# ASSESSMENT OF FUNCTIONAL NEUROPROTECTION IN A RAT MODEL OF NEONATAL STROKE

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## ABSTRACT

One type of neonatal stroke, hypoxic-ischemic encephalopathy (HIE), represents a major cause of brain damage in the human term neonate. There have been relatively few studies published to date that assess the neurobehavioural consequences associated with HIE, or use behavioural studies in rats to investigate potential therapies for HIE. To address this issue, we conducted two separate studies. First, we subjected neonatal Sprague-Dawley (SD) rats to an episode of unilateral hypoxia-ischemia (HI) (modified Levine method) and tested both neonatal and adult animals in a behavioural battery developed "in house." This battery consisted of various physical and sensory developmental, neuromotor and cognitive assessments. Preliminary analysis revealed a significant difference between male and female animals on a number of these assessments and all subsequent analyses were conducted separately for males and females. Results revealed a significant difference between HI and sham animals on neuromotor (pivoting, forelimb grip strength and, wire mesh ascending) and cognitive tests (spontaneous alternation, radial arm maze and, Morris Water Maze) with HI animals exhibiting deficits in each of these assessments.

Second, using the same surgical method and behavioural test battery deemed sensitive to detecting functional deficits following neonatal HI from the first study, we assessed the neuroprotection offered by a single high dose of erythropoietin. Immediately following hypoxia, postnatal day (pnd) 7 SD rats were administered recombinant murine erythropoietin (rmEPO; 5,000 U/kg i.p.). Results revealed that EPO offered only moderate protection in physical developmental and cognitive assessments and that these effects were different between male and female animals. Further, there was no observed protection offered in assessments of sensorimotor (both male and female) or neuromotor (males; negative geotaxis) abilities. Post-mortem measures of lesion area and brain morphology revealed that EPO did not offer significant structural neuroprotection although there was a trend towards moderate protection offered by EPO in male animals.

In conclusion, the results from these two studies show that neonatal SD rats subjected to HI exhibit various functional deficits and structural damage that can be used to measure neuroprotection. Administration of the putative neuroprotective compound, EPO, in the present dosage regime however, does not appear to offer significant long-term functional or structural neuroprotection.

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## TABLE OF CONTENTS

## APPENDICIES

## LIST OF FIGURES

## LIST OF TABLES

## ABBREVIATIONS

| Abbreviation | Term |
|---|---|
| 2-VO | 2-Vessel-occlusion |
| 4-VO | 4-Vessel-occlusion |
| ANOVA | Analysis of variance |
| AMPA | ∝-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| Apaf-1 | Apoptotic protease-activating factor 1 |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| BCM | Barnes' Circular Maze |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| CCA | Common carotid artery |
| CCAo | Common carotid artery occlusion |
| CD | Cluster of differentiation |
| CD11b/CD18 | Cluster of differentiation 11b & 18; another name for CR3 |
| CPP | 3-(carboxypiperazin-4-yl)-propyl-1-phosphonate |
| CR3 | Complement receptor-3 |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| -COOH | Carboxyl |
| COX-2 | Cyclooxygenase-2 |
| EPO | Erythropoietin |

| Abbreviation | Term |
|---|---|
| g | Gram |
| HI | Hypoxia-ischemia |
| HIE | Hypoxia-ischemia encephalopathy |
| HIF-1 | Hypoxia-inducible factor-1 |
| ICE | IL-1β converting enzyme |
| iEPO | Inactive erythropoietin |
| IFN-β | Interferon-β |
| IL-1β | Interleukin-1β |
| iNOS | Inducible nitric oxide synthase |
| i.p. | Intraperitoneal |
| i.v. | Intravenously |
| JAK | Janus tyrosine kinase |
| KA | Kainate |
| kg | Kilogram |
| LSD | Least significant difference |
| m | Meter |
| MCA | Middle cerebral artery |
| MCAo | Middle cerebral artery occlusion |
| MHC II | Major histocompatibility complex II |
| MK-801 | Dizocilpine |
| mRNA | Messenger ribonucleic acid |
| MWM | Morris Water Maze |

| Abbreviation | Term |
|---|---|
| $N_2$ | Nitrogen |
| NIH | National Institutes of Health |
| NFκB | Nuclear factor κ B |
| NMDA | *N*-methyl-*D*-asparate |
| NMDAR | *N*-methyl-*D*-aspartate receptor |
| NO | Nitric oxide |
| nNOS | Neuronal nitric oxide synthase |
| NR2 | *N*-methyl-*D*-asparate receptor 2 subunit |
| $O_2$ | Oxygen |
| PBS | Phosphate buffered saline |
| pnd | Postnatal day |
| RAM | Radial Arm Maze |
| RM ANOVA | Repeated measures ANOVA |
| rhEPO | Recombinant human erythropoietin |
| rmEPO | Recombinant murine erythropoietin |
| s | Seconds |
| SEM | Standard error of the mean |
| STAT | Signal transducers and activators of transduction |
| TNF-α | Tumor necrosis factor-α |
| TUNEL | Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling |
| tPA | Tissue plasminogen activator |
| U | Units |

| Abbreviation | Term |
|---|---|
| VEGF | Vascular endothelial growth factor |

# 1.0 General Introduction

## 1.1 Overview

Cerebrovascular disease is currently the most common cause of neurological disability in Western countries (Beers and Berkow 2005). Stroke is the most common type of cerebrovascular accident. There are two major types of strokes: Ischemic stroke, that results from an interruption in arterial blood flow that ultimately leads to neuronal cell death and occurs in approximately 88% of cases, and; Hemorragic stroke, that results from a rupture of a cerebral blood vessel within the brain and occurs in the remaining 12% of cases (American Heart Association 2005). In Canada, about 50,000 individuals are affected by stroke each year (Heart and Stroke Foundation of Canada 2002). About 16,000 of these cases result in mortality, thus, the remaining 30,000-35,000 Canadians survive this episode and are often left with various functional deficits. Financial costs to the Canadian health care system to care for these survivors is upwards of $3 billion yearly, with the average acute care costs resulting in about $27,500 per stroke (Heart and Stroke Foundation of Canada 2002). United States statistics are similar to Canadian, with a total financial burden to US society of $30-50 billion and estimates of the cost of medical treatment and rehabilitation per patient averaging $50,000 per annum (Legos *et al.* 2002). Since stroke results in such massive hospital, rehabilitative and emotional costs, there is a considerable need for intervention. Intervention itself may be targeted at any or all of the various time points surrounding stroke: prevention, neuroprotection and rehabilitation. The focus of this thesis is on post-stroke intervention in terms of: 1)

reduction of stroke-related cell death and, 2) characterizing functional deficits that may correlate with the human condition.

## 1.2 Animal Models of Ischemia

Within the experimental stroke literature there are numerous animal models that have been used to evaluate the effects and mechanisms of stroke and stroke-related damage. The advantages of using animal models to study stroke are that one is able to control the experimental conditions in which stroke occurs, such as the severity and duration of ischemia. Also, one is able to analyze the effects of ischemia by following the pathology and conducting assessments on the tissue of interest. Different strains of animals can be used to model specific human ischemic conditions, and these models can be standardized for consistency and reproducibility (Yamori *et al.* 1976; Corbett and Nurse 1998; Koch and Britton 2004). The usefulness of consistency and reproducibility between animal models is that measuring the pathophysiology, as well as the neuroprotective efficacy, of various compounds for the treatment of stroke can be evaluated more effectively. Generally, rodents (mainly rats and gerbils) have been used as animal models (Ginsberg and Busto 1989; Corbett and Nurse 1998) for several reasons: 1) rodents are obtained at relatively low cost and the experimental procedures used can be performed at a lower cost; 2) a close resemblance in the cerebrovascular anatomy and physiology to that of higher animal species as well as a consistency within particular strains of animals due to inbreeding, and; 3) a greater ethical acceptability than using 'higher' species such as non-human primates.

Animal models of stroke can broadly be categorized into 2 different types of ischemia: global and focal (MaCrae 1992). Global ischemia, the absolute reduction of cerebral blood flow, is thought to model the human case of cardiac arrest and is used to study the vulnerability of the brain to cardiocirculatory failure (Pulsinelli and Brierley 1979; Hossmann 1991). Since 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; Karpiak *et al.* 1989; Ginsberg and Busto 1989). In the 2-vessel-occlusion (2-VO) model of global forebrain ischemia, the two common carotid arteries (CCA) are occluded and either combined with systemic hypotension (in rats) or not (in gerbils due to lack of a complete formation of the circle of Willis) that reduces the blood flow to the forebrain below a critical threshold and results in initial swelling and ultimately to an area of infarction (Levine and Klein 1960; Catania *et al.* 2002). An extension of the 2-VO model is the 4-vessel-occlusion model (4-VO). Similar to the 2-VO model, both CCA are occluded 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 is much more variability in rates of mortality and extent of ischemia within the 4-VO model when compared with that of the 2-VO model (Volpe *et al.* 1984; Ginsberg and Busto 1989). Although the 2-VO and 4-VO models of ischemia are the most commonly used models to assess effects of cardiac arrest, there are several additional models of global ischemia that

have been less widely reported within the experimental literature: bihemispheral forebrain compression-ischemia (Kramer and Tuynman 1967), neck tourniquet inflation to very high pressures (Siemkowicz and Gjedde 1980), and decapitation ischemia for metabolic assessments (Yoshida *et al.* 1985).

In accord with their similarity to the human stroke condition, focal models of cerebral ischemia have increasingly gained acceptance within the experimental stroke literature (Karpiak *et al.* 1989; McRae *et al.* 1995). To date, the models that have received the most attention are models that work with occlusions of the middle cerebral artery (MCAo models) (Tamura *et al.* 1981a; Laing *et al.* 1993; Belayev *et al.* 1996; Renolleau *et al.* 1998). These models can take one of two forms: permanent or transient occlusion. In a permanent occlusion model, the artery of interest is permanently occluded usually by a cut between two ligatures or by electrocauterization (Tamura *et al.* 1981a; Tamura *et al.* 1981b; Markgraf *et al.* 1992; Yonemori *et al.* 1999). In the transient models of stroke, the artery of interest is occluded either by a clip or by insertion of an intra-luminal thread for a defined period; the occlusion is then removed to allow the recirculation of blood or a period of reperfusion similar to the situation observed in many human ischemic cases (Laing *et al.* 1993; Belayev *et al.* 1996).

Additional focal models of stroke include the introduction of cerebral embolism and thrombosis. In the blood clot embolization model, blood clots are injected either into one of the CCA or in the territory of the MCA in order to occlude neuronal blood flow (Papadopoulos *et al.* 1987). Blood 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). Although 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 tissue plasminogen activator (tPA), the emboli are unpredictably placed which make it difficult to assess the pathophysiology of cerebral ischemia (Ginsberg and Busto 1989; MaCrae 1992). A more commonly used model now is the endothelin-1 injection model. In this model, the vasoconstrictive peptide, endothelin-1, is injected locally into the area of interest and results in moderate neuronal infarcts (Riek-Burchardt *et al.* 2004; Windle and Corbett 2005). The advantage of this 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 intra-luminal thread) which may closely resemble particular human situations. Although there is no animal model that exactly replicates the clinical situation of human stroke, each of these animal models offers advantages and disadvantages and all of which depend on the specific research question being asked (Karpiak *et al.* 1989).

Another stroke model receiving a lot of attention within the past decade is the neonatal model of stroke, or neonatal hypoxia-ischemia encephalopathy (HIE) (Levine 1960; Rice *et al.* 1981; Vannucci and Vannucci 1997; Ikeda *et al.* 2002; Mishima *et al.* 2004). This model is a modification of a model originally described by Levine in the 1960's where adult rats underwent a permanent unilateral CCA occlusion followed by exposure to anoxic air (Levine 1960). In the modified model, neonatal rats at the ages of postnatal days (pnd) 7-9, undergo permanent unilateral CCA occlusion combined with exposure to hypoxic air at 7.6% or 8.0% oxygen ($O_2$) – and a balanced nitrogen ($N_2$) mix (Rice *et al.* 1981; Grafe 1994). The neonatal ages were selected in accordance with the similarity of brain development in the rat at this time with that of a term human infant,

since this is the age most commonly affected by HIE (Dobbing and Sands 1979; Rutherford *et al.* 1998; al-Naqeeb *et al.* 1999). Additionally, it is the combination of the CCA occlusion and the exposure to hypoxic air that produces neuronal damage since either in isolation does not produce structural or functional deficits (Towfighi *et al.* 1997; Nagata *et al.* 2000; Ikeda *et al.* 2001; Arteni *et al.* 2003).

There are several advantages to the rat neonatal hypoxia-ischemia (HI) model. This model resembles the human HIE condition and can be reliably reproduced with minimal animal mortality (Balduini *et al.* 2000). Although one of the CCA is permanently occluded, following hypoxia, there is still complete reflow to the ipsilateral hemisphere, reflecting a similarity with that observed in cases of neonatal HIE (Silverstein *et al.* 1984; Vannucci *et al.* 1988). Researchers who use this model are able to observe the animal for long-term assessments of neuropathology and functional abilities as well as evaluate the neuroprotective efficacy of various compounds on both a short- and long-term time scale (Han *et al.* 2002; Ikeda *et al.* 2002; Kumral *et al.* 2004; Spandou *et al.* 2004).

## 1.3 Cellular Death in Ischemia

### *1.3.1 Ischemia and Glutamate:*

Studies of post-stroke mechanisms of 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). Following an ischemic episode, massive amounts of

glutamate are released within the CNS (Benveniste *et al.* 1984; Hagberg *et al.* 1987; Takagi *et al.* 1993). Specific glutamate receptors involved in the neuronal excitatory response include: N-methyl-D-aspartate (NMDA), ∝-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA) receptors (Ozawa *et al.* 1998). The role of the NMDAR is probably the best established with respect to ischemia. Binding of glutamate to the NMDAR initiates channel opening allowing the influx of $Ca^{2+}$ and other divalent cations (Martin *et al.* 1998; Lynch and Guttmann 2002). Although $Ca^{2+}$ is an important molecule for normal cellular functioning (Olney *et al.* 1989; Ikonomidou *et al.* 1999), excess intracellular $Ca^{2+}$ can result in detrimental effects (see below), the most immediately severe of which is excitotoxicity and necrotic cell death which occurs during an ischemic episode (Ford *et al.* 1989; Olney *et al.* 1991; Streit *et al.* 1992).

