration by 20% (Figure 4.3) on day 30 relative to animals fed the control diet. The decrease in food intake, body weight and serum albumin in combination with the elevation in liver lipid is indicative that PEM was successfully induced by the low-protein diet (Table 5.1). The magnitude of PEM-induced changes in food intake, body weight, serum albumin and liver lipid are in line with those reported in previous experiments conducted within our laboratory (Alaverdashvili et al. 2015a; Andrade Ramos 2015).

Table 5.1: Summary of major anthropometric and biochemical outcomes.

Variable	Body Weight	Food Intake	Serum Albumin	Serum Alpha-2-Macroglobulin	Liver Lipid
PEM	✓ (↓)	✓ (↓)	✓ (↓)	✓ (↑)	✓ (↑)
ISCH	–	–	–	✓ (↑)	–

✓Indicates the presence of an effect. Arrows indicate the direction of the statistically significant main effect of the independent variable (PEM or ISCH) on each endpoint.

The cylinder task was used to quantify the influence of protein-energy malnutrition on changes in forelimb use asymmetry during spontaneous exploration after stroke. The most common approach to the evaluation of cylinder data is to score affected forelimb use as a percentage of the number of total wall contacts (Clark et al. 2008; Kim et al. 2012; MacLellan et al. 2013; Mestriner et al. 2013). The present study design attempted to increase sensitivity using an expanded approach to evaluate forelimb use asymmetry after stroke to include a second method for evaluating limb use during exploratory behaviour (wall exploration) in addition to initiation of exploration (takeoff and first touch), and termination of exploration (landing). Scoring methods 1 and 2 evaluated the preferred paw used to contact the cylinder wall with different definitions of independent paw use. The term takeoff refers to the paw used to push off of the cylinder floor to initiate a bout of exploration. First touch was defined as the first paw used to contact the cylinder wall during a bout of exploration. The paw used to terminate a bout of exploration by contacting the cylinder floor was referred to as the preferred paw used for landing.

In the photothrombotic stroke model employed, forelimb asymmetry scores during initiation of exploration (first touch and takeoff) and exploratory behaviour (methods 1 and 2) were found to be sensitive endpoints for detecting deficits in preferred forepaw use (Table 5.2). Paw use during landing to terminate exploration was the only endpoint that was not sensitive to the effects of stroke. The CON-ISCH group made a complete recovery in affected forepaw use in each of the aforementioned measures (Figures 4.7, 4.8 & 4.9), with the exception of takeoff (Figure 4.10), by the end of the experiment. Although takeoff may be useful for detecting the effects of stroke, especially in the chronic period, this endpoint was not sensitive to the effects of PEM on ischemia. Thus, it can be concluded that an in-depth analysis of cylinder behaviour does not confer any additional sensitivity for detecting the effects of stroke (with the exception of takeoff); however, this expanded approach may be useful for the evaluation of the impact of comorbidity factors such as protein-energy malnutrition in larger animal studies.

Table 5.2: Summary of major findings for infarct volume and the cylinder test.

Variable	Scoring Method 1	First Touch	Takeoff	Infarct Volume
PEM	–	–	–	↔
ISCH	–	✓	✓	✓
PEM x ISCH Interaction	✓	–	–	–

✓Indicates the presence of a statistically significant effect or interaction.

Ideally, behaviour tests should be sensitive enough to detect deficits in the chronic period as opposed to exhibiting complete recovery; the latter was the case for the majority of cylinder endpoints in the thesis study. Recovery in behavioural tasks is largely influenced by the type of stroke model, the brain region affected, the size of the infarct and time point. Thus, some experiments demonstrate a full recovery (Li et al. 2004; MacLellan et al. 2013; Andrade Ramos 2015), whereas others do not (Ploughman et al. 2009; Mestriner et al. 2013). The cylinder task in particular is more susceptible to spontaneous recovery relative to tasks that require more skilled movements such as the Montoya staircase task (Murphy and Corbett 2009).

Protein-energy malnutrition depressed the use of the affected forelimb in the cylinder task during exploratory behaviour after stroke relative to control diet fed counterparts, as assessed by scoring method 1 (Figure 4.7). It is proposed that this represents a specific effect of PEM on post-stroke motor function, since the decreased rate of recovery in preferred forepaw use was not due to an overall decline in activity relative to that of the CON-ISCH group; this was measured by the total number of touches with any forepaw. Since PEM does not appear to exacerbate brain injury, it can be hypothesized that protein-energy malnutrition interferes with the building blocks, signalling molecules, and/or enzymes involved in the formation of novel synaptic connections that compensate for lost neural circuitry after stroke (Murphy and Corbett 2009). Our laboratory has previously reported an effect of post-stroke PEM on such mechanisms in the hippocampus in a model of global brain ischemia (Smith et al. 2014).

Method 2 (exploratory behaviour) and first touch (initiation of exploration) demonstrated similar patterns for preferred limb use, although these endpoints were found to be less sensitive to the effects of PEM. Method 1 (Figure 4.7), relative to Method 2 (Figure 4.8), places emphasis on a strict definition of independent use. In scenarios where the unaffected paw is used for

postural support while the affected paw is used to contact the cylinder wall, scoring method 1 equalizes the use of the paw used for postural support with the paw used for exploration by tracking these contacts as simultaneous use. Conversely, scoring method 2 would categorize the same scenario as sole use of the affected limb, thereby biasing the limb use score towards recovery. Quantitative assessment of affected forepaw use during initiation of exploration (takeoff) did not reveal any effects of PEM on post-stroke performance. Unfortunately, the present study was limited by sample size. The low n may have prevented some measurements, first touch in particular (Figure 4.9), from reaching statistical significance.

It is possible that qualitative analysis of movements would be more sensitive to the effects of PEM on stroke-induced deficit. A recent study aimed at increasing the sensitivity of the cylinder task for detecting forelimb asymmetry in mice determined that the quantification of paw dragging behaviour was more sensitive than limb use asymmetries for these rodents (Roome and Vanderluit 2015). The animals in the present study also exhibited similar paw dragging type behaviour to varying degrees. Tracking minute aberrations in paw use during exploration through qualitative analysis may further increase sensitivity of the cylinder task to forelimb motor deficits.

Although a minimum of 20 forepaw contacts with the cylinder wall has been recommended for evaluating limb use during wall exploration in the cylinder, our observation is that the period of time over which 20 contacts occurs would not consistently allow for the sufficient collection of data related to takeoff, landing or first touch. This is primarily the case where initial cylinder exposure is concerned. As mentioned previously, Schallert and Tillerson (2000) suggest recording until the animal has made a minimum of 10 landing movements when the cylinder test is evaluated for forepaw use during landing in addition to wall contacts. This may occasionally

extend the filming period beyond 5 minutes, particularly after the animal has been exposed to the cylinder repeatedly. The number of movements recorded influences the sensitivity of an endpoint for detecting a stroke deficit as well as for detecting an effect of nutritional status on stroke deficit, as more data increases the likelihood of an existing impairment reaching statistical significance. Another methodological obstacle is that repeated exposure to the cylinder familiarizes the animal with the apparatus and decreases exploratory activity over time. This phenomenon, referred to as habituation, is a common problem with the cylinder test (Schallert et al. 2002) and the present study was no exception. Although the number of test days was minimized to reduce the extent to which the rats habituated to the cylinder, the total number of contacts or movements decreased over time with successive cylinder trials. Some animals had to be filmed beyond 5 minutes in order to satisfy the minimum standard of 20 contacts with the cylinder wall. Another interesting observation was that exposure to stroke independently decreased activity in the cylinder as measured by wall contacts scored by both methods 1 and 2. Nonetheless, having the control-ischemia group (the brain ischemic group fed the control diet) allowed me to tease out the effect of the low protein diet on motor outcome using scoring method 1 after ischemia. The activity level for first touch, landing and takeoff remained relatively stable over the four study trials conducted. This indicates that although the animals continue to rear after the stroke, the number of wall contacts within each bout of exploration decreases.

Two additional endpoints suggested by Schallert and Tillerson (2000) are the determination of an overall forelimb asymmetry score and an overall ipsilateral (or non-affected) limb use ratio. The overall asymmetry score is calculated by subtracting the percent contralateral (preferred) limb use from the percent ipsilateral limb use for each endpoint. The overall

ipsilateral limb use ratio is calculated by combining the scores for non-affected limb use during wall behaviour and landing. Schallert et al. suggest combining these scores by averaging them together in order to reflect equal emphasis on contributions from asymmetries in forelimb use during wall exploration and landing. This combined score may help to correct for variability in the number of wall movements between animals or groups. Either of these scoring methods will produce scores that represent overall asymmetry by providing adequate weighting among ipsilateral, contralateral and simultaneous limb uses (Schallert and Tillerson 2000). These scores should be considered in the evaluation of future projects that involve the cylinder task.

Protein-energy malnutrition may have hindered recovery of spontaneous use of the forelimb affected by stroke in numerous ways. Protein-energy malnutrition that develops after stroke has been shown to modify proteins that are involved in post-ischemic repair mechanisms (Smith et al. 2014). Protein-energy malnutrition also alters muscle structure and function (Alaverdashvili et al. 2015a), which in turn may hinder mobility and impede recovery. Alternative mechanisms by which PEM may affect stroke recovery are through modification of the brain's inflammatory response to ischemia (Bobyn et al. 2005; Ji et al. 2008) or an independent PEM-induced systemic acute-phase response (Smith et al. 2013; Smith et al. 2014); the latter of which was again observed in the thesis study. Inflammation plays a role in both brain injury and repair processes (Lo 2008; Kriz and Lalancette-Hebert 2009). Thus, an increase or decrease in systemic inflammation may hinder neuronal repair and/or exacerbate the initial damage caused by stroke.

