NEUROINFLAMMATION AND COGNITIVE PLASTICITY FOLLOWING EXPERIMENTAL STROKE

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by

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ABSTRACT

Stroke is one of the leading causes of morbidity and mortality in Canada and only relatively few individuals avail of pharmacological treatment. Consequently, most stroke patients are left with permanent disabilities. Post-stroke rehabilitation is beneficial but is often incomplete. Animal models of stroke have helped in our understanding of the mechanisms involved in the recovery of sensorimotor function but little attention has been paid to cognitive impairments, which are common following stroke.

Importantly, patients with cognitive problems are less likely to be reintegrated into society and benefit less well from rehabilitation. Animal models of ischemia have not demonstrated the lasting cognitive impairments that are apparent in human stroke. In the second chapter of this thesis I describe a cognitive assay that detected long-term (~9 mo) alterations in learning, working and reference memory function following global ischemia. Further, I identified a sustained neuroinflammatory state confined to area CA1 at a protracted time point of 270 days post-ischemia, a time when neuroinflammation is typically thought to have subsided.

In the third chapter I assessed the impact of increased neuroinflammation caused by systemic inflammation on ischemic outcome. Systemic inflammation 24-hours post-ischemia significantly increased neuroinflammation at 3 days post-ischemia as indicated by increases in microglia/macrophages and infiltrating neutrophils. This resulted in significant increases in functional deficits and infarct volumes assessed 30 days post-ischemia. These results confirm for the first time, that systemic inflammation at such a

delayed time point, similar to what occurs in a clinical setting, has a profound impact on ischemic outcome.

The fourth chapter attempted to develop a post-stroke intervention to enhance cognitive function. A combination of 2 hours of physical activity (wheel running) and 2 hours of cognitive activity (Hebb-Williams maze exposure) significantly improved working memory in normal rats compared with either physical or cognitive activity alone, independent of significant changes in neuronal BDNF or pCREB levels. These results are the first to suggest that cognitive training in rats, when combined with only 2 hours of wheel running, can significantly improve working memory function, a finding that may be useful in developing cognitive rehabilitation strategies following stroke in humans.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Term
2-VO	2-Vessel occlusion
4-VO	4-Vessel occlusion
AMPA	5-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDNF	Brain-derived neurotropic factor
CA	Cognitive activity
CA1	Cornu ammonis area 1
Ca^{2+}	Calcium
CCA	Common carotid artery
CD11b	Cluster of differentiation 11b
cm	Centimeters
CNS	Central nervous system
^{o}C	Degrees Celsius
EE	Environmental enrichment
ELISA	Enzyme-linked immunosorbant assay
ET-1	Endothelin-1
FGF2	Basic fibroblast growth factor
GFAP	Glial fibrillary acidic protein
H&E	Hematoxylin and eosin

H_2O_2	Hydrogen peroxide
HSD	Honestly significant difference
ICH	Intracerebral hemorrhage
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
i.p.	Intraperitoneal
KA	Kainic acid
kg	Kilogram
LPS	Lipopolysaccharide
LSD	Least significant difference
MAP2	Microtubule-associated protein 2
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
min	Minutes
mL	Milliliter
μg	Mircograms
μm	Micrometers
mg	Milligram
mmHg	Millimeters of mercury
mRNA	Messenger ribonucleic acid

MWM	Morris water maze
n	Number
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
N_2O	Nitrous oxide
O_2	Oxygen
OD	Optical density
p	Probability
PA	Physical activity
PBS	Phosphate buffered saline
pCREB	Phosphorylated cyclic AMP response element binding protein
PFA	Paraformaldehyde
pg	Picogram
pmol	Picomole
R	Receptors
RAM	Radial arm maze
ROD	Relative optical density
s	Seconds
SEM	Standard error of the mean
TNFα	Tumor necrosis factor-α
tPA	Tissue plasminogen activator

CO-AUTHORSHIP STATEMENT

Chapter 2

I designed this experiment with helpful comments from Drs. Dale Corbett and Carolyn Harley. I performed all surgeries, behavioural testing and histological analyses except for staining which was performed by Shirley Granter-Button and CA1 cell counts which were done by Dr. Corbett. I wrote the manuscript, with the assistance of Dr. Corbett, entitled "Persistent behavioral impairments and neuroinflammation following global ischemia in the rat", published in *European Journal of Neuroscience,* 28: 2310-2318 (2008) by KD Langdon, S Granter-Button and D Corbett. Garry Chernenko assisted with the figure presentation of this manuscript.

Chapter 3

This study was designed with input from Drs. Crystal MacLellan and Dale Corbett. I performed the core probe telemetry surgeries but the MCAo surgeries were performed by Dr. MacLellan. I did the majority of injections and blood sampling with some assistance from Dr. MacLellan, Shirley Granter-Button and Sue Evans. I carried out the behavioural testing with some help with the beam (Dr. MacLellan) and cylinder analysis (Julia Curtis). I analyzed cytokine levels (ELISA) with the assistance of Garry Chernenko and Shirley Granter-Button. I sectioned and analyzed the brain tissue but Shirley Granter-Button and Garry Chernenko helped with the immunostaining. I wrote

the manuscript with input from Drs. MacLellan and Corbett entitled "Prolonged, 24-hour delayed peripheral inflammation increases short- and long-term functional impairment and histopathological damage following focal ischemia in the rat" published in the *Journal of Cerebral Blood Flow and Metabolism* (2010) by KD Langdon, CM MacLellan and D Corbett (in press).

Chapter 4

I contributed to the design of this study, along with input from Dr. Corbett. I conducted the majority of behavioural testing and rehabilitation interventions with some help from Julia Curtis. Brain preparation for ELISA measurement was performed by a team consisting of myself, Sue Evans, Shirley Granter-Button, and Garry Chernenko. I performed all analyses and wrote the manuscript with input from Dr. Corbett entitled "Improved working memory following novel combinations of physical and cognitive activity" currently in preparation to be submitted to the *Journal of Neuroscience* by KD Langdon and D Corbett.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Approximately 50,000 Canadians are affected by stroke each year (Canadian Institute for Health Information, 2007). Roughly 16,000 of these cases result in mortality, while the remaining 30,000-35,000 survivors are often left with permanent disabilities. Financial costs to the Canadian health care system to care for these survivors is upwards of $3 billion annually, with the average acute care costs resulting in nearly $27,500 per stroke (Canadian Institute for Health Information, 2007). Since stroke results in such massive economic and emotional costs there is an urgent need for effective interventions. Because permanent disabilities are often the most devastating consequence of stroke, research needs to focus on reducing its long-term functional consequences. The current thesis addresses this issue using animal models to: **1)** characterize permanent cognitive impairments and long-term neuroinflammation following global forebrain ischemia; **2)** determine the consequences of increased neuroinflammation following ischemia, using a model of systemic inflammation, on functional and histological outcome, and; **3)** assess the efficacy of physical and cognitive exercise on learning and memory abilities and neuroplasticity-associated proteins.

1.2 Animal Models of Stroke

Animal models are used throughout the experimental stroke literature to evaluate the effects and mechanisms of stroke-related damage. Animal models allow one to study

stroke and control the experimental conditions in which stroke occurs. Also, the cellular and molecular effects of ischemia can be analyzed by conducting assessments on the tissue of interest. A variety of species and strains of animals can be used to model stroke conditions, and these models can be standardized for consistency and reproducibility (Corbett and Nurse, 1998; Koch and Britton, 2004). Consistency and reproducibility between animal models is essential for assessing the efficacy of potentially beneficial neuroprotective drugs or post-stroke rehabilitative interventions. Generally, rodents (mainly rats, mice and gerbils) have been used as animal models (Ginsberg and Busto, 1989; Corbett and Nurse, 1998) for several reasons: 1) the relatively low cost; 2) similarity in the cerebrovascular anatomy and physiology to that of higher animal species; 3) consistency within strains of animals due to inbreeding, and; 4) a greater ethical acceptability than using 'higher' species such as non-human primates.

Animal models of stroke can be broadly categorized into 3 different types: intracerebral hemorrhage (ICH), global forebrain ischemia and focal ischemia (MaCrae, 1992; Traystman, 2003; MacLellan et al., 2008).

1.2.1 Intracerebral Hemorrhage

There are two widely used models of ICH: autologous whole blood and bacterial collagenase infusion (MacLellan et al., 2008). The whole blood model involves withdrawing the animal's blood and then injecting the blood intracerebrally, usually in the striatum, to model the most common form of ICH in humans (Bullock et al., 1984). The collagenase model involves injecting bacterial collagenase directly into the target

structure, resulting in disruption of the basal lamina of cerebral blood vessels and spontaneous bleeding (Rosenberg et al., 1990). The whole blood model was originally thought to more closely resemble human pathophysiology, however it was found that ongoing, spontaneous bleeding occurs in humans up to 24-hours following hemorrhage (Fujii et al., 1994). In contrast to the whole blood model, blood brain barrier breakdown occurs to a greater extent, tissue loss continues over 4 weeks post-hemorrhage and functional deficits resolve much more slowly and incompletely in the collagenase model, thus making this model arguably more like human hemorrhage (MacLellan et al., 2008).

1.2.2 Global Forebrain Ischemia

Global ischemia mimics the human case of cardiac arrest (Pulsinelli and Brierley, 1979; Hossmann, 1991). Cardiac arrest affects not only neuronal tissue but peripheral organs as well possibly having a secondary detrimental effect on the brain. A number of models have been developed to measure the effects of a total reduction of cerebral blood flow without the complications of peripheral organ ischemia (Molinari and Laurent, 1976; Ginsberg and Busto, 1989; Karpiak et al., 1989). In the 2-vessel-occlusion (2-VO) model of global ischemia, the two common carotid arteries (CCA) are occluded and either combined with systemic hypotension (in rats) (Smith et al., 1984) or not (in gerbils due to lack of a complete formation of the circle of Willis) (Kirino, 1982). This reduces the blood flow to the forebrain below a critical threshold and results in delayed, selective pyramidal cell death of cornu ammonis area 1 (CA1) neurons.

An extension of the 2-VO model is the 4-vessel-occlusion model (4-VO). Similar to the 2-VO model, both CCAs are occluded however, this is combined with the permanent occlusion of both vertebral arteries (Pulsinelli and Brierley, 1979). The advantage of this model over the 2-VO model is that it can be produced in the awake animal so that assessment of functional alterations immediately following occlusion is possible (Pulsinelli and Brierley, 1979). However, there are also disadvantages of this model. There is much more variability in terms of mortality and extent of CA1 cell loss with the 4-VO model when compared with that of the 2-VO model (Volpe et al., 1984; Ginsberg and Busto, 1989), although much of this variability is related to temperature regulation and post-ischemic survival periods (Colbourne et al., 1999b). Further, the 4-VO model is more invasive and technically challenging than the 2-VO surgical model. Although these models of global ischemia closely mimic the neuropathological consequences of cardiac arrest (Zola-Morgan et al., 1986), there is a relative lack of consensus on the functional consequences. Many authors indicate that there is an initial deficit in learning and memory, but that these resolve over time (Bendel et al., 2005; von Euler et al., 2006; Bueters et al., 2008), which is contrary to the permanent anterograde amnesia found clinically (Zola-Morgan et al., 1986). We address this dichotomy in Chapter 2 where we developed a long-term behavioural assay that was sensitive in detecting sustained and permanent learning and memory dysfunction up to 8-months post-ischemia.

1.2.3 Focal Ischemia

In accordance with their similarity to human stroke, focal models of cerebral ischemia have increasingly gained acceptance (Karpiak et al., 1989; Corbett and Nurse, 1998). To date, the most widely utilized models involve either permanent or transient occlusions of the middle cerebral artery (MCAo models) (Tamura et al., 1981; Longa et al., 1989; Laing et al., 1993; Sharkey et al., 1993; Belayev et al., 1996). In the transient MCAo model the middle cerebral artery is occluded either by a clip or by insertion of an intra-luminal thread (e.g. 60-120 min occlusion). The occlusion is then removed to allow reperfusion similar to the situation observed in human stroke (Laing *et al.* 1993; Belayev *et al.* 1996). There are several difficulties with these models of MCAo injury. They usually require substantial surgical expertise such that reliability and reproducibility are not always maintained between laboratories and may depend on the skills of the surgeon. Further, these models are invasive and usually require the exposure and cauterization of additional arteries that may have residual detrimental effects on long-term outcome, independent of ischemia. With respect to the intraluminal suture models, there may also be significant damage to arteries in the process of inserting a foreign body into the animal's blood vessels that leads to exacerbation of the inflammatory response, known to increase injury (McColl et al., 2007, 2008). In order to achieve consistent injury, long occlusion times must be used (e.g. 90-120 min) which results in near total hemispheric damage often including the hypothalamus (Li et al., 1999; Ryan et al., 2006). This model has the additional disadvantage of immediate cerebral reperfusion that models

only a small number of patients who receive tissue plasminogen activator treatment (tPA) (Barber et al., 2001; Laloux et al., 2007).

A model of MCAo that more closely resembles that of human stroke involves the injection of a vasoconstrictive peptide, endothelin-1 (ET-1) (Sharkey et al., 1993). Infusions of ET-1 applied adjacent to the MCA results in approximately 50% reduction in cerebral blood flow for 22-24 hours (Biernaskie et al., 2001; Windle et al., 2006). This is likely the case in the majority of cases of human stroke that do not receive tPA-treatment, where cerebral blood flow is significantly reduced for a period that eventually resolves over time (Carmichael, 2005). The ensuing histopathology and functional deficits in ET-1 MCAo are similar to the other more invasive models, but can be reproduced with more consistency and reliability due to its standard stereotaxic nature and animals recover more quickly (Biernaskie and Corbett, 2001; Biernaskie et al., 2001; Windle et al., 2006). Another advantage of the ET-1 model is that one can study the slow progressive morphological and functional damage (as opposed to the relatively rapid occurring infarct from the MCAo by clip or intraluminal thread), which may closely resemble particular human situations. In contrast to the intraluminal suture model, injection of ET-1 can be used to target specific neuronal areas of interest (Biernaskie and Corbett, 2001; Windle et al., 2006; Ploughman et al., 2007; Clarke et al., 2009). This is especially important when specific functional abilities are to be assessed. Traditional models of ischemia (e.g. suture model) damage significant portions of the brain, often sparing regions of brain damaged in human stroke, instead encroaching upon area rarely affected in people (e.g. hypothalamus). This, in turn, creates additional complications and confounding variables for functional assessments. However, ET-1 receptors are not only localized to endothelial

cells but also to neurons and astrocytes in the brain (Nakagomi et al., 2000; Naidoo et al., 2004). It is therefore possible that ET-1 acts directly on cell types additional to endothelial cells thus potentially confounding the interpretation of the pathophysiology corresponding to this model of stroke.

Other focal models of stroke include the introduction of a cerebral embolism or thrombosis. In the blood clot embolization model, blood clots are injected either into one of the CCAs or in the territory of the MCA in order to reduce cerebral blood flow (Papadopoulos et al., 1987). Emboli can also be formed by irradiating the CCA with a laser to create an area of infarction within the cortex, hippocampus, striatum, and thalamus (Futrell et al., 1988). The cerebral embolism models of ischemia have proven useful in the study of clot formation and its ramifications, as well as in the discovery of human recombinant tPA. However, the emboli are unpredictably placed and of varying sizes which make it difficult to assess the pathophysiology of cerebral ischemia as well as the ensuing functional deficits (Ginsberg and Busto, 1989; MaCrae, 1992). Further, as a result of the unpredictability of infarct size and location, the use of these models is limited in their predictive value for neuroprotection afforded by experimental treatments (Carmichael, 2005).

The injection of a photothrombotic dye, rose-bengal, has also been used to create ischemic damage to the cortex (Watson et al., 1985). In this model the photosensitive dye is injected peripherally and allowed to circulate throughout the animal's body. A small 'window' is created in the skull overlying the cortical region of interest. A laser beam directed towards the surface of the brain interacts with the dye creating platelet aggregration and eventually a blood clot in the cortex. Subsequently, researchers are able

to closely monitor the evolution of infarct formation and determine changes in cerebral blood flow and structural changes that result from cerebral ischemia (Carmichael, 2005; Schaffer et al., 2006). Advantages of the photothrombosis model include the small, consistent infarcts within functional subdivisions of the cortex with minimal surgical intervention (e.g. cortical thinning). However, these infarcts can only be placed in the cortex and thus the effects of subcortical damage (e.g. striatum) cannot be assessed. Additionally, the infarct consists primarily of an ischemic core and a relative lack of penumbral tissue making it less like human stroke conditions (Carmichael, 2005).

Although there is no animal model in particular that exactly replicates human stroke, each of these animal models offers advantages and disadvantages, all of which depend on the specific research question of interest (Karpiak et al., 1989; Murphy and Corbett, 2009).

1.3 Pathophysiology of Ischemic Stroke

1.3.1 Ischemia and Glutamate:

Studies of mechanisms of ischemic cell death indicate a significant role for the excitatory amino acid glutamate (Hagberg et al., 1987; Ikonomidou et al., 1989). Glutamate is the most abundant excitatory amino acid in the mammalian central nervous system (CNS) (Ozawa et al., 1998) and following an ischemic episode, massive amounts of glutamate are released within the brain (Benveniste et al., 1984; Hagberg et al., 1987; Takagi et al., 1993). Specific glutamate receptors (R) involved in the excitatory response include: N-methyl-D-aspartate (NMDA), 5-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid (AMPA), and kainic acid (KA) receptors (Ozawa et al., 1998). The role of the NMDAR is probably most characterized with respect to ischemia. Glutamate binds to the NMDAR and initiates the influx of Ca^{2+} and other divalent cations (Martin et al., 1998; Lynch and Guttmann, 2002). Although Ca^{2+} is instrumental for normal cellular functioning, excess intracellular Ca^{2+} results in excitotoxicity and cell death following ischemia (Ford et al., 1989; Olney et al., 1991; Streit et al., 1992).

The mechanisms of excitotoxicity are not fully understood and likely consist of a combination of factors (Westbrook et al., 2000). One excitotoxic mechanism linked to ischemia is the association of NMDARs with neuronal nitric oxide synthase (nNOS). In cases of excessive glutamate stimulation, NMDARs are activated causing an opening of a cation channel and subsequently allowing an abundant influx of Ca^{2+} (Martin et al., 1998; Lynch and Guttmann, 2002). Calcium passing through the NMDAR channel activates intracellular nNOS resulting in the increased production of the free radical nitric oxide, ultimately leading to cell death (Dawson et al., 1991; Kornau et al., 1995; Sattler et al., 1999).

Other mechanisms of cell death linked to increased intracellular Ca^{2+} concentrations include the activation of destructive intracellular Ca^{2+}-dependent enzymes such as phosphatases, caspases and various lipases (Westbrook et al., 2000). Mitochondrial function may also be compromised due to increased intracellular Ca^{2+}, leading to further cell stress and cell death (Choi, 1995; Martin et al., 1998). One type of cell death, necrosis, occurs primarily in the infarct core and causes a destruction of cellular organelles as well as a disruption of the cellular membrane, ultimately leading to neuroinflammation (see below) (Li et al., 1995).

1.3.2 Necrosis and Apoptosis:

Cell death following ischemia is thought to occur as a result of two processes: necrosis and apoptosis (Colbourne et al., 1999a; Dirnagl et al., 1999). Apoptosis and necrosis are thought to lie along a continuum, where it is difficult to distinguish between the two types of cell death. Typically, apoptotic cell death is characterized by the condensation of chromatin into dense masses within the nucleus (Martin et al., 1998). Following this, condensation of the cytoplasm occurs where the cell shrinks in size while the membrane remains intact. Next, the cell membrane begins to bud, forming cellular debris and is soon phagocytosed by microglia or macrophages, typically without generating a substantial inflammatory response (Martin et al., 1998). In contrast, necrosis usually results from membrane permeability and subsequent dysfunction of ion transport proteins as well as additional mechanisms such as oxidative stress and depletion of ATP (Farber et al., 1981). Necrosis is characterized by clumping of the chromatin, swelling and degeneration of organelles, and the destruction of membrane integrity. The cell eventually swells and ruptures causing the organelles to be expelled into the extracellular fluid. During stroke, the relative cell death balance is altered, with necrosis thought to be initially responsible for cell death and a subsequent increase in apoptotic cell death as a secondary response (see below) (Hagberg et al., 1987; Ikonomidou et al., 1989; Nakajima et al., 2000; Geddes et al., 2001).

1.4 Neuroinflammation

1.4.1 CNS Immunospecialization:

In the past, the CNS has been characterized as an immunologically privileged site (Janeway et al., 2001). It should, however, be viewed as an immunologically specialized site (Ransohoff et al., 2003). Immune reactions do indeed occur within the CNS, however they are manifested in a somewhat distinctive manner. Some of the most important morphological features that differentiate the CNS immune reactions from those of its peripheral counterpart, and limits the exchange of immune cells and mediators, include: 1) the relative lack of lymphatic drainage of the parenchyma; 2) the presence of the blood brain barrier (BBB), and; 3) with the exception of microglia, a relative lack of endogenous antigen-presenting cells (Ransohoff et al., 2003). As a result, researchers have had difficulty in defining the specific mechanisms that support immune reactions within the CNS. Further, researchers have only relatively recently begun to appreciate the complexity of the interaction between the central and peripheral immune systems, especially following injury. This is important because following ischemic injury there is disruption of the BBB that enables inflammatory cells in the peripheral system to directly interact with cells within the central immune system.

1.4.2 Cerebral Ischemia and Inflammation

Microglia represent the resident tissue macrophage of the CNS where they play an active role in brain inflammation following neuronal injury (Wood, 2003).