The cellular and molecular mechanisms of excitotoxicity are not fully understood and likely consist of a combination of factors (Westbrook 2000). One excitotoxic mechanism linked to ischemia, that has received attention recently is the association of NMDARs with neuronal nitric oxide synthase (nNOS). In cases of excessive glutamate stimulation, NMDARs become stimulated, consequently opening the 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 subsequently activates intracellular nNOS resulting in the increased production of the free radical nitric oxide (NO), which ultimately leads to cellular death (Dawson *et al.* 1991; Kornau *et al.* 1995; Sattler *et al.* 1999).

Other mechanisms of cellular death that have been 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 2000). The role of mitochondrial function in maintaining cellular homeostasis may also be compromised as a result of high intracellular concentrations of $Ca^{2+}$, further leading to cellular stress, and ultimately to cellular death (Choi 1995; Martin *et al.* 1998). One type of cell death, necrosis, occurs around the penumbral region (area of reduced or cessation of blood flow) and, causes a destruction of cellular organelles as well as a disruption to the cellular membrane of these neurons leading to an innate inflammatory response (see below) (Li *et al.* 1995).

*1.3.2 Necrosis and Apoptosis:*

Cellular death is a prominent feature of normal central nervous system (CNS) development. As the CNS is developing and organizing, neurons receive stimulation from neurotrophic factors acting on specific cell surface receptors (Jessell and Sanes 2000). Neurotropic signaling promotes neuronal survival by suppressing a latent suicidal pathway present in neurons. Not all neurons receive this signal however, and those that do not receive adequate neurotropic signaling activate this suicide pathway and are killed by apoptosis (Jessell and Sanes 2000).

Cell death following neonatal HI is thought to occur as a result of two processes: necrosis and a relative increase in apoptosis (McDonald *et al.* 1988; Ikonomidou *et al.* 1989; Nakajima *et al.* 2000). 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 wherein 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 macrophages, typically without generating an inflammatory response (Martin *et al.* 1998). In contrast, necrosis usually results from deficits in membrane permeability and ion transport proteins as well as additional mechanisms such as oxidative phosphorylation and depletion of high-energy phosphates (Farber *et al.* 1981). Therefore, energy in the form of ATP is not required. Necrosis is characterized by clumping of the chromatin, swelling and degeneration of organelles, and destruction of membrane integrity. The cell eventually swells and ruptures causing the organelles to be expelled into the extracellular fluid. According to Martin and colleagues (1998), neuronal death immediately following cerebral ischemia is not apoptotic, based on the absence of an apoptotic morphological phenotype and the fact that particular protein synthesis becomes abnormal during the cell death process. Therefore, under stroke-related pathological conditions, the relative cell death balance is altered, with necrosis thought to be primarily responsible, at least initially, for the cell death process and a relative 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).

The neonatal rat is most sensitive to excitotoxic cell death by necrosis around pnd 6-7 (McDonald *et al.* 1988; Ikonomidou *et al.* 1989). This corresponds with the time at which the NR1 and NR2B subunit of the NMDAR are transiently elevated in the striatum and hippocampus, likely contributing to the sensitivity of the neonatal brain to excitotoxic injury at this time (Nansen *et al.* 2000; Ritter *et al.* 2002). Further, this also

corresponds with the time that animals in the neonatal HI model are exposed to the ischemic episode.

Simple blockade of the NMDAR at this time during development by pharmacological antagonism has been shown to cause deleterious morphological effects such as an increase in apoptosis (Haberny *et al.* 2002). In a study of traumatic brain injury in pnd7 rats, antagonism of NMDAR with 3-(carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP) or dizocilpine (MK-801) decreased the amount of necrotic cell death, however, the apoptotic cell death was more severe (Pohl *et al.* 1999). Therefore, the development of effective neuroprotective compounds that target the NMDAR have been slow and have only been viewed with limited success to date.

## 1.4 Neuroinflammation in Ischemia

### *1.4.1 Leukocyte Migration into the CNS:*

In the past, the CNS has been characterized as an immunologically privileged site (distinct from the peripheral immune system) (Janeway *et al.* 2001). It should, however, be viewed as an immunologically specialized site (Ransohoff *et al.* 2003). Immune reactions do occur within the CNS, but take on a distinctive character. Probably the most important morphological features that differentiate CNS immune reactions from that of the periphery, and which limit the exchange of immune cells and mediators, include: the relative lack of lymphatic drainage of the parenchyma, the lack of endogenous antigen-presenting cells, and the blood brain barrier (BBB) (Ransohoff *et al.* 2003). As a result of this limited array of immune-defense components within the CNS,

researchers have had difficulty in defining the specific mechanisms that support immune reactions within the CNS.

*1.4.2 Inflammatory Cells and Brain Ischemia:*

There is increasing evidence to suggest that cerebral ischemia elicits an unrestrained inflammatory response, which is also associated with a delayed, but significant, secondary ischemic episode (Morioka *et al.* 1993; Li *et al.* 1995; Gehrmann *et al.* 1995). For many researchers, inflammation has now become a target in the development of therapeutic interventions in order to prevent or attenuate the consequent neuronal damage that is associated with ischemia.

Since microglia represent the resident tissue macrophage of the CNS, they play an active role in brain inflammation following neuronal injury (Wood 2003). In neonatal HI (and other CNS trauma), microglial cells start to proliferate and migrate into the damaged area (Gehrmann *et al.* 1992; McRae *et al.* 1995; Ivacko *et al.* 1996). 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.* 1993). As resting microglia encounter various activating cytokines and chemokines, there is increased expression of the constitutive complement type-3 receptor (CR3, Mac-1, CD11b/CD18) on the cellular membrane (Morioka *et al.* 1992; Gehrmann *et al.* 1992; Gehrmann and Kreutzberg 1993). Additionally, there is induction of 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 has been shown to occur 2-3 hours following neonatal HI (McRae *et al.* 1995). This early activation leads to activation of additional microglial cells, presumably via the release of various cytokines and chemokines. 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 (McRae *et al.* 1995; 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. In an adult MCAo model there was evidence, however, that activation of microglia may lay dormant for several weeks (four- to five-weeks) post-ischemia, peak shortly thereafter, and then disappear six- and 7½ - 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.

### *1.4.3 Functional Roles of Inflammatory Cells in the CNS:*

One of the functional roles of microglia, as previously mentioned, is that they have phagocytic properties, which is an important mechanism to remove cellular debris. Activated, phagocytic microglia can be found adjacent to dying neurons, their dendrities, and synapses (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 penumbra, or the area surrounding the necrotic area (Gehrmann *et al.* 1992; Morioka *et al.* 1993).

Second, it has been shown that activated microglia produce several proinflammatory cytokines such as interleukin-1β (IL-1β) (as well as IL-1β converting enzyme or ICE) and tumor necrosis factor-α (TNF-α) (Bhat *et al.* 1996; Koistinaho and Yrjanheikki 2003). The sustained induction of these proinflammatory cytokines following ischemia suggests a primary role in microglial cytotoxicity. Additionally, IL-6 is produced by microglia in the penumbra and 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. On the contrary, 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).

Third, a variety of destructive enzymes are released following microglial activation including, mitogen-activated protein kinases (MAPKs), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), each of which in turn, yield reactive nitrogen and oxygen species that cause damage to cellular macrophages (Bhat *et al.* 1996; Nogawa *et al.* 1997; Kim and Ko 1998). The functional significance of these enzymes in pathological conditions, especially iNOS, is that blocking their activation following an ischemic episode, leads to a reduction in the infarct lesion (Aarts *et al.* 2002).

It is interesting to note that microglial activation does not always lead to phagocytosis and neuronal death. There is evidence that following ischemia some activated microglia lie adjacent to neurons that do not die (Banati *et al.* 1996). Instead, 'synaptic stripping' occurs. This is a process where microglia displaces synapses, leading to a situation where the neuron may remain functional; however, its synaptic connections may be altered (Koistinaho and Yrjanheikki 2003). Therefore, activation of microglial

cells following ischemia may lead to a variety of events ranging from an alteration in neuronal communication to phagocytosis of damaged and dying neurons.

Following neonatal HI, the microglia response is maximum between 24-72 hours post-ischemia but there is still evidence of activated microglia up to two weeks post-ischemia (McRae *et al.* 1995; Ivacko *et al.* 1996). Similarly, apoptotic levels were highest during 24-72 hours post-ischemia, but remained elevated up to seven days post-stroke with apoptotic markers (caspase-3) evident 17 days post-stroke (Nakajima *et al.* 2000). There is also recent evidence that cell death (most likely apoptosis), in the neonatal HI model of stoke, continues throughout the animal's life, with the area of infarction extending to the contralateral hemisphere in a progressive fashion up to 57 weeks post-HI, thus showing similarity with neourdegenerative diseases (Geddes *et al.* 2001; Mishima *et al.* 2005).

*1.4.4 Neuroinflammation and Apoptosis:*

Apoptosis is regulated by the Bcl-2 family of proteins, the adaptor protein apoptotic protease-activating factor 1 (Apaf-1), and the cysteine proteases caspase family (Yuan and Yankner 2000). Caspase-3 has been implicated in normal programmed cell death during development, but also in delayed neuronal cell death (Han *et al.* 2000). Caspases are enzymes that cleave substrate proteins at the amino acid carboxy-terminal to aspartate residues (Jessell and Sanes 2000). Activation of the precursor Apaf-1 results in the cleavage of caspase-3 and ultimately cell death. Following neonatal HI, levels of caspase-3 are significantly elevated within the ipsilateral hemisphere and inhibiting the

cleavage of this enzyme results in a reduction in apoptosis and subsequent neuronal cell death (Han *et al.* 2000; Han *et al.* 2002).

There are intrinsic anti-apoptotic proteins present in the normal animal, and activation or upregulation of these proteins results in a decrease in the levels of apoptosis following insult (Sirén *et al.* 2001; Wen *et al.* 2002). This reduction in levels of apoptosis leads to a decrease in the area of neuronal infarction following neonatal HI and may, thus, be a potential mediator in neuroprotection. Since the most significant contributor to long-term neuronal cell death following an ischemic attack is apoptosis (Renolleau *et al.* 1998; Nakajima *et al.* 2000), and apoptotic cell death follows a similar timeframe to microglial activation and neuroinflammation (McRae *et al.* 1995; Ivacko *et al.* 1996; Benjelloun *et al.* 1999), it is natural to suspect that they are intertwined (Fellman and Raivio 1997). Therefore, novel mechanisms that target these processes should theoretically, result in neuroprotection following ischemic episodes.

## 1.5 Neuroprotection Strategies for Ischemia

To date, there has only been one pharmacological intervention approved in Canada for treating ischemic strokes in adults, tissue plasminogen activator (tPA) (Legos *et al.* 2002). Tissue plasminogen activator is a thrombolytic agent that has been shown to be effective in treating some patients who present to the hospital with an ischemic stroke within three hours of initial onset. This drug, however, is not recommended for perinatal stroke and so there is a pressing need to develop neuroprotective strategies for stroke in general, including HIE. The following sections discuss a few of the pharmacological

interventions that have been studied in both adult and neonatal humans and animal models of stroke.

*1.5.1 Anti-Excitotoxic Interventions:*

With the understanding that ischemia-related excitotoxic cell death results largely from abnormal increases in intracellular $Ca^{2+}$ concentrations following excessive presynaptic release of glutamate (Martin *et al.* 1998; Lynch and Guttmann 2002), approaches have been undertaken to evaluate the efficacy of blocking the influx of $Ca^{2+}$ into neurons following ischemia. One intervention utilized the $Ca^{2+}$ channel blocker, nimodipine, in patients six hours post-stroke. But, after a three month follow-up, nimodipine failed to provide any beneficial effects on outcome for the patient (Legos *et al.* 2002). In experimental studies, NMDAR antagonists, such as MK-801 and CPP, initially seemed promising, however, long-term studies showed that although blocking the NMDARs resulted in a reduction of necrotic cell death, an increase in apoptotic cell death following stroke occurred (Pohl *et al.* 1999), ultimately providing little overall neuroprotection (Olney *et al.* 1989; Olney *et al.* 1991). One explanation for the lack of neuroprotection provided by NMDA antagonists, is that glutamate may initially be responsible for the cellular destruction, however, after this short period, it assumes its normal physiological functions, one of which is promotion of neuronal survival. Blocking the NMDAR may actually contribute to further neuronal cell loss (Ikonomidou and Turski 2002). Further, with respect to HIE, blocking these receptors early in development appears to have a detrimental effect (Ikonomidou *et al.* 1999). Recently, a synthetic peptide, Tat-NR2B9c, has been shown to disrupt the NMDAR-mediated

activation of nNOS resulting in a decrease in cellular death both *in vitro* and *in vivo* (Aarts *et al.* 2002; Aarts and Tymianski 2003). Although this compound shows promise, it is still in the experimental phase and has yet to be tested in a clinical stroke trial.

### *1.5.2 Anti-Inflammatory Interventions:*

The secondary ischemic episode resulting from apoptotic cell death has been shown to occur for several weeks and possibly even continue throughout life, if left untreated (Li *et al.* 1995; Nakajima *et al.* 2000; Mishima *et al.* 2005). This cell death is thought to result from the activation of inflammatory cells which result in the release of various pro-inflammatory cytokines and destructive enzymes (section 1.4.3). Studies have examined the neuroprotective efficacy of anti-inflammatory compounds following stroke. One class of these compounds is the tetracyclines, minocycline and doxycycline. There are several studies that show that administration of both of these compounds results in structural neuroprotection following ischemia (Yrjanheikki *et al.* 1998; Yrjanheikki *et al.* 1999; Arvin *et al.* 2002; Jantzie *et al.* 2005), however, there has yet to be published evidence for functional neuroprotection provided by tetracyclines. Other anti-inflammatory interventions tested to date include the administration of anti-inflammatory cytokines (IFN-β), proinflammatory cytokine antagonists, inhibitors of neutrophil activators, and antibodies against various cellular adhesion molecules (Nogawa *et al.* 1997; La *et al.* 2001; Liu *et al.* 2002). To date, none of these agents have been approved for the treatment of stroke.