The observed differences in behavioural outcome between the PEM-ISCH and CON-ISCH groups may have been affected by several of the aforementioned mechanisms. Altered muscle structure and function may have contributed to a decrease in the ability to recover from or

compensate for functional losses in the affected forepaw. It is possible that adequately nourished animals are better able to compensate for weaknesses in the affected forelimb by adjusting their posture or compensating for altered balance (Li et al. 2011). Alternatively (or perhaps in addition), the elevated systemic acute phase response as a result of protein-energy malnutrition may have altered the brain's response to stroke (Dziedzic 2015). This may in turn have had a detrimental impact on post-ischemic neuroplastic repair and compensatory pathways within the brain.

The second major objective of this study was to evaluate the impact of PEM developing after stroke on skilled reaching, using the Montoya staircase task. Unfortunately, it was not possible to answer this question because an insufficient number of rats met training criteria prior to stroke. In order to use this task reliably to assess the effect of stroke on forepaw reaching, these animals need to be very well trained prior to the stroke to the point where they can obtain the majority of the pellets. Two previous small pilot studies had indicated that some rats can meet and even surpass our set minimum pellet retrieval standard of 12 pellets +/- 2 standard deviations in the Montoya staircase without food restriction. This plus the findings of the present study suggest that while a selected group of rats is sufficiently motivated to reach for the pellets without being food restricted, this is not a reliable method for successfully training large groups of rats.

Future work that evaluates Sprague Dawley rats in the staircase apparatus during daylight hours, when rats are less active, should use food restriction to motivate the rats to learn the task. In addition to studying skilled reaching, further work is required to examine how PEM might impact other motor functions such as gait and sensorimotor deficits (as assessed by the ladder walking and adhesive tests, respectively). Prospective studies should also evaluate the interaction

between nutritional status and rehabilitation strategies in determining post-stroke recovery, given that the standard in stroke care is to engage patients in intensive physical rehabilitation programs (Veerbeek et al. 2014). An important unanswered question is whether optimizing nutritional status after stroke is essential to maximize the benefit of rehabilitation-assisted brain recovery.

In order to address whether infarct size was influenced by PEM developing after stroke, the volume of damage to the cortex and corpus callosum was evaluated. Given that there was no difference in infarct volumes between the protein-energy malnourished and control diet fed stroke groups (Table 4.2), the degree of brain injury does not appear to explain the difference in functional outcome between the two groups. The similarity in infarct volume was anticipated given that diets were assigned on Day 4 post-stroke, which is after the time point during when one would expect a major effect on infarct size. However, infarct volume assessment in the present study was limited by the approach that was employed. Typically, infarct volume is computed by subtracting the volume of the remaining healthy tissue in the damaged hemisphere or cortex from the volume of the intact hemisphere (or cortex) (Jang et al. 2014; Nishibe et al. 2015). This approach helps to compensate for the distorting effects of edema on the injured hemisphere. Due to some imperfections in sectioning and adherence of the sections to the slides, I estimated infarct area by measuring the area of infarct. This may have over-estimated infarct volume, as the process of mounting the sections may expand the damaged tissue beyond its cavity. In addition, the remaining healthy tissue in the damaged hemisphere may become distorted from its original shape, making it difficult to trace the outline of the damaged cavity. However, an error in the estimate due to edema after stroke is much less of a concern in the chronic period studied than in the acute period (Overgaard and Meden 2000; Sommer 2010).

The final study objective was to evaluate the effect of post-stroke PEM on the acute phase response in the chronic period. PEM caused an independent decrease in serum albumin concentration and increase in serum alpha-2- macroglobulin on Day 30 (Figure 4.4), which together provides evidence for an acute phase response. As noted above, serum albumin concentrations are influenced by both dietary amino acid supply and inflammation (Qu et al. 1996). The present study found no effects of stroke on the serum albumin concentration. This is in agreement with a report that the decrease in serum albumin after stroke is transient and most predominant in the acute period (Dziedzic et al. 2004). This suggests that the decrease in serum albumin is a reflection of nutritional status in the present study. The induction of an acute phase response by protein-energy malnutrition independent of other comorbid disease processes has been previously reported (Ling et al. 2004; Smith et al. 2013; Alaverdashvili et al. 2015a). Increasing stroke severity in clinical studies has been associated with a more pronounced acute phase response (Dziedzic 2008), and an increased acute phase response is associated with poor functional outcome and survival rates after stroke (den Hertog et al. 2009; Whiteley et al. 2009). Thus, the acute phase response is another mechanism, among many, by which PEM could have diminished functional recovery after stroke in the current study. Whether the acute phase response reflects increased neuroinflammation or systemic inflammation would require more specific measurements of key players in the inflammatory response (Gabay and Kushner 1999). It is of note that there was a trend towards an interaction between PEM and stroke for serum alpha-2-macroglobulin concentration (Figure 4.4). Whether PEM that goes untreated has the potential to exacerbate and lengthen the acute phase response to stroke is an important question to address in future research.

A particularly interesting observation in the current study was that an acute phase reaction (increase in serum alpha-2-macroglobulin concentration) to stroke, albeit small, was detected in the chronic period in our preclinical model of stroke. This finding is consistent with the clinical study by Nezu et al. (2013), which demonstrated an association of increased serum alpha-2-macroglobulin with ischemic brain damage in human subjects in both acute (24 hours to 7 days) and chronic (2 months) periods. Whether preclinical models of stroke can be used to study the acute phase reaction that arises after stroke has received little attention. A major challenge is that the surgery required to induce stroke in virtually all the preclinical models induces a large acute phase response (Lowry and Coyle 2014) that is much greater than what arises after stroke. While the inclusion of a surgical sham group has partially controlled for this problem, the addition of a nonsurgical control group would assist further with interpretation. In addition, measuring the profile of several acute phase proteins would help to unravel these complex interactions.

5.1 Conclusion & Impact

In summary, feeding a low protein diet until day 30 after stroke results in protein-energy malnutrition as measured by a decrease in body weight, food intake and serum albumin concentration and increase in liver lipid and serum alpha-2-macroglobulin concentrations. Post-stroke PEM negatively impacts recovery of some, but not all types of forelimb use during spontaneous exploration, and this is independent of infarct size. Both stroke and the low protein diet elicited independent, acute-phase responses in the chronic period. The latter provides one mechanism by which PEM could influence post-stroke recovery.

The Canadian Stroke Best Practice Recommendations for acute inpatient stroke care endorse the evaluation of stroke patients by an inter-professional team that includes a dietitian within 48 hours of admission to a hospital in order to facilitate the creation of a management plan for each patient (Casaubon et al. 2016). This thesis research provides evidence to support this recommendation, and demonstrates some of the potential consequences of not providing early dietary intervention for patients who are at risk of developing protein-energy malnutrition following stroke.

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Appendix A

Appendix A illustrates reaching success in the Montoya staircase for individual rats that were tested after surgery. Each graph demonstrates reaching success (±SEM) as a percentage of baseline (pre-surgery) performance in the Montoya staircase. Each data point is a 2 day performance average consisting of 4 trials. Data are shown for the forelimb targeted by the infarct (ischemia) or the preferred forelimb (sham) as determined during staircase training. PEM = Low protein diet, CON = Control diet, ISCH = Ischemia, SHAM= Sham surgery.

Although Montoya staircase data from rats not meeting the pre-stroke baseline criteria indicative of sufficient learning of the task are unreliable for predicting forepaw function after cortical stroke, the data have been presented here to illustrate some specific patterns. The data for the animals in the following figures met training criteria for the staircase: A3, A5, A6, A8, A12, A15, A18, and A19. The remaining figures represent animals that did not meet criteria for the staircase. Many of the animals that did not meet baseline criteria for subsequent testing in the staircase appear to exhibit learning (achievement of a reaching success rate of greater than 100% of baseline performance) upon re-exposure to the task during post-surgical trials. An example of the aforementioned phenomenon is a PEM-SHAM rat shown in Figure A14 in Appendix A. Figure A2 shows a PEM-ISCH rat who showed insufficient proficiency in the task prior to stroke, and thus we would predict that the results are unreliable. Figure A18 shows a CON-ISCH rat that met training criteria in the Montoya staircase; he shows a typical pattern of post-stroke deficit in the staircase for a well-trained rat. That is, forepaw reaching is reduced to 66% of baseline at Day 3 and shows mild recovery to 71% at Day 15.