Following ischemia, microglial cells start to proliferate and migrate into the damaged area (Gehrmann et al., 1992a; Gehrmann et al., 1992b). The resident, resting microglia retract their processes and become more amoeboid-like in shape and then, once at the damaged site, become reactive phagocytic brain macrophages (Morioka et al., 1991). As resting microglia encounter activating cytokines and chemokines, there is increased expression of the constitutive complement type-3 receptor (CR3, Mac-1, CD11b/CD18) on the cellular membrane (Gehrmann et al., 1992b; Morioka et al., 1992; Gehrmann and Kreutzberg, 1993). Additionally, there is induction of the major histocompatibility complex (MHC) class II antigens and of transforming growth factor-β1, as well as more potent and localized alterations in morphology, surface antigens and cytokine mRNA expression (Morioka et al., 1992; Koistinaho and Yrjanheikki, 2003).

Microglia activation occurs within hours of ischemic injury (Gehrmann et al., 1992a; Gehrmann et al., 1995). This early activation is presumed to occur via the release of various cytokines and chemokines of damaged and dying neurons (Morioka et al., 1992; Schnell et al., 1999; Aloisi, 2001). The number of activated microglial cells continues to increase following ischemia until its peak at 24-72 hours in most brain areas, with the exception of the thalamus, where a delayed microglia response is observed up to 14 days post-injury (Gehrmann et al., 1995). At this point, many of the necrotic neurons (as a result of excitotoxicity caused by ischemia) are phagocytosed and microglia can then assume their resting, ramified state. One study, using the MCAo model, found evidence, that activation of microglia may lay dormant for several weeks (four- to five-weeks) post-ischemia, peak shortly thereafter, and continue until 6- or 7-weeks post-ischemia thus indicating that cell death may represent a continuous process (Morioka et

al., 1993). This delayed activation may occur as a result of late cytokine stimulation from surrounding glial cells serving as a protective measure to prevent further necrotic cellular death. It is also noteworthy that the inflammatory response continues for such a prolonged period post-ischemia, potentially indicating a sustained inflammatory response. These results were confirmed in one of the experiments outlined in this thesis (Chapter 2) (Langdon et al., 2008). In this study we observed a significant 'early' neuroinflammatory response at 14-days post-ischemia, but also sustained inflammation at 270-days post-ischemia. The results from this study were pursued further and the role of an exaggerated inflammatory response was assessed using a clinically-relevant model of systemic infection (Chapter 3).

1.4.3 Functional Roles of Inflammatory Cells in the CNS:

The phagocytic property of microglia is one of the most important functional roles of this cell type. Activated, phagocytic microglia can be found adjacent to dying neurons, their dendrities, and synapses in order to remove cellular debirs (Morioka et al., 1993). Their acute activation occurs directly in the necrotic core, where the ischemic lesion is thought to occur. Subsequently, a more selective phagocytosis is thought to occur in the penumbral tissue (Gehrmann et al., 1992b; Morioka et al., 1993). It has also been shown that astrocytes may contribute to the phagocytosis of cellular debris, albeit to a lesser extent, in addition to their role in the formation of a glial scar (al-Ali and al-Hussain, 1996; Bechmann and Nitsch, 1997; Sofroniew, 2009).

Activated microglia secrete proinflammatory cytokines including interleukin-1β (IL-1β) (as well as IL-1β converting enzyme), IL-6 and tumor necrosis factor-α (TNF-α) (Bhat et al., 1996; Perini et al., 2001; Koistinaho and Yrjanheikki, 2003). The sustained induction of these proinflammatory cytokines following ischemia suggests a primary role in microglial cytotoxicity. Additionally, IL-6 production may play a dual role in ischemia (Perini et al., 2001). It can act as an antagonist, blocking the action of IL-1 and TNF-α at their respective receptors. In contrast, sustained elevation of IL-6 has been reported to stimulate gliosis and BBB leakage, possibly leading to an increase in leucocyte infiltration into the CNS (Koistinaho and Yrjanheikki 2003). The effect of exaggerated neuroinflammation on ischemic outcome was the basis of Chapter 3. Specifically, I assessed the effects of increasing the above cytokines (TNFα, IL-6 and, IL-1), using a model of systemic infection (Spencer et al., 2007), on both functional and histopathological outcome following MCAo.

1.4.4 Infection and Stroke

Following ischemia there is significant activation of the peripheral immune system (Offner et al., 2006; Offner et al., 2009). Anti-inflammatory cytokines such as interferon-γ and IL-2, are initially upregulated (Offner et al., 2006). Within hours, however, the cytokine profile is switched and there are significant increases in serum proinflammatory cytokines (TNFα and IL-6) and a reduction in anti-inflammatory cytokines (Offner et al., 2006). This switch may leave an individual susceptible to developing an infection. As such, there is considerable evidence that infections

commonly occur in patients following stroke (up to 35%) (Chamorro et al., 2007; Emsley and Hopkins, 2008). To further complicate matters, stroke-associated infections have also been associated with a poorer long-term outcome (Emsley and Hopkins, 2008; Hong et al., 2008).

Interestingly, there is substantial evidence that infection is a significant risk factor for stroke and antecedent infections may also result in poorer long-term stroke outcome (Bova et al., 1996; Macko et al., 1996; Grau et al., 1998). The interpretation of the results from preceding and post-stroke infections is complicated by the febrile response associated with infection. Fever is associated with significantly poor neurological scores on admission and poorer outcome following stroke (Grau et al., 1999). Further, in the vast majority of patients assessed by Grau and colleagues (1999), presenting with a fever was associated with a positive test for an infection. It is therefore difficult to determine the role of infection by itself, independent of a febrile response, on stroke outcome. Fortunately, experimental studies are able to control for this confound. One study elegantly measured post-ischemic temperatures following peripheral inflammation (Spencer et al., 2007). In this study, independent of a significant febrile response, there was an acceleration of cell death and an increase in functional deficits at 3-days post-ischemia. Data from this study emphasize the importance of controlling stroke-associated infections, whether they occur antecedent to or post-stroke. Consequently, I examined the differential effects of either preceding inflammation or clinically-relevant post-ischemic systemic inflammation (model of infection) on functional and pathological outcomes (Chapter 3).

1.5 Rehabilitation Therapies Following Experimental Ischemia

With the exception of tPA and hypothermia (Papadopoulos et al., 1987; Colbourne and Corbett, 1994), neuroprotective strategies have not been successfully translated from experimental studies to treating clinical stroke. The most efficacious treatment in dealing with the long-term consequences of stroke is rehabilitation (Robertson et al., 1997; Ploughman and Corbett, 2004; DeJong et al., 2005). Experimental studies corroborate clinical data, and have the additional advantage of revealing the underlying mechanisms associated with the positive effects of rehabilitation. One of the demonstrated benefits of rehabilitation has been the 'rewiring' of the brain to reduce functional deficits (Nudo et al., 1996; Biernaskie and Corbett, 2001). One example of this is the demonstration of cortical neurons in the contralesional hemisphere undergoing increased dendritic sprouting and arborization in response to enriched rehabilitation (Biernaskie and Corbett, 2001). More importantly, in this study the increases in dendritic complexity were associated with improved functional outcome. Additionally, the timing of rehabilitation following stroke has proved to be instrumental in recovery. Studies have shown that initiating rehabilitative treatment within the first week following ischemia is more efficacious than delaying the treatment until 30-days post-ischemia (Biernaskie et al., 2004). This also holds true for long-term rehabilitative treatment, even when more intense therapy regiments are implemented, limited recovery occurs at later time points (Clarke et al., 2009). These experimental results closely model clinical findings where earlier and more intense rehabilitation provides better stroke outcomes (DeJong et al., 2005).

1.5.1 Neuronal Plasticity

It is evident that the brain is highly plastic (Kolb, 2003). For example, neuronal plasticity is apparent in normal daily functioning as demonstrated by learning and memory (Bliss et al., 2007; Gould, 2007). New proteins are synthesized on an ongoing basis and these proteins help in the formation of new connections throughout the brain. Although the entire underlying molecular and neuroanatomical basis for neuroplasticity is not yet defined, many of these mechanisms are starting to be discovered.

Following ischemia, there is an upregulation of numerous restorative proteins (e.g. brain-derived neurotrophic factor (BDNF) & phosphorylated cyclic AMP response element binding protein (pCREB)), similar to the pattern of protein upregulation observed during early brain development (Jessell and Sanes, 2000). Interestingly, these proteins have also been linked to recovery of motor function following ischemia. In one study, antagonism of BDNF resulted in abolishment of the efficacy of rehabilitation, thus emphasizing the importance of this growth factor in the recovery of function mechanisms (Ploughman et al., 2009). Because hemiparesis is such a common physical manifestation of stroke (Mayo et al., 2002), many of the experimental research efforts have focused on motor dysfunction following ischemia. As mentioned above, this has led to several advances in our understanding about the importance of timing and intensity of post-stroke rehabilitation (Biernaskie and Corbett, 2001; Biernaskie et al., 2004).

1.5.2 Cognitive Rehabilitation

Although the physical consequences of stroke are evident and commonly treated by rehabilitation, the cognitive deficits (i.e., spatial neglect, deficits in attention, learning, and working memory) often go unnoticed and untreated. It is, however, the cognitive deficits that are reported to be the most detrimental to the patient, interfering with post-stroke rehabilitation efforts (see below) as well as reintegration into the community (Barrett et al., 2006). As a result, the presence of cognitive deficits has a major impact at an individual, social, and economic level. Thus, research to understand and treat cognitive deficits is critical to improving stroke outcome and burden. Deficits in attention and executive control of attention (e.g., working memory) are commonly associated with vascular cognitive impairment and stroke (Rockwood, 2002) and create significant difficulties in recovering function and in responding to rehabilitation. In fact, the severity of attention dysfunction is directly associated with recovery in physical abilities, such as upper and lower limb function (Robertson et al., 1997; Cirstea et al., 2006). To complicate matters, there is limited evidence for effective treatment and no standard care guidelines to help ameliorate attention deficits. Recent studies have provided some evidence for the positive effects of attention training in healthy older individuals (Basak et al., 2008) and in those with stroke (Sturm et al., 1997; Sturm et al., 2002; Barker-Collo et al., 2009; Tang and Posner, 2009). However, further research is needed to determine whether these positive gains can be transferred to the re-learning of physical skills and other functional abilities (Lincoln et al., 2000).

Chapter 4 of this thesis describes an animal model of cognitive rehabilitation that uses a combination of physical and cognitive activity. An attempt was also made to relate cognitive outcome to changes in BDNF which has been implicated in learning and memory and recovery of function following stroke (Gomez-Pinilla et al., 2001; Gomez-Pinilla et al., 2008; Ploughman et al., 2009). One difficulty with human studies of 'normal' participants is the lack of transfer from the trained cognitive task to other cognitive domains or situations. This was one area of consideration taken into account in the development and assessment of the cognitive rehabilitation model outlined in Chapter 4.

1.6 Behavioural Paradigms Used in this Thesis

1.6.1 Tests of Cognition

Although the physical consequences of stroke are evident and commonly treated by rehabilitation, the cognitive deficits (i.e., executive function, learning, memory, attention) often go untreated. It is imperative that animal models of stroke incorporate assessments of cognition in order to better validate the clinical condition. Accordingly, multiple tests of learning, working and reference memory were used in this thesis and are outlined below.

1.6.1.1 Morris Water Maze

The Morris water maze (MWM) was developed as an aversively motivated water-based assessment of spatial learning and memory (Morris et al., 1982). Depending on the paradigm, animals use either proximal or distal spatial cues to locate a submerged platform. The hippocampus is instrumental in locating the hidden platform, especially with respect to distal cue place-learning paradigms (Morris et al., 1982; Whishaw, 1987; Morris et al., 1990). Acquisition of information from the Morris water maze is thought to involve procedural, working, and reference memory (Save and Poucet, 2005). Procedural memory is required in order for the rat to acquire information about swimming and that it must locate a hidden platform and climb onto the platform in order to be removed from the maze (i.e., the maze procedure). Working memory is required to remember the spatial location of the platform from one trial to the next, based on distal cue locations (i.e., a short-term memory store while the animal is working or using the information). Reference memory is required for the rat to store, retain, and retrieve the spatial platform location from one day to the next (i.e., long-term memory storage of information).

Many different MWM paradigms have evolved over the years to assess learning and memory. Similarly, different procedural paradigms of varying difficulty were used in this thesis (Chapter 2) in order to either challenge the animals' learning and memory capacity or to assess different aspects of learning and memory. For example, one can examine the rat's reference memory abilities by keeping the platform location constant over trials and days of testing. A decrease in latency to locate the platform location over days would indicate the long-term storage, retention, and retrieval of information (Morris

et al., 1982). However, if the platform location is altered each day, the previous day's location information would no longer be accurate and new information would need to be acquired. Tasks that use this paradigm would assess an animal's working memory abilities because a new platform location has to be acquired each day, although this information stays the same over trials on each day (Morris et al., 1986). A decrease in latency to locate platform location during trials each day in this paradigm would indicate intact working memory abilities.

There are numerous procedural considerations to take into account when using the MWM to assess learning and memory, all of which can affect outcome measures. Water temperature (Sandi et al., 1997), start and platform locations (Save and Poucet, 2005), number of trials (Hartman et al., 2005) and length of testing (Morris, 2007) are all important maze configuration considerations. Rats can 'solve' the MWM using various strategies, and researchers must therefore be cautious and observant when implementing this task.

1.6.1.2 T-Maze

The T-maze was originally developed in 1925 by Edward Tolman (Tolman, 1925). He was the first to describe rats' tendency to alternate arm choices between trials when exposed to a T-maze. This behaviour is thought to have evolved from an optimization of rats' foraging behaviour so that they do not enter an arm where the food storage may have been depleted (Save and Poucet, 2005). When used as an appetitively motivated task the rat is placed in the start box at the base of the maze and allowed access

to the maze. Rats will then run up the stem/alley and enter one of the arms to receive a reward. On the next trial the animal is granted access to the maze and will normally enter the opposite arm (arm not previously visited) to receive the reward. This is the normal behaviour of the rat and will occur in the absence of a reward (spontaneous alternation). This maze has been used in studies of working memory because the rat must remember information from the previous trial. Increasing the delay between train and test trials (e.g. 15 s to 5 min) increases the difficulty of the paradigm by taxing the working memory system (Dudchenko, 2004). There are several paradigms of the T-maze that take the form of either non-matching-to-place or matching-to-place paradigms. The non-matching-to-place paradigm ('win-shift') was described above. The rat is reinforced to enter the non-entered arm on the previous trial, a behaviour thought to be naturalistic and therefore more easily acquired (Save and Poucet, 2005). In the matching-to-sample paradigm ('win-stay'), rats are required to enter the arm previously entered. That is to say, if a rat enters the left arm on trial 1, then it must enter the left arm on the next trial. This behaviour is not natural to the rat and is therefore more difficult and requires more trials to be acquired (Langdon et al., 2008). Studies of rodent hippocampal injury resulting from global ischemia have used this test to assess working memory (Colbourne and Corbett, 1995; Farrell et al., 2001). In order to further investigate the working memory capacity of animals, a delay between training and test trials can be implemented of up to 5 minutes where the animal has to hold in working memory the choice on the previous trial. This has also been shown sensitive at detecting hippocampal damage (Colbourne and Corbett, 1995).

1.6.1.3 Radial Arm Maze

The radial arm maze (RAM) was also developed to assess rats' working and reference memory abilities (Olton and Samuelson, 1976). This maze consists of a number of elevated (most often 8) runways/arms that may or may not be covered with transparent Plexiglas® walls. A food pellet is usually placed at the end of the runways to motivate animals to explore the maze (appetitive reinforcement). As with the T-maze, due to rodents' natural foraging habits, they are more likely to enter an arm not previously entered. Over time, rats will successfully learn to navigate the maze error-free. That is to say, they will use either, or both, intramaze (scents) or extramaze (room) cues to remember arms previously or not previously entered. The 8-arms baited configuration of the RAM, where all 8 arms are baited, taxes rats' working memory abilities in that they have to remember either what arms they have previously entered or not entered within a given trial. This changes each day and the rat is not required to remember arm choices between days.

In a different configuration of the RAM, however, fewer arms may be baited. As used in this thesis (Chapters 2 & 4), one may bait only 4 (or some combination thereof from 1-7) arms. If these arms remain baited throughout testing, the rat must remember not only the arms visited on each particular trial (working memory), but must also learn and remember the location of the 4 baited arms that remain unchanged over trials (reference memory). Over time, rats learn both strategies indicated by a reduction in the number of working and reference memories over trials.

1.6.2 Tests of Motor Function

Weakness, or hemiparesis, of the body contralateral to the lesion is the most common deficit after stroke with over 50% of stroke patients suffering from residual motor impairment (Mayo et al., 2002). It is therefore important that tests of both upper and lower extremity (limb) function be developed in animal models in order to reflect the most commonly observed motor impairments following clinical stroke. Several of those motor tasks were used in the current thesis and are outlined below.

1.6.2.1 Beam Traversing Task

The beam task is used to assess both forelimb and hindlimb coordination (Feeney et al., 1982). In this task, rats are required to traverse an elevated beam to enter the safety of a darkened chamber. Rats with CNS motor damage will exhibit foot faults where either the forepaw or hindpaw, or both, slips off the beam. Therefore, forelimb and hindlimb paw slips are recorded as dependent measures. Different beams have been developed and used to assess motor function following various types of CNS injury (Schallert and Woodlee, 2005). These include flat or tapered beams with or without ledges (Schallert et al., 2002; Schallert and Woodlee, 2005; Clarke et al., 2009).

1.6.2.2 Staircase Test

The staircase test was developed by Montoya and colleagues (Montoya et al., 1991) to assess skilled reaching abilities. Animals are required to climb onto a central

platform within a Plexiglas® rectangular container. Food pellets are placed on 7 steps (3 pellets/step) on each side of the central platform where the pellets can only be retrieved by the ipsilateral forepaw (Montoya et al., 1991). This is important because following injury the contralateral limb is affected due to the descending cortical innervation of the opposite limb. Animals, like humans, will try to compensate by using their ipsilesional paw ('good' paw) to retrieve the pellets. With extensive training, animals are able to achieve a consistent level of performance of generally 17-18 pellets out of a possible 21. Although this test requires substantial training (generally 2-15 minute trials/day for at least 10 days), when animals are well trained on the staircase task, persistent deficits in skilled reaching forelimb function can be detected many months following injury (Biernaskie and Corbett, 2001; Clarke et al., 2009) indicating the extreme sensitivity of this task and most importantly, reflecting the persistent deficits that are characteristic of human stroke.

1.6.2.3 Cylinder Test of Forelimb Asymmetry

The cylinder test examines innate asymmetries in forelimb function following brain injury (Schallert and Woodlee, 2005). Rats are inquisitive animals and when placed in a novel environment will begin to explore. In this test, animals are placed in a Plexiglas® cylindrical tube and simply observed. Because the cylinder is a relatively small environment (20 cm diameter), animals will rear and place forepaws on the walls of the transparent cylinder. A normal rat without damage to the motor cortex will use both left and right paws equally to support itself while rearing. However, an animal with

damage to the forelimb motor system will use the ipsilesional paw more frequently than the affected contralesional paw (Schallert and Woodlee, 2005; Schallert, 2006). As mentioned above, this test has the advantage of assessing animals' natural rearing behaviour and does not require extensive training as in the staircase test. Further, the test is relatively easy to administer and score and has a high interrater reliability (Tillerson et al., 2001). One disadvantage of this test, however, is that although there are profound deficits immediately following injury, significant spontaneous recovery occurs and can make the identification of sustained, long-term treatment effects difficult to demonstrate (Hicks et al., 2008).

1.7 Overview of Experimental Chapters

1.7.1 Chapter 2

This chapter describes the development and validation of a behavioural assay sensitive for detecting long-term functional deficits following global ischemia. In contrast to previous studies, permanent, sustained cognitive deficits were evident 270 days post-ischemia, a time point well beyond those typically used. Further, these behavioural deficits were associated with significant neuroinflammation, a time point (i.e., 9 mo) when inflammation is normally thought to have subsided. The data from this chapter has been presented in the paper "Persistent behavioral impairments and neuroinflammation following global ischemia in the rat", published in *European Journal of Neuroscience,* 28: 2310-2318 (2008) by KD Langdon, S Granter-Button and D Corbett.

1.7.2 Chapter 3

This chapter builds upon the findings of long-term neuroinflammation in Chapter 2. A model of prolonged infection either prior to or 24-hours post-ischemia produced increases in markers of neuroinflammation and histopathological damage at both early (3 days) and long-term (30 days) survival times post-ischemia. This histopathological profile was accompanied by severe deficits in sensorimotor abilities at 7 and 30 days post-ischemia. Interestingly, the histological damage and functional deficits were significantly more pronounced in the 24-hour delayed treatment condition. This chapter is based on the manuscript "Prolonged, 24-hour delayed peripheral inflammation increases short- and long-term functional impairment and histopathological damage following focal ischemia in the rat" published in the *Journal of Cerebral Blood Flow and Metabolism* (2010, in press) by KD Langdon, CM MacLellan and D Corbett.