*1.5.3 Anti-Apoptotic Interventions:*

Additional approaches to ameliorating the secondary ischemic episode have evaluated the efficacy of various anti-apoptotic compounds. In several studies, the inhibition of caspase-3 following neonatal HI resulted in structural neuroprotection (Han *et al.* 2000; Han *et al.* 2002). Further, administration of the anit-apoptotic protein, Bcl-$x_L$, results in inhibition of the pro-apoptotic protein Bax, and ultimately reduced apoptotic cell death (Finucane *et al.* 1999). There is currently a clinical trial underway in Germany, which is evaluating the neuroprotection afforded by administration of the hematopoietic cytokine erythropoietin (EPO) following an ischemic episode (Ehrenreich *et al.* 2004). Erythropoietin is thought to provide neuroprotection by stimulating the transcription of various anti-apoptotic proteins such as Bcl-2 and Bcl-$x_L$ (Sirén and Ehrenreich 2001; Digicaylioglu and Lipton 2001; Wen *et al.* 2002). Although there are only limited reports within the experimental literature with respect to the effects that EPO has in a perinatal model of stroke, these reports indicate that EPO may also protect the developing brain from ischemic damage (Kumral *et al.* 2003; Aydin *et al.* 2003; Kumral *et al.* 2004; Spandou *et al.* 2004; Spandou *et al.* 2005).

## 1.6 Functional Outcomes Following Perinatal Stroke

*1.6.1 Clinical Literature:*

Birth asphyxia, or cerebral HI, represents a major cause of brain damage in the human term neonate. Hypoxic-ischemic insults are the most common cause of brain lesions in the full-term newborn infant (Volpe 1995). Diagnosing infants with HIE

has been limited due to the variations in the clinical presentation of the event. Additionally, some infants do not manifest symptoms that lead to a diagnosis in the neonatal period and the short postnatal hospital stays may prevent a HIE diagnosis by a qualified professional (Biagioni *et al.* 2001; Lynch and Nelson 2001). As a result, epidemiological reports of the incidence of HIE have been limited and, in some cases, only children who suffer seizures were considered to suffer from perinatal stroke, resulting in an estimate of 28.6/100,000 live births (Perlman *et al.* 1994). Recent reports, however, place the incidence of neonatal stroke at 1/4,000 live births, but state that this estimate is based on those strokes that are recognized and still may represent a conservative figure (Lynch and Nelson 2001).

The major risk factors for perinatal stroke differ from those for adults. In adults, the major risk factors include atherosclerosis, diabetes, and hypertension. In neonates the major risk factors include cardiac, blood, and maternal disorders, infections, trauma and catheterization procedures and birth asphyxia (deVeber *et al.* 2000; Lynch and Nelson 2001). The causes and treatment of perinatal stroke have not been well studied and have only recently received attention within the experimental literature, possibly due to the lack of diagnosing abilities within the clinic, making it difficult to model a human phenomenon where very little is known (Perlman 1997).

Although there are differences between adults and neonates with respect to the major risk factors associated with stroke, there is overlap in the functional consequences of ischemia where perinatal stroke can also result in motor and cognitive disability and even death (Robertson and Finer 1993; Rutherford *et al.* 1998; Biagioni *et al.* 2001). For example one study that followed term neonates who suffered perinatal asphyxia found

that 25% of individuals who had suffered from moderate or severe neonatal encephalopathy were disabled when evaluated at eight years of age (Robertson and Finer 1993). These disabilities included cerebral palsy, severe cognitive delay, seizure disorders, and vision loss. Neonatal encephalopathy may also extend to affect subsequent cerebral blood flow, motor coordination, and various measures of recall or short-term memory (Gray *et al.* 1993; Robertson and Finer 1993). These statistics may even be conservative estimates, since the proportion of infants recorded as suffering from severe neonatal encephalopathy decreases from preschool to school years as a result of early death or the changing diagnosis of the type of cerebral palsy (Robertson *et al.* 1989).

To date, there has only been one drug approved in Canada for the treatment of adult stroke, the thrombolytic agent tPA, but unless this drug is administered within three hours of ischemic onset it seems that the risks outweigh the benefits that can be obtained (Legos *et al.* 2002). Given the fact that most patients do not present to the hospital until well after the three hour tPA intervention window, only a small percentage of patients actually receive treatment. With respect to neonatal stroke, however, since a diagnosis of HIE does not usually occur within the first three hours of the ischemic attack, this intervention window for thrombolytic agents may well be lost (Rutherford *et al.* 1998; al Naqeeb *et al.* 1999). Additionally, due to the risk of serious bleeding following systemic tPA administration for systemic clots, combined with the lack of safety data in childhood stroke, tPA is not recommended for children with stroke (deVeber *et al.* 2000).

Neuronal damage during this period may have significant long-term effects as evident from the various chronic neurologically-related diseases listed above. As a result, the health care system is required to treat and attempt to rehabilitate these individuals

throughout their entire lives, consequently placing a substantial economic burden on the system. Therefore, there is an imminent need for a neuroprotective intervention that may offset or attenuate the deficits caused by HI and lead to 'normal' functioning of those affected.

### *1.6.2 Experimental Literature:*

The goal of developing an animal model of a human clinical disease is to ultimately develop an intervention that will help ameliorate the consequences of that disease state. Neonatal rats undergoing the hypoxia-ischemia procedure have been shown to be similar to HIE children with respect to the neuronal structures affected (Rice *et al.* 1981; Robertson and Finer 1993; Ikeda *et al.* 2001; Biagioni *et al.* 2001). These structures (and primary functions) include the hippocampus (learning and memory) (Morris *et al.* 1982; Chou *et al.* 2001), striatum (gross volitional movements) (Felt *et al.* 2002; Lubics *et al.* 2005), thalamus (primary sensory relay center) (McHugh and McHugh 2000; Ikeda *et al.* 2001), and cortex (higher-order sensory, volitional movements, thought processing, reasoning and memory processing) (Kandel 2000; Mishima *et al.* 2004; Spandou *et al.* 2005). Therefore, in further developing the neonatal HI model of stroke, one must also model the functional deficits that are observed in children such as the somatosensory, neuromotor, and cognitive deficits. These functional abilities have been assessed in their various forms throughout the experimental literature, albeit mainly in adult animals.

*1.6.2.1 Neonatal Testing:*

There are only a limited number of studies that have assessed the behavioural abilities of neonatal rats following HI. Since some higher-order cognitive abilities occur later in development, probably due to a latent development of the hippocampus (Richman *et al.* 1986), physical development, reflexive behaviour, motor coordination and sensorimotor abilities are usually the most common assessments made during the neonatal period in rats (Altman and Sudarshan 1975; Hall and Oppenheim 1987; Lubics *et al.* 2005; Spandou *et al.* 2005). With respect to the neonatal HI studies, most fail to conduct assessments of neonatal behaviour. Of those that do assess neonates, there are varying results, with many authors reporting that there are no differences between control and HI animals in the ontogeny of reflexive behaviour (auditory startle, surface righting, negative geotaxis, and bar holding), motor coordination (rota-rod; sensitive to striatial damage) or sensorimotor abilities (sticky label; sensitive to sensorimotor cortical damage) (Young *et al.* 1986; Balduini *et al.* 2000; Felt *et al.* 2002; Grow *et al.* 2003). Further, most authors report that there are no differences in physical developmental measures such as incisor eruption, fur development, or ear unfolding between HI and control animals (Felt *et al.* 2002; Lubics *et al.* 2005). A recent study, however, that closely measured neonatal behaviours and conducted extensive assessments following the induction of HI, did find significant differences between many of the behaviours assessed (Lubics *et al.* 2005). For example, these authors found significant differences between HI and control animals in physical developmental measures such as weight gain (HI animals lighter) and day to eye opening (HI animals delayed). Also, reflexive behaviours such as surface righting, negative geotaxis, ear

twitch, and hindlimb grasp were developmentally delayed in the HI animals, but this was only a transient effect. These, and other authors, have also found HI animals to exhibit deficits when forced to use both their contralateral fore- and hind-paws in tests such as the footfault (ladder) and forelimb placing but not on the rota-rod (Young *et al.* 1986; Felt *et al.* 2002; Lubics *et al.* 2005). Other authors, however, have reported that HI animal's abilities to perform on a rota-rod may be affected when tested at pnd21 (Jansen and Low 1996; Wagner *et al.* 2002).

*1.6.2.2 Adult Testing:*

Analysis and description of the long-term functional deficits following neonatal HI have only been recently studied in depth. Cognition is the most commonly tested function in the majority of studies that assess animal's long-term functional abilities in this model of stroke. Cognitive assessments in the HI rat usually include tests of learning and memory such as the spontaneous alternation and T-test (Balduini *et al.* 2000), choice reaction time task (Ikeda *et al.* 2001; Mishima *et al.* 2004), radial arm maze (RAM) (Ikeda *et al.* 2002; Mishima *et al.* 2005) and the Morris water maze (MWM) (Chou *et al.* 2001; Kumral *et al.* 2004) (all of which have been shown to be sensitive to hippocampal damage).

There seems to be a general consensus within the literature that the cognitive abilities of the rat are affected following stroke. The most common cognitive test implemented in the HI literature is the MWM. In this task, animals are required to locate a hidden platform within a circular water maze by navigating its way according to extramaze cues (Morris *et al.* 1982). Although animals exposed to neonatal HI exhibit

evidence of learning of the maze and a memory for the location of the platform from day to day, they generally have an increased latency to locate the hidden platform when compared to control animals (Ikeda *et al.* 2001; Chou *et al.* 2001).

It has been shown that the extent of neuronal injury following this model of stroke is progressive, likely due to a prolonged apoptotic response (see section 1.4.3), thus resembling that of a neurodegenerative disease (Mishima *et al.* 2005). Corresponding to the progression of brain damage, there is also evidence of an abnormal decline in memory function with age. Mishima et al. (2005) have shown that rats exposed to the RAM at six weeks post-stroke make significantly more working memory errors than control animals (defined as choosing an arm which had previously been visited), similar to that previously reported (Ikeda *et al.* 2001; Ikeda *et al.* 2002). When rats were tested at 15 weeks post-stroke, however, not only did HI animals make significantly more working memory errors than their age-matched controls, they also made significantly more working memory errors than the stroke group that was tested at six-weeks post HI (Mishima *et al.* 2005). These results indicate that the evaluation of a neuroprotective intervention should not only incorporate short-term histological or reflexive behaviour assessments, which may only be transiently affected, but also include pathological and functional assessments throughout life and into adulthood. To date, however, there has yet to be published a report describing a long-term comprehensive test battery that is sensitive to detecting functional deficits in rats subjected to this model of stroke.

## 1.7 Thesis Objectives

The objectives of this thesis are threefold:

1) To develop and validate a comprehensive behavioural test battery based on the current experimental literature that is sensitive to detecting both short- and long-term functional deficits following exposure to neonatal hypoxia-ischemia;

2) To use histopathology to verify brain damage in this model of stroke; and

3) To determine if administration of a single high dose of erythropoietin following neonatal HI confers structural and/or functional neuroprotection.

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## 2.0 Development of a Functional Test Battery Following Neonatal Hypoxia-Ischemia

### 2.1 Introduction

#### 2.1.1 Overview of Hypoxic-Ischemic Encephalopathy

Hypoxic-ischemic encephalopathy (HIE) in neonates is a known cause of long-term neurological disability in children. Its manifestations include, but are not limited to, vision loss, seizure disorders, cerebral palsy, and abnormalities in various cognitive abilities (Gray *et al.* 1993; Robertson and Finer 1993). The most commonly used animal model of HIE uses neonatal rats on pnd 7 (Rice *et al.* 1981) and is a modified version of the Levine model originally described for adult rats (Levine 1960). Many of the experimental studies conducted to date have focused much of the attention on the biochemical and morphological changes that occur in response to the HI episode (Hagberg *et al.* 1987; Ikonomidou *et al.* 1989; McRae *et al.* 1995; Nakajima *et al.* 2000; Cowell *et al.* 2002). Over the past couple of decades we have learned much about these molecular processes in the neonate, and recently, more emphasis has been placed on learning about the functional consequences that are associated with this animal model of stroke. This is important because it is the functional deficits that are the most costly and devastating to family members and to society, so the clinical utility of this animal model requires assessments of long-term function. Similarly, measures of the effectiveness of therapeutic interventions on the long-term effects of neonatal stroke require long-term behavioural studies. Although this is becoming more common today, most researchers

still rely on only limited functional assessments that compare one aspect of an animals' abilities (such as sensory, motor, or cognitive) (Liu *et al.* 2001; Felt *et al.* 2002; Spandou *et al.* 2005). There is a need for implementation of more comprehensive functional measurements when assessing the efficacy of neuroprotective interventions in order to gain a more complete understanding of their potential value.