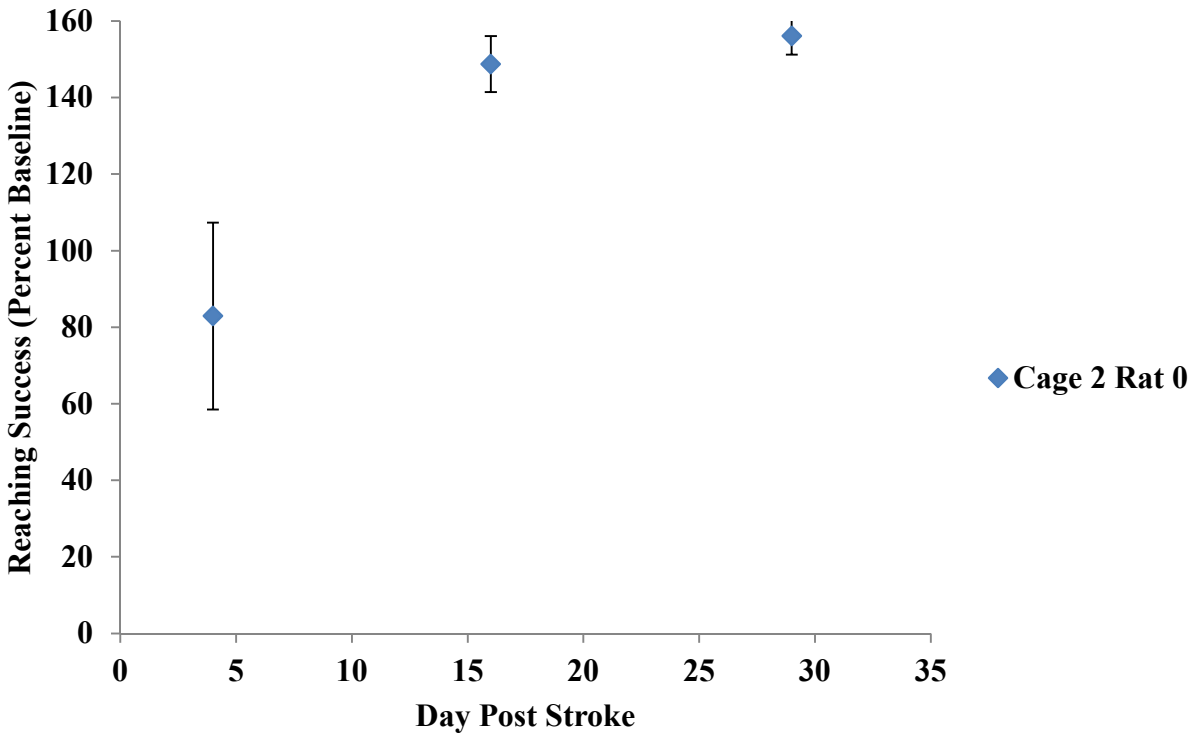


Figure A1: PEM-ISCH

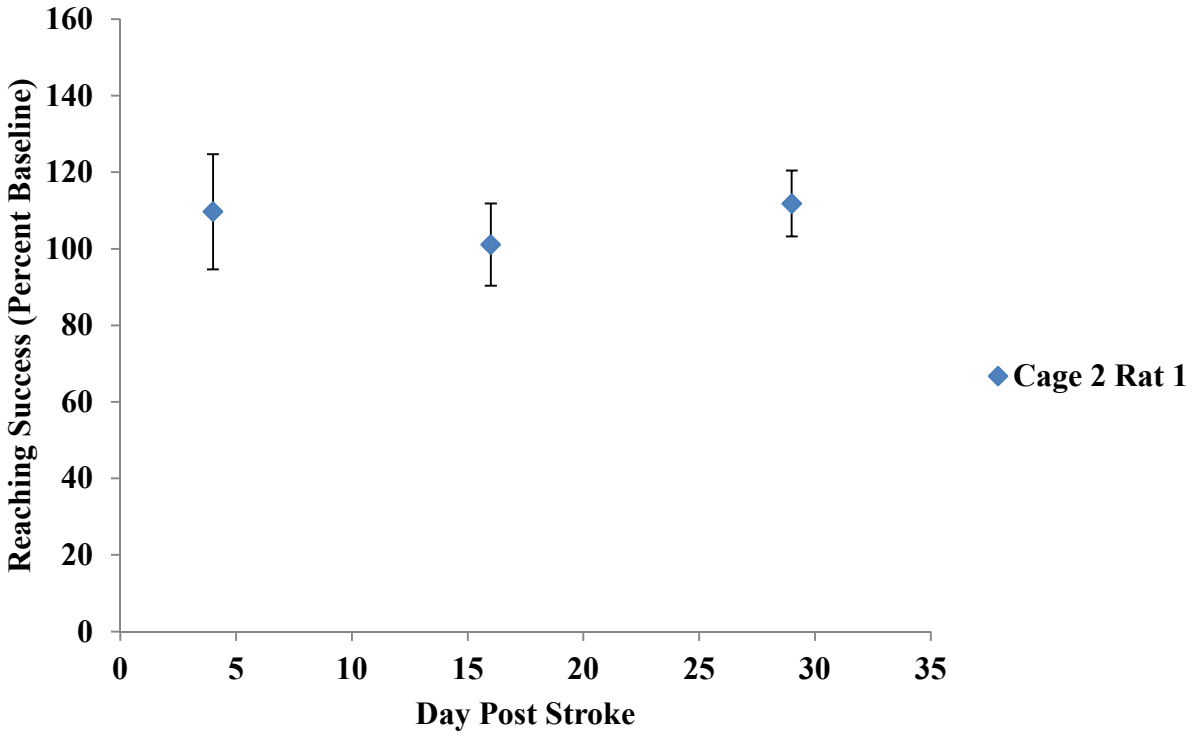


Figure A2: PEM-ISCH

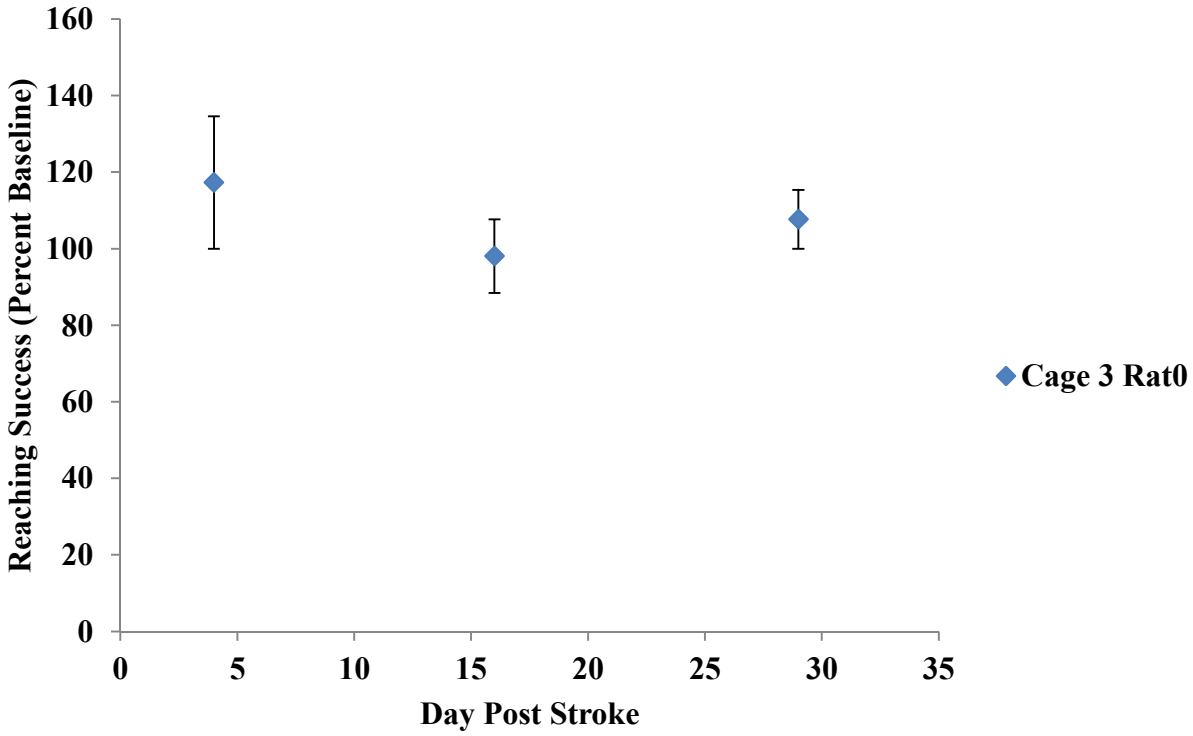


Figure A3: CON-SHAM