1.7.3 Chapter 4

This Chapter describes the effects of the combination of physical and cognitive activity on learning and memory outcome. This chapter is also an extension of Chapter 2 where the need for interventions (e.g. cognitive rehabilitation) to improve cognitive function and to ameliorate cognitive deficits following ischemia was identified as a priority. Clinical studies have demonstrated that cognitive training improves abilities within the specific cognitive domain being trained but there is limited data on the transfer of the positive effects to other cognitive domains. A combination of 2 hours of physical activity with 2 hours of cognitive activity resulted in significant improvements in working

memory abilities as assessed in both the 8- and 4-arms baited configurations of the RAM. These data are particularly interesting because the improvements were in a different cognitive domain (working memory in RAM) than the cognitive rehabilitation condition (i.e., Hebb-Williams maze exposure). This chapter is based on the manuscript titled "Improved working memory following novel combinations of physical and cognitive activity" currently in preparation to be submitted to the *Journal of Neuroscience* by KD Langdon and D Corbett.

CHAPTER 2: PERSISTENT BEHAVIOURAL IMPAIRMENTS AND NEUROINFLAMMATION FOLLOWING GLOBAL ISCHEMIA IN THE RAT

2.1 Introduction

Although the clinical consequences of cardiac arrest have been thoroughly documented (e.g. memory problems) (Zola-Morgan et al., 1986; Bartsch et al., 2006), deficits have not been well characterized in animal models of global ischemia. Indeed, the magnitude and type of deficit appear to vary considerably (Nunn and Hodges, 1994; Corbett and Nurse, 1998; Block, 1999) depending on the behavioural tests, the animal model used, and survival times employed. For example, open field and T-maze appear to be the most sensitive tests for detecting CA1 global ischemia injury in the gerbil (Colbourne and Corbett, 1995; Babcock and Graham-Goodwin, 1997; Farrell et al., 2001), whereas the Morris water maze (MWM) has been widely used in assessing global ischemia-associated deficits in both 2-and 4-vessel occlusion models in rats (Nunn and Hodges, 1994; Block, 1999). In studies using these latter models CA1 cell loss ranges from 30-80%, and frequently the MWM conducted within the first days or weeks after ischemia is the only test used. Variation in task difficulty along with these factors likely account for the inconsistencies in behavioural impairment reported in global ischemia studies (Auer et al., 1989; Nunn and Hodges, 1994; Corbett and Nurse, 1998; Nakatomi et al., 2002; Hartman et al., 2005).

Another important consideration is that in recent studies using longer survival times of three or four months, ischemic animals exhibit less CA1 cell loss (40-50% of control values) than at survival times of 2-3 weeks (Nakatomi et al., 2002; Bendel et al., 2005; Hartman et al., 2005). It has been proposed that CA1 cells may, under the right circumstances, repopulate the affected area (without exogenous intervention), become integrated in the hippocampal circuitry, and contribute to functional recovery (Bendel et al., 2005; von Euler et al., 2006). Obviously, the potential for repopulation complicates the interpretation of global ischemia behavioural studies because many studies only report short-term (e.g. 2-3 weeks) functional assessments without long-term follow up. Further, in the studies noting CA1 repopulation it is unclear whether the "behavioural recovery" was due to neurogenesis or spontaneous recovery especially since these studies relied heavily on the MWM which may not be sensitive to detecting residual deficits after ischemia (Corbett et al., 1992).

Consequently it seemed imperative to employ a more comprehensive battery of cognitive assessments over a much longer time period than previously employed. In view of the possibility that newly formed CA1 neurons may make detection of behavioural deficits more difficult, we varied the task difficulty of some of the tests to maximize the likelihood of detecting even subtle impairments. We report here the results of such an analysis in which both short- (16 days) and long-term (~270 days) histological and immunohistochemical analyses were combined with long-term assessments of learning and memory ability in rats exposed to global ischemia.

2.2 Materials and methods

2.2.1 Subjects

Forty male Sprague-Dawley rats (Charles River, PQ, Canada) weighing approximately 250 g at the time of surgery were used in this study. Animals were pair-housed and maintained on 12:12 h light-dark cycles with *ad libitum* access to food and water unless otherwise indicated. All procedures were approved by the Memorial University Animal Care Committee and conformed to the Canadian Council on Animal Care guidelines.

2.2.2 Induction of global ischemia

Global forebrain ischemia was induced by occluding (10 min, n=26) both common carotid arteries (CCAs) combined with systemic hypotension (2-VO) (Hartman et al., 2005). Animals were anesthetized with isoflurane (4.0% induction, 2.0% maintenance) in a 30/70% O_2/N_2O mixture. A tail artery was catheterized to continuously monitor mean arterial blood pressure (MABP), to measure blood-gases (pO_2, pCO_2 and pH), and to exsanguinate blood to induce hypotension (40-45 mmHg) (Table 2.1). Tympanic temperature was maintained at ~37.3 ± 0.3°C. Blood-gas samples were taken prior to, during, and 10-min post-occlusion. Sham surgery (n=14) consisted of identical surgical conditions, including systemic hypotension, except for CCA occlusion.

2.2.3 Experimental groups

Twenty-one rats were used for the short-term histological assessment (7 sham and 14 ischemic animals) and 16 animals were used for the long-term behavioural and histological assays (7 sham and 9 ischemic animals). Three ischemic animals were excluded due to surgical (n=2) or post-operative (n=1) complications.

2.2.4 Behavioural assessment

2.2.4.1 Morris Water Maze

The behavioural assay and timeline used in this study is presented in Figure 2.1. Three paradigms of the Morris water maze were used in this study similar to published reports (Hartman et al., 2005). Animals were habituated to the water (22°C) before the 'easy' and 'difficult' paradigms using a visible platform paradigm (4 trials with the visible platform in a new location on each trial). In all other MWM paradigms the platform (100 cm^2) was submerged 2.5 cm below the surface of the water. Animals were tested in a relatively 'easy' water maze paradigm at 10 days and 28 weeks post-surgery (different platform location and room cues). This paradigm consisted of four trials/day (with varied start points) over four consecutive days (acquisition). After locating the platform on each trial, animals remained there for 30 s and were then removed from the pool (180 cm diameter) for 60 s. A probe trial (platform removed) was conducted on day 5 (60 s) and a re-test one-week later for long-term memory retention. Latency to locate the platform, swim speeds and number of platform crosses (probe trial) were recorded.

Surrounding the pool were numerous salient extramaze cues in order to facilitate the ease of locating the hidden platform. A 'difficult' Morris maze paradigm was used at 26 weeks post surgery, where the majority of extramaze cues were removed from the room walls. Further, in order to increase the difficulty of this paradigm, animals were trained for only two trials/day for five consecutive days (Hartman et al., 2005). Animals remained on the platform for 10 s and were then removed from the maze for 30 s. A probe trial and re-test were conducted as with the easy paradigm. In the third paradigm (32 weeks post-surgery) the platform was moved to a new location each day (platform reversal). Animals were tested for 4 consecutive days, 4 trials/day and remained on the platform for 15 s with a 30 s intertrial interval.

2.2.4.2 T-Maze

Four weeks after surgery animals were tested in a 'forced-choice' win-shift T-maze (Colbourne and Corbett, 1995; Farrell et al., 2001). Rats were food restricted to approximately 90% *ad libitum* body weight and acclimated to the apparatus (47 cm stem; 30 cm arms; 10 cm wide) for 2 consecutive days with food pellets (TestDiet AIN-76A Rodent Tablet, 45 mg, Richmond, IN) scattered throughout the maze. The following day animals were forced to choose either a right or left arm (opposite arm blocked by a guillotine door; equal number of left and right forced choices) to access the food reward. Animals were then immediately removed from the arm, placed into the start box for 15 s, and then had access to the entire maze. Animals received 10 trials/day with a minimum of 5 min between trials until a criterion of 85% correct (choosing the opposite arm) on 3

consecutive days was reached. Animals' choice responses (arm entered) and latency were recorded. When animals reached criterion on the 15 s delay, the delay between forced-choice and test-choice was increased to 60 s (criterion of >85% correct on 2 consecutive days, 6 trials/day), 3 and then 5 min (criterion of >85% correct on 2 consecutive days, 4 trials/day). Furthermore, on each day of testing all animals were tested with a 15 s delay between the forced-choice and test-choice for the first 2 trials and last 2 trials to ensure memory retention for the goal of the maze. Nine weeks after surgery, animals were tested in a forced choice 'win-stay' version of the T-maze. This was similar to the win-shift, 15 s delay-paradigm, except animals were required to choose the arm previously baited on the forced-choice trial (Colbourne and Corbett, 1995) (same criterion as above).

2.2.4.3 Radial Arm Maze

Two configurations of the radial arm maze (RAM; 70 cm arms; 12 cm wide; 35 cm centre platform; 20 cm clear Plexiglas walls) were used: 8- and 4-arms baited (Olton and Samuelson, 1976). Beginning 15 weeks after surgery, animals were tested in an 8-arms baited configuration similar to that previously reported (Hartman et al., 2005). Briefly, animals were acclimated to the maze for 2 consecutive days with food pellets scattered throughout the maze. During testing, which began on the third day the pellets were restricted to the food cups at the end of each of the 8 arms. Latency to obtain all rewards (maximun of 5 min), number of re-entry errors (working memory error), and number of correct choices on the first eight arm selections were recorded. Animals were tested once per day on consecutive days until they achieved a criterion of <2 errors/trial

on 4 consecutive days (maximum of 25 days). Twenty weeks post-surgery, animals were tested in the 4-arms baited configuration of RAM. Testing parameters were similar to those above, however, only 4 of the 8 arms were baited, and the same arms remained baited throughout testing. All animals were tested for 30 consecutive days and the latency to obtain all 4 rewards, number of re-entry errors (working memory errors), number correct on the first 4 arm selections, and number of entries into unbaited arms (reference memory errors) were recorded. Animals were food restricted to 90% of *ad libitum* body weight throughout testing.

2.2.5 Histology

Animals were transcardially perfused with 0.9% saline followed by ice-cold 4.0% paraformaldehyde (PFA). Animals were decapitated, heads post-fixed for 4 hours at 4°C, the brains removed and placed in PFA overnight at 4°C. Brains were then placed in a 20% sucrose solution in phosphate buffered saline (PBS) at 4°C until saturated, then frozen and stored at -20°C until sectioning.

Coronal sections were cut (14 μm thick for slide-mounted cresyl-violet, GFAP, NG2, OX-42 and ED-1; and 40 μm for free-floating MAP2) at levels corresponding to the rostral and middle hippocampus (bregma -3.80 and -4.70). For immunohistochemistry, sections were washed in PBS, treated with 1.0% H_2O_2, blocked with normal goat serum (5.0%, Jackson Immunoresearch Laboratories, West Grove, PA, USA) and subsequently incubated overnight at 4°C with either polyclonal rabbit anti-glial fibrillary acidic protein (GFAP, for astrocytes, 1:1,000, DakoCytomation, Z0334, Mississauga, ON, Canada),

polyclonal rabbit anti-NG2 (1:150, oligodendrocyte precursor, Chemicon, AB5320, Temecula, CA, USA), monoclonal mouse anti-rat CD11b (OX-42, for microglia, 1:500, Serotec, MCA275G, Raleigh, NC, USA), monoclonal mouse anti-rat CD68 (ED-1, for activated microglia, 1:1000, Serotec, MCA341R), or polyclonal rabbit anti-microtubule associated protein-2 (MAP2, 1:2000, Chemicon, AB5622). The sections were then exposed to either goat anti-rabbit or anti-mouse biotinylated secondary antibodies (GFAP, 1:500; NG2, 1:1,000; OX-42, 1:2,000; ED-1, 1:1,000; and MAP2, 1:2,000; Jackson Immunoresearch Laboratories, Inc.), incubated in 10μg/mL extravadin (Sigma-Aldrich, Oakville, ON, Canada), and reacted for 3-5 min in a 3,3'-diaminobenzidine tablet set (Sigma-Aldrich). Histological procedures were carried out concurrently on all animals of the same survival time in order to minimize variation of staining intensity.

Viable cells within a grid (75 μm X 250 μm) from medial, middle, and lateral sectors of the CA1 region in rostral-hippocampal cresyl-violet stained sections were counted as previously described (Colbourne and Corbett, 1995). ED-1 positive cells were counted as above, on adjacent sections. Relative optical density (ROD) of the CA1 pyramidal layer in a rostral and mid-hippocampal section was used to analyze the remaining stains (GFAP, NG2, OX-42, and MAP2) using the corpus callosum as a background measurement ((OD CA1 - OD corpus callosum) / OD corpus callosum) due to the intensity of these stains. MAP2 staining was also used to assess CA1 atrophy over time (distance from the apex of CA1 to the hippocampal fissure). Data obtained from the left and right hemispheres were averaged and presented as the mean ± SEM.

2.2.6 Statistical Analyses

All data are presented as mean ± SEM. All behavioural and histological assessments were conducted blind. Most behavioural data were analyzed using one-tailed independent t-tests or repeated measures analysis of variance (ANOVA). The T-maze delay data were analyzed using a 2-factor Chi Square. All histological data were analyzed by either an independent t-test (cell counts) or a 2 X 2 (condition X section) ANOVA. To control for multiple t-test comparisons a Bonferroni correction was used. In cases where the homogeneity of variance was violated, the Brown-Forsythe correction factor was used. A probability value of ≤ 0.05 was considered statistically significant.

2.3 Results

2.3.1 Morris water maze

There were no differences between ischemic and sham animals with respect to swim speeds in either testing period ($p>0.05$). As a result, latency to locate the platform was conducted and presented. There was no difference between conditions with respect to latency to locate the hidden platform during the 'easy' paradigm acquisition trials at either 10-days or 28-weeks post-surgery ($p>0.05$; Figure 2.2A and D). Similarly, there were no differences between conditions at either time point with respect to memory abilities as assessed in the probe test ($p>0.05$; Figure 2.2B and E) or the re-test ($p>0.05$; Figure 2.2C and F).

Analysis of the 'difficult' paradigm conducted at 26-weeks post-surgery revealed no effect of day, no interaction, and no difference between conditions ($p>0.05$; Figure 2.3A). However, in the probe trial ($t_{14}=1.849$, $p<0.05$) ischemic animals made significantly fewer crosses (2.67 ± 0.41) than sham animals (3.86 ± 0.51; Figure 2.3B). There was no significant difference between conditions on average latency to locate the platform during the re-test ($p>0.05$; Figure 2.3C).

To analyze the platform reversal test, all trial 1 data, trial 2 data, and so on over four days were averaged, as previously described (Hartman et al., 2005). A repeated measures analysis revealed the latency to locate the platform on subsequent trials decreased ($F_{3,42}=13.74$, $p<0.01$), however there was no interaction and no significant difference between conditions ($p>0.05$) (Figure 2.3D).

2.3.2 T-Maze

Analysis of the win-shift paradigm in the T-maze revealed no differences in the average number of trials to reach criterion between ischemic and sham animals ($p>0.05$; Figure 2.4A). Furthermore, there were no differences in the percentage of animals that completed the delay trials ($p>0.05$). However, in the win-stay paradigm ($t_{11.404}=2.05$, $p<0.05$) ischemic animals required significantly more (169 ± 19.0) trials to meet criterion compared to sham animals (126 ± 9.0 trials) (Figure 2.4B), thus indicating a learning/memory deficit.

2.3.3 Radial arm maze

Analysis of the average number of trials to reach criterion in the 8-arms baited configuration of the RAM revealed a significant difference between ischemic and sham animals (t_{14}=2.18, $p<0.05$). On average, ischemic animals took longer (18 ± 2.0 trials) to reach criterion compared to sham animals (11 ± 2.0 trials) thus indicating a working memory deficit (Figure 2.5A).

The data from the 4-arms baited configuration of the RAM were blocked into five-day bins and are presented as such. Repeated measures ANOVA of the average number of entries into unbaited arms (reference memory errors) revealed a significant effect of blocks ($F_{5,70}$=26.42, $p<0.01$), condition ($F_{1,14}$=5.12, $p<0.05$), and a significant interaction ($F_{5,70}$=2.35, $p=0.05$) (Figure 2.5B). Post-hoc analysis using independent t-tests revealed that the interaction occurred over blocks 5 and 6 (days 21-30) where the ischemic animals made significantly more errors (1.8 ± 0.18 and 1.7 ± 0.22 reference memory errors/day) compared to sham animals (1.0 ± 0.21 and 0.66 ± 0.25 reference memory errors/day). Repeated measures ANOVA of the average number of re-entry errors (working memory) revealed a significant effect of block ($F_{3.861,54.054}$=2.88, $p<0.05$) where animals improved performance over blocks. There was no effect of condition and no interaction ($p>0.05$), although there was a similar plateau in ischemic animals' performance over blocks 5 and 6 compared to sham animals (Figure 2.5C).

2.3.4 Histology

There were no differences between sections (rostral and middle dorsal hippocampus) and no interaction, therefore ROD histological data were collapsed across section and presented as such. Further, there were no differences in background staining in the corpus callosum of sham or ischemic animals in either the short- or long-term conditions. Representative micrographs of the histological and immunohistological stains of sham, short-term (16-day ischemia) and long-term survival (approximately 270 days post-surgery) animals are shown in Figures 2.6 and 2.7. Global ischemia resulted in a significant reduction of CA1 cells at both the short- (34% of sham; $t_{15.346}$=5.915, $p<0.01$) and long-term (59% of sham; $t_{11.079}$=2.796, $p<0.05$) survival periods (Figure 2.6A-D). Further, ROD analyses revealed a corresponding significant reduction in MAP2 staining within the stratum radiatum in the ischemic animals at both 16 ($F_{1,34}$=4.188, $p<0.05$) and 270 days compared to sham animals ($F_{1,28}$=5.656, $p<0.05$) (Figure 2.6E-H).

Analysis of the glial stains (GFAP, OX-42, ED-1, and NG2) revealed significant increases in staining between ischemic and sham animals (Figure 2.7). Global ischemia significantly increased the ROD ratio in GFAP staining at 16 ($F_{1,39.948}$=32.127, $p<0.01$) and 270 days ($F_{1,24.263}$=29.387, $p<0.01$) in ischemic animals compared to shams (Figure 2.6A-D). Similarly, OX-42 staining was more intense in ischemic animals at both time points (16 days – $F_{1,35}$=15.9, $p<0.01$; 270 days – $F_{1,21.101}$=11.8, $p<0.01$) (Figure 2.7E-H). There was also a significant increase in the number of ED-1 positive microglia short-term (t_{10}=4.407, $p<0.01$) and a somewhat attenuated response of ED-1 staining, but nonetheless, a continued elevation of cell number in the long-term survival animals

($t_{8.017}$=3.015, $p<0.05$) compared to shams (Figure 2.7I-L). NG2 staining followed the same pattern of staining as GFAP and OX-42 where staining was more intense in ischemic animals at the 16 ($F_{1,39.9}$=44.126, $p<0.01$) and 270 day ($F_{1,22.063}$=25.397, $p<0.01$) timepoints (Figure 2.7M-P).

Finally, there was significant atrophy in the CA1 area of the 270-day ischemic animals compared to sham animals as assessed from the apex of CA1 to the hippocampal fissure ($F_{1,24.221}$=27.135, $p<0.01$; Figure 2.8). There was no atrophy in the 16-day ischemic animals ($p>0.05$).

2.4 Discussion

Severe and permanent memory impairments characterize patients (e.g. patient R.B.) suffering cardiac arrest with damage primarily to the CA1 sector of the hippocampus (Zola-Morgan et al., 1986). Surprisingly, in rat models of global ischemia with comparable CA1 damage, there is relatively little evidence suggesting that disturbances in learning and memory are persistent. Indeed, many studies report an initial modest impairment in the first days or weeks post-injury characterized by significant recovery to near normal levels by the end of testing (Nunn and Hodges, 1994; Corbett and Nurse, 1998; Block, 1999). Unlike these and more recent studies (Elsersy et al., 2004; Bendel et al., 2005; von Euler et al., 2006; Bueters et al., 2008), our results clearly demonstrate sustained functional deficits lasting up to 8-months post-ischemia. These deficits do not reflect a general decline in cognition over time because ischemic animals perform similarly in 'easier' paradigms of MWM at two and 28 weeks post-surgery,

indicating that behavioural test sensitivity is more likely the explanation for these long-term deficits.

Others have suggested that the use of more challenging versions of the MWM paradigm are sensitive at detecting CA1 injury whereas easier paradigms fail to demonstrate differences (Auer et al., 1989; Nakatomi et al., 2002; Hartman et al., 2005). However, even this claim is not without controversy. One group has reported that ischemic animals are impaired on an 'easy' version of the MWM 14-days post-ischemia, but the difference subsides at 90- or 125-days post-ischemia (Bendel et al., 2005; von Euler et al., 2006), a time when there was significant repopulation of CA1 pyramidal cells. These data suggest that the new neurons were responsible for the observed behavioural recovery. In a subsequent study by the same authors there were similar numbers of CA1 cells at 14- and 250-days post-ischemia although the learning deficits at 250 days are relatively minor and there are no reported memory deficits at this time compared to earlier reports (Bendel et al., 2005; von Euler et al., 2006). A possible explanation for this is that the repopulated cells in the CA1 pyramidal layer eventually die but initially contribute to a functional reorganization of learning and memory function akin to the reorganization of motor maps in the penumbral cortex following focal ischemia (Nudo et al., 1996; Dancause et al., 2005; Williams et al., 2006).

Another explanation for the apparent recovery of learning and memory behaviour is that the water maze tasks selected do not have sufficient sensitivity to detect differences between ischemic and sham animals. The data reported in this study and others (Auer et al., 1989; Hartman et al., 2005) suggest that increasing the difficulty of the MWM by reducing the number of salient extramaze cues, reducing the number of trials each day,

increasing the intertrial interval to 5 or 10 min, or using a non-matching to position paradigm are required to identify learning and memory deficits in the 2-VO model. Indeed, data from the MWM is often difficult to interpret because different search strategies can be used in the maze following hippocampal injury (Kiyota et al., 1991), other brain areas play an important role in solving the paradigm (Gerlai et al., 2002), and recovery can occur (Corbett et al., 1992).