### 2.1.2 Neonatal HI Experimental Considerations

The method of HI used in this study (modification of Levine's procedure (Levine 1960)) is similar to those used in many other studies (Rice *et al.* 1981; Balduini *et al.* 2000; Ikeda *et al.* 2001; Chou *et al.* 2001; Felt *et al.* 2002; Ikeda *et al.* 2002; Lubics *et al.* 2005). Throughout these studies there has been variability in reports of functional deficits and neuronal lesion size associated with this model of stroke. Differences in the severity of brain damage, or in the variability associated with the behavioural findings, may be due to a number of factors. For example, different studies use different hypoxic exposure time following CCAo ranging from 1 hour (Wang *et al.* 2002; Spandou *et al.* 2005), 1.5 hours (Chou *et al.* 2001; Felt *et al.* 2002; Grow *et al.* 2003), 2 hours (Ikeda *et al.* 2001; Ikeda *et al.* 2002; Lubics *et al.* 2005), 2.5 hours (Jansen and Low 1996; Almli *et al.* 2000; Liu *et al.* 2001), 3 hours (Ivacko *et al.* 1996; Nagata *et al.* 2000; Balduini *et al.* 2000), to 4 hours (Young *et al.* 1986). Further, although the majority of studies using the neonatal HI model use a humidified gas mixture of 8% $O_2$ / 92% $N_2$ for the hypoxic exposure (Nagata *et al.* 2000; Almli *et al.* 2000; Mishima *et al.* 2005), others use a 7.6-7.7% $O_2$ / balance $N_2$ mixture (Grafe 1994; McRae *et al.* 1995) to induce the hypoxic

episode, which may result in a difference in the subsequent neuronal loss and consequent functional deficits. Another factor that may contribute to the variability within the experimental HI literature is the temperature control of the pups during the HI period where it has been shown that reducing the pups' temperature by $6^0$C during the hypoxic exposure results in significant neuroprotection, and that increasing the pups' temperature by only $4^0$C results in increased neuronal loss and functional deficits when compared with animals kept at normal nesting temperatures (Mishima *et al.* 2004). Also, it has been shown that the CCA which is occluded (i.e., left or right) may have a subsequent effect on the outcome of the behavioural analysis, in that contingent on the artery which is occluded, hemispheric differences in functional abilities may occur (Arteni *et al.* 2003).

Since all of these factors play a role, one might expect variability in the results across various studies conducted in different laboratories. One goal in the present study, as indicated, was to take into account all of the factors mentioned above that were associated with the induction of hypoxia-ischemia and develop a consistent surgical protocol that addressed these concerns and a long-term follow up in order to avoid potential confounds. In this study, pnd 7-8 rats were exposed to left CCAo and were exposed to three hours of hypoxia with a mixture of 8% $O_2$ / balanced nitrogen since this combination has been shown to result in more consistent neuronal infarcts and produces the maximum amount of injury before significant mortality is experienced (Grafe 1994; Nagata *et al.* 2000).

The behavioural test battery which was selected to assess functional deficits in rats following neonatal HI was a compilation of behavioural tests that were shown to be

sensitive to detecting HI deficits by other laboratories (such as spontaneous alternation, RAM, and MWM) (Balduini *et al.* 2000; Ikeda *et al.* 2001), as well as tests and assessments that have been used previously to quantify the effects of exposure to trauma early in development (pivoting, swimming ontogeny, and negative geotaxis) (Ryan and Pappas 1985; Pizzi *et al.* 1998; Ohta *et al.* 1998). Further, additional assessments included the observation of the normal development of the rat such as eye opening, incisor eruption and auditory startle as well as additional cognitive assessments such as the T-maze and the BCM, which have also been shown sensitive to detecting early trauma (Hass *et al.* 1999; Balduini *et al.* 2000; Patin *et al.* 2004). The rationale behind selecting such a wide range of functional tests was to address more than simply one aspect of a rats' behavioural repertoire, unlike much of the neonatal HI literature that usually looks at either cognitive *or* motor abilities as well as to develop a comprehensive battery reflecting the diversity within the clinical literature. In the present study physical and sensory development, neuromotor and cognitive abilities of the rat were all assessed in various forms and at various time points throughout the animals' life. By conducting these assessments we were able to gain a more complete understanding of the deficits associated with this model of stroke in rats.

### 2.1.3 Study Goals

The goals of this current study were to develop a comprehensive functional test battery that was sensitive to detecting stroke effects following neonatal HI and to describe damage to the particular brain areas that are affected by this model of stroke. In

the current study we analyzed rat behaviour using a variety of tests for both neonates and adults. The range of functional assessments included physical and sensory developmental measures, neuromotor, sensorimotor and cognitive abilities. Further, we tested both male and female animals but separated the sexes when analyzing the data. The rationale behind this stems from the experimental literature stating there are differences in the neuronal development and neurochemistry (Ross *et al.* 1981), and maze solving abilities of male and female rats (Roof 1993; Ulloa *et al.* 2004). Further, female rats complete the estrous cycle in 4-5 days which contributes to differences in hormonal levels and may contribute to differences in behavioural performance (Jenkins and Becker 2004). These differences may be manifested as differences in the way in which stroke affects a particular sex.

We hypothesized that animals exposed to neonatal HI on pnd 7 would experience deficits in neuromotor, sensorimotor and cognitive abilities and also experience neuronal loss when compared to animals not exposed to neonatal HI.

## 2.2 Materials and Methods

### 2.2.1 Animals

Untimed, pregnant Sprague-Dawley rats (Charles River Laboratories; Montreal, Canada) were housed in individual cages at Dalhousie University (Nova Scotia, Canada). The animal colony room was kept at $22^0$C and maintained on a 12:12 hour light:dark cycle (lights on at 07:00) and animals were provided with food (Purina Lab Chow) and water *ad libitum.* Except for routine cage maintenance, cages were left undisturbed until the day of delivery which was considered postnatal day 0 (pnd 0). Litters were culled on the day of surgery (pnd 7-8), at which time litters were randomly reduced to five males and five females where possible. There were two conditions represented within each litter selected by pseudo-randomization: one that underwent the hypoxia-ischemia procedure (5 males and 5 females) and another, the control group, which underwent a sham procedure (3 males and 7 females).

### 2.2.2 Animal Transportation Procedures

Animals were transported from Dalhousie University to the University of Prince Edward Island at ages pnd 11-12. Rat pups and dams were transported in individual cages with filter cage lids in a temperature controlled University vehicle. Upon arrival at the University of Prince Edward Island the animals were placed in a colony room with similar environmental conditions as at Dalhousie University (i.e., 12:12 hour light cycle

with light on at 07:00 and temperature controlled at approximately 22$^0$C). As at Dalhousie University, animals were provided with *ad libitum* food (Purina Lab Chow) and water upon arrival to the University of Prince Edward Island.

2.2.3 Surgery

The procedure used was a modification from that first described by Levine in adult animals and later revised by Rice and colleagues to accommodate neonatal animals (Levine 1960; Rice *et al.* 1981). Pups (pnd 7-8) were anesthetized with 2% isoflurane in $O_2$ and a 1 cm incision was made along the midline of the neck and the left common carotid artery (CCA) was exposed and isolated using a dissecting microscope. The CCA was coagulated using a unipolar electrocauterizer (Aaron RAM$^{TM}$ cauteries; AA04) (CCA occlusion; CCAo), closed by suturing and Xylocaine® (topical anesthetic) was applied to the incision site. Pups were then returned to the dam for 2 hours to recover and nurse. After 2 hours of recovery, pups were removed from the dam and rendered hypoxic by placement in humidified chambers (max. 2 pups / chamber) (Figure 2.1) and exposed to 8% oxygen and 92% nitrogen for 3 hours at 34.0± 0.5°C. Pups were monitored by visual inspection throughout this period. Only one litter was subjected to the HI procedure at a time. After exposure to this hypoxic air, animals were exposed to normoxia at 34.0 ± 0.5°C and observed for 15 minutes, then returned to the dam. Control animals underwent a similar surgical procedure with the exception of the carotid ligation. In this group of animals, the left CCA was isolated but was not cauterized. Control animals were then returned to the dam for the same two hour recovery period. Following

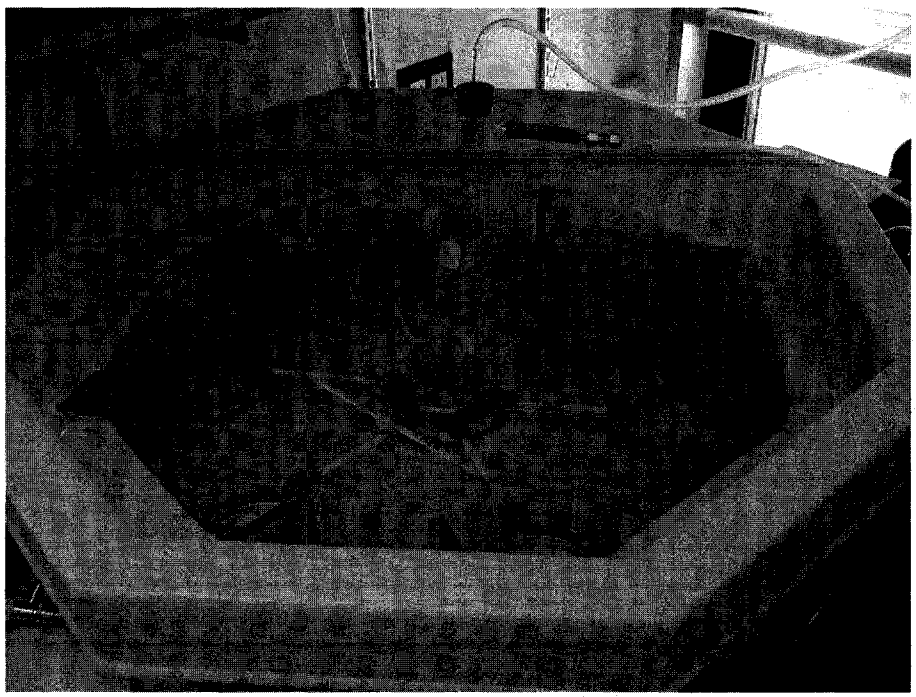


*Figure 2.1.* Neonatal hypoxia chambers. Pnd 7-8 rat pups were placed in chambers and exposed to hypoxic air mixture (8% $O_2$ / 92% $N_2$) for a period of 3 hours.

recovery, control pups were removed from the dam and exposed to normoxia for 3 hours at 34.0± 0.5°C and then returned to the dam until testing on the following day.

2.2.4 Behavioural Tests

All behavioural assessments were conducted by an experimenter blind to the individual condition of each animal. The timeline for all behavioural testing is shown in Figure 2.2.

2.2.4.1 Physical Development:

The physical development measures that were recorded included day on which eye opening occurred (slit in the eye suture) and top and bottom incisor eruption (recorded the day on which both the top and bottom incisors erupted). Additionally, pup weight was recorded immediately following surgery and again once per day until pnd 30 and then subsequently at various intervals (pnd 37, 44, 125 and 177).

2.2.4.2 Sensory Function:

*Olfactory Orientation* (pnd 9-12): Pups were placed in the center of a cage. At one end of the cage there was clean bedding and at the other was soiled bedding from the home cage. The pup's initial directional orientation, the latency to orient to a particular bedding and latency to reach the selected bedding were recorded (Altman and Sudarshan 1975).

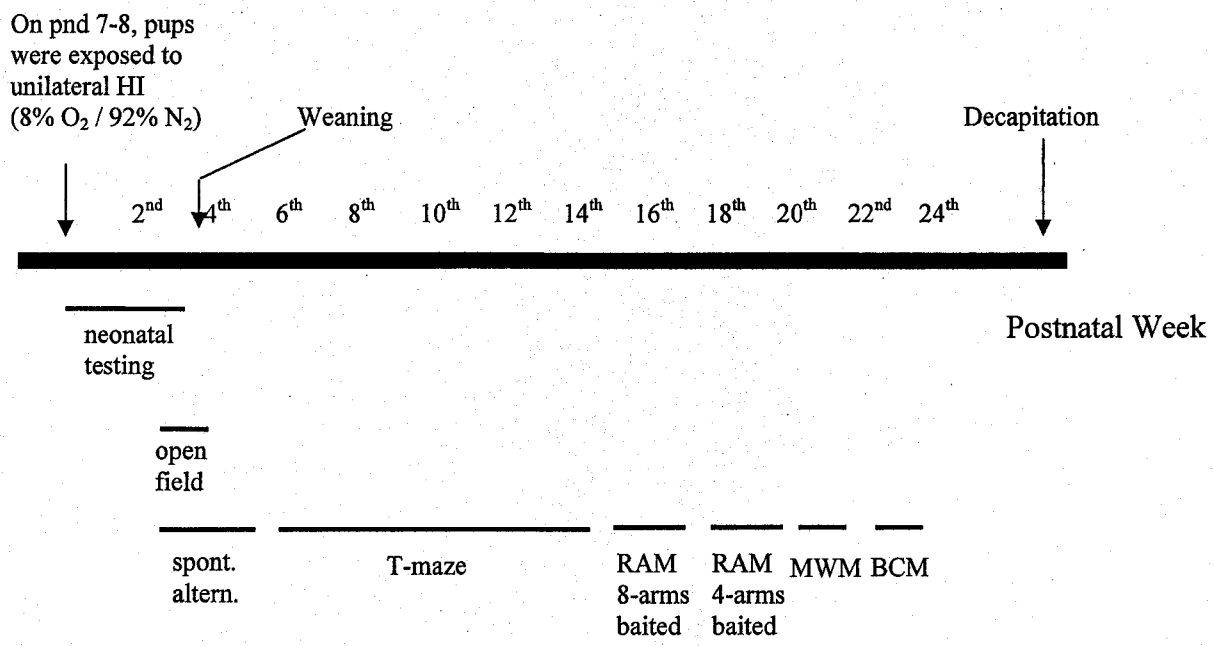



*Figure 2.2.* Experimental schedule for all behavioural tests

*Auditory Startle Reflex* (pnd 10 - until criterion): Animals were placed on the table top and subjected to an audible click approximately 5 cm directly above the head and the animal's response was recorded. The criterion response consisted of an animal exhibiting a startle response to the sound.

2.2.4.3 Neuromotor Tests:

Due to the nature of development (i.e., rostral-caudal development), forelimb function develops more rapidly than hindlimb function, manifested as differences in various aspects of neuromotor behaviour (Altman and Sudarshan 1975). Further, limb coordination continues to improve throughout development and this is thought to be reflected in improved scores on neuromotor tests. All neuromotor rating scales are described in Table I.

*Surface Righting* (pnd 8): Latency to turn over from a supine position on to all four legs (maximum of 30s) was recorded. Each pup was given two trials and the average latency to turn was recorded.