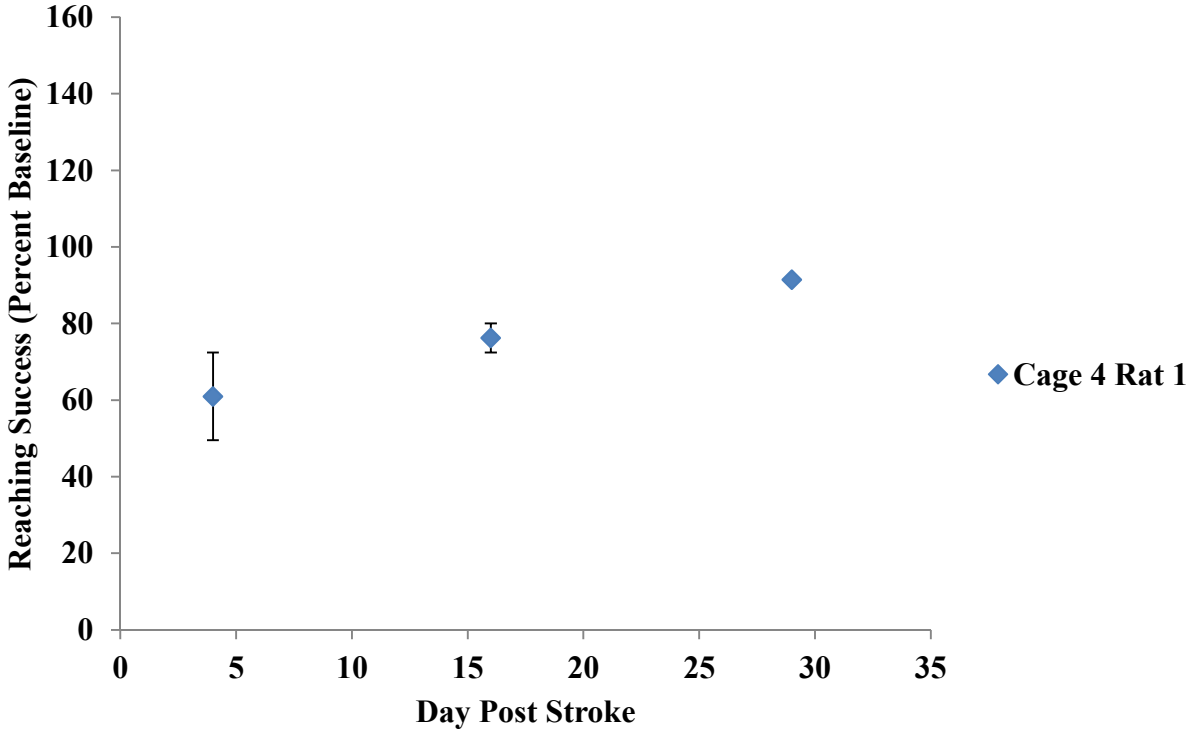


Figure A4: CON-ISCH

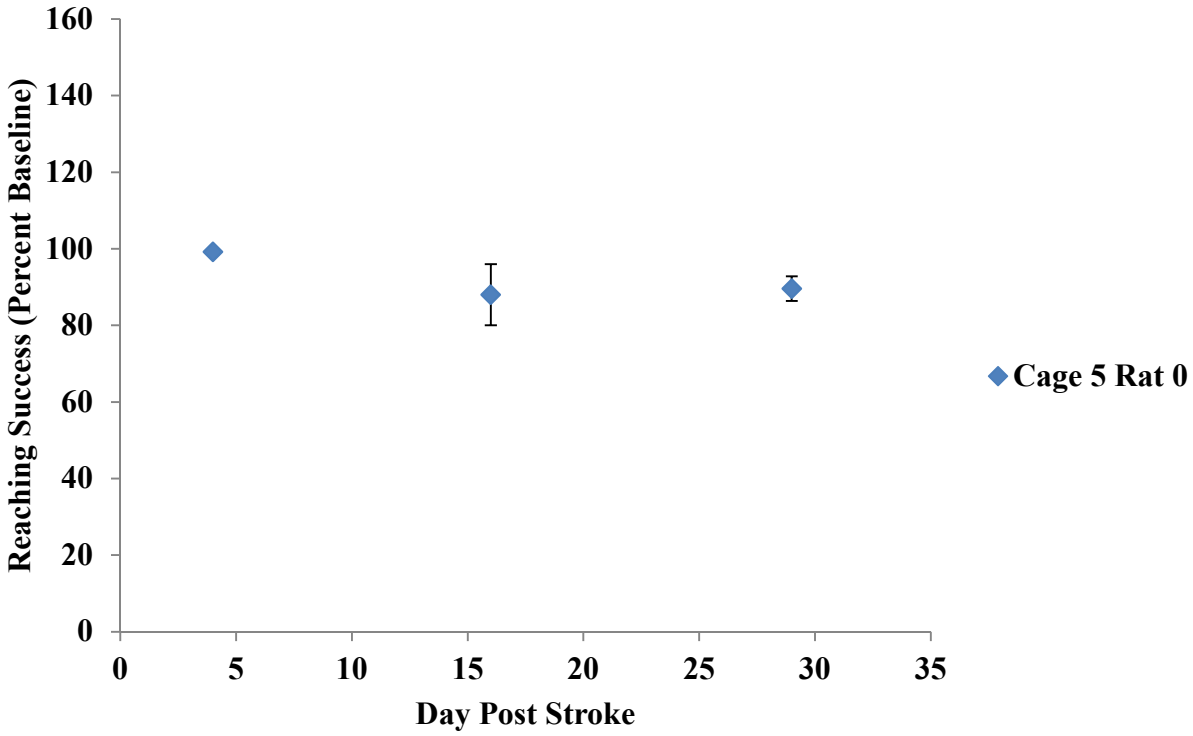


Figure A5: CON-SHAM

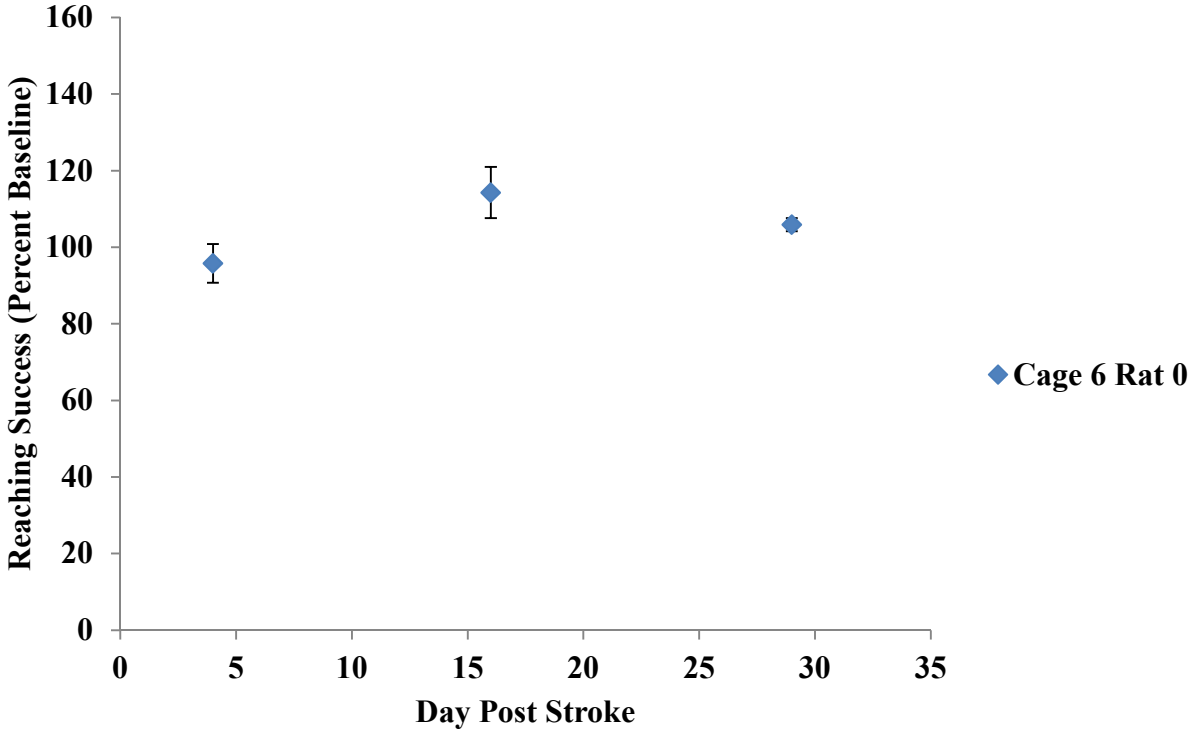


Figure A6: PEM-SHAM

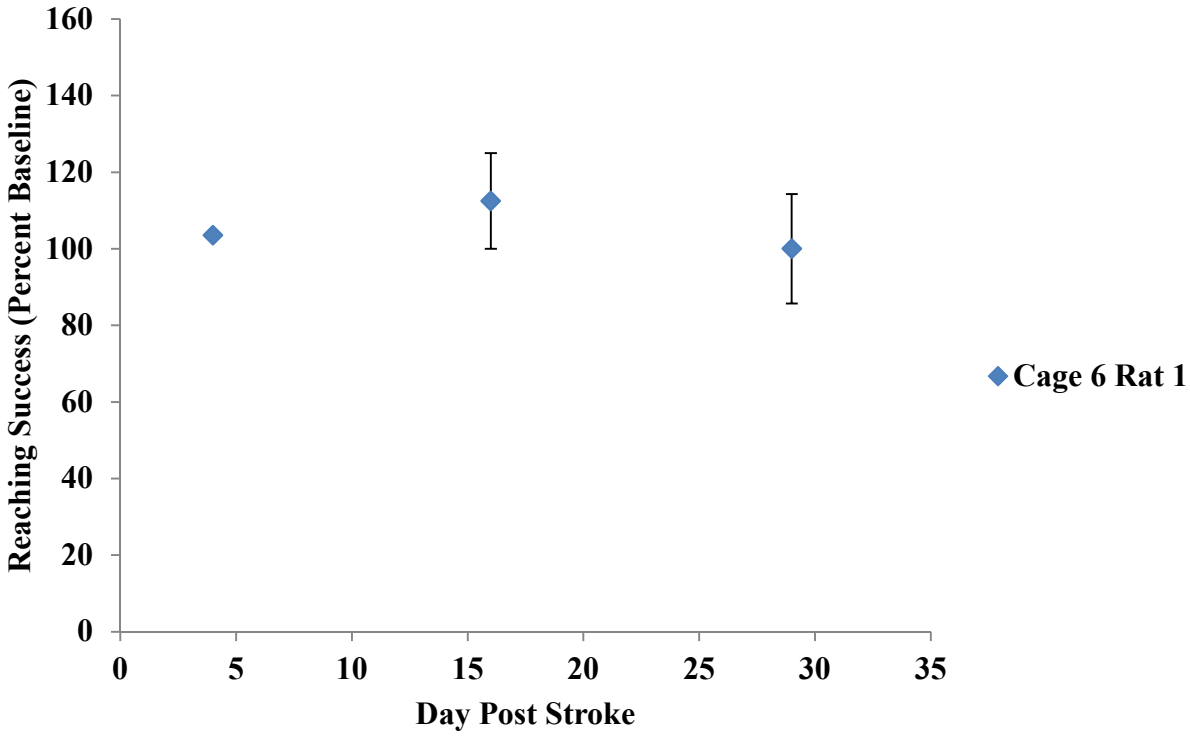