In view of the limitations of the MWM task we used a battery of both aversively and appetitively motivated cognitive tasks of increasing difficulty, rather than a single test, to reveal long-term functional deficits arising from global ischemia. Accordingly, we found working and reference memory deficits in ischemic animals in the RAM and a learning/memory deficit in the T-maze (win-stay). Because T-maze and RAM are extremely time consuming and labour intensive they are often avoided in studies of learning and memory. However, as reported here and elsewhere (Volpe et al., 1984; Volpe et al., 1988; Colbourne and Corbett, 1995; Farrell et al., 2001; Hartman et al., 2005), these appetitively motivated tasks are more sensitive in detecting global ischemia-induced deficits than water maze tasks.

With respect to our histological findings, we are among the first (Mudrick and Baimbridge, 1989; Bueters et al., 2008) to report on the long-term neuropathological sequelae following global ischemia. We found a significant increase in all glial markers assessed (astrocytes, microglia, and NG2-positive glia) at both the 16-day and 270-day survival periods. However, the pattern of staining within the CA1 area changed between 16 and 270 days. Glial staining in the 16-day ischemic animals was uniformly expressed throughout the CA1 area (stratum oriens and radiatium, and pyramidal layer; Figure 2.7),

whereas 270-day ischemic animals exhibited glial staining concentrated only within the pyramidal layer. We found significantly fewer CA1 cells at both 16-days and 270-days post-ischemia, consistent with others (Bueters et al., 2008), with a corresponding reduction in MAP2 staining at both time points and CA1 atrophy in the long-term ischemic animals. Importantly, there was a strong trend for an increased number (~25%) of CA1 cells surviving at 270 days (Figure 2.6 D), although our MAP2 immunohistochemical data suggest that these cells do not have normal dendritic processes and possibly do not function 'normally'. This may reflect a partial, albeit abortive, repopulation of the CA1 region in response to the original injury as noted recently by Bueters and colleagues (Bueters et al., 2008). This interpretation is supported by the finding that ED-1 staining was robust at day 270, a time well beyond the point when the inflammatory response should have subsided. This suggests an ongoing injury process with relatively late cell death. There is evidence that neuroinflammation increases both functional and histological damage in models of neurotrauma (McColl et al., 2007; Spencer et al., 2008) and may have contributed to the lasting functional deficits in this study. While it is possible that the extensive testing regime could have encouraged CA1 neurogenesis, this seems unlikely because individual testing of a particular animal required less than five min of testing each day.

Assessments of animals' learning and memory abilities at 28- and 32-weeks post-surgery using easy and moderately difficult paradigms of the MWM revealed no differences between conditions in these tasks. However, in a 'difficult' version of the MWM at 26-weeks post-surgery there was a significant memory deficit in the probe trial where ischemic animals crossed the platform zone fewer times than sham animals.

Furthermore, in the re-test trial ischemic animals required twice as much time compared to sham animals to reach the platform, although this difference was not statistically significant. In contrast, the more sensitive RAM and T-maze testing paradigms were able to reveal subtle learning and memory differences in this model of global ischemia. Clearly the MWM should not be used by itself for assessing impairment and recovery of cognitive function after ischemia.

In conclusion, we report for the first time that rats exposed to 2-VO experience lasting impairments in learning and memory abilities as revealed using a battery of challenging cognitive assessment paradigms. Surprisingly, following global ischemia there is a significant neuroinflammatory response within the CA1 area that is sustained up to 270-days post-injury. Importantly, the present results describe a profile of long lasting cognitive impairments after 2-VO that is consistent with the deficits reported in humans following resuscitation from cardiac arrest (Zola-Morgan et al., 1986). It remains to be determined if the transitory repopulation of CA1 neurons can be enhanced and maintained and in so doing effect a more robust recovery after this type of brain insult.

Table 2.1. Physiological Variables During Surgery

Condition	pH	pCO_2[§]	PO_2[§]	MABP[§]	Temp. (^{O}C)
Sham (n=14)	7.42 (.02)	40.4 (1.9)	123.6 (4.7)	43.4 (.52)	37.4 (.02)
Ischemia (n=23)	7.43 (.01)	38.7 (1.0)	113.7 (3.9)	43.4 (.34)	37.3 (.04)

Data represent mean ± SEM. There were no differences between conditions in any of the physiological variables assessed. [§] Units: mmHg

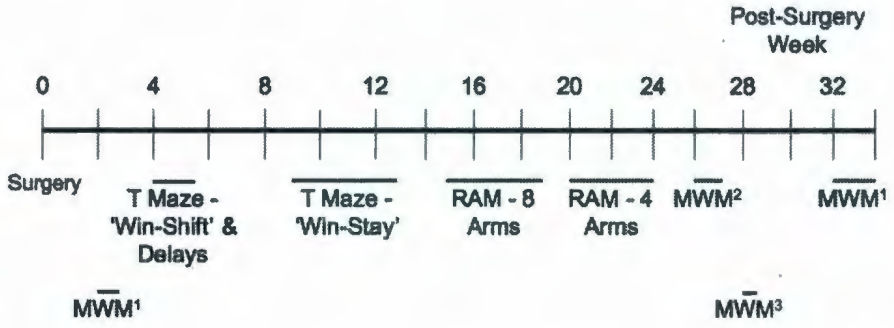


Figure 2.1 Timeline for behavioural testing. Numbers indicate weeks post-surgery. Morris water maze paradigm 1 (MWM^1): four trials per day for 4 days with 30 s on the platform and numerous, salient extramaze cues. MWM^2: two trials per day for 5 days with 10 s on the platform and a reduced number of extramaze cues. MWM^3: four trials per day for 4 days with 15 s on the platform and numerous, salient extramaze cues. The platform was moved to a new location on each of the 4 days. RAM, radial arm maze.

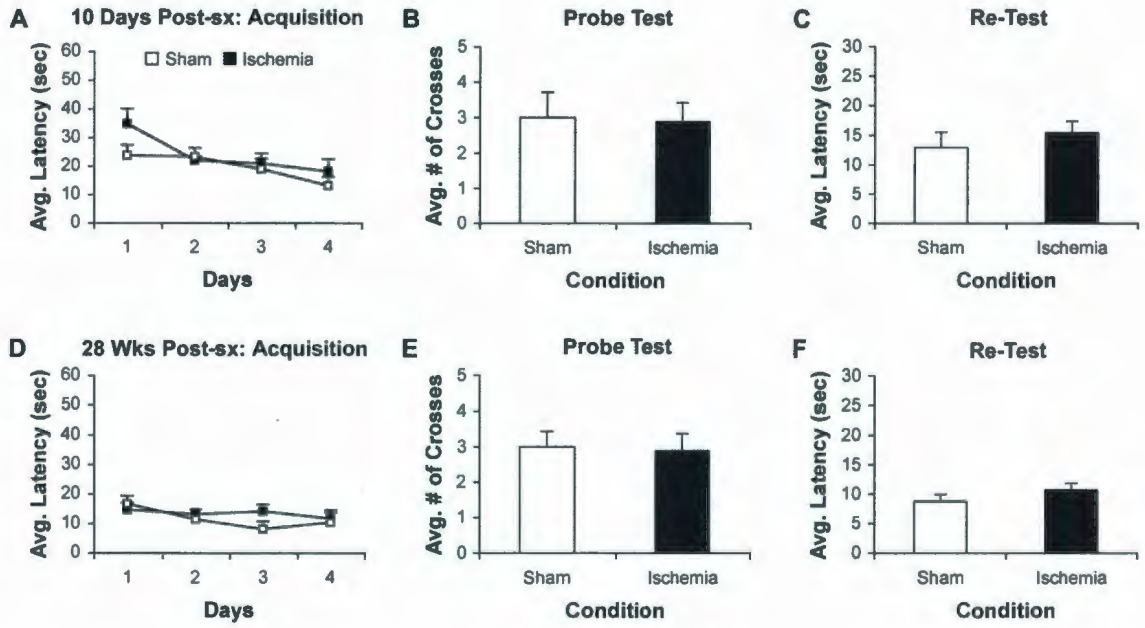


Figure 2.2. 'Easy' Morris water maze paradigms assessing animals' learning and memory abilities at 10 days (A-C) and 28-weeks post-surgery (D-F). Data represent means ± SEM (sham n = 7; ischemia n = 9; animals were used in all behavioural assessments, Figures 2.2 – 2.5). There were no significant differences between conditions at either time point with respect to learning (A and D) or memory abilities as assessed in the probe (B and E) and re-test (C and F) trials.

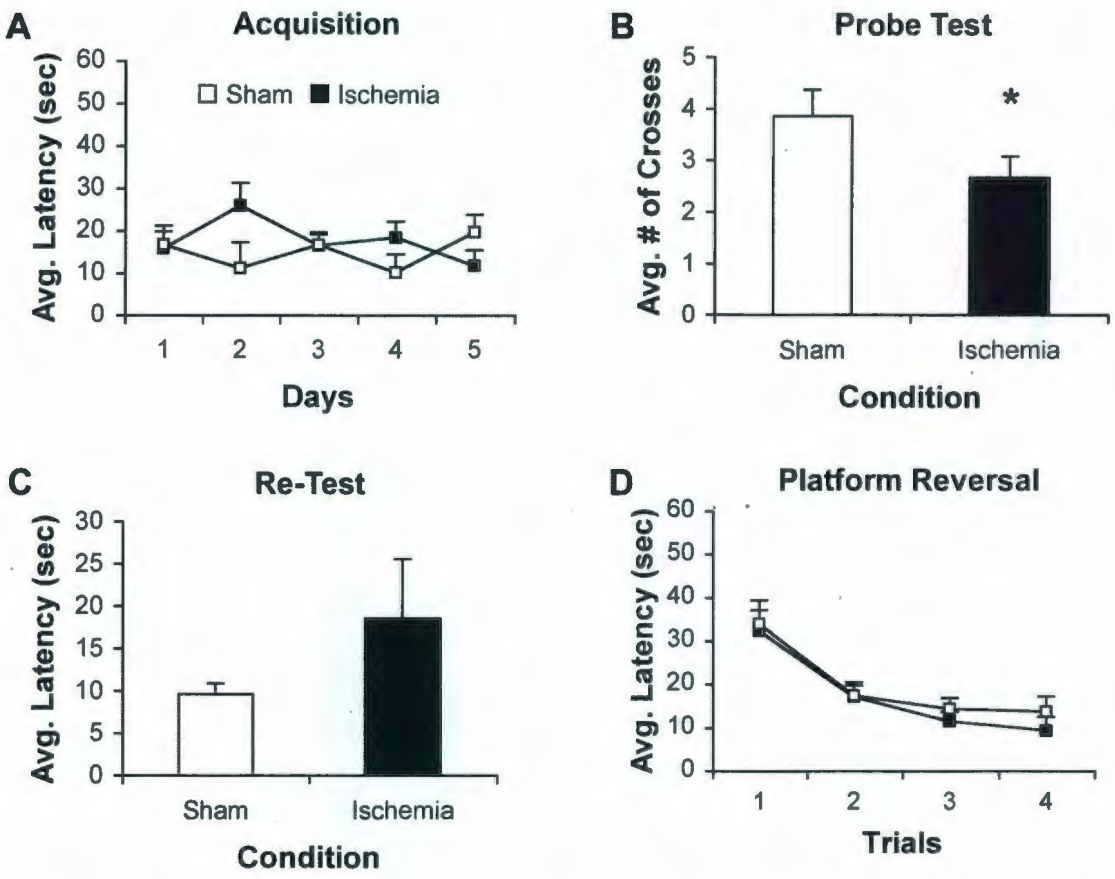


Figure 2.3 'Difficult' Morris water maze paradigms assessing animals' learning and memory abilities at 26- (A-C) and 32-weeks post-surgery (D). Data represent means ± SEM. There was no difference between conditions with respect to learning abilities (A), but ischemic animals had significantly fewer platform crosses (*$p<0.05$) than sham animals in the probe trial (B). There was no difference between conditions in the re-test (C) or in working memory abilities as assessed in the platform reversal paradigm (D).

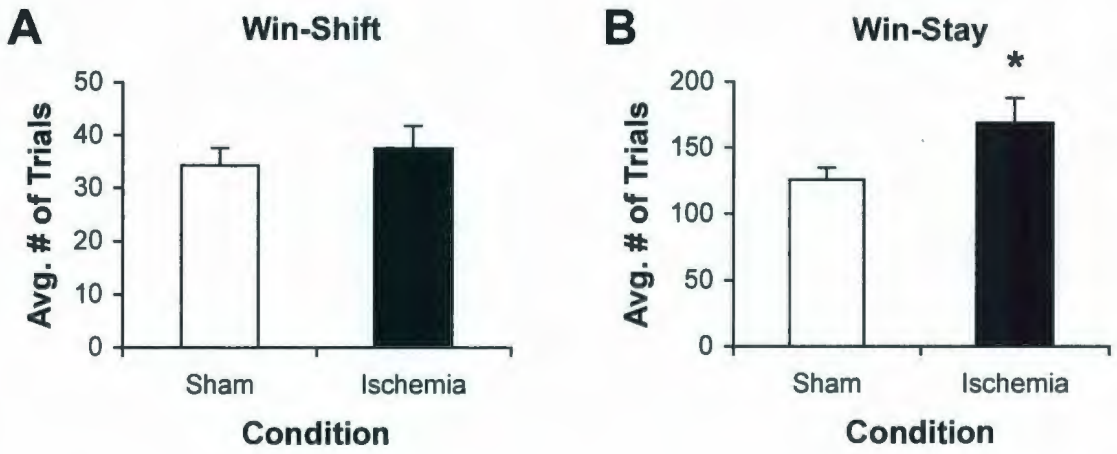


Figure 2.4. Performance in the T-maze. Average (mean ± SEM) number of trials to reach a criterion of 85% over 3 consecutive days in the T-maze. There was no difference between conditions in the number of trials to reach criterion in the 'win-shift' paradigm (A). However in the 'win-stay' paradigm (B), ischemic animals required significantly (*$p<0.05$) more trials to reach criterion than sham animals.

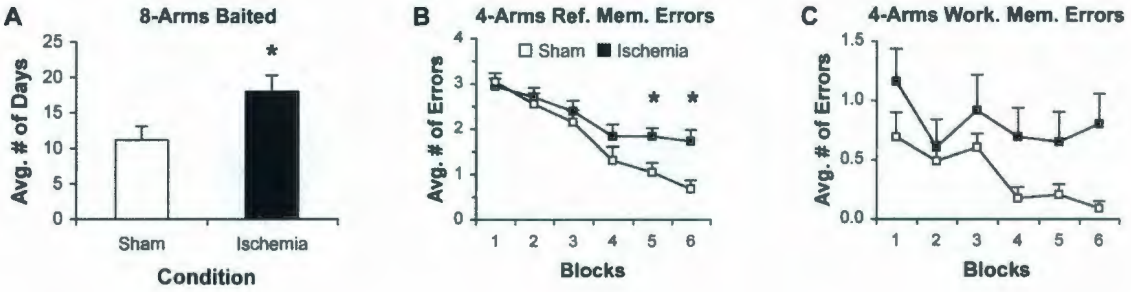


Figure 2.5. Radial arm maze performance. Performance of animals in the 8- (A) and 4-arms baited (B and C) configurations of the RAM. Ischemic animals required significantly (*$p<0.05$) more days to reach criterion than sham animals (working memory deficit; A). Ischemic animals made significantly (*$p<0.05$) more reference memory errors (entry into unbaited arms) than sham animals in the 4-arms baited paradigm on blocks 5 and 6 (days 21-30; B). There were no differences between conditions in working memory abilities (re-entry into unbaited arms) in this paradigm (C).

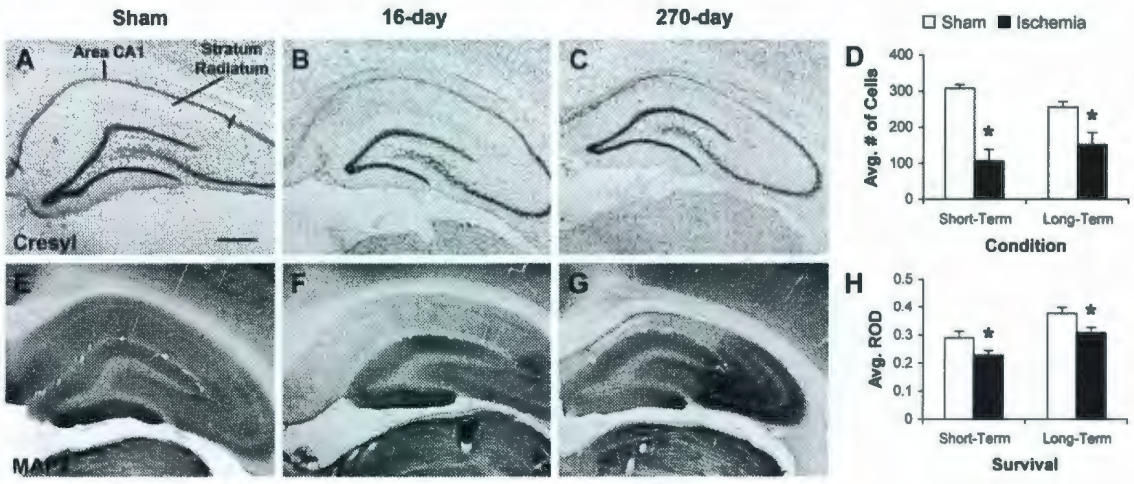
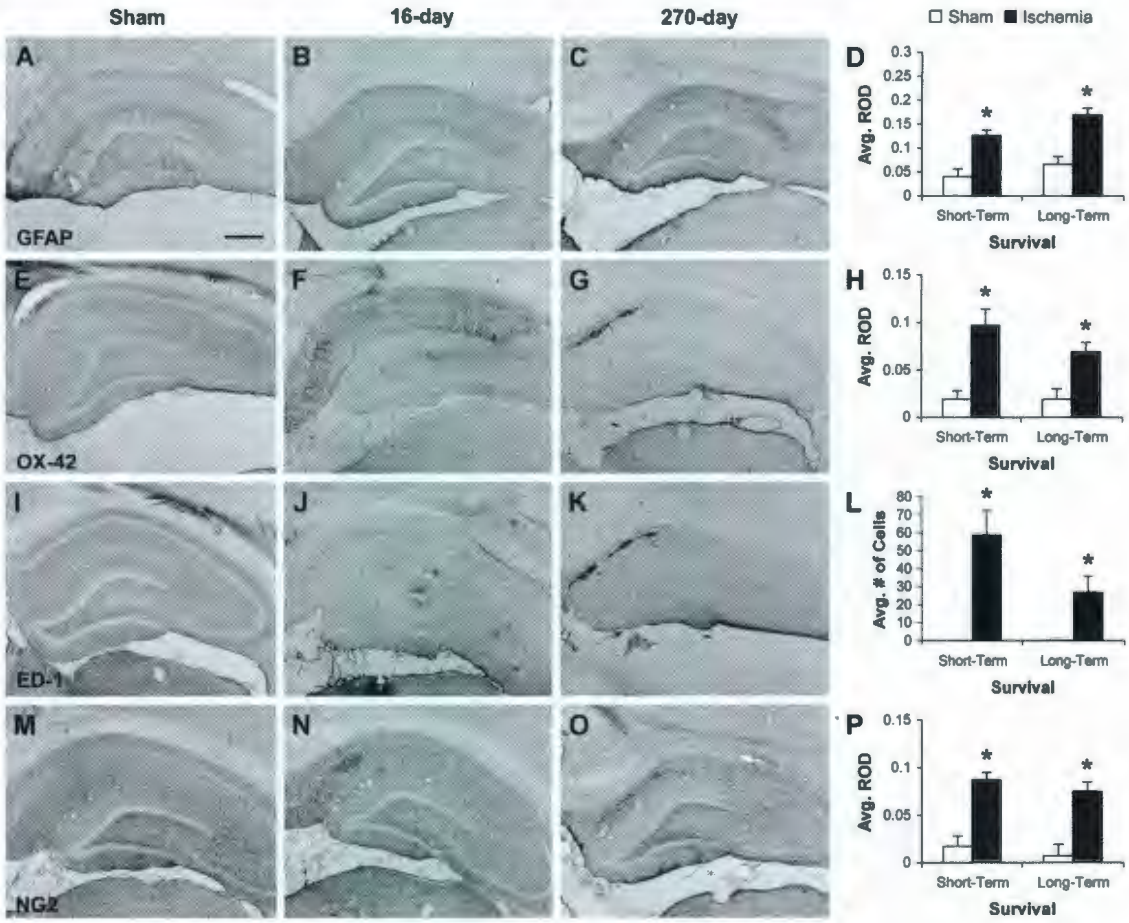


Figure 2.6. Histological and immunohistochemical characterization of hippocampal CA1 area in sham animals (A and E), 16-day (B and F), and 270-day (C and G) ischemic animals. There was a significant reduction in CA1 cell counts in ischemic versus sham animals counts (A-D, cresyl violet stain). There was also a significant decrease in MAP2 ROD staining of the stratum radiatum in both ischemic conditions compared to sham animals (E-H) (*$p<0.05$). Scale bar = 500μm.

Figure 2.7. Immunohistochemical characterization of the neuroinflammatory response in CA1 region of sham animals (A, E, I, and M), 16-day (B, F, J, and N), and 270-day (C, G, K, and O) ischemic animals. There was a significant increase in ROD measurements within the pyramidal layer of CA1 at both ischemic survival times compared to sham animals with respect to GFAP (A-D), OX-42 (E-H), and NG2 (M-P) staining. There were significantly more ED-1 positive cells in both ischemic conditions compared with shams (I-L). Also note the change in staining patterns of the ischemic animals. Neuroinflammation was detected throughout all layers (stratum oriens, pyramidal layer, and stratum radiatum) of CA1 in the 16-day survival animals, however, staining was limited to the pyramidal layer in the 270-day ischemic animals. (*$p<0.01$). Scale bar = 500μm.