*Negative Geotaxis* (pnd 8-12): Pups were placed facing downward on an inclined rough surface. The degree of inclination has varied throughout the literature: 25° (Adams 1986), 30° (Ohta *et al.* 1998; Mikulecka and Mares 2002), and 45° (Coleman *et al.* 1999). Due to the purpose of this study, a higher degree of inclination (i.e. 45°) was selected in order to create a maximal differentiation between hypoxia-ischemia and control animals.

Table I.

*Neuromotor Tests Scoring Regime*

| Behavioural Test | Score | Description |
|---|---|---|
| *Negative Geotaxis* | **0** | pup falls off or remains head down for entire time |
| | **1** | pup turns > 40s |
| | **2** | pup turns > 20s |
| | **3** | pup turns ≤ 20s |
| *Swimming Ontogeny* | | |
| ▪ Direction | **0** | pup sank |
| | **1** | pup floated |
| | **2** | swam in a circle |
| | **3** | swam in a straight line |
| ▪ Nose Angle | **0** | nose submerged |
| | **1** | nose at water surface |
| | **2** | nose and top of head at/above surface |
| | **3** | nose and head elevated so that water level at mid-ear or below |
| ▪ Limb Usage | **0** | no limb paddling |
| | **1** | paddling with front limbs only |
| | **2** | paddling with all 4 limbs |
| | **3** | paddling with hind limbs only |
| *Forelimb Grip Strength* | **0** | no hind limbs on bar |
| | **1** | places 1 hindlimb on bar |
| | **2** | places both hindlimbs on bar |
| *Wire Mesh Ascending* | **0** | pup remains at the bottom or fails to reach platform |
| | **1** | pup reaches platform > 60 sec. |
| | **2** | pup reaches platform 30-59 sec. |
| | **3** | pup reaches platform < 30 sec. |
| *Mid-Air Righting* | **0** | pup fails to right |
| | **1** | pup rights |

Coleman *et al.* (1999) reported that control animals were capable of turning 180° by pnd 9. Pups were given a maximum time limit to turn 180° of 60s.

*Pivoting* (pnd 9-12): Animals were placed in an empty cage on which lines were drawn that delineated 90° quadrants. The total number of turns/pivots for each completed quadrant (i.e., 0[<90°], 1[90°], 2[180°], 3[270°], etc.) was recorded within a one minute testing session. Any movement of less within a quadrant or retracing less than 90° were discarded (Pizzi *et al.* 1998; Ohta *et al.* 1998). Testing consisted of one session per day.

*Swimming Ontogeny* (pnd 16, 17, 18, 19, 21, 23 & 31): Animals were placed in a rectangular pool with glass on one side in order to observe swimming behaviour for a single trial on each of the days. Three aspects of swimming were measured on a scale of 0-3 (direction, angle of the nose, and limb usage; (Schapiro *et al.* 1970; Ryan and Pappas 1985; Vorhees 1986).

*Forelimb Grip Strength* (pnd 10-17, 19 & 23): Animal's forelimbs were placed on a wire horizontal bar [0.2cm diameter; (Coleman *et al.* 1999)] and then released. The duration of holding onto bar and the paw which first slipped off of the wire was recorded (maximum of 60s; adapted from Barlow and Sullivan battery in (Adams 1986)).

*Wire Mesh Ascending* (pnd 12-17): Pups were placed on a surface consisting of 10mm wire mesh, 45cm high and 15cm wide surface on a 70° inclination (Mikulecka and Mares 2002). Littermates were placed in a cage at the top of the platform in order to

provide sufficient motivation for the pups to climb to the top of the apparatus. Pups were removed from the cage and placed at the bottom of the wire mesh (which was immersed in cold water) with all four paws in contact with the surface, and given 120s to reach the platform.

*Mid-Air Righting* (pnd 13 & 14): Animals were released from a height of approximately 60 cm onto a well padded surface (Altman and Sudarshan 1975). Animals' landing position was observed and recorded. Righting ability was determined based on two out of three landings.

*Open Field* (pnd 18, 20, 22, 27 & 29): Animals were placed in an open arena (60 cm x 60 cm) with grids for a 5 minute trial. Total number of grid crosses and inner grid crosses verses outer grid crosses were recorded.

2.2.4.4 Cognitive Tests:

*Spontaneous Alternation* (pnd 20, 25, 30, 35) (Figure 2.3): The spontaneous alternation test measures rats' innate exploratory behaviour (Richman *et al.* 1986). In this maze, normal rats select alternating arms over different trials. In order for the rat to achieve this, it must remember the arm which was previously selected. Therefore, if there is damage to the memory systems in the brain, the rats' alternating behaviour will be affected.

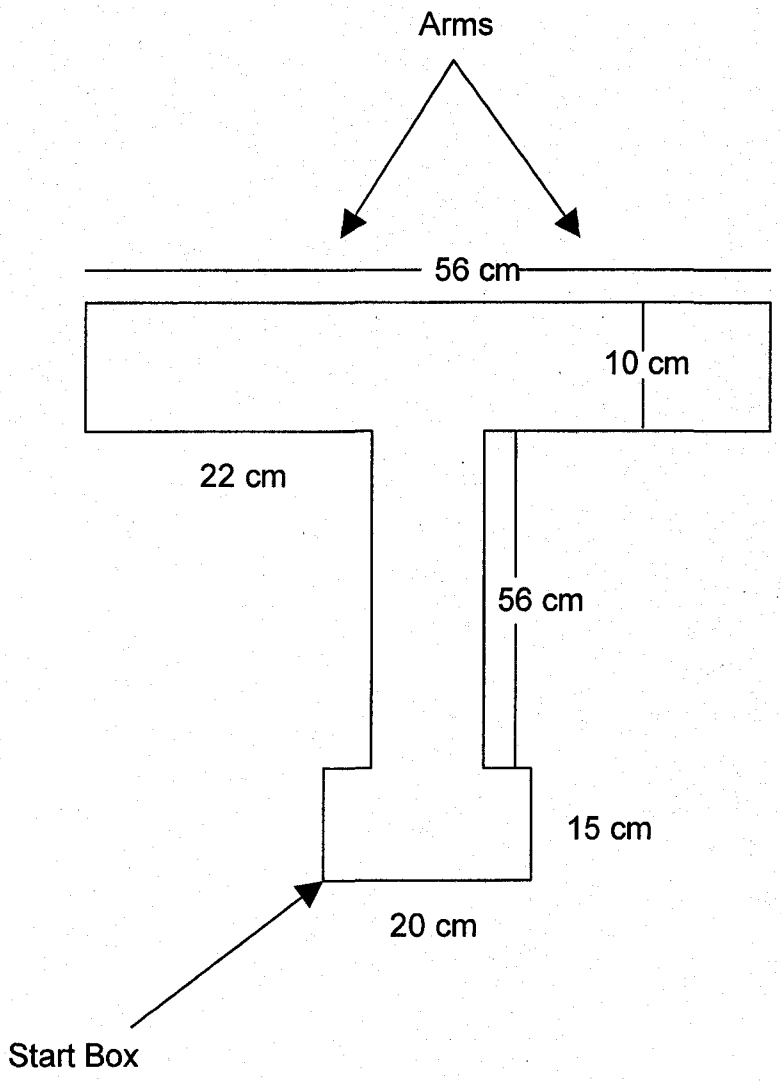



*Figure 2.3.* Equipment for spontaneous alternation and T-maze tests.

Animals were placed in the start box of a T-Maze for 60 seconds. Following this 60 second period, the guillotine door which blocked access to the maze was raised. Animals were given a maximum of 120 seconds to choose an arm. Once all four paws were in either the right or left arm, another guillotine door was closed and access to the maze was therefore blocked. Arm selection and latency to arm selection was recorded. There were three trials per session, separated by an intertrial interval of 30 sec. where the maze was cleaned to remove odor trails, with four separate testing sessions separated by five days each.

*T-Maze* (pnd 48-100) (Figure 2.3): Rats were placed in the apparatus for two 5 minute exploratory sessions (on separate days) each before test trials begin for habituation purposes. In these acclimation sessions, 20 banana pellets (Noyes pellets) were placed throughout the maze. Animals were not food deprived on these two days (pnd 43-47). Following these two habituation days, animals were tested using a 'forced choice' procedure (Balduini *et al.* 2000) that reinforced a win-stay strategy for 15 days with each day consisting of 8 'forced choice' trials. Each trial had a maximum time limit of 60 seconds. When T-maze testing began, animals were tested every 3$^{rd}$ day (two groups of 10 animals each day). Animals were given ad libitum food for a 24 hour period followed by 24 hours of food deprivation prior to testing.

Animals were given a 'forced choice' beginning on day 3. Animals were placed in the start box of the T-maze for a 15 second period. One arm was blocked and the rat was required to leave the start box and retrieve the reward from the unblocked arm. Once the reward was obtained, animals were removed from the baited arm and returned to the

start box for a 15 second period. During this time, the block was removed from the arm so that there were two available choices, but the previously baited (Noyes pellets) arm (forced choice arm) was re-baited (reinforcing a win-stay strategy). Following the 15 second period, animals were allowed access to the maze. Arm choice (left or right) as well as the latency to select an arm was recorded. A correct choice was defined as selecting the arm that was rewarded in the forced arm trial. Baiting of the forced arms was pseudo-randomly selected.

Following the choice selection trial, animals were removed and placed back into the home cage for a 5 minute period. Following this period, animals were placed into the start box and the second trial began using the procedure as described above. If an animal failed to select an arm during a trial it was removed and returned to the start box (or home cage depending on the step of the procedure).

*Criterion:* The criterion for this procedure was that animals make at least seven correct choices out of eight trials (i.e., 87.5) on two consecutive sessions. Once the animal reached criterion, the latency between the forced choice and test trial was increased from 15 seconds to 30 seconds. Additionally, when the animals reached this criterion (7/8 for two consecutive sessions), this latency was further increased to 60 seconds.

*Radial Arm Maze* (pnd 106-139) (Figure 2.4): Animals were placed in a standard radial arm maze (Lafayette Instrument®, IN, USA). This maze consisted of an apparatus with eight arms projected from an octagonal center platform with clear plexiglas® rails on the sides and ends (Olton and Samuelson 1976; Volpe *et al.* 1984). At the ends of

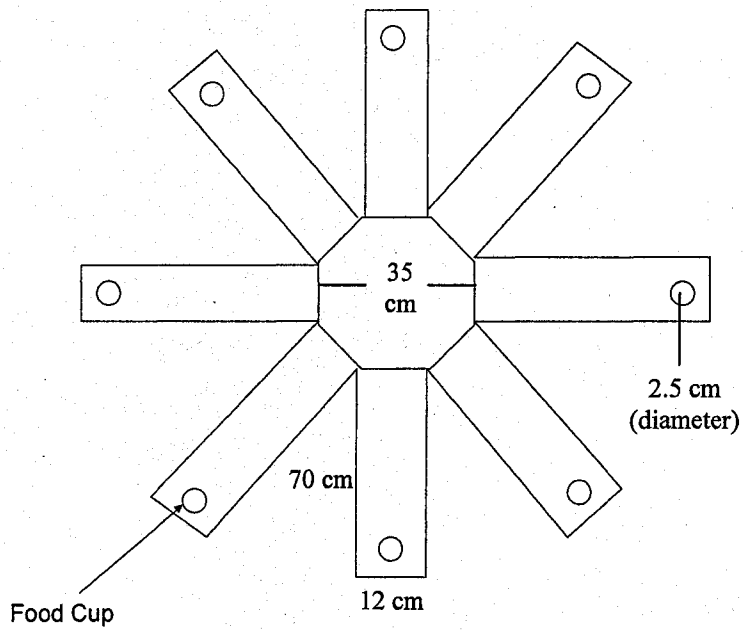



*Figure 2.4.* Equipment for Radial Arm Maze testing. Each arm of the maze had 20 cm high walls. The maze was elevated 90 cm above the experimental room floor.

each arm there was a food cup where food rewards (Noyes pellets) were located. Animals are food restricted prior to testing in order to increase motivation levels and are reinforced for choosing the correct arm by obtaining the food reward.

Animals were placed in the radial 8-arm maze (RAM) for two sessions (one session / day) with each session consisting of a maximum of five minutes, or until all pellets were consumed. There were 24 pellets scattered throughout the maze (3 pellets / arm) and latency to consume all pellets was recorded. Animals were food restricted to a maximum of 95% *ad libitum* body weight throughout the RAM procedure.

Following the habituation procedure, only one pellet was placed in each arm so that all eight arms were baited. Animals were placed in the maze and allowed access to all eight arms. The trial was completed when the animal had visited all eight arms or if five minutes had elapsed. The latency to visit all eight arms was recorded as well as the number of omission errors (failure to enter an arm) and the first eight arm selections (number recorded represented the number of correct choices made, i.e., visiting an arm not previously visited during the same session). Additionally, the number of re-entry errors (commission errors) were also recorded. Animals were tested one session per day using this procedure for a 10 day period (pnd 106-116).

Animals were next tested on the RAM using a modified version of the procedure described above (pnd 126-139). In this procedure, only four of the eight arms were baited (randomly selected), and kept constant throughout the procedure (Huang *et al.* 2004). The session was completed when the animal visited the four baited arms or if a period of five minutes had elapsed. Latency to visit the four baited arms was recorded. Additionally, the unbaited arm entries and re-entries, omission errors (failure to visit a

rewarded arm), baited arm re-entries, and first four choices were recorded. Similarly as above, animals were tested one session per day for a period of 13 days.