Figure A7: PEM-SHAM

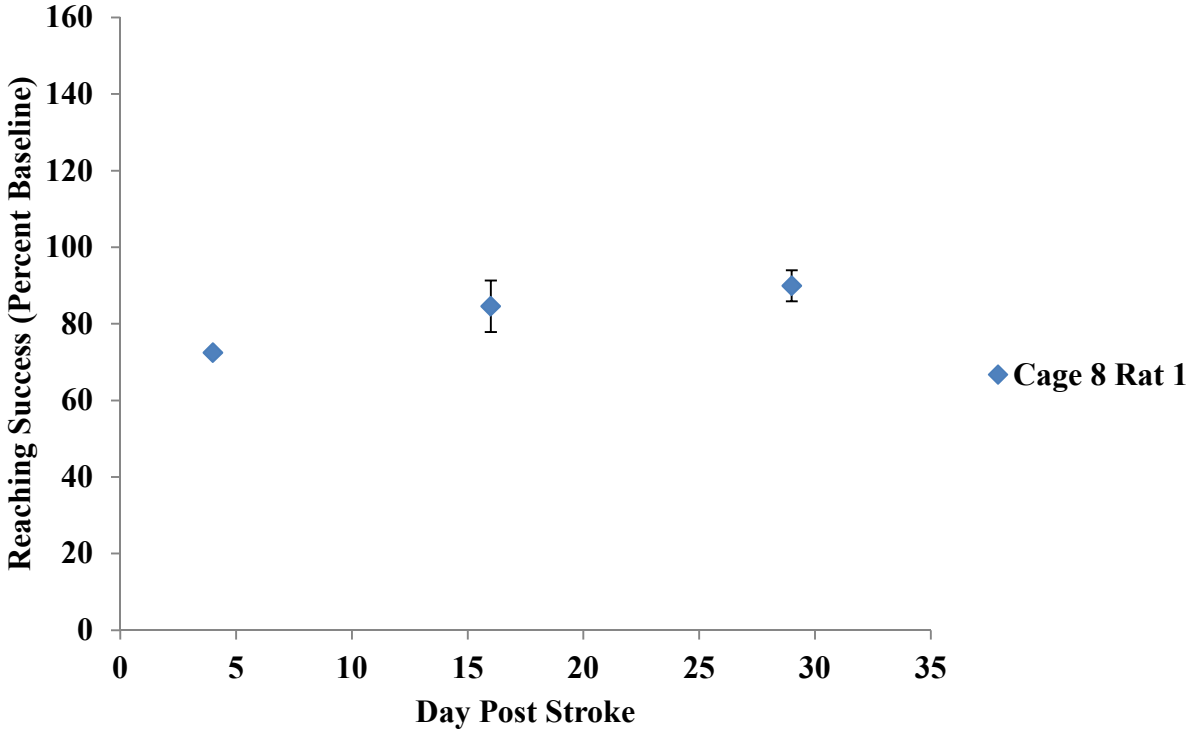


Figure A8: PEM-ISCH

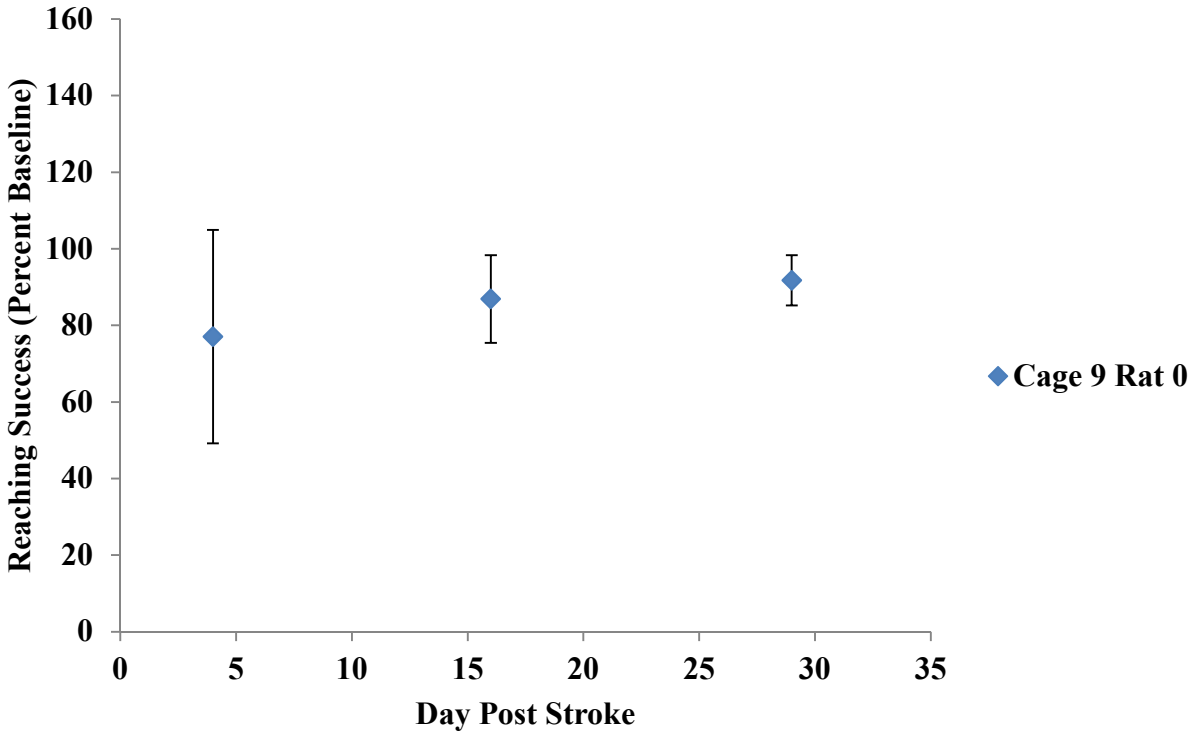


Figure A9: CON-SHAM

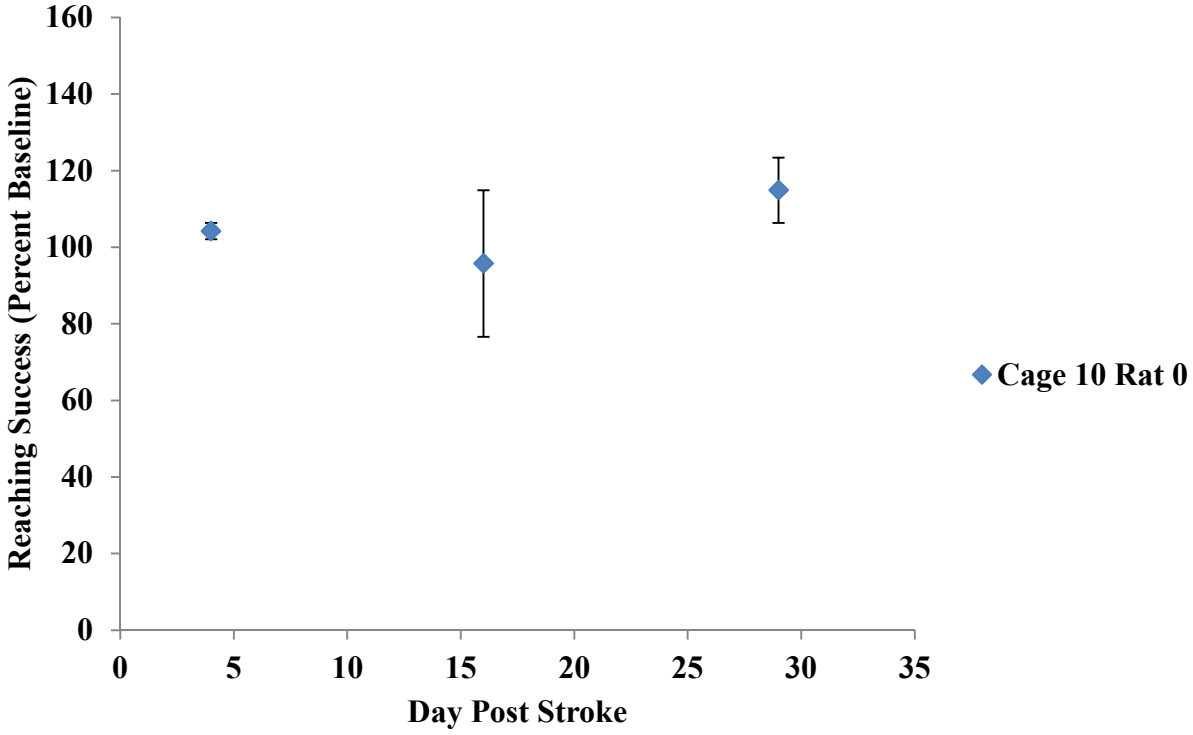