Sham
16-day
270-day
A
B
C
GFAP
D
□ Sham ■ Ischemia
Avg. ROD
0.3
0.25
0.2
0.15
0.1
0.05
0
Short-Term
Long-Term
Survival
E
F
G
OX-42
H
Avg. ROD
0.15
0.1
0.05
0
Short-Term
Long-Term
Survival
I
J
K
ED-1
L
Avg. # of Cells
80
70
60
50
40
30
20
10
0
Short-Term
Long-Term
Survival
M
N
O
NG2
P
Avg. ROD
0.15
0.1
0.05
0
Short-Term
Long-Term
Survival

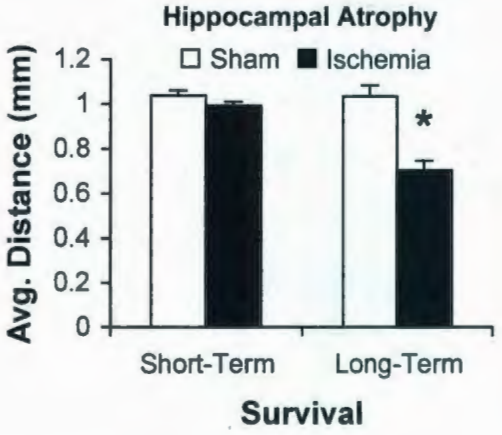


Figure 2.8. Measure of hippocampal atrophy. Average distance (mean ± SEM) between the corpus callosum at the apex of area CA1 and the hippocampal fissure in a rostral and middle section assessed using the MAP2 immunohistochemical stain. There was no difference between ischemic and sham animals at 16-days post surgery, however, at 270-days post-surgery, there was a significant reduction in distance between the corpus callosum and fissure indicating significant atrophy of CA1 (*$p<0.01$).

CHAPTER 3: PROLONGED, 24-HOUR DELAYED PERIPHERAL INFLAMMATION INCREASES SHORT- AND LONG-TERM FUNCTIONAL IMPAIRMENT AND HISTOPATHOLOGICAL DAMAGE FOLLOWING FOCAL ISCHEMIA IN THE RAT

3.1 Introduction

Infection in stroke patients, either prior to or following injury, worsens functional outcome when assessed at delayed time points (Emsley and Hopkins, 2008). It is estimated that ~30% of ischemic stroke patients present with an antecedent infection, and that infection is a significant risk factor for ischemic stroke (Bova et al., 1996; Grau et al., 1998). A further 30% of patients develop an infection while in hospital, most commonly urinary or respiratory tract infections, which are also associated with poorer functional outcomes (Grau et al., 1999). This poor prognosis may be due to an immunosuppression following stroke leaving the patient susceptible to developing an infection soon after ischemia (Offner et al., 2006; Prass et al., 2006; Chamorro et al., 2007).

Several experimental studies have examined the effects of acute ischemic inflammation (immediately prior to or post-ischemia) using a low dose injection of an endotoxin, lipopolysaccharide (LPS) to model the systemic effects of an infection. Spencer and colleagues (2007) injected a single low dose of LPS immediately following global cerebral ischemia and found an acceleration of cell death at three days. Further, they found significant elevations of the proinflammatory cytokines TNFα and IL-6 at

four hours post-ischemia, independent of significant temperature elevations. This is important because mild hyperthermia worsens outcome in experimental and clinical ischemia (Busto et al., 1987). Additional studies conducted by McColl and colleagues (2007, 2008) demonstrated increased ischemic damage following LPS or IL-1 injection that was related to increased IL-6 and peripheral immune cell infiltration through breakdown of the blood brain barrier, although post-ischemic temperature was not assessed. Additional studies have used higher doses of LPS injections at prolonged time points prior to ischemia (>24 hours) and have found equivocal results ranging from neuroprotection (e.g. due to preconditioning) to increased injury (Tasaki et al., 1997; Rosenzweig et al., 2004; Spencer et al., 2006).

Proposed mechanisms for increased neuronal damage following acute ischemic infection include increases in proinflammatory cytokines (TNFα, IL-6 and IL-1β) as well as increases in neuroinflammation as measured by microglial activation, and macrophage, neutrophil and T cell infiltration (Zheng and Yenari, 2004; McColl et al., 2007; Shichita et al., 2009). Reductions of these cytokines or neuroinflammation lead to reduction in cerebral damage and improved function (Hewlett and Corbett, 2006; McColl et al., 2007). In the current study we evaluated a number of ramifications of concurrent ischemia and systemic inflammation. First, we used more prolonged systemic inflammation than that used by others. Second, we compared the effects of pre-ischemic inflammation to delayed ischemic inflammation, 24 hours following MCAo. Third, we repeatedly assessed serum cytokine levels for 72 hours following ischemia instead of just four, eight or 24 hours post-ischemia. Fourth, we assessed long-term functional outcome and both short- and long-term histopathology. Based on previous reports (McColl et al., 2007; Spencer et al.,

2007; McColl et al., 2008), we hypothesized that animals exposed to LPS either before or post-ischemia would have higher levels of proinflammatory cytokines, increased functional deficits and increased neuronal injury.

3.2 Materials and Methods

3.2.1 Subjects

Sixty-three male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing ~200-225g upon arrival were used in this study. Animals were housed in pairs on a 12 hour light:dark cycle and were fed food and water *ad libitum* except during staircase training and testing. All procedures were approved by the Memorial University of Newfoundland Animal Care Committee and conformed to the Canadian Council on Animal Care guidelines.

3.2.2 Surgery

3.2.2.1 Telemetry Core Temperature Probe Implantation

All animals were implanted with a core temperature telemetry probe (TA10TA-F20, Data Sciences International, St. Paul, MN, USA) as previously described (MacLellan et al., 2006). Briefly, animals were anesthetized with Isoflurane (4.0% induction, 2.0% maintenance) in a 30:70 O_2/N_2O mixture, and a sterile core temperature probe was inserted into the peritoneal cavity using aseptic techniques. Animals were

individually housed over a telemetry receiver (RPC-1, Data Sciences International, St. Paul, MN, USA) and temperature recorded every five minutes. Temperature was averaged every hour and the complete 24-hour period prior to surgery served as a baseline. One animal died following telemetry probe surgery.

3.2.2.2 Middle Cerebral Artery Occlusion

One week following telemetry probe surgery, animals underwent middle cerebral artery occlusion (MCAo) surgery by infusing 1,200 pmol of the vasoconstrictive peptide, ET-1, adjacent to the MCA at coordinates anterioposterior +0.9, mediolateral -5.2, dorsoventral -9.1 from the skull at bregma into the hemisphere opposite of paw of best performance in the staircase. Animals were anesthetized with Isoflurane as above. Rectal temperature was maintained at ~37.0°C throughout surgery using a self-regulating heating blanket (Harvard Apparatus, Holliston, MA, USA). Following MCAo surgery, animals were singly housed and temperature was monitored for 48-hours. One animal died following MCAo surgery and resultant data were collected from the remaining 61 animals.

3.2.3 Prolonged Systemic Infection

Whereas most researchers use a single large bolus dose of LPS to produce acute inflammation (Rosenzweig et al., 2004; McColl et al., 2007; Marsh et al., 2009), we sought to produce more prolonged, clinically relevant inflammation as might occur in stroke patients. Lipopolysaccharide (LPS; *Escherichia coli*; serotype 026:B6, Sigma, St.

Louis, MO, USA) was injected intraperitoneally at a concentration of 50 μg/kg so as not to induce a febrile response that may occur at higher concentrations (Spencer et al., 2007). In order to simulate a more prolonged inflammatory condition, two additional injections of equal concentration were given, separated by four-hour intervals. Animals in the control condition received three injections (separated by four hours) of the pyrogen-free saline vehicle.

3.2.4 Experimental Conditions

Eighteen animals were used for short-term (3 day survival) cytokine and histological assessments and 43 animals were used for long-term (30 days) functional and histological assessments. All animals underwent both telemetry and MCAo surgeries. Animals were randomized into the following conditions: pre-ischemia inflammation animals (n=6 short-term; n=15 long-term) were administered LPS (50 μg/kg, i.p.) at 8, 4 hours, and immediately before MCAo (Pre); delayed post-ischemia inflammation animals (n=6 short-term; n=14 long-term) were administered LPS (50 μg/kg, i.p.) at 24, 28, and 32 hours post-MCAo (Post); and Saline control animals (n=6 short-term; n=14 long-term) received saline injections immediately following MCAo, and at 4 and 8 hours post-surgery.

3.2.5 Cytokine Assessment

Repeated blood sampling occurred by a tail nick procedure (Canadian Council on Animal Care, 1993) under brief (~5 minutes), light anesthesia (2.0% Isoflurane). Blood sampling was conducted during telemetry surgery (baseline), MCAo surgery (time 0), 12, 24, 36, and 72 hours post-MCAo. At each time point, 500 μL of blood was collected into a Microtainer® (Becton, Dickinson and Co, Franklin Lakes, NJ, USA) and allowed to clot for 30 min. Blood was then centrifuged at 14,000 rpm for 2 min and serum was decanted and stored at -20°C until further processing. Serum TNFα, IL-6, and IL-1β concentration levels were quantified using standard enzyme-linked immunosorbent assay kits (ELISA; R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

3.2.6 Behavioural Assessments

Three separate motor assessments sensitive to detecting MCAo induced ischemic injury were used in this study (Windle et al., 2006).

3.2.6.1 Beam Walking Test:

Animals were trained to traverse a tapered beam (widest portion, 6 cm; narrowest portion, 2 cm), elevated 75 cm from the floor, to enter a dark chamber. Performance was videotaped and the number of errors (fore- and hind-limb slips) made with each limb was

recorded as a percent of the total steps made and averaged over three independent crosses prior to surgery and at each time point post-MCAo.

3.2.6.2 Staircase Test of Skilled Reaching:

Animals were mildly food-restricted (~90-95% of free-feeding body weight) and trained to reach for 45 mg food pellets (TestDiet, Richmond, IN, USA) in the Montoya staircase task prior to telemetry surgery (Montoya et al., 1991). Training occurred over a 10-day period with animals receiving two 15-minute trials/day until they reached a pre-set criteria of at least 12 out of a possible 21 pellets on an arm with a standard deviation ≤ 2 over a period of eight trials (the average number of pellets successfully reached was 17/21). On post-surgery testing, animals received two trials/day for two days at each time point.

3.2.6.3 Spontaneous Limb use (Cylinder) Test:

Animals' forelimb use was calculated prior to surgery using the cylinder test of limb asymmetry (Schallert, 2006). Briefly, animals were placed in a clear Plexiglas© cylinder (20 cm in diameter) on a glass tabletop and videotaped from below. Animals were required to make 20 independent contacts with the cylinder wall and the number of single (ipsilateral or contralateral) or bilateral contacts were calculated. Contralateral forelimb usage was calculated using the equation: [(number of contralateral contacts + ½ number bilateral contacts) / (number of ipsilateral + contralateral + bilateral contacts)] x 100 (Schallert and Woodlee, 2005). All animals were assessed prior to surgery (baseline) on each of the functional assessments and all post-MCAo behaviour is presented as a

percentage of baseline abilities. Animals were assessed in each behavioural test 7 and 30 days post-MCAo.

3.2.7 Histology and Immunohistochemistry

Animals were deeply anesthetized with 4.0% isoflurane, transcardially perfused with ice-cold heparinized 0.9% saline and 4.0% paraformaldehyde (PFA) and quickly decapitated. The heads were stored in PFA at 4°C for 4 hours, the brains removed and then stored overnight in PFA at 4°C. Brains were then transferred to 20% sucrose in phosphate buffered saline, stored at 4°C until saturated, and then frozen and stored at -20°C until further processing.

Coronal slices were cut at 20 μm using a cryostat and sections were slide-mounted for H&E or leukocyte stains, and the immunohistological ED-1 stain for activated microglia as previously described (Langdon et al., 2008). Leukocytes were stained using the naphthol AS-D chloroacetate esterase procedure as outlined by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). ED-1 staining was as follows: sections were washed in PBS, blocked with 1.0% H_2O_2 for endogenous peroxidase, blocked with normal goat serum (5.0%; Jackson Immunoresearch Laboratories, West Grove, PA, USA), incubated overnight with monoclonal mouse anti-rat CD68 (ED-1; 1:1,000, MCA341R; Serotec, Raleigh, NC, USA) at 4°C, exposed to goat anti-mouse biotinylated secondary antibody (1:1,000; Jackson Immunoresearch Laboratories), incubated in 10 μg/mL extravadin (Sigma-Aldrich, Oakville, ON, Canada) and reacted for 5 min in a 3,3'-diaminobenzidine tablet set (Sigma-Aldrich).

To quantify the infarct volume, the area of contralateral and ipsilateral tissue remaining were calculated from the H&E stained tissue (ImageJ 1.36b software for Mac, downloaded from the public domain, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). Tissue was measured at 800 μm intervals throughout the brain and the volume of intact tissue was calculated as: (average area of intact tissue in each section) x (number of sections analyzed) x (distance between sections (800 μm)). The volume of infarction was calculated as: volume of contralateral hemisphere – volume of intact ipsilateral hemisphere. Infarct volume was corrected for edema at the earlier time point by normalizing the ipsilateral hemispheric volume to the non-affected contralateral hemisphere and calculating volume of intact tissue as above (Belayev et al., 2005). The average number of activated microglia/macrophages and neutrophils in each brain were estimated using a Leica DMRXE microscope and the Fractionator method of Stereo Investigator® (MBF Bioscience, Williston, VT, USA). An average of 25 sampling sites (100 μm x 100 μm) were randomly superimposed on the circumscribed area of damage and positive cells were counted in a 20 μm section, every 800 μm throughout the injury. The average number of cells between each counted section was calculated and the sum of those averages was multiplied by 40 (800 μm / 20 μm) to estimate a total number of cells.

3.2.8 Statistical Analyses

All analyses were conducted using the statistical package for the social sciences (SPSS; v 13.0.0 Grad Pack for Mac OS X, SPSS Inc., CITY, USA) All data from the cytokine and histological assessments are presented as means ± SEM. Baseline

cytokine and histological data were analyzed with a univariate analysis of variance (ANOVA) and subsequent cytokine data were analyzed using repeated measures ANOVA. Post-hoc tests were conducted using the Tukey's honestly significant difference (HSD) test (unequal group sizes) or the least significant difference (LSD) test (equal group sizes). All data from functional assessments are presented as percentage of baseline and analyzed using repeated measures ANOVA. In cases where the homogeneity of variance or sphericity assumptions were violated, the Brown-Forsythe or Huynh-Feldt correction was used, respectively. Statistical significance was considered at $p \leq 0.05$.

3.3 Results

3.3.1 Temperature

Post-ischemic core temperature was recorded for 48 hours post-surgery and averaged over one hour bins (Fig. 3.1). Over the eight-hour period prior to MCAo surgery, Pre animals' temperature was on average 37.7 °C (± 0.07°C), but was maintained at ~37.0 °C during surgery. Immediately following MCAo surgery, Pre animals were significantly warmer than Saline animals ($p<0.01$; 0.45°C) for a period of four hours. Similarly, at 27-hours post-ischemia, Post animals exhibited a temperature spike, lasting eight hours, that was significantly higher ($p<0.01$; 0.47°C) than Saline animals. In both cases, the increases in temperature were mild and sustained for short periods.

3.3.2 Cytokine Assessment

There were no differences among conditions at baseline for any of the cytokines analyzed ($p>0.05$). All data are presented as means ± SEM (Fig. 3.2).

TNFα: Repeated measures ANOVA revealed a significant effect of Time ($F_{1.52,22.77} = 8.42$, $p<0.01$), Condition ($F_{2,15} = 15.02$, $p<0.01$) and a significant Time X Condition interaction ($F_{3.04,22.78} = 10.57$, $p<0.01$) (Fig. 3.2A). Follow-up analysis of this interaction showed that TNFα was significantly elevated in the Pre condition (vs. Saline and Post) at surgery ($p<0.01$), 12 and 24 hours post-ischemia ($p<0.05$). Further, at 36 hours post-MCAo, animals in the Post condition had significantly higher plasma TNFα levels than both the Pre and Saline animals ($p<0.01$).

IL-6: One animal (Pre) was excluded because baseline IL-6 levels were >2 standard deviations above the group mean. There was a significant effect of Time ($F_{2.54,35.57} = 3.01$, $p=0.05$), showing a general increase in plasma IL-6 levels over time following MCAo surgery (Fig. 3.2B). There was no effect of Condition and no Time X Condition interaction.

IL-1β: Two animals (one Pre and one Saline) were excluded from further analyses because both were >2 standard deviations above group means at baseline measurement. Subsequent analysis of IL-1β showed that there was no effect of Time or Condition and no Time X Condition interaction (Fig. 3.2C; $p>0.05$).

3.3.3 Neuroinflammatory Response

There was a significant difference among conditions in the assessment of the microglial/macrophage response three days post-MCAo both in the cortex ($F_{2,15}$ = 3.81, p<0.05) and striatum ($F_{2,10.6}$ = 4.15, p<0.05). There were significantly more ED-1+ cells in the cortex (p<0.02) and striatum of 24-hour Post animals than Saline animals (p<0.03) and significantly more positive cells in the striatum of 24-hour Post animals than Pre animals (p<0.03; Fig. 3.3A & B). There were no differences among conditions with respect to the number of infiltrating leukocytes ($F_{2,15}$ = 1.06, p>0.05). However, there was a trend, similar to the microglia/macrophage response where there was ~100% increase in leukocyte infiltration in the delayed infection animals (Post; Fig. 3.3C & D).

3.3.4 Functional Assessments

There were no differences among conditions at baseline for any dependent measures of the functional assessments (p>0.05).

3.3.4.1 Beam

Repeated measures ANOVA of forelimb walking behaviour showed that there were no effects of Time or Condition and no Time x Condition interaction (p>0.05; Fig. 3.4A). There was, however, a significant effect of Condition on hindlimb function ($F_{2,40}$ = 9.86, p<0.01) but no effect of Time or Time x Condition interaction. Tukey's HSD test showed that animals in the Post condition made significantly more hindlimb foot faults (~17%) than both Pre and Saline animals p<0.01; Fig. 3.4B).

3.3.4.2 Staircase

Repeated measures ANOVA of skilled forelimb reaching on post-ischemia day 7 and 30 revealed a significant effect of Time ($F_{1,40}$ = 11.52, p<0.01) but no effect of Condition and no Time x Condition interaction (p>0.05; Fig. 3.4C).

3.3.4.3 Cylinder

Repeated measures ANOVA of forelimb asymmetry revealed a significant effect of Time ($F_{1,40}$ = 6.33, p<0.01) but no effect of Condition and no Time x Condition interaction (p>0.05; Fig. 3.4D).

3.3.5 Infarct Assessment

Analysis of post-ischemia day 3 animals showed no significant differences among conditions with respect to infarct volumes corrected for edema (p>0.05; Fig. 3.5A). There was, however, a trend towards larger infarct volumes in animals in the Post condition compared to the other conditions. At 30 days post-ischemia, there was a significant difference among conditions in cortical infarct volume ($F_{2,40}$ = 4.15, p<0.03) and hemispheric infarction ($F_{2,40}$ = 3.41, p<0.05) but not in the striatum (p>0.05). Tukey's HSD post hoc test revealed that Post animals had significantly larger infarct volumes than Saline animals (Fig. 3.5C). There were no differences among any of the other conditions, although animals treated with LPS prior to ischemia had ~30% larger infarcts than Saline animals.

3.4 Discussion

Here we describe the differential effects of prolonged pre- and post-stroke systemic inflammation on ischemic outcome. This is one of the first studies to assess the effects of delayed and prolonged systemic inflammation following experimental stroke (Prass et al., 2006) and the first to show such detrimental effects on neurological outcome. Short- and long-term effects of systemic inflammation were assessed using multiple outcome measures including histology, behavioural analyses, and cytokine assays. The cytokine profile shown in Figure 3.2 demonstrates that there were significant changes in serum cytokine levels over time following MCAo and that these changes were further exacerbated by LPS. Delayed systemic inflammation in the Post animals increased functional impairments which were especially evident in hindlimb function, further indicating the devastating effects of prolonged, delayed inflammation on ischemic outcome. It remains possible that there were also delayed inflammatory events not detected at 3 days that contributed to the increased neuronal damage observed at 30 days but confirmation awaits further studies.

Lipopolysaccharide administered prior to ischemia significantly increased serum TNF-α levels for 24 hours, while LPS administered 24 hours post-ischemia increased these levels for 12 hours. Interleukin-6 levels were significantly increased over 48 hours post-ischemia, and animals injected with LPS tended to have more elevated serum levels. These changes are similar to those reported in other studies at shorter time points of four (Spencer et al., 2007) and eight hours post-ischemia (McColl et al., 2007).