*Morris Water Maze* (pnd 141-149) (Figure 2.5; procedure modified and adapted from Morris *et al.* (1982)): Animals were placed in a circular pool of water (1.50 m diameter) with a platform submerged approximately 2.0 cm below the water. The water was darkened with non-toxic, black liquid paint and was kept at $21.8^0 \pm 0.29^0$C (mean ± S.E.M.). Animals were placed into the pool, facing the wall of the pool, and were required to locate the platform based on extra-maze cues. Surrounding the maze was a series of connecting curtains in order to concentrate the area of extra-maze stimuli which consisted of a large white triangle and square on one side, horizontal and vertical white lines on another, towels on the third, and the forth consisting of the curtain itself. Each animal was given 4 trials per day (placed once at each quadrant) for four days where the starting positions were pseudo-randomly determined and kept constant throughout testing. The hidden platform remained in the same position throughout the entire procedure. Each trial lasted for a maximum of 60 seconds. If the animal failed to locate the platform within the allotted 60 seconds it was removed from the maze and placed on the platform. Once the animal was on the platform, it was given 60 seconds to further observe the location of the platform relative to the extra-maze stimuli. Following this 60 second period, the animal was removed from the maze and placed in a drying cage for 30 seconds. The animal was then removed from the drying cage and placed back into the maze for another trial. Latency to locate the hidden platform was recorded.

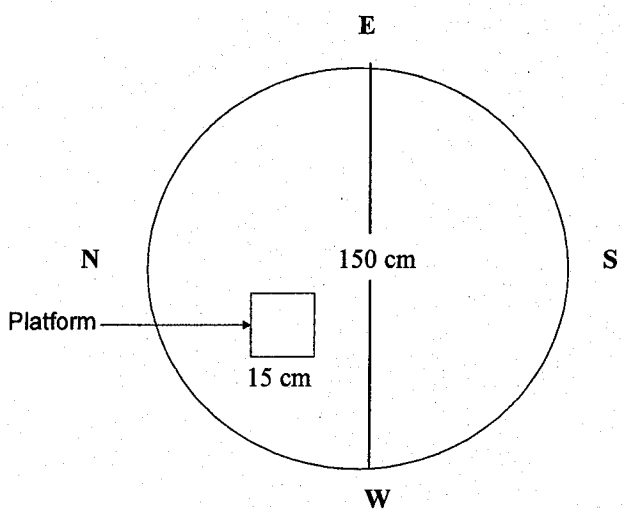



*Figure 2.5.* Equipment for Morris' Water Maze testing. Pool was filled with approximately 25 cm of water. Platform was submerged approximately 2.5 cm below the surface of the water.

Following this four day procedure, the platform was removed from the maze and a probe trial procedure was used. In this procedure the animal was placed into the maze, opposite to where the platform was located, for one trial lasting 60 seconds. Following this, the animal was removed from the maze and returned to its home cage.

Three days post-probe trial, animals were re-tested in the maze using the original testing procedure. The platform was submerged and returned to its original location. Each animal was given four trials to locate the platform and latency to locate the hidden platform was recorded. Following completion of each trial the animal remained on the platform for 60 seconds and then removed from the maze for 30 seconds (as described above).

The next day all animals were tested in a visible platform test trial. In this procedure, animals were placed into a pool of clear water with a dark platform 0.5 cm above the water surface. Animals were first placed on the platform for 60 seconds. Following this, the animal was placed in the center of the maze and allowed to swim to the platform. Immediately following successful completion of this task, animals were removed from the platform and placed in the maze at the pool wall directly opposite the platform and allowed to swim to the platform (two trials). Following this animals were removed from the maze and returned to the home cage.

All MWM trials were video recorded for later analysis using the computer program Ethovision© (Noldus, Wageningen, Netherlands) to calculate swimming velocity and distance.

*Barnes' Circular Maze* (pnd 154-162) (Figure 2.6; procedure modified and adapted from (Barnes 1979)): Animals were placed on an elevated circular platform. Around the perimeter of this platform there were 18 escape holes, with only one hole representing the *true* escape hole where the animals' home cage was located. This maze is thought to represent a land-based version of the Morris Water Maze, with the additional capability of separating the working and reference memory component of the behaviour and without subjecting the animals to a stressful swimming procedure (McLay *et al.* 1998). In order to motivate the animals to locate and enter the escape hole, this maze utilized an aversively motivated task represented by bright lights surrounding the maze (painted white in order to maximize light reflection) combined with a loud "white" noise from directly above the maze. The animals were initially placed in the centre of the maze under a box in order to prevent orientation confounds. The trial began when the lights and white noise were turned on and the box was raised allowing animals access to the maze.

Animals were acclimated to this procedure before the testing began. The acclimation trial consisted of exposing each animal to the maze, bright lights, loud noise, and that entering the escape hole was associated with the termination of this aversive environment. Surrounding the maze were cues such as a computer in one corner, wall dividers, a clear wall and, the door to the experimental room. Following the acclimation trials, the animals were tested on four consecutive days, one session per day, and four trials per session. Further, one week (seven days) post-testing, animals were tested again in order to evaluate reference memory abilities. Throughout testing, the escape hole remained in same location with respect to external room cues. Between each trial, the

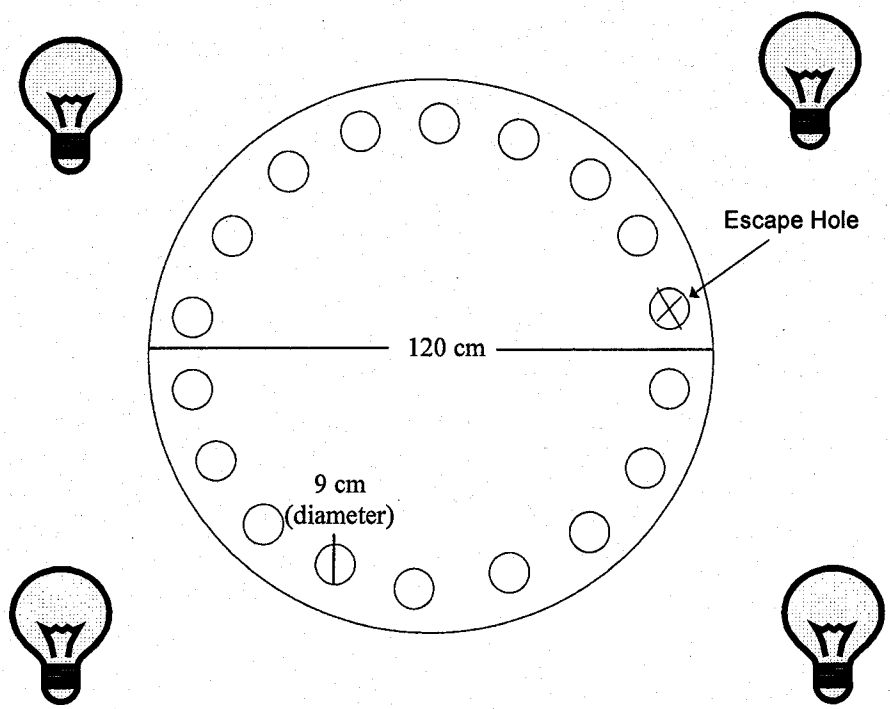



*Figure 2.6.* Equipment for Barnes' Circular Maze testing. A box was placed directly beneath the escape hole. The maze was elevated approximately 1 m above the experimental room floor.

maze was rotated in order to minimize the role of odor cues in locating the escape hole. In addition, the maze was cleaned between each animal in order to avoid potential odor confounding.

Trials consisted of a maximum of 180 seconds with the trial ending when the animal entered the escape hole, and the latency recorded. As previously mentioned, trials began when the box was raised allowing the rat access to the maze. The intertrial interval was 60 seconds: 30 seconds in the escape box and an additional 30 seconds removed from the maze while it was rotated; following the maze rotation, animals were placed back into the box to begin the next trial. Four dependent measures were recorded, including: latency to enter escape hole; number of times animal visited an incorrect hole; the number of time animal re-visited an incorrect hole within the same trial; and the number of times an animal visited the correct escape hole but failed to enter. Animals were tested four trials per day for four days, followed by a re-test one week later. Following testing, animals were removed from the testing room and returned to the home cage in the colony room.

### 2.2.5 Brain Removal

Following completion of all functional assessments, rats were euthanized by $CO_2$ exposure and decapitation. Brains were removed and fixed in 10% buffered neutral formalin (Fisher Scientific Co., Ottawa, ON) for 4-6 days and then photographed.

2.2.6 Statistical Analysis

Results were expressed as mean ± standard error of the means (S.E.M.). Data from the physical, neuromotor and cognitive tests were analyzed by two-way analysis of variance (ANOVA) with repeated measures, where applicable. The sphericity assumption was measured using Mauchly's Test and in instances where the sphericity assumption was violated, the Huynh-Feldt test was used to correct for this violation (Crowder and Hand 1990; Weinfurt 2000). Homogeneity of variance was assessed following univariate ANOVA using Levene's Test, however, since the Levene's test is considered very sensitive, the alpha value was set at 0.001 (Tabachnick and Fidell 1996). In cases where the Levene's test result exceeded a probability value of 0.001, the Welch correction was used (Dr. Andy Field, personal communication). For all other analyses, the alpha value was set at 0.05. Analyses of condition X day interactions were conducted using independent t-tests with the Bonferroni correction. In cases where parametric tests were not applicable, the Chi-Square nonparametric test was used. Separate analyses were used for male and female animals to analyze the effects of condition on the functional effects of a hypoxic-ischemic episode in the absence of potential sex x condition interactions. The computer software Statistical Package for the Social Sciences (SPSS for Windows 11.5.1, 2002, SPSS Inc., USA) was used to analyze all of the data obtained.

## 2.3 Results

Only those measures showing a *condition* or a *condition X day* effect are reported in detail in text. Data from all behavioural tests are presented in tabular format in Appendix A.

### 2.3.1 Physical Development:

There were no significant differences between groups on measures of weight, day to eye opening (right: male HI-pnd 13.0; male control-pnd 12.7; female HI-pnd 13.0; female control-pnd12.9) (left: male HI-pnd 14.4; male control 12.7; female HI-pnd 13.2; female control-pnd 13.3), nor incisor eruption (top: male HI-pnd 9.2; male control-pnd 9.0; female HI-pnd 9.2; female control-pnd 9.9) (bottom: male HI-pnd 10.4; male control-pnd 9.3; female HI-pnd 10.2; female control-pnd 10.6) (see Appendix A).

### 2.3.2 Sensory Function:

There were no significant differences between groups on measures in the olfactory orientation test or day on which auditory startle first developed (male HI-pnd 12.6; male control-pnd 11.7; female HI-pnd 12.2; female control-pnd 12.0) (see Appendix A).

2.3.3 Neuromotor Tests:

There were no significant differences between groups in surface righting, negative geotaxis, swimming ontogeny, mid-air righting and, olfactory orientation (see Appendix A). Two-way ANOVA (condition x day) with repeated measures revealed that there was a significant difference between both male [$F(1,6) = 6.474$, $p<0.05$] and female [$F(1,10) = 5.510$, $p<0.05$] HI and control animals in the number of pivots in the clockwise (right) direction with HI animals making more right turns (Figure 2.7A & C, respectively). There were however, no differences in the number of counterclockwise (left) pivots between male and female HI and control animals [$F(1,6) = 3.675$, $p>0.05$; $F(1,10) = 0.519$, $p>0.05$, respectively], or in the total number of pivots [$F(1,6) = 0.062$, $p>0.05$; $F(1,10) = 1.285$, $p>0.05$] (Figure 2.7B & D, respectively).

There were no differences between conditions in the average latency to hold on to wire in the forelimb grip strength test, however there was a significant difference between female HI and control conditions in paw slips on pnd 23 [$\chi^2(2) = 8.571$, $p<0.05$] with female HI animals making significantly more right paw slips than control females (Figure 2.8B). There was no corresponding difference between male HI and control conditions (Figure 2.8A).

Wire mesh ascending was evaluated at pnd 12-17. With respect to the male animals, two-way ANOVA with repeated measures of latency to reach platform revealed that there was no effect of condition [$F(1,6) = 0.383$, $p>0.05$] or interaction [$F(3.921,23.527) = 0.347$, $p>0.05$], but a significant effect of day [$F(3.921,23.527) = 5.250$, $p<0.01$] (Figure 2.9A). With respect to female's latency, there was a significant