Figure A10: CON-ISCH

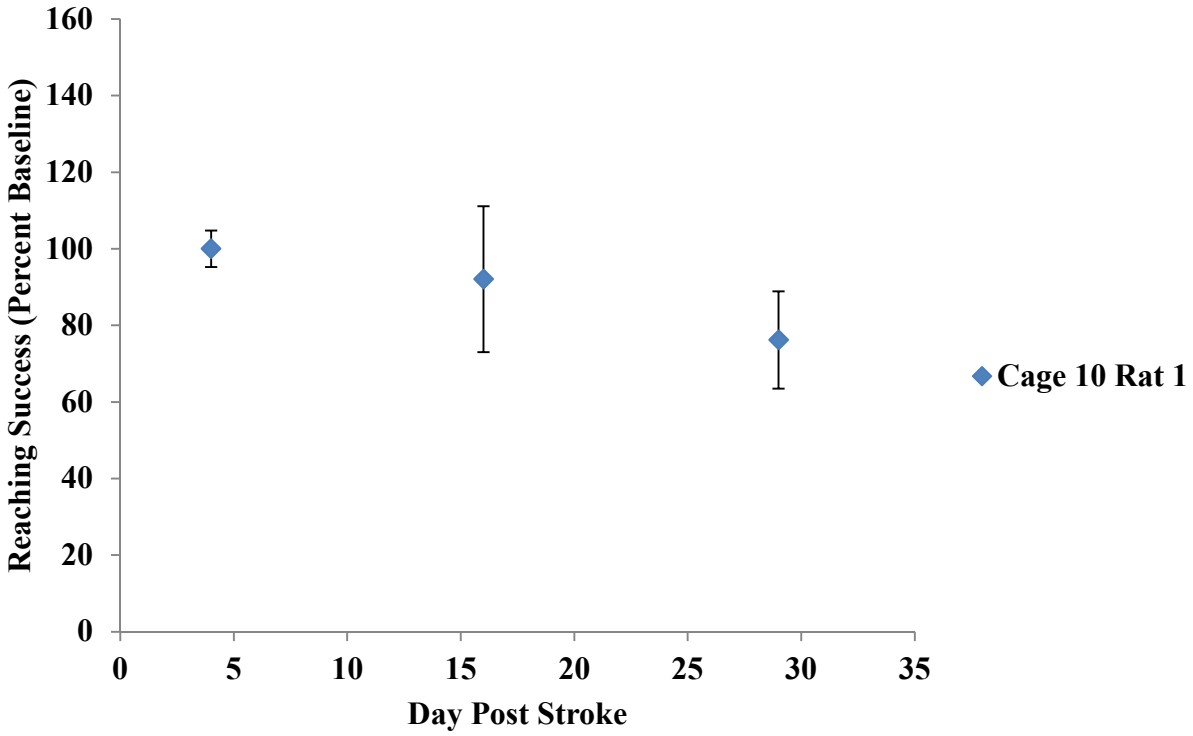


Figure A11: CON-SHAM

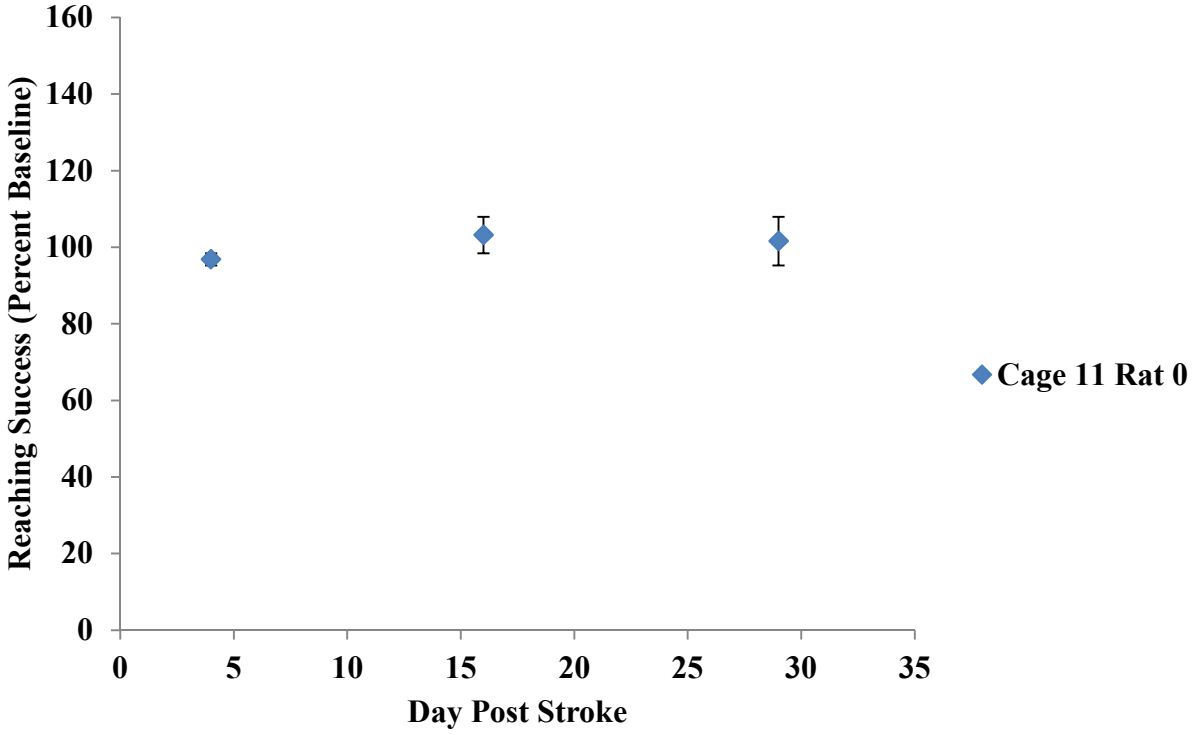


Figure A12: CON-ISCH

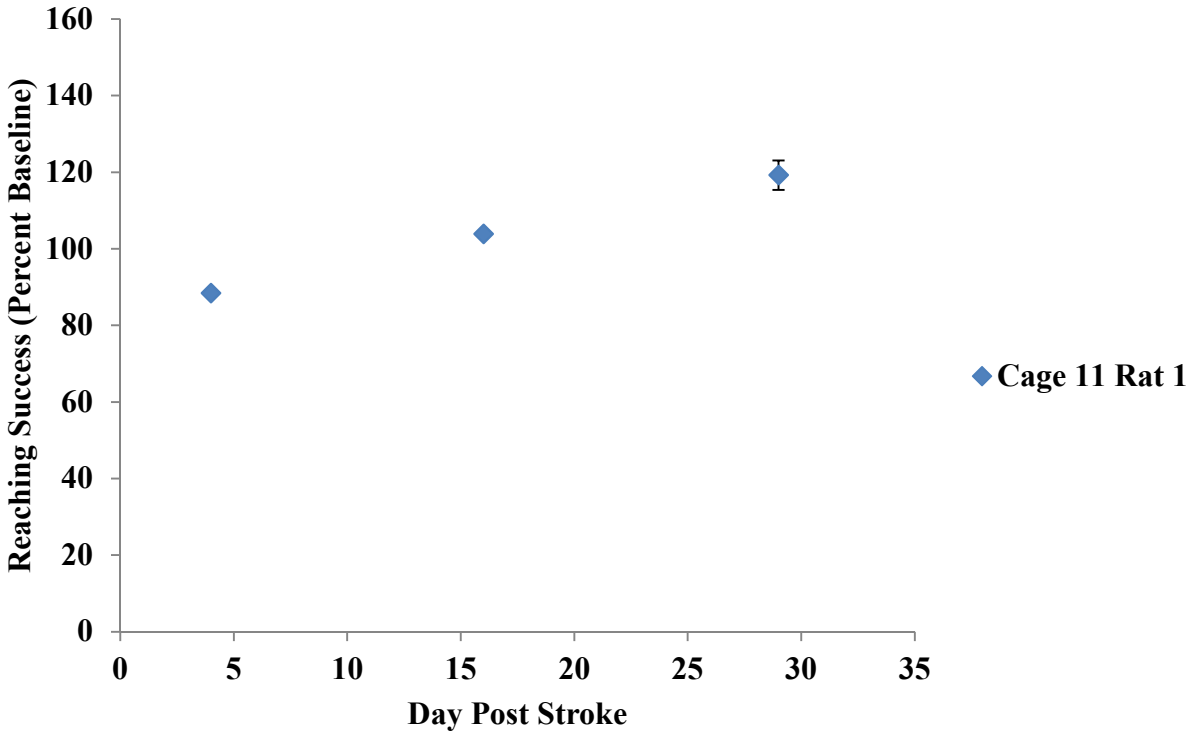


Figure A13: CON-ISCH