Histopathological analysis at 30 days post-ischemia showed that delayed inflammation significantly increased brain injury by ~85%. Animals injected with LPS prior to ischemia had ~30% larger infarct volumes than saline-treated animals. Post-ischemia day three infarct volumes showed a similar trend where 24-hour Post animals had larger infarct volumes than either Pre or Saline animals. The neuroinflammatory response following LPS injection may partially explain this increased infarction. There was a significant increase in the number of microglia/macrophages at three days post-MCAo in 24-hour Post animals compared to Saline animals, corresponding to the infarct volume difference at 30 days post-MCAo. Additionally, there were ~70% more ED-1+ cells in the Pre-treated than saline-treated animals, a difference that may have resulted in a further 30% increase in infarct volume at 30 days post-ischemia. There was a similar increase in the leukocyte infiltration response. Animals with delayed infection had ~100% increase in leukocyte counts, corresponding to a similar increase in microglia/macrophage counts, undoubtedly further exacerbating neuronal injury.

The infarct volumes also corresponded to the functional deficits observed in this study, whereby animals in the Post condition had more severe deficits than Pre or Saline animals. Others have shown that LPS increases functional deficits post-ischemia, but only at relatively short survival times (e.g. 24-72 hours post-ischemia) (McColl et al., 2007; Spencer et al., 2007), and in these studies it is difficult to know whether injury would have been greater at longer survival times or whether neuronal injury may have been accelerated. Our use of both delayed and prolonged inflammation paradigms, allowed sufficient recovery time before functional assessments began ensuring that we did not assess animals while systemic inflammation was still developing.

In this study, LPS administered 24-hours post-ischemia significantly impaired hindlimb function as demonstrated in the tapered beam-traversing task. Additionally, forelimb function in both the beam and staircase tasks was also compromised, although the differences did not reach statistical significance. This may be because even with the increased injury induced by delayed LPS in this study much of the forelimb motor cortex was spared and two of our three behavioural tests (i.e. staircase and cylinder) are most sensitive for detecting forelimb impairments. Animals with delayed inflammation had forelimb function reduced by ~15% in both the beam and staircase tasks when compared to animals treated with saline. Animals treated with LPS prior to MCAo performed similar to saline-treated animals in the beam test with both forelimbs and hindlimbs. Although not statistically different, Pre animals had ~15-20% increased forelimb deficits in the skilled reaching staircase task. Interestingly, these animals also had 30% larger infarct volumes than Saline animals. In spite of the increase in infarct size produced by LPS there was no significant impairment in the staircase and cylinder tasks, likely reflecting compensation resulting from the sparing of forelimb cortex. Nonetheless, there is converging evidence between our short- and long-term histopathological data and functional outcomes in that greater injury was associated with more substantial functional impairments. Delayed inflammation 24 hours post-ischemia increased the neuroinflammatory response 72 hours post-surgery, the tissue loss at 30 days post-surgery and the functional deficits assessed at both seven and 30 days post-surgery. Further, pre-ischemic inflammation caused a trend for similar effects both at 72 hours and 30 days post-ischemia.

Post-ischemic temperature is one of the most important factors in determining stroke outcome (Busto et al., 1987; Colbourne and Corbett, 1994; Kim et al., 1996). Because infection is often accompanied by fever, determining whether the increased injury and residual deficits result from the infection, per se, or simply from hyperthermia, is clinically important. In this study we recorded each animal's post-ischemic core temperature for 48 hours (Fig. 3.1). Animals in the Pre condition had slightly higher temperatures (0.45°C) initially following surgery, but quickly returned to normal within four hours. Injection of LPS 24-, 28- and 32-hours post-MCAo slightly increased core temperature as well, but only by ~0.5°C, and only for eight hours (27-35 hours post-ischemia). It is unlikely that such brief increases in core temperature for several hours, especially at remote time points post-ischemia, would worsen functional outcome and histopathological damage. Indeed, a 24 hour post-ischemic brain temperature of 40°C significantly increased tissue loss whereas increases to 39°C had no effect (Kim et al., 1996). These temperature elevations were much greater than in the present study. Reports noting detrimental effects of hyperthermia usually involve temperature elevations during or shortly after stroke, not at such prolonged post-ischemic time points (MacLellan et al., 2009). A more plausible explanation for the increase in damage is that microglia and macrophages recognize LPS, and secrete proinflammatory cytokines such as TNF-α and IL-6 when stimulated leading to the production of free radicals and oxidative stress and exacerbation of damage (Boje and Arora, 1992; Bhat et al., 1998).

One of the most interesting and important findings in our current study is the assessment of a delayed post-ischemic inflammation. Other studies have modeled acute ischemic infections by injecting LPS either immediately prior to or post-surgery. This

represents only ~50% of the clinical population (Emsley and Hopkins, 2008). The rest develop an infection while in hospital 24-48 hours post-stroke. Our data indicate that delayed post-ischemic inflammation is more detrimental to long-term function and neuropathology than a pre-ischemic inflammation potentially resulting from post-ischemic immunosuppression (Offner et al., 2006; Prass et al., 2006). Although serum cytokine levels were increased in animals exposed to LPS prior to MCAo, cerebral blood flow is typically reduced for a number of hours in this model (Biernaskie et al., 2001; Windle et al., 2006), potentially reducing cytokine levels in the brain. In contrast, animals injected with LPS 24-hours post-MCAo had normalized cerebral blood flow. At this time TNFα and IL-6 levels were highest in the Post condition, potentially reperfusing significantly higher levels of proinflammatory cytokines to the MCA territory. These data taken together may explain why delayed systemic inflammation significantly increased the early neuroinflammation following MCAo as well as the long-term residual functional deficits and histological damage. It is interesting to note that a recent report has indicated a positive relationship between infarct size and the occurrence of infection (Hug et al., 2009). This creates an even more complex dynamic between stroke and infection, both of which appear to be detrimental to outcome.

In summary, using repeated serum sampling, this study documents significant changes in proinflammatory cytokine levels soon after ischemia and systemic inflammation, that correspond to an increase in neuroinflammation at three days post-ischemia. Further, we demonstrated that these early effects lead to sustained functional and neuropathological deficits at 30 days survival times. This study also emphasizes the importance of the interaction between the peripheral immune and central nervous

systems. Following injury, the central nervous system becomes increasingly susceptible to peripheral challenges, such as infection and inflammation, and the resulting consequences may have dramatic effects on both neuronal damage and subsequent function. Peripheral inflammation, independent of a febrile response, that occurs at delayed periods following ischemia, significantly increases functional deficits and histopathological damage. Clinicians should vigilantly monitor and aggressively treat patients suffering from a post-ischemic infection in the hours and days following injury to avoid the devastating results that accompany this condition.

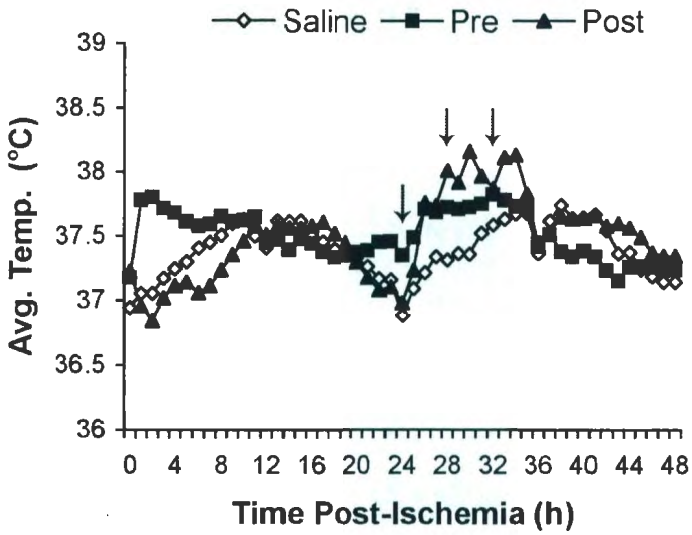
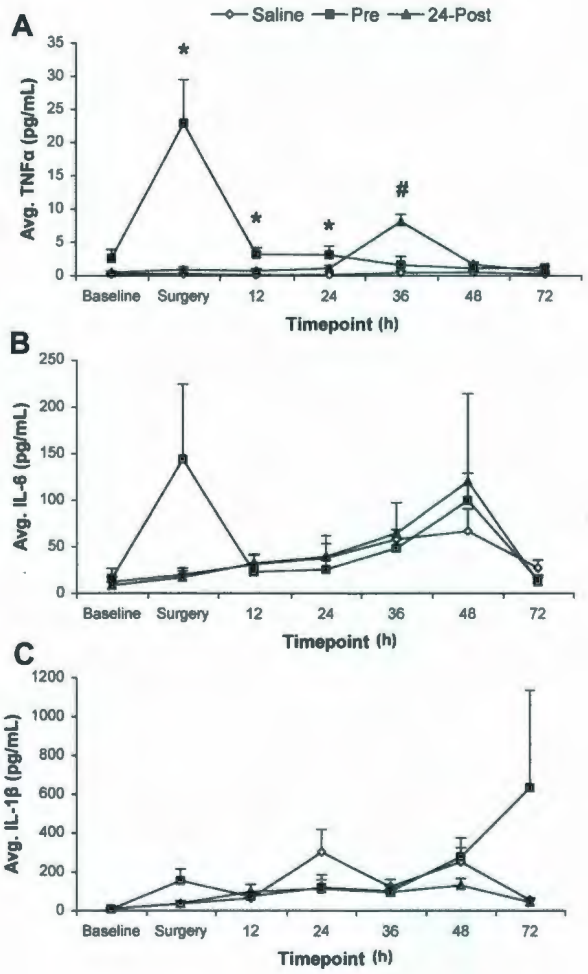


Figure 3.1. Post-ischemic temperatures (°C) averaged over one hour time bins for 48 hours. Arrows indicate LPS administration (50 μg/kg, i.p.) in the 24-hour Post condition. Pre animals were administered LPS (50 μg/kg, i.p.) 8, 4, and 0 hours prior to MCAo surgery and mean core temperature over this period was ~37.7 °C (data not shown).

Figure 3.2. Repeated sampling of serum proinflammatory cytokines by ELISA measurement. Three separate injections of saline or LPS (50 μg/kg) were administered at four-hour intervals either prior to MCAo (Pre; 8, 4, and 0 hours pre-MCAo) or 24-hours post-MCAo (Post; 24, 28, and 32 hours post-MCAo). Data represent average (mean ± SEM) serum concentration of the proinflammatory cytokines at baseline, MCAo surgery, 12, 24, 36, 48, and 72 hours post-ischemia. (A) Lipopolysacchride administered prior to MCAo surgery significantly increased the serum concentrations of TNFα at the time of surgery and remained elevated for 24 hours post-ischemia (*$p<0.05$ vs. Saline and Post). Further, LPS also significantly elevated TNFα levels in the 24-hour Post condition for 12 hours compared to both Saline and Pre concentrations (#$p<0.01$). (B) Serum concentrations of IL-6 showed similar trends as TNFα, but there were no significant differences among conditions, nor was there a difference among conditions in IL-1β serum concentrations (C).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Baseline
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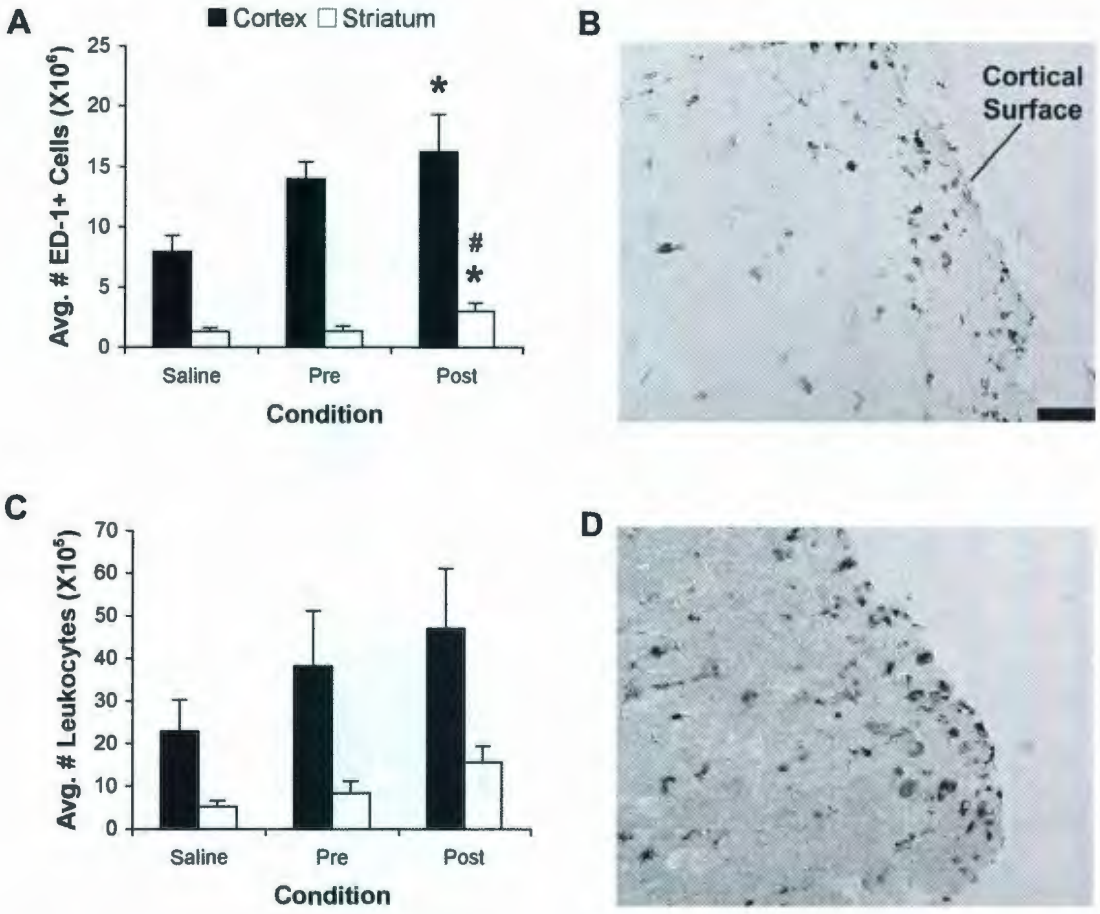


Figure 3.3. Quantification of the neuroinflammatory response. (A) Number (mean ± SEM) of activated microglia/macrophages quantified by ED-1 cell counts in the injured hemisphere. Delayed injection of LPS (50 μg/kg) significantly increased the number of ED-1+ cells compared to saline-treatment in both the cortex and striatum (*p<0.03) and compared to Pre infection in the striatum (#p<0.03). (B) Example of cortical ED-1+ staining within the ischemic core. (C) Number (mean ± SEM) of infiltrating leukocytes in the injured hemisphere. (D) Example of cortical leukocyte staining within the ischemic core. Scale bar represents 50 μm.

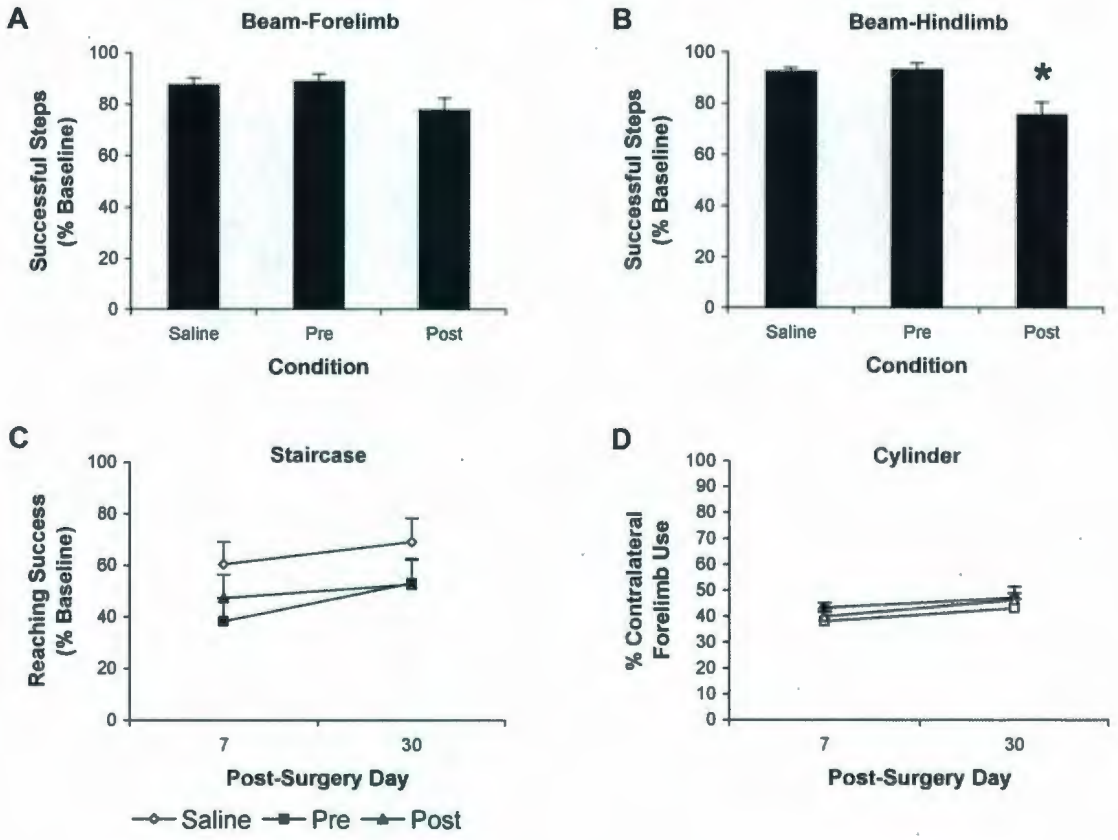
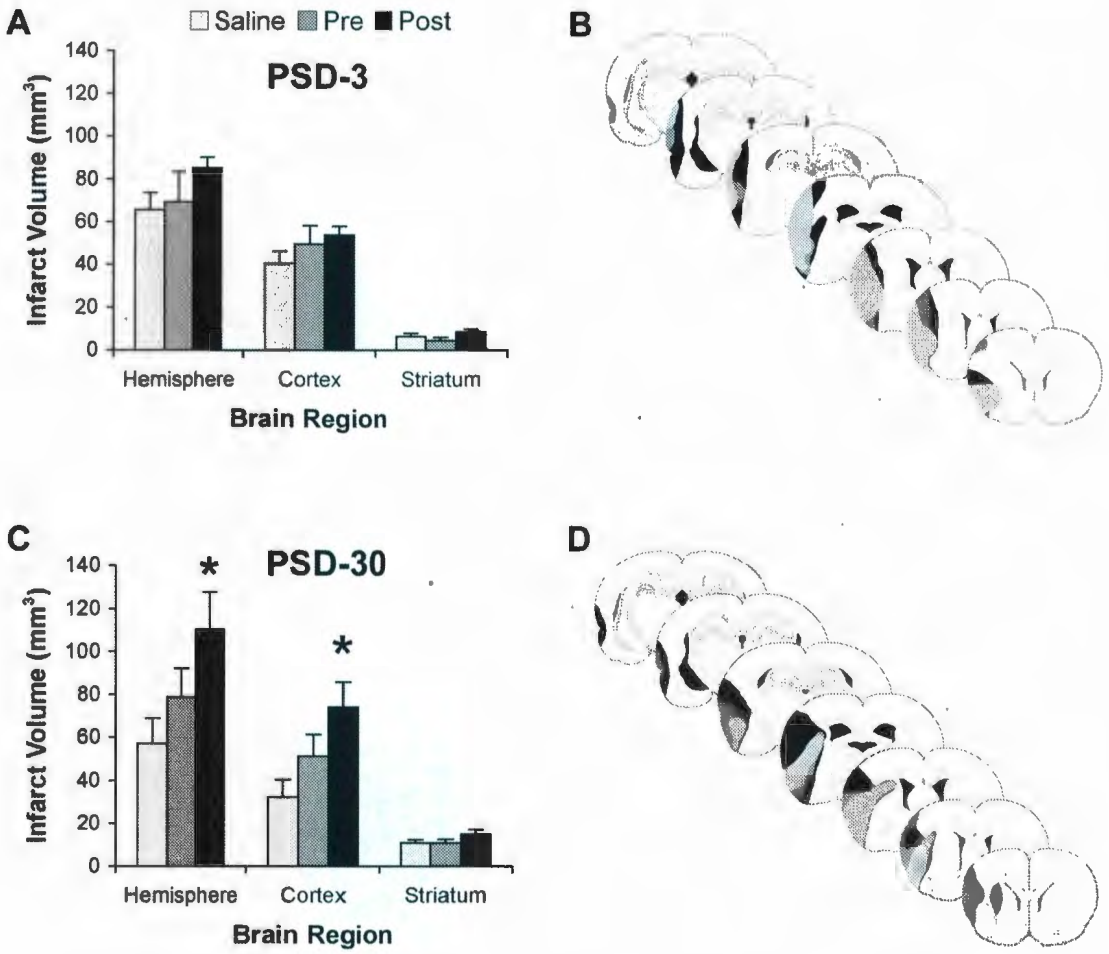


Figure 3.4. Post-ischemic functional assessments. Forelimb and hindlimb function were assessed at seven- and 30-days post-ischemia in the (A & B) tapered beam; (C) staircase; and (D) cylinder tasks. There was no effect of Time or Time X Condition interaction on the beam task and thus data collected on days 7 and 30 are represented as the average of both days (A & B). Data are presented as percentage (mean ± SEM) of baseline performance. Delayed post-ischemic administration of LPS (50 μg/kg) significantly increased the percentage of hindlimb paw slips as assessed in the beam walking task when compared to both Saline and Pre animals (B; $*p<0.01$).