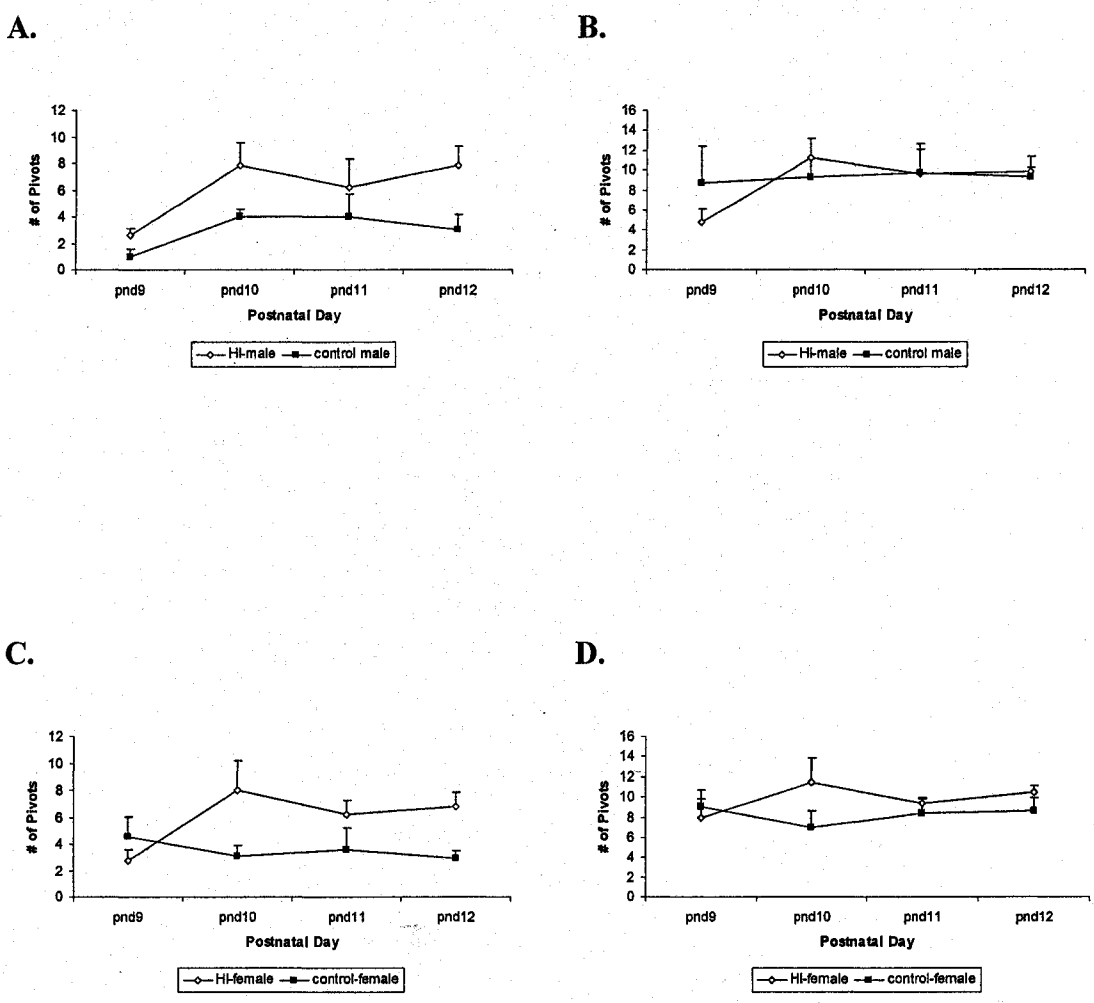
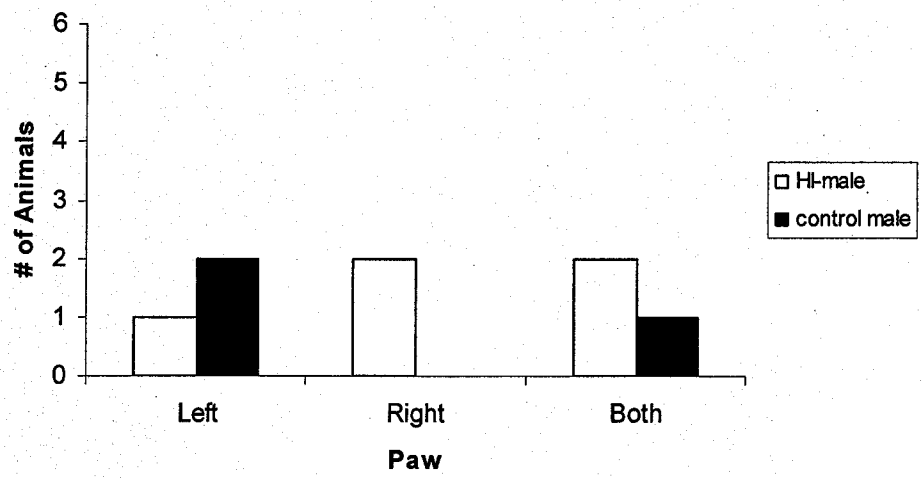



*Figure 2.7.* Performance on the pivoting task. Average number of pivots from postnatal day 9-12 (means ± SEM). (A) Male average number of right pivots. Significant difference between conditions ($p<0.05$). There was no difference between male conditions in the total number of pivots (B). (C) Female average number of right pivots. Significant difference between conditions ($p<0.05$). There were no differences between female conditions in the total number of pivots (D).

A.




B.

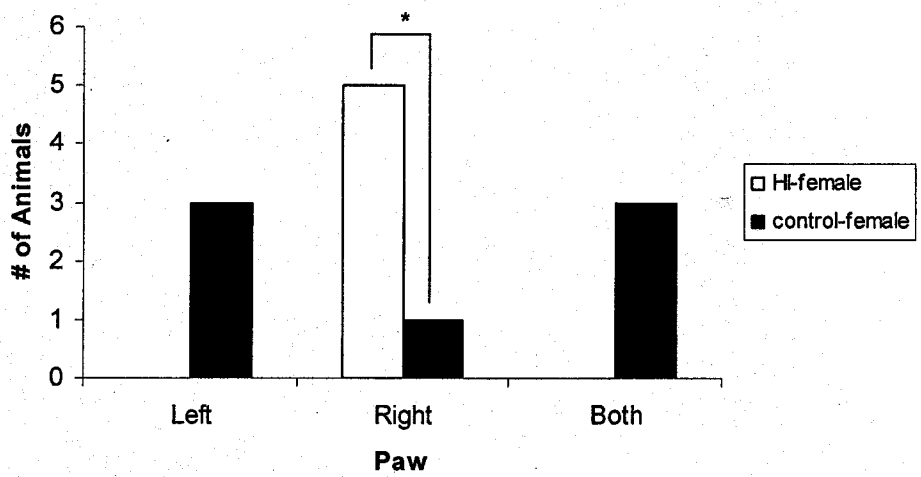
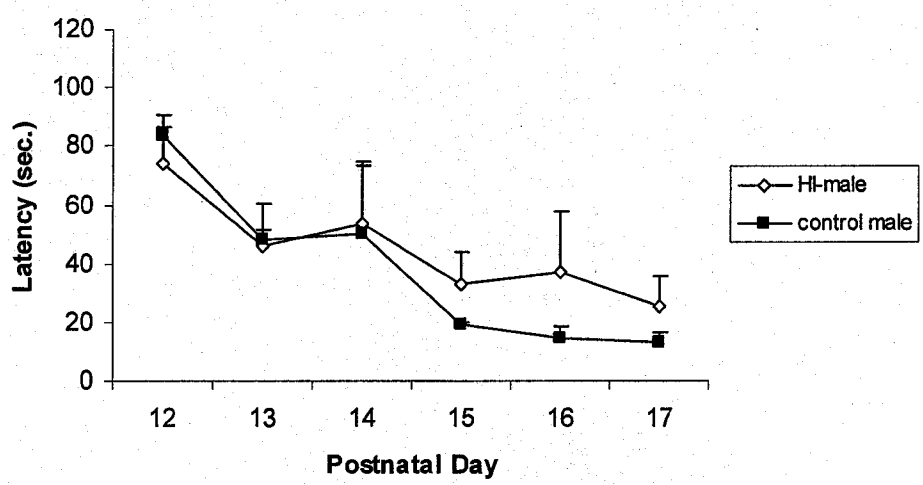



*Figure 2.8.* Forelimb grip strength and paw slip data. Paw slips on the forelimb grip strength test for male (A) and female (B) conditions. * indicates a significant difference between conditions ($p<0.05$).

A.




B.

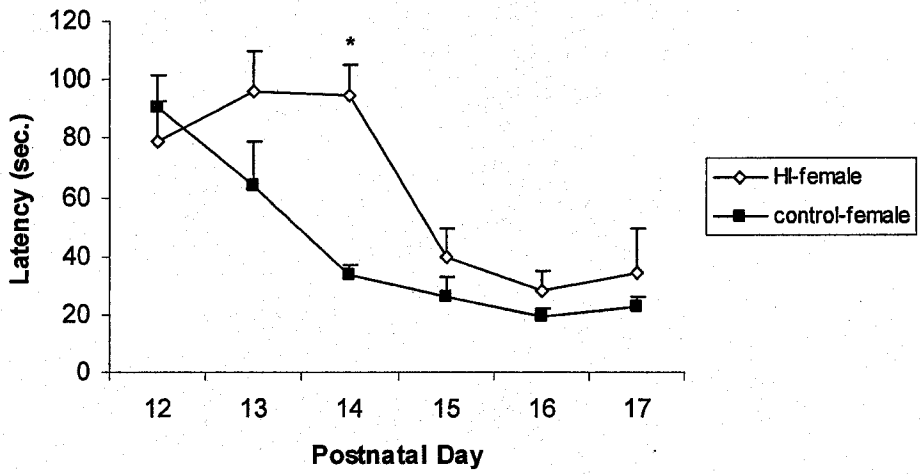



*Figure 2.9.* Wire mesh ascending performance. Average latency to reach the platform of the wire-mesh ascending test on postnatal days 12-17 for male (A) and female (B) conditions (means ± SEM). (B) RM ANOVA revealed a significant interaction between female HI and control conditions. Analysis of this interaction revealed that HI animals had a significantly higher latency on pnd 14 than control animals (* $p<0.01$).

effect of condition [$F(1,10) = 5.882$, $p<0.01$], day [$F(5,50) = 18.054$, $p<0.01$], and a significant interaction [$F(5,50) = 3.685$, $p<0.01$] in the latency to reach the goal platform whereby HI animals had a significantly longer latency to reach the platform on pnd 14 ($p<0.01$) (Figure 2.9B).

There were no significant differences between either the male or female conditions in the total number of grid crosses during the pre-weaning (pnd 18, 20 and, 22) or post-weaning period (pnd 27 and 29). Further, there were no differences between male or female conditions during either testing period with respect to the number of inner or outer grid crosses (see Appendix A).

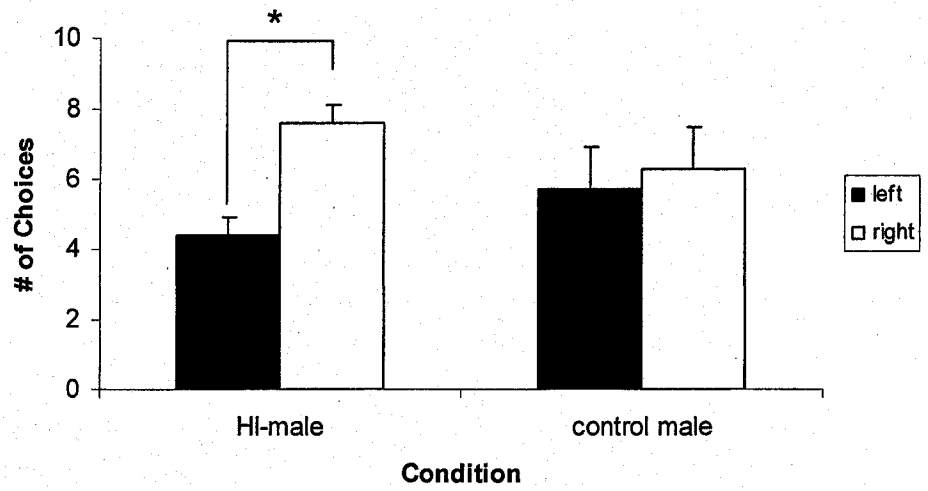
2.3.4 Cognitive Tests:

*2.3.4.1 Spontaneous Alternation:*

Males: Analysis of the total number of alternations over all days revealed that there were no differences between conditions [$t(6) = 1.177$, $p>0.05$]. There was however, a significant difference in the total number of left versus right choices over all days for HI males [$t(4) = 3.138$, $p<0.05$] where HI males made significantly more right choices (Figure 2.10A). There was no difference in latency to make a choice or the total number of left or right choices between conditions.

Females: Analysis of the total number of alternations over all days revealed a significant difference between conditions where control animals made significantly more alternations than HI animals [$t(10) = 3.191$, $p<0.05$] (Figure 2.10B). There was no

A.




B.

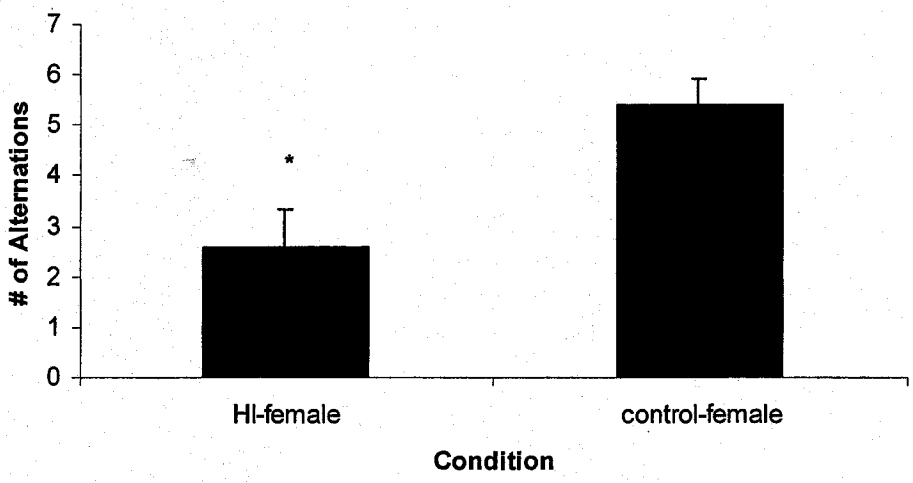



*Figure 2.10.* Spontaneous alternation test scores (means ± SEM) that resulted in significant differences between conditions. (A) male total number of left versus of right choices throughout testing. Male HI animals made significantly more right choices than left choices (* $p<0.05$). (B) female total number of alternations over all days. Control females made significantly more alternations than HI anaimls (* $p<0.05$).

difference in latency to make a choice or the total number of left or right choices between or within conditions.

*2.3.4.2 T-Maze:*

Male: Analysis of the number of correct choices made by male animals by two-way ANOVA with repeated measures revealed a significant effect of day [$F(14,84) = 8.207$, $p<0.01$] but no effect of condition [$F(1,6) = 3.941$, $p>0.05$] or interaction [$F(14,84) = 1.368$, $p>0.05$] (Figure 2.11A). Analysis of latency to choose an arm revealed a significant effect of day [$F(14,84) = 2.029$, $p<0.05$], but no effect of condition [$F(1,6) = 1.996$, $p>0.05$] or interaction [$F(14,84) = 0.858$, $p>0.05$] (Figure 2.11B).

Female: As with the male data, analysis of the average number of correct choices made by female animals revealed a significant effect of day [$F(14,140) = 11.075$, $p<0.01$], but no effect of condition [$F(1,10) = 0.030$, $p>0.05$] or interaction [$F(14,140) = 1.619$, $p>0.05$] (Figure 2.11C). Similarly, analysis of females' latency to select an arm revealed a significant effect of day [$F(14,140) = 2.437$, $p<0.05$], but no effect of condition [$F(1,10) = 0.769$, $p>0.05$] or interaction [$F(14,140) = 0.797$, $p>0.05$] (Figure 2.11D).