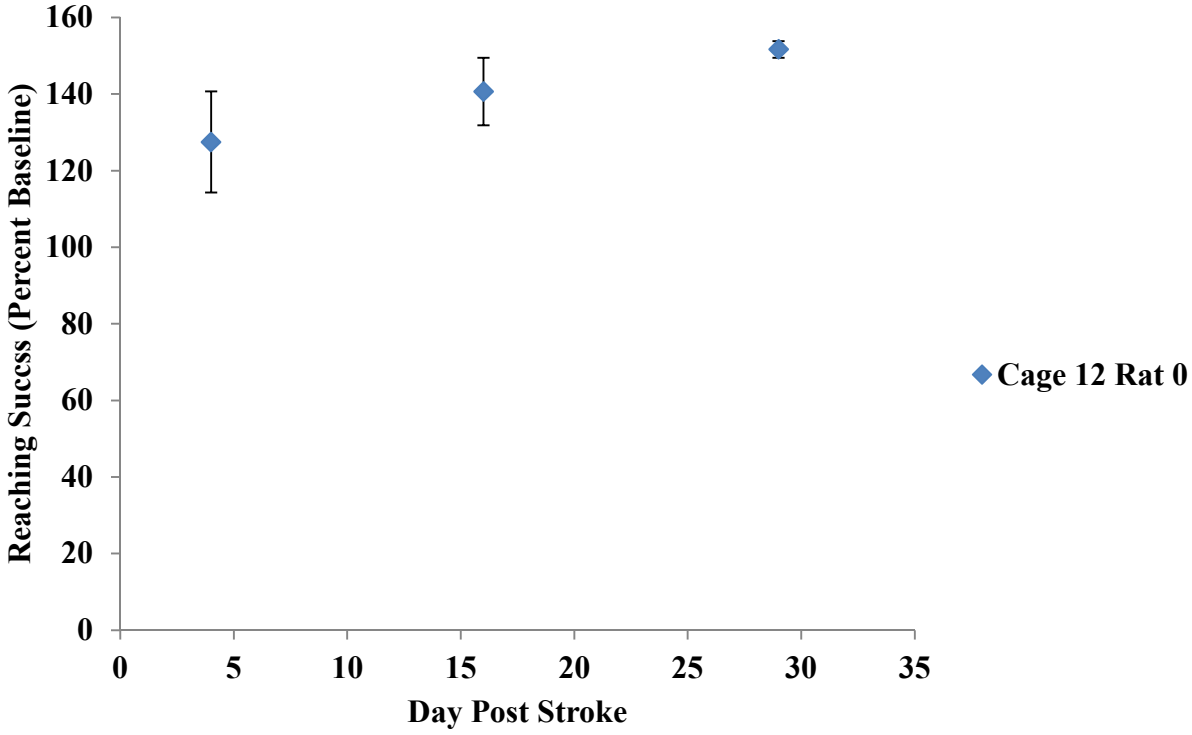


Figure A14: PEM-SHAM

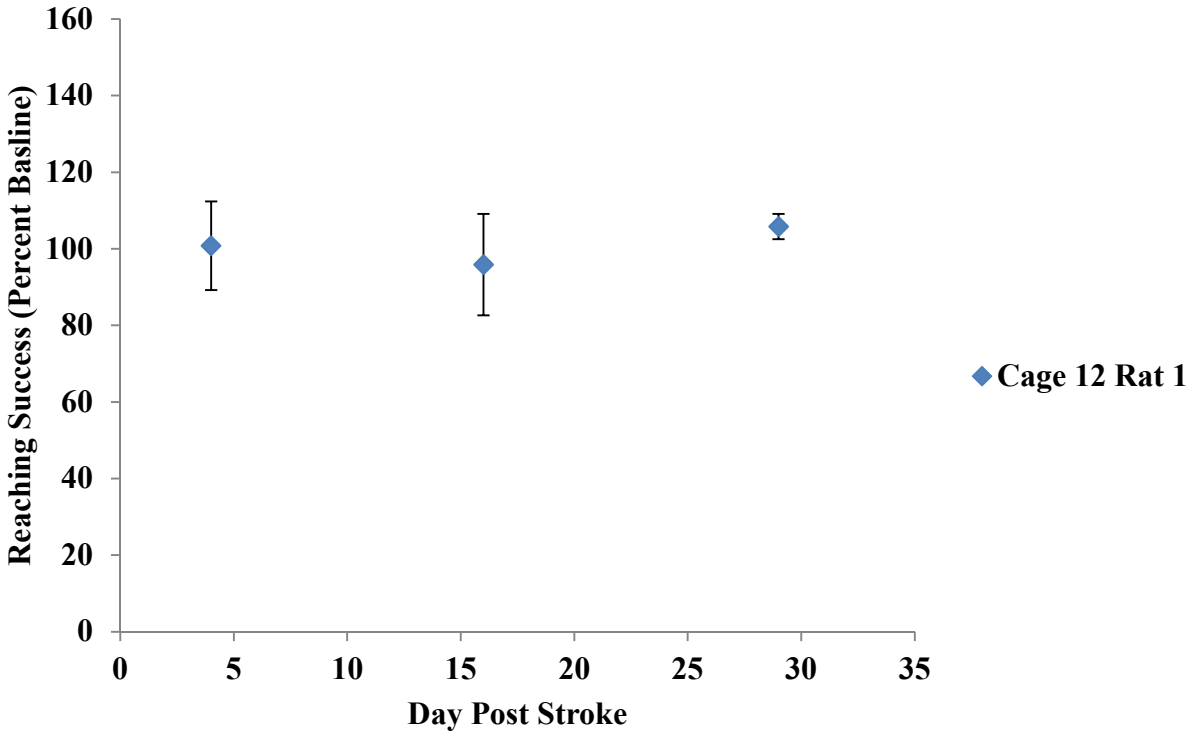


Figure A15: PEM-SHAM

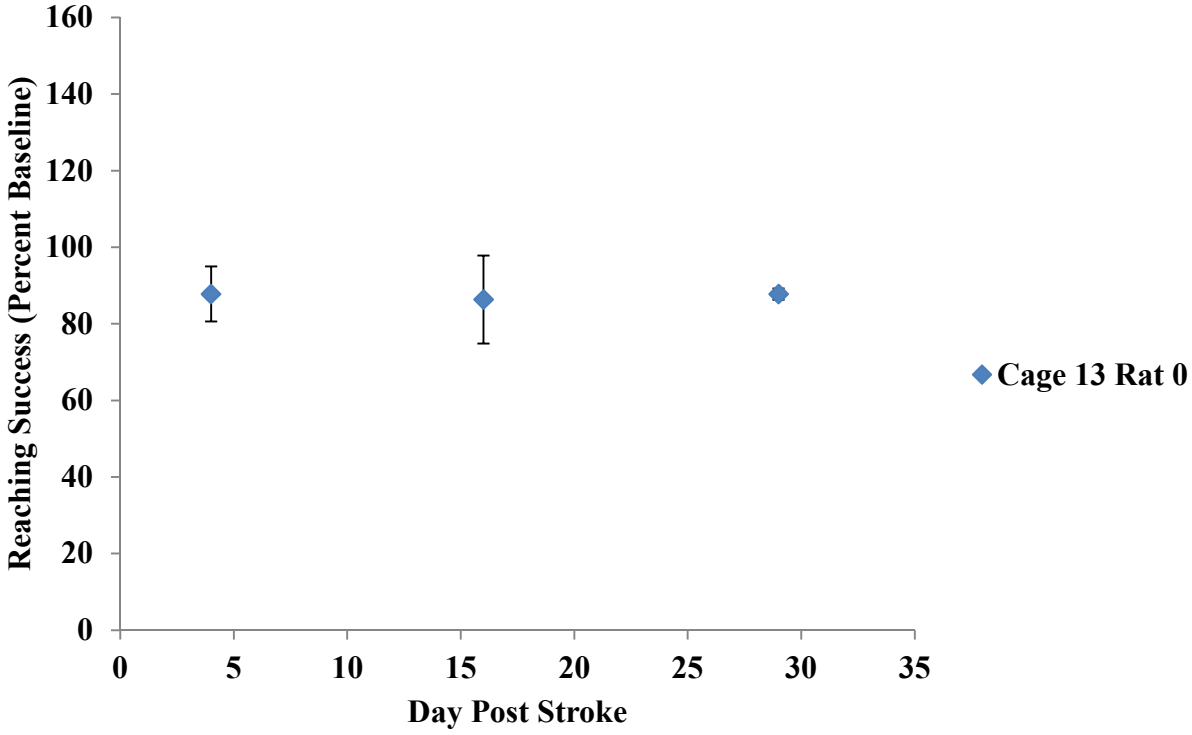
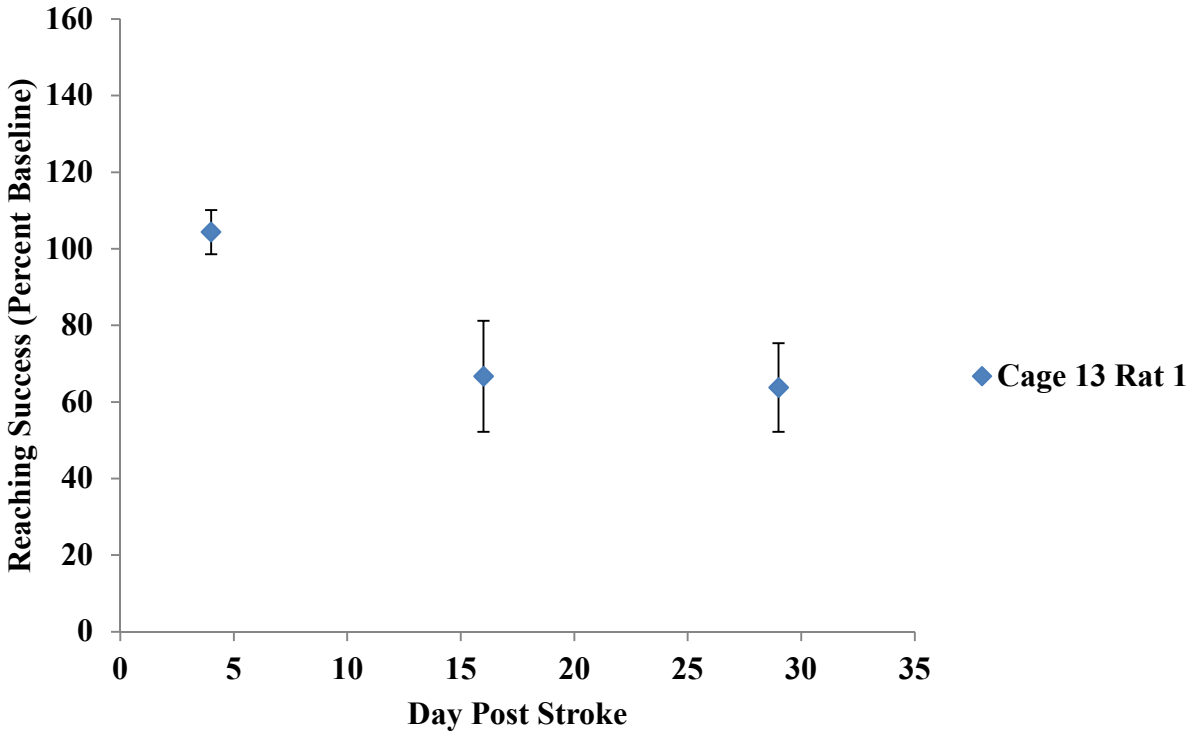