Figure 3.5. Assessment of infarct volumes. Animals were euthanized at either three (A & B) or 30-days (C & D) following MCAo surgery and infarct volumes were calculated. (A) At three days following MCAo there was a trend for 24-hour Post animals to have larger infarct volumes than both Saline and Pre animals. (B) Representative injury profile of animals at three days post-MCAo. (C) At 30-days post-surgery, the three day trend continued and animals in the Post condition had significantly larger infarcts than Saline animals (*$p<0.05$), most evident in the cortex. Of note, animals in the Pre condition had ~30% larger infarct volumes than Saline animals at 30-days post-MCAo. (D) Representative injury profile of animals at 30 days post-MCAo.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Saline
Pre
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PSD-3
Infarct Volume (mm³)
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Cortex
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Brain Region
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PSD-30
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Infarct Volume (mm³)
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120
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Hemisphere
Cortex
Striatum
Brain Region
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CHAPTER 4: IMPROVED WORKING MEMORY FOLLOWING NOVEL COMBINATIONS OF PHYSICAL AND COGNITIVE ACTIVITY

4.1 Introduction

Physical activity improves cardiovascular health and decreases the occurrence of obesity, diabetes mellitus, osteoporosis and other diseases (Rodriguez-Colon et al., 2009). Recently, attention has focused on the ability of exercise to enhance brain health (Cotman et al., 2007; Kramer and Erickson, 2007). For example, in animal studies, physical exercise increases levels of growth factors (Gomez-Pinilla et al., 2002), angiogenesis (Swain et al., 2003), neurogenesis and, improves learning and memory (van Praag et al., 1999). Further, in humans, physical exercise has been linked to attenuating cognitive decline with aging (Friedland et al., 2001; Laurin et al., 2001; Andel et al., 2008). One difficulty in extrapolating exercise data derived from animal studies to the clinical population is that animals are typically housed in isolation throughout experimentation and provided with 24-hour exposure to wheel running. This is problematic for several reasons. Rodents are highly social animals and individual housing results in isolation stress, decreased neurogenesis, and structural changes in cortical and hippocampal neurons (Stranahan et al., 2006; McEwen, 2007). Additionally, isolated rats given 24-hour access to running wheels often develop pathological patterns of running whereby they may run as much as 48 km in one 24-hour period (Farmer et al., 2004). Clearly there

is a serious mismatch between commonly used animal exercise paradigms and exercise guidelines in humans that recommend 30-45 minutes of aerobic exercise per day.

Cognitive activity has begun to attract attention in treatment of mild cognitive impairment and dementia. Several retrospective reports indicate that individuals who actively participate in cognitive activity such as board games, reading, and writing are less likely to develop dementia (Snowdon et al., 1996; Friedland et al., 2001; Hall et al., 2009). Limited animal studies indicate that learning in the Morris water maze results in increases in levels of brain-derived growth factor (BDNF), TrkB, and basic fibroblast growth factor (FGF2) mRNA levels, all of which are thought to enhance neuroplasticity and improve learning and memory (Gomez-Pinilla et al., 1998, 2001). Interestingly, BDNF and other molecular markers are also increased with physical activity (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002). Further, whereas physical activity may increase the proliferation and division of precursor cells, cognitive activity may promote the survival of these cells indicating an important link between both forms of activity (Kempermann et al., 1997; Kronenberg et al., 2003; Kempermann, 2008)

It is very difficult to determine the amount and intensity of physical and especially cognitive activity in human subjects since most studies rely on self-reports (Kramer and Erickson, 2007). However, in animals these variables can be more precisely regulated and potentially beneficial mechanisms investigated. We examined a novel combination of physical and cognitive activity and its effects on proteins (e.g. BDNF, pCREB) implicated in neuroplasticity processes including learning and memory. We found that this novel, clinically-relevant paradigm that combines both physical and cognitive activity

resulted in improved cognitive performance as reflected by enhanced learning and working memory abilities.

4.2 Materials and Methods

4.2.1 Subjects

Forty-one Sprague-Dawley rats (Charles River, Quebec, Canada) weighing approximately 250 g on arrival were used in this study. Animals were socially housed on a 12:12 hour reverse light:dark cycles (lights off at 8:00) and fed food and water *ad libitum*, unless otherwise indicated. All procedures were approved by the Memorial University of Newfoundland Animal Care Committee and conformed to the Canadian Council on Animal Care guidelines.

4.2.2 Experimental Conditions

There were four experimental conditions in this study (Table 4.1). Physical activity (PA) animals (n=8) were exposed to voluntary running wheels (115 cm diameter) for two hours/day, five days/week. Running distances were recorded with CatEye cyclocomputers (CC-MT400, Osaka, Japan). An additional eight animals underwent similar physical activity training and were assessed in the radial arm maze (RAM) to measure the effects of PA alone on learning and memory abilities. Cognitive activity (CA) animals (n=9) were placed in novel Hebb-Williams mazes each day for two hours, five days/week (similar exposure durations as the PA animals). Following two weeks of

daily novel Hebb-Williams exposure, CA animals were trained in an 8-arm baited version of the RAM for an additional two weeks (two trials/day for 10 consecutive days) as previously described (Langdon et al., 2008). Following the RAM procedure, CA animals were again exposed to novel Hebb-Williams mazes as above for two additional weeks, then exposed to a 4-arm baited configuration of the RAM (see below). Radial arm maze testing served both as a training paradigm as well as an outcome measure. Physical- and cognitive-activity (PA/CA) animals (n=8) were exposed to both procedures as outlined above, where these animals were exposed to four hours of daily treatment for five days/week (unless otherwise indicated), counterbalanced for treatment (Table 4.1). Control animals (n=8) were housed in pairs in standard cages and handled similarly as the other conditions throughout the study.

4.2.3 Hebb-Williams Maze Training

Animals in the CA and PA/CA conditions were placed with a cagemate in modified Hebb-Williams mazes (100 x 100 x 20 cm) (Hebb and Williams, 1946) for two hours/day, five days/week during weeks one, two, five and six (Table 4.1). Hebb-Williams maze configurations were changed daily, so that animals had only one repeated configuration throughout the entire study. Numerous objects were scattered throughout each maze and these were replaced each week to increase novelty.

4.2.4 Radial Arm Maze Training

Two configurations (8- and 4-arm baited paradigms) of the RAM (70-cm arms; 12-cm width; 35-cm center platform; 20-cm clear Plexiglas walls) were used as previously described (Olton and Samuelson, 1976; Langdon et al., 2008). All animals, regardless of condition, were mildly food restricted (~90-95% *ad libitum* body weight) while the CA and PA/CA animals underwent RAM training. A 45 mg food pellet (TestDiet AIN-76A Rodent Tablet, 45 mg; Richmond, IN, USA) was placed at the end of each arm during testing (at the end of four arms for the 4-arm baited paradigm). Briefly, animals were acclimated to the maze for two consecutive days with food pellets scattered throughout the maze. Following acclimation, each animal was placed in the center platform and allowed access to the maze until all eight (or four) arms were visited. Working memory errors were recorded in the 8-arm paradigm (re-entry into a previously visited arm), and working and reference memory errors (entering a non-baited arm) were recorded in the 4-arm baited paradigm. Further, animals' accuracy was determined by recoding the number of correct choices on the first eight or four arm selections in the 8- or 4-arm RAM, respectively. The 8- and 4-arm paradigms occurred at week three and seven of the experimental study, respectively (Table 4.1). Cognitive activity consisted of two trials/day for 10 consecutive days.

4.2.5 Tissue Processing

Following completion of the behavioural training, animals were immediately removed from their respective training session, anesthetized (4.0%

isoflurane in 30:70% O_2:N_2O mixture) and decapitated between the hours of 10:00-14:00 to control for the diurnal effects of BDNF (Bova et al., 1998). Brains were quickly removed and the left hippocampus was dissected as previously described (Ploughman et al., 2005). In addition, a wedge section of cortex corresponding to the prefrontal and motor cortex extending ventrally to the corpus callosum (Paxinos and Watson, 1997) was also dissected and both were weighed, rapidly frozen in liquid nitrogen and stored at -80°C.

4.2.6 Enzyme-Linked Immunosorbant Assay

Tissue from the hippocampus and cortex was homogenized in three times volume ice-cold lysis buffer for phosphorylated cyclic AMP response element binding protein (pCREB) as per manufacturer's instructions, incubated on ice for approximately 30 minutes and centrifuged at 14,000 g for 30 minutes at 4°C. Supernatants were collected and either immediately returned to -80°C until further processing for pCREB or further diluted 120 times in ice-cold BDNF lysis buffer (100 mM Tris/HCl, pH 7, containing 2% bovine serum albumin, 1 M NaCl, 4 mM EDTA.Na^2, 2% Triton X-100, 0.1 μg/mL pepstatin A, 17 μg/mL phenylmethyl-sulphonyl fluoride, and one protease inhibitor tablet per 50 mL (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Laval, QC, Canada)) then stored at -80°C until further processing. Duplicate samples of supernatants were quantified for pCREB using ELISA (TransAM™ CREB/pCREB Transcription Factor Assay Kit, Active Motif, Carlsbad, CA, USA) and for BDNF using ELISA (ChemiKine™ BDNF Sandwich ELISA KIT, Millipore,

Temecula, CA, USA) according to manufacturers' protocols. BDNF concentrations (pg/mL) were calculated using known standards. All data are presented as percentage of control animal pCREB and BDNF protein concentrations.

4.2.7 Statistical Analyses

All analyses were conducted using the statistical package for the social sciences (SPSS; v 13.0.0 Grad Pack for Mac OS X, SPSS Inc., Chicago, IL, USA). All behavioural data are presented as mean ± SEM and were analyzed using repeated measures analysis of variance (ANOVA). In cases where the sphericity assumption was violated, the Huynh-Feldt correction was used. Post-hoc analyses for repeated measures ANOVA were conducted using independent t-tests with Bonferroni correction for multiple comparisons. Further, protein concentrations are presented as percentage of control animals and were analyzed using one-way ANOVA. Post-hoc analyses were conducted using Tukey's honestly significant difference test. Statistical significance was considered at $p \leq 0.05$.

4.3 Results

4.3.1 Running Distances

Repeated measures ANOVA of animals' running distances revealed a significant effect of Time ($F_{3.36,47} = 13.19$, $p<0.01$) where animals ran greater distances over weeks, no effect of condition ($p>0.05$), and a significant Time X Condition

interaction ($F_{3.36,47}$ = 2.97, $p<0.04$) (Figure 4.1). Follow-up analyses of this interaction showed that during week three, PA/CA animals ran significantly greater distances than PA animals ($p<0.02$). Analysis at week four showed a similar trend, although not statistically significant ($p=0.053$).

4.3.2 Radial Arm Maze: 8-Arm Baited

Animals' performance was averaged over two consecutive days for the 10-day assessment period. Because there were two trials/day, each block of data represent four trials in the RAM and are presented as such. Repeated measures ANOVA of working memory errors in the RAM revealed a significant effect of Time ($F_{4,60}$ = 8.53, $p<0.01$), Condition ($F_{1,15}$ = 6.85, $p<0.02$) and no interaction ($p>0.05$). All animals' performance improved over time as indicated by a reduction in the number of working errors over blocks. Further analysis of the effect of Condition showed that PA/CA animals made significantly fewer working memory errors than CA animals ($p<0.02$) (Figure 4.2A & B).

Analysis of animals' choice accuracy revealed a similar effect where there was a significant effect of Time ($F_{4,60}$ = 7.95, $p<0.01$) and Condition ($F_{1,15}$ = 6.97, $p<0.02$) and no interaction. Follow-up analyses determined that all animals' accuracy improved over trials, however PA/CA animals exhibited superior accuracy when compared to CA alone (Figure 4.2C & D).

In order to assess the effects of physical activity-alone on cognitive abilities, an additional group of animals (n=8) exposed to running wheels for two weeks prior to RAM (similar to PA and PA/CA animals) were assessed in the 8-arm baited configuration of the

RAM. Analyses revealed a significant effect of Time ($F_{3.74,38.6} = 11.81$, $p<0.01$) and Condition ($F_{1,14} = 4.99$, $p<0.05$), and no interaction (Figure 4.2A & B). As with CA-alone, animals exposed to PA-alone made significantly more working memory errors than PA/CA animals in the 8-arm baited configuration of the RAM.

As above, analysis of PA animals' choice accuracy revealed a significant effect of Time ($F_{4,56} = 13.65$, $p<0.01$) and Condition ($F_{1,14} = 8.84$, $p<0.02$) and no interaction. As with animals exposed to CA alone, PA animals' accuracy was inferior to animals exposed to a combination of PA and CA (Figure 4.2C & D).

4.3.3 Radial Arm Maze: 4-Arm Baited

Repeated measures ANOVA revealed a significant effect of Time ($F_{4,60} = 3.64$, $p<0.02$) and Condition ($F_{1,15} = 8.88$, $p<0.01$) and no interaction in the number of working memory errors in the 4-arm baited RAM. As with the 8-arm baited configuration, PA/CA animals made significantly fewer working memory errors than CA animals (Figure 4.3A & B). There were no differences in the number of reference memory errors (Figure 4.3C), nor was there a difference between PA/CA and CA animals' accuracy (Figure 4.3D) in the 4-arm baited configuration ($p>0.05$), although there was a significant effect of Time for each measure indicating that all animals' performance improved over trials ($p<0.05$). For each measure, however, there was a trend for PA/CA animals to exhibit fewer reference memory errors and have superior accuracy throughout the training period (Figure 4.3C & D). This was especially true with respect to choice accuracy, where on Block 5, one-tailed independent t-test showed that PA/CA

animals were making significantly more correct selections than CA-alone animals (t_{15} = 1.81, $p<0.05$).

4.3.4 ELISA Measurements

Univariate ANOVA showed a significant difference among conditions in hippocampal BDNF concentrations ($F_{3,19.62} = 3.24$, $p<0.05$). Tukey's HSD revealed that PA animals had significantly higher concentrations of hippocampal BDNF than control animals. There were no differences among other conditions (Figure 4.4). Additionally, there were no differences among conditions with respect to cortical BDNF concentrations ($p>0.05$; Figure 4.4). Similarly, there were no differences among conditions in hippocampal or cortical pCREB concentrations ($p>0.05$; data not shown).

4.4 Discussion

In the present study we investigated the effects of a moderate amount of physical activity-alone, cognitive activity-alone, or a combination of physical and cognitive activity on learning and memory. Additionally, we assessed neuronal BDNF and pCREB protein levels of animals exposed to the above conditions compared to sedentary negative controls. We found that animals exposed to the combination of two hours of physical and two hours of cognitive activity displayed superior performance in both working memory and choice accuracy in the 8-arm baited configuration of the RAM compared to either cognitive activity- or physical activity-alone animals, and in working memory abilities in

the 4-arm baited paradigm than cognitive activity-alone animals. Cortical and hippocampal BDNF levels were slightly elevated in all experimental conditions compared to sedentary controls at the end of eight weeks of training, however two hours of physical activity was the only condition that significantly elevated BDNF levels in the hippocampus, but not in the cortex. Further, neither cortical nor hippocampal pCREB levels were altered by any of the treatment conditions.

Combining the Hebb-Williams and RAM maze training with physical activity translated into improvements in learning and memory function as assessed in the RAM. Although the RAM served primarily as a training paradigm, we were also able to use the RAM to assess animals' acquisition of information (learning) and retention as well as choice accuracy. Because there was a significant difference between PA/CA and CA-alone animals in both of the above dependent measures, we sought to determine whether this was due solely to the effects of physical activity contributing to an improvement in learning and memory (van Praag et al., 1999) or a result of a more complex interaction between both physical and cognitive activity. A separate group of animals were exposed to voluntary running wheels for two weeks and then tested in the RAM (without simultaneous wheel exposure). Results indicated that these animals perform similarly to CA-alone animals with respect to the number of working memory errors and choice accuracy, but significantly poorer than PA/CA animals. This important finding indicates that the combination of physical and cognitive activity has a synergistic effect on learning and memory outcome.

Both retrospective and prospective clinical studies reported a lower incidence of cognitive deficits and dementia among those individuals who regularly engaged in

physical exercise (Friedland et al., 2001; Laurin et al., 2001; Andel et al., 2008). Numerous animal studies have also shown that physical exercise promotes improvements in cognitive function (van Praag et al., 1999; van Praag et al., 2005). One difficulty in the translation between the animal and clinical data presented above is the interpretation of physical activity. Physical activity in the clinical literature often refers to chronic activity over a 5-year period where moderate to high levels corresponded to once per week to ≥3 times per week of activity, respectively (Laurin et al., 2001). However, in preclinical studies, animals are often housed in isolation and have 24-hour access to running wheels throughout the entire study (Gomez-Pinilla et al., 2002; van Praag et al., 2005; Gomez-Pinilla et al., 2008), sometimes running distances of up to 48 km per day (Farmer et al., 2004). These running paradigms induce changes in reward pathways that are similar to compulsive stimulant use (Werme et al., 2002), and therefore may have little in common with typical exercise patterns in humans.

Our use of physical activity paradigms that include much shorter durations and less frequent access to running wheels lend themselves better to clinical translation. Interestingly, with our shorter duration exercise paradigms we observed only slight elevations in neuronal BDNF and pCREB levels in all of our experimental conditions. The observation that hippocampal BDNF was significantly increased in PA but not in PA/CA animals suggests that elevation of BDNF is not a necessary condition for cognitive enhancement in intact animals. Based on previous work (Berchtold et al., 2002; Farmer et al., 2004), longer running wheel exposure (e.g. 24 h) would likely have produced larger increases in BDNF levels, however such exposure is of limited relevance to human exercise patterns.

In addition to physical activity, cognitive activity is also thought to protect the brain by delaying or preventing the onset of dementia. Snowdon and colleagues (1996) reported in their analysis of nun's diaries that those nuns who had higher linguistic abilities, idea and grammatical complexity in early life were less likely to suffer from dementia than their counterparts who had lower neuropsychological scores in these areas (Snowdon et al., 1996). Further, one retrospective report of intellectual activity in midlife showed that those who participated in activities with more diversity and intensity were less likely to be diagnosed with Alzheimer's disease in later life (Friedland et al., 2001). Additional evidence derived from elderly patients 75 years of age, showed that participating in leisurely activities such as playing board games, reading and playing musical instruments decreased the likelihood of developing dementia (Verghese et al., 2003).

Interestingly, learning in the Morris water maze increases BDNF, FGF2, synapsin-I, and TrkB mRNA levels in the hippocampus (Gomez-Pinilla et al., 1998; Kesslak et al., 1998; Gomez-Pinilla et al., 2001). Whether these changes also correspond to changes in protein levels or are sustained is unknown. Further, it is unclear whether the molecular changes observed in these learning paradigms are associated with a transfer to and subsequent improvement in other paradigms of learning and memory. It is possible that both physical and cognitive activity interventions share underlying molecular mechanisms given that physical and cognitive activity both improve cognitive functioning in humans and animals as well as slow age-related cognitive decline (Kempermann et al., 2002; Kramer and Erickson, 2007).

It is difficult to determine the extent to which intellectual or physical activity independently contribute to improved cognitive outcome in older adults (Friedland et al., 2001). It is more likely the result of a complex combination of variables. Although these variables can be controlled within the confines of laboratory testing, surprisingly few animal studies exist on the effectiveness of the combination of both physical and cognitive activity on brain health (Gomez-Pinilla et al., 1998). In the current study we have developed a rodent physical and cognitive activity paradigm that results in superior performance in a task of learning and memory. Although levels of BDNF were significantly higher only in PA animals, there was a trend for levels to be elevated in all conditions in both the cortex and hippocampus. These results suggest that the functional improvements observed may not simply be due to elevated levels of BDNF. Clearly, further studies are needed to identify the underlying mechanisms responsible for the cognitive enhancing effects of the combination therapy used in this study. For example, exercise increases dendritic length and complexity in the dentate gyrus (Redila and Christie, 2006). Such changes may also contribute to the benefits arising from our PA/CA combination.

An additionally important finding from this study is the transfer of improvements from training in the Hebb-Williams maze to performance of working memory abilities in the RAM. Working memory errors were significantly reduced in both paradigms of the RAM in animals with combined physical and cognitive activity compared to either alone. The paradigm of modified Hebb-Williams maze training likely involved more reference memory training (e.g. object recognition) (Bevins and Besheer, 2006), than working memory or spatial memory training as originally designed (Hebb and Williams, 1946),

although we cannot exclude the possibility that these cognitive processes were indeed activated (Rolls and Kesner, 2006). Animals were placed in a new maze each day, in a different location within the room and with novel objects in the maze. Notably, our results contrast with clinical data where only cognitive training was employed. In such studies the transfer of abilities from one cognitive domain (e.g. attention) to another (e.g. working memory) are often difficult to demonstrate (Buschkuehl et al., 2008; Dahlin et al., 2008; Green and Bavelier, 2008) and any improvements in cognitive abilities appear transient (Buschkuehl et al., 2008).

A growing body of evidence indicates that physical activity improves cognition in both animals and humans (Cotman and Berchtold, 2002; Hertzog et al., 2009; van Praag, 2009) paralleled by anecdotal literature suggesting similar benefits can be derived by engaging in life-long intellectual activities (e.g. reading, cross-word puzzles). This is important because it is predicted that with an aging population worldwide the incidence of cognitive decline will increase to epidemic proportions in the coming decades (Kramer and Erickson, 2007; Fotuhi et al., 2009). Consequently, therapies that can reverse or delay cognitive decline are urgently needed. The results of our study suggest that a promising therapeutic approach for improving cognitive ability and brain health is a combination of physical and cognitive activity.

Table 4.1 Experimental Paradigm and Timeline

Condition	**Weeks**			
	1-2	**3-4**	**5-6**	**7-8**
PA*	Run	RAM-8		
PA#	Run	Run	Run	Run
CA	HW	RAM-8	HW	RAM-4
PA/CA	Run/HW	RAM-8/Run	Run/HW	RAM-4/Run
Control	Sed	Sed	Sed	Sed

* - Physical activity animals assessed in the eight arm baited radial arm maze

- Physical activity animals used *only* for protein assessment

Run – Two-hour voluntary running wheel exposure / day

HW – Two-hour Hebb-Williams maze exposure / day

RAM-8 – Radial arm maze (eight arm baited paradigm); two trials / day

RAM-4 – Radial arm maze (four arm baited paradigm); two trials / day

Sed – Sedentary control animals used *only* for protein assessment

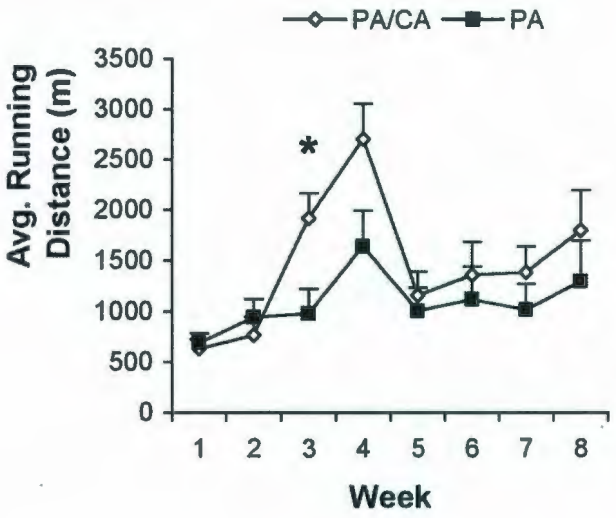

Figure 4.1 Average (±SEM) running distance in meters (m). Physical activity (PA) and the combined physical and cognitive activity (PA/CA) animals were exposed to voluntary running wheels for two hours/day, five days/week over a period of eight weeks. PA/CA animals ran significantly greater distances during week three (*$p<0.02$) and showed a tendency to run greater distances during week four ($p=0.053$) than PA-alone animals. Physical activity animals in this figure were used *only* for protein measurements not for behavioural assessments.

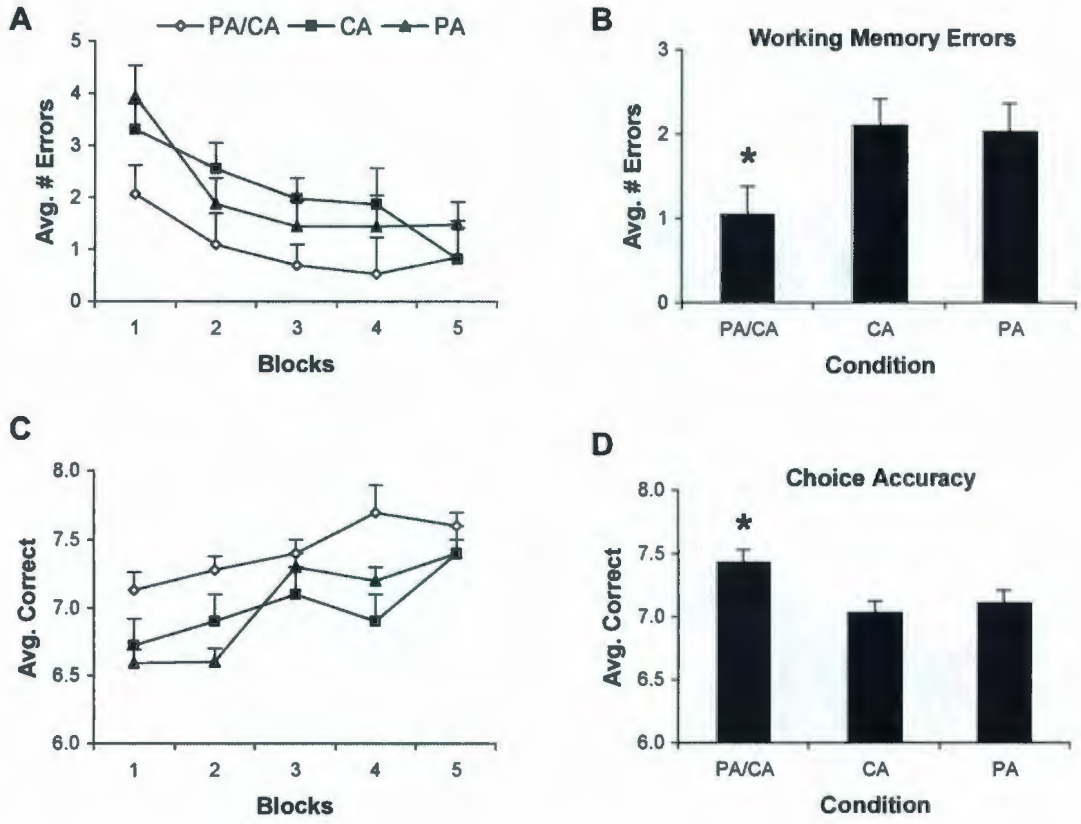

Figure 4.2 Performance (mean ± SEM) in the 8-arm baited configuration of the RAM. (A & B) Animals in the PA/CA condition made significantly fewer working memory errors over the entire training period than either CA-alone or PA-alone animals (*$p<0.05$). This was also accompanied by an increase in choice accuracy (C & D), where PA/CA animals were significantly more accurate in choosing non-entered arms in the first eight choices than either CA-alone or PA-alone animals (*$p<0.05$).

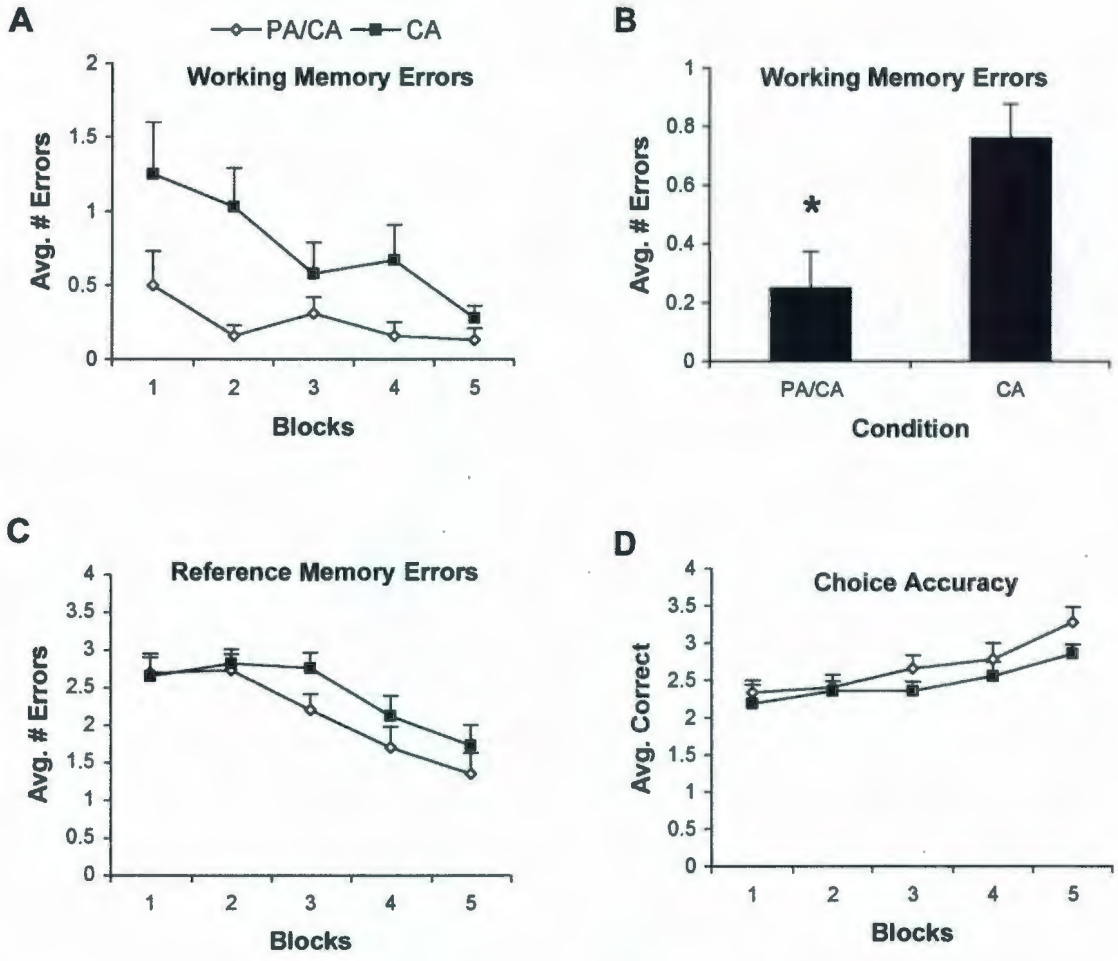

Figure 4.3 Performance (mean ± SEM) in the 4-arm baited configuration of the RAM. (A & B) Similar to the 8-arm baited configuration, PA/CA significantly increased the working memory abilities of animals compared to CA-alone in the 4-arm baited RAM configuration (*$p<0.01$). There were no statistical differences in reference memory abilities (C) or in choice accuracy (D), although in both measures PA/CA animals had a tendency to outperform CA-alone animals.

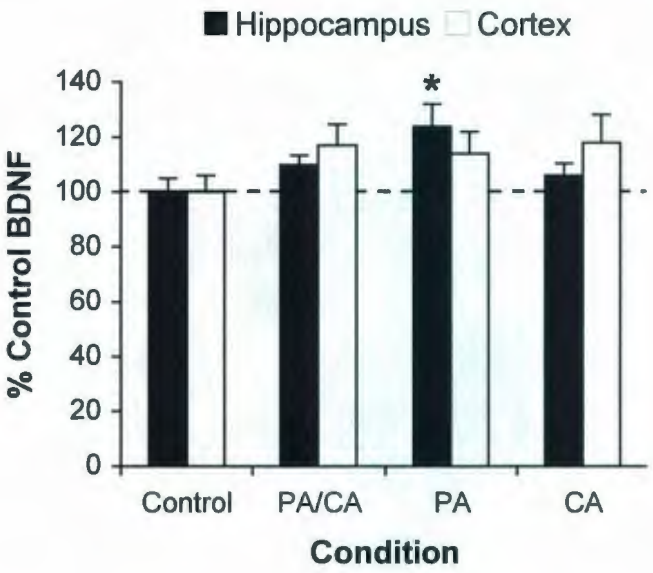

Figure 4.4 Quantification of hippocampal and cortical BDNF levels by ELISA measurement (% control ± SEM). There was a general increase in concentrations of BDNF in both the hippocampus and cortex of all conditions when compared with control animals. This, however, reached statistical significance only in the hippocampus of PA-alone animals (*$p<0.05$). There were no other differences among conditions.

CHAPTER 5: SUMMARY

5.1 Overview of Main Findings

5.1.1 Permanent cognitive deficits and inflammation after global ischemia

Numerous studies have used global cerebral ischemia to model cardiac arrest in humans. This model results in a similar neuropathological sequele as found with human cardiac arrest, affecting primarily area CA1 of the hippocampus (Pulsinelli and Brierley, 1979; Smith et al., 1984; Zola-Morgan et al., 1986). In humans with CA1 cell loss, severe anterograde amnesia is often present (Zola-Morgan et al., 1986; Bartsch et al., 2006). Gerbil models of global ischemia have produced similar long lasting cognitive impairments where affected gerbils have extreme difficulty in acquiring a 'win-shift' version of the T-maze and are unable to acquire the 'win-stay' paradigm (Colbourne and Corbett, 1995; Farrell et al., 2001). Unfortunately, the cerebrovasculature of the gerbil has changed such that bilateral occlusion of the carotid arteries no longer leads to consistent CA1 injury (Laidley et al., 2005). Studies that use rats have yielded inconsistent deficits in learning and memory (Volpe et al., 1984; Volpe et al., 1988; Auer et al., 1989; Nakatomi et al., 2002; Bendel et al., 2005; Hartman et al., 2005; von Euler et al., 2006). The apparent functional recovery following global ischemia in the rat may be due to neurogenesis or sensitivity of testing paradigms (Bendel et al., 2005; von Euler et al., 2006; Bueters et al., 2008).

This thesis describes a behavioural test battery that is sensitive at detecting chronic if not permanent learning and memory dysfunction following global ischemia.

Unlike previous studies (e.g. Hartman et al., 2005), it is clear that these deficits are not a result of a general decline in cognitive ability with age, but due to the sensitivity of the tests used. It is also interesting to note that there was a profound neuroinflammatory response early (14 days) following ischemia, however there was also a sustained inflammatory response 270 days post-ischemia. This is important because neuroinflammation is traditionally thought to have subsided within weeks following stroke (Morioka et al., 1993; Gehrmann et al., 1995). Additionally, most ischemia studies use only short survival times (e.g. < 7 days) and would therefore fail to observe this phenomenon. The results from the study outlined in Chapter 2 emphasize the importance of using a battery of functional assessments as well as using long-term protracted survival times to assess outcome following global ischemia.

5.1.2 Delayed peripheral inflammation increases damage following ischemia

The main findings from the study outlined in Chapter 3 is that 24-hour delayed systemic inflammation results in significant increases in neuroinflammation (microglia/macrophages and neutrophil infiltration), histological damage and functional impairment 30 days post-ischemia. Previous studies that used models of systemic inflammation around the time of ischemia (McColl et al., 2007; Spencer et al., 2007; McColl et al., 2008) demonstrated significant exacerbation of injury, but this study is the first to show that delayed inflammation has such deleterious functional and histological consequences. This study is consistent with clinical evidence indicating that the development of an infection, resulting in peripheral inflammation, after hospitalization

from a stroke results in a poorer prognosis (Chamorro et al., 2007; Emsley and Hopkins, 2008). Importantly, the increases in damage are independent of a prolonged febrile response, a finding not easily tested clinically. Clearly, more care and caution needs to be taken with patients to avoid the detrimental effects of stroke-associated infections.

5.1.3 Combination of physical and cognitive activity improves working memory

In this study a combination of cognitive and physical activity was found to improve learning and memory abilities in normal animals. Other studies have shown that learning and memory abilities are improved in animals exposed to physical activity (van Praag et al., 1999; Cotman and Berchtold, 2002), however, these studies often use 24-hour running wheel exposure throughout the experimental study and simplistic behavioural outcome measures. One difficulty in interpreting and translating these data is that of clinical relevancy. Twenty-four hours of continuous running wheel access often results in a compulsive form of running resembling addictive states in humans (Werme et al., 2002). Consequently, in this study rats were provided with 2 hour access to running wheels in order to better approximate human exercise patterns. The data from this study demonstrates the beneficial effects of combining both physical and cognitive activity on working memory outcome. The combination therapy produced significant improvements in working memory abilities when compared with either therapy alone. Interestingly, there were no corresponding increases in levels of BDNF or pCREB, which has been suggested to underlie the positive effects of exercise on learning and memory (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002; Cotman et al., 2007; Gomez-Pinilla et

al., 2008). It is possible that the relatively limited wheel running exposure or cognitive activity (2 hours each) experienced by the animals in this study in comparison to the 24-hour exposure used in other studies (Berchtold et al., 2002; Farmer et al., 2004) was not sufficient to upregulate BDNF. Regardless of the results from our protein measurements, the functional outcomes of improved working memory using clinically-relevant therapy paradigms are the most important findings and it may be that the increases in BDNF and pCREB are not necessary for cognitive improvement.

Another important aspect of this study is that the functional improvements were demonstrated using a different behavioural outcome measure. There is little evidence within the clinical literature that cognitive training transfers to aspects of cognition outside the tasks used for training (Green and Bavelier, 2008). The Hebb-Williams maze training used novelty as a key feature of the cognitive training experience. In order for an animal to recognize novelty from day-to-day, it had to use long-term recognition memory or reference memory. Importantly, improvements were noted in working memory abilities as assessed in the RAM. These results demonstrate the transfer of cognitive abilities into another functional domain. This study creates the framework for using combination therapies in order to improve cognitive outcome (e.g. cognitive rehabilitation) in animal models of stroke.

5.2 Implications For Stroke Patients

The studies that were undertaken in this thesis were all designed with careful consideration of the clinical relevancy of each experimental paradigm. For example, in

Chapter 3 I examined the effects of peripheral inflammation on ischemic outcome. Peripheral inflammation caused by systemic infection is common following stroke, manifested primarily by gram-negative bacteria that leads to pneumonia, urinary tract infections, or upper respiratory tract infections (Emsley and Hopkins, 2008). Additionally, infections occur prior to stroke in up to 30% of patients and a similar percentage of patients develop an infection 24-48 hours post-stroke (Bova et al., 1996; Macko et al., 1996; Grau et al., 1998; Grau et al., 1999). Although there were several preclinical studies indicating that systemic inflammation occurring immediately prior to, or at the time of ischemia worsens outcome (McColl et al., 2007; Spencer et al., 2007; McColl et al., 2008), there were no studies assessing the effects of delayed post-ischemic systemic inflammation. To address this, a slightly different model of systemic infection was used. Instead of an acute infection (one injection of LPS) (Spencer et al., 2006; Spencer et al., 2007), a model of a more chronic infection (three injections of LPS) over an eight-hour period was utilized because clinical infections are likely to occur over a longer period. The data indicates that delayed post-ischemic systemic inflammation results in poorer outcomes following ischemia, independent of a prolonged febrile response. These findings corroborate the clinical results and emphasize the need for increased vigilance in treating stroke-associated infections.

Results from combined cognitive and physical activity interventions on cognitive ability (Chapter 4) also have important clinical implications. There are retrospective and prospective studies that relate physical exercise with a decreased likelihood of developing dementia (Friedland et al., 2001; Laurin et al., 2001; Andel et al., 2008). Further, there are also reports that cognitive exercise may decrease the chances of developing dementia

(Snowdon et al., 1996; Verghese et al., 2003). Following stroke there is even less evidence indicating the positive effects of cognitive training on outcome (Barker-Collo et al., 2009). There are even fewer animal studies assessing the potential efficacy of cognitive training on the improvement of learning and memory abilities (Gomez-Pinilla et al., 1998). To address this shortcoming I used a novel training paradigm that consisted of Hebb-Williams maze exposure for only 2 hours/day, for 5 days/week. Similarly, the physical activity paradigm consisted of 2 hours/day for 5 days/week of running wheel exposure. Both of these paradigms have human analogues such as problem solving (e.g. crossword puzzles, exposure to new environments) and limited periods of physical exercise (e.g. treadmill running). The findings indicate that combining physical and cognitive activity together for only 4 hours/day total, each day of the week (excluding weekends) results in significant improvements in working memory abilities. This model can be used as a starting point to explore the possible beneficial effects of cognitive rehabilitation following CNS injury. While more basic research needs to be conducted in order to confirm this potential efficacy, the possibilities of improving cognitive function following ischemia appears hopeful.

5.3 Implications For Basic Research

Throughout the studies outlined in this thesis, numerous outcome measures have been used to evaluate the question of interest. Indeed, this is the primary strength of this thesis. By using multiple measures of learning and memory abilities I have identified a sensitive, comprehensive test assay to detect permanent, albeit subtle cognitive deficits

following global ischemia (Chapter 2), and improvements in working memory abilities after combined physical and cognitive activity (Chapter 4). I was also able to dissociate the detrimental effects of ischemia-associated infections on motor outcome (Chapter 3). In addition to functional assessments, I demonstrated alterations in neuropathology associated with functional endpoints using both histological and immunohistochemical measures (Chapters 2 and 3) and changes in cytokine and neuroplasticity-associated protein levels using molecular techniques (Chapters 3 and 4).

It is of utmost importance that studies use multiple outcome measures (e.g. behaviour, electrophysiology, histology) when assessing therapeutic efficacy, especially in animal stroke models. Too many studies have used only single outcome measures (e.g. histological outcome or acute behavioural test outcomes) and have found positive effects that were later found ineffective when more clinically relevant outcome measures and timeframes were examined (Corbett et al., 1990; Corbett and Nurse, 1998).

5.4 Future Directions

As I have outlined throughout this thesis, there are several exciting possibilities for future research. One avenue of experimentation would be to assess the efficacy of the physical and cognitive activity paradigm (Chapter 4) on learning and memory outcome following stroke (e.g. global and focal) as well as related models of brain injury. It is important that future studies investigate the cellular and molecular basis underlying the improvement produced by this cognitive rehabilitation paradigm (e.g. altered neurogenesis or changes in spine density). If we are able to delay or treat cognitive

problems in our animal models, then the translation into clinical practice becomes more likely as has been seen previously with motor recovery (Biernaskie and Corbett, 2001; Biernaskie et al., 2004).

Specific treatments for stroke-associated infections (Chapter 3) need to be given more careful consideration. Stroke-associated infections potentially impede patients' recovery by directly increasing neuronal damage. In addition, a microenvironment characterized by heightened neuroinflammation may offset endogenous repair processes (e.g. enhanced growth factor levels, etc.) (Murphy and Corbett, 2009). Finally, due to infections, patients may be less able to participate in rehabilitation during the first few weeks after stroke, which may be a critical time period for optimal recovery (Biernaskie et al., 2004; Murphy and Corbett, 2009).

Effective treatments for stroke are routinely reported in the experimental literature yet very few have been translated to the clinic, hypothermia being a notable exception. The reasons for this are numerous, however many studies fail to consider the clinical-relevancy of the particular animal model or treatment approach used. In fact, many of those studies have impeded progress by creating the impression that therapies are effective in animal models and not effective in humans. By developing and utilizing more clinically relevant animal models and treatment paradigms there is a much greater likelihood that findings from the laboratory will be successfully translated to the clinic.

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