Figure A16: CON-ISCH



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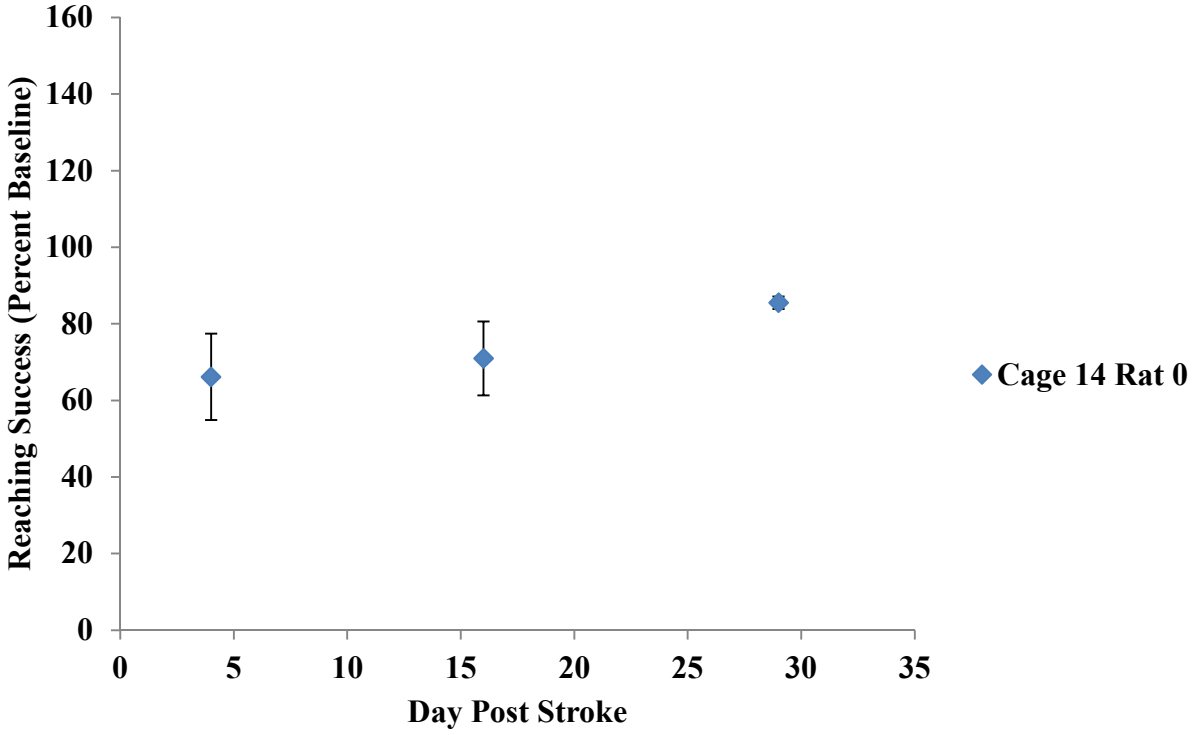

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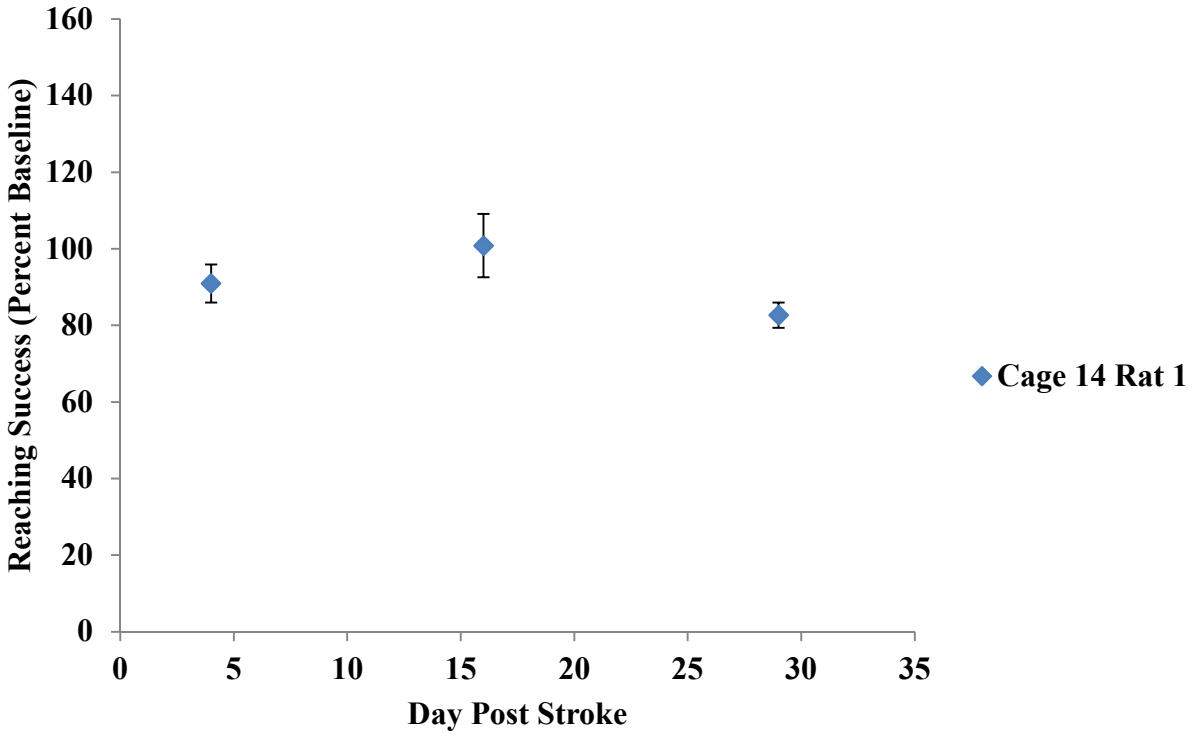

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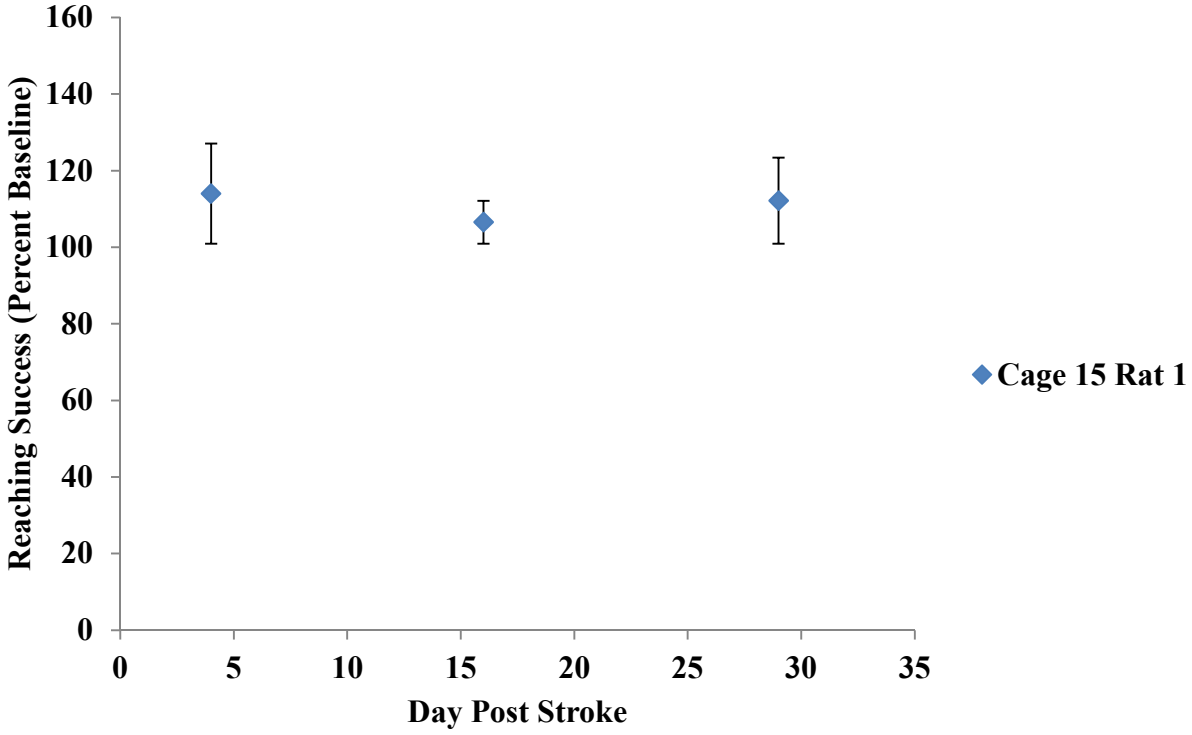

Figure A20: PEM-SHAM