*2.3.4.3 Radial Arm Maze: 8 Arms Baited:*

Males: Two-way ANOVA (day x condition) with repeated measures was used to analyze the RAM data. Analysis of males' latency to obtain all eight rewards revealed a significant effect of condition [$F(1,6) = 19.443$, $p<0.01$] (HI animals having

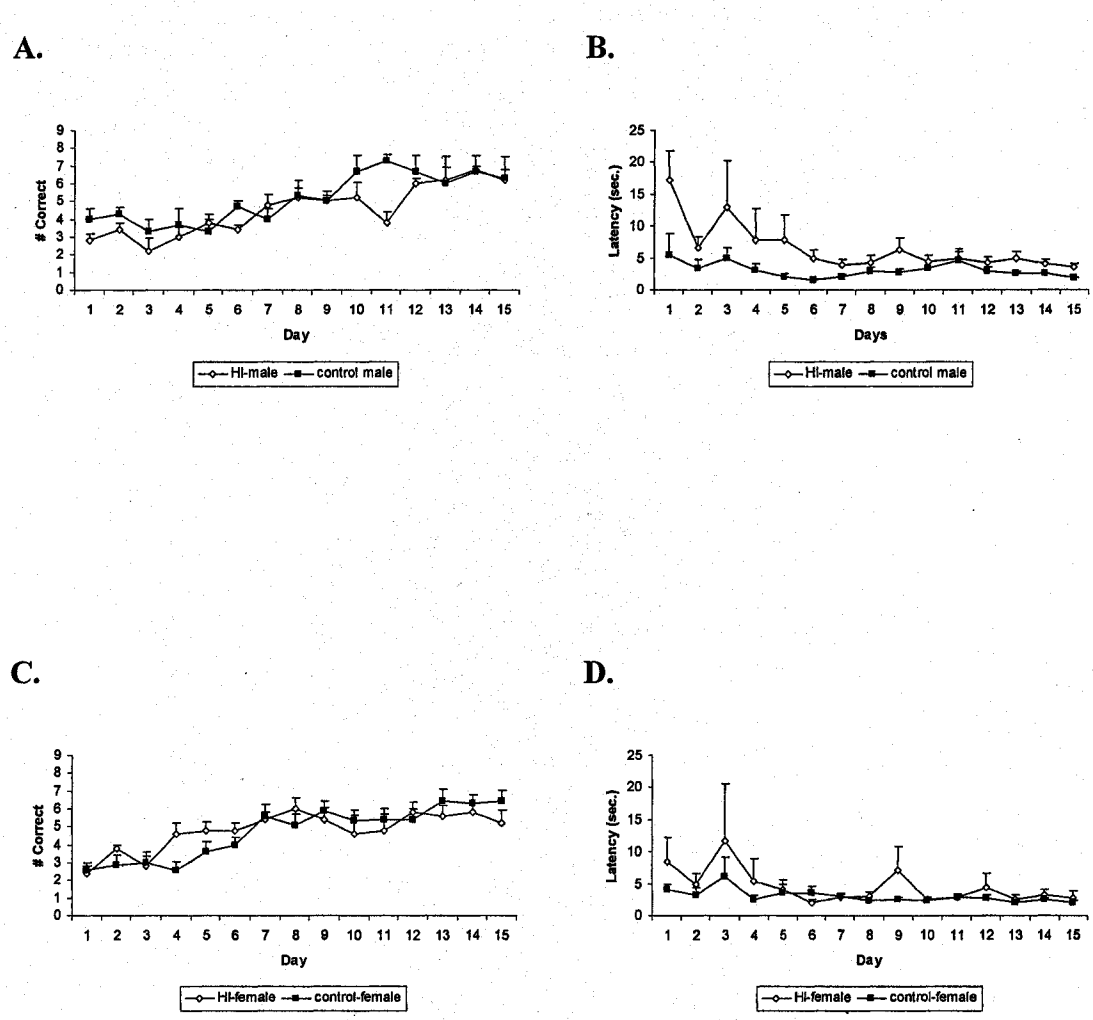
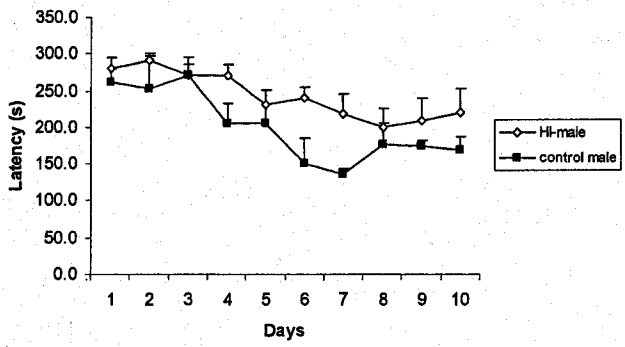



*Figure 2.11.* Performance in the T-maze. (A) number of correct choices made by male animals in the T-maze and (B) latency to choose an arm. (C) number of correct choices made by female animals and (D) latency to choose an arm. There were no significant differences between conditions in either of the dependent measures.

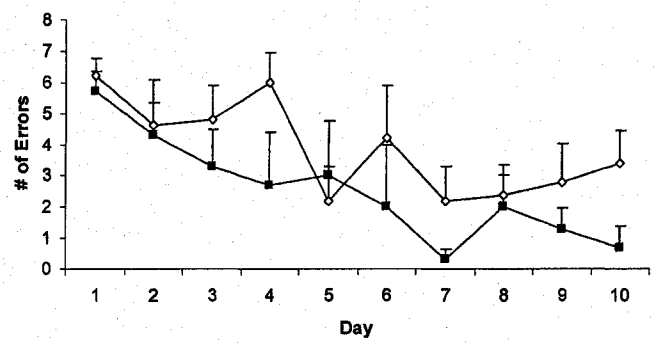
longer latencies to obtain all eight rewards) and day [$F(9,54) = 4.619$, $p<0.01$], but no interaction [$F(9,54) = 0.694$, $p>0.05$] (Figure 2.12A). Analysis of the average number of errors made in the eight arms baited configuration revealed no effect of condition [$F(1,6) = 2.229$, $p>0.05$] or interaction [$F(9,54) = 0.647$, $p>0.05$], but a significant effect of day [$F(9,54) = 3.430$, $p<0.05$] (Figure 2.12B). Analysis of the average number of commission errors revealed no effect of condition [$F(1,6) = 1.557$, $p>0.05$] or interaction [$F(9,54) = 0.823$, $p>0.05$], but again, a significant effect of day [$F(9,54) = 3.176$, $p<0.01$] (Figure 2.12C).

Females: Analysis of females' latency revealed no effect of condition [$F(1,10) = 2.212$, $p>0.05$] or interaction [$F(7.157,71.574) = 1.831$, $p>0.05$], but a significant effect of day [$F(7.157,71.574) = 5.604$, $p<0.01$] (Figure 2.13A). With respect to females' average number of errors made, there was a significant effect of condition [$F(1,10) = 9.759$, $p<0.05$], with HI animals making more errors, but no effect of day [$F(4.596,45.956) = 0.769$, $p>0.05$] or interaction [$F(4.596,45.956) = 0.740$, $p>0.05$] (Figure 2.13B). When the errors were further divided into commission and omission errors, it was noted that errors of commission made a larger contribution to the total errors where analysis of females' average number of commission errors revealed a significant effect of condition [$F(1,10) = 9.569$, $p<0.05$], with HI animals making significantly more errors of commission than control animals, but no effect of day [$F(4.662,46.618) = 0.673$, $p>0.05$] or interaction [$F(4.662,46.618) = 0.841$, $p>0.05$] (Figure 2.13C).

A.




B.




C.

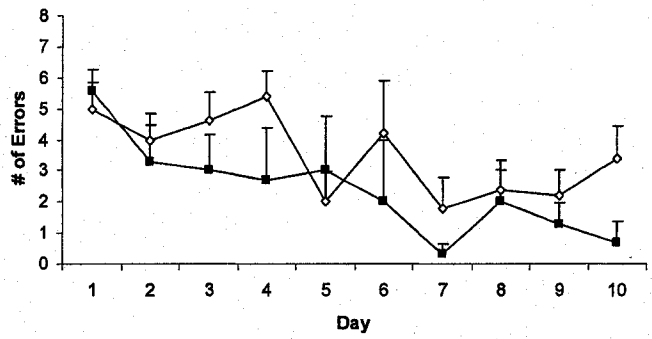
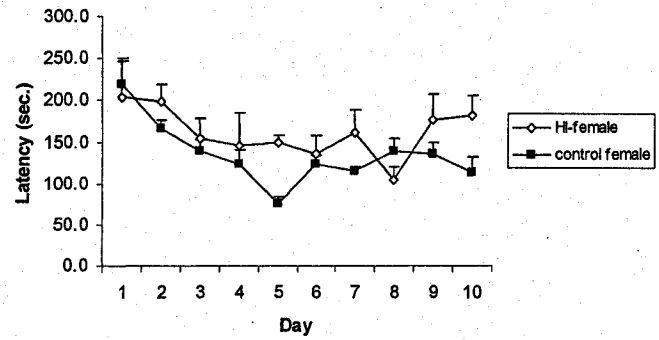
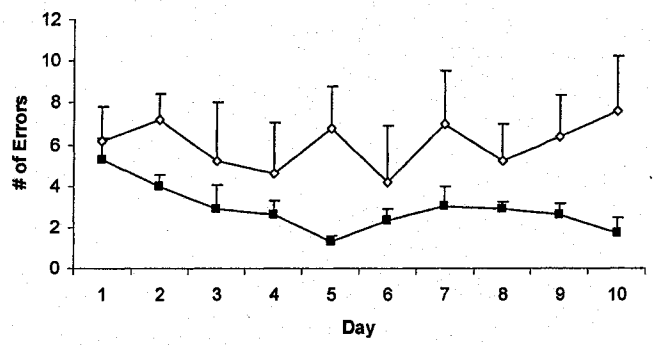



*Figure 2.12.* Performance in the RAM 8 arms baited configuration - Males (mean ± SEM). (A) Average latency to obtain all eight rewards. HI animals had significantly longer latencies to obtain all rewards ($p<0.01$). (B) Average number of errors (commission and omission errors combined). (C) Average number of commission errors. There were no differences between conditions in the average number of errors made throughout RAM 8 arms baited testing.

A.




B.




C.

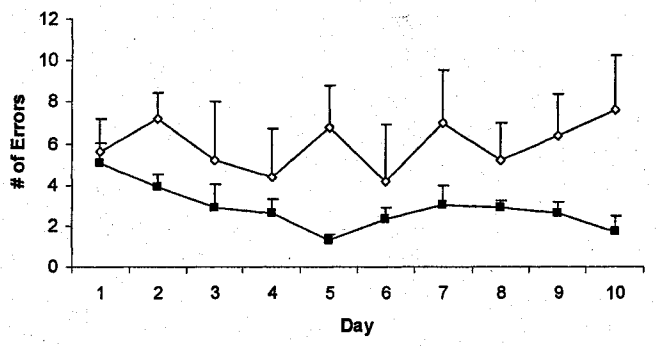



*Figure 2.13.* Performance in the RAM 8 arms baited configuration - Females (mean ± SEM). (A) Average latency to obtain all eight rewards. There were no differences between conditions. (B) Average number of errors (commission and omission errors combined). (C) Average number of commission errors. In both cases, HI animals made significantly more errors than control animals throughout RAM 8 arms baited testing ($p<0.05$).

*2.3.4.4 Radial Arm Maze: 4 Arms Baited:*

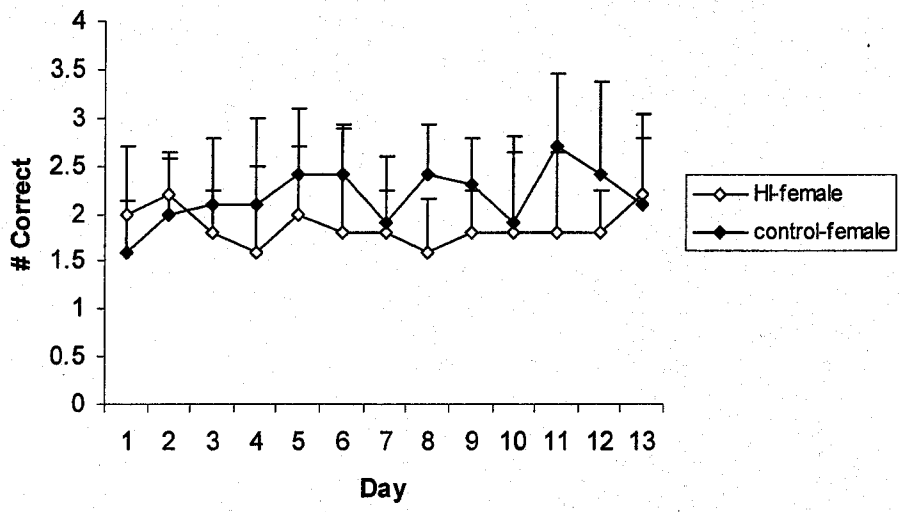
Males: There were no significant differences between male conditions in the average latency to obtain all four rewards, the number of unbaited arm entry errors, unbaited arm re-entry errors, omission errors, or the number correct on the first four selections.

Females: There were no significant effects between female conditions in the average latency to obtain all four rewards, the number of unbaited arm entry errors, omission errors, or the number correct on the first four selections (although this measure approached significance with the control females making more correct choices than HI females [$F(1,10) = 4.401$, $p=0.062$] (analyzed using two-way ANOVA with repeated measures) (Figure 2.14A). Analysis of females' re-entry errors to unbaited arms revealed a significant effect of condtion [$F(1,10) = 13.846$, $p<0.01$], where HI females made significantly more re-entry errors (working memory errors) than control animals, but no effect of day [$F(12,120) = 1.229$, $p>0.05$] or interaction [$F(12,120) = 1.479$, $p>0.05$] (Figure 2.14B).

*2.3.4.5 Morris Water Maze:*

Males: There was no difference in swimming velocity between conditions in either the visible (analyzed using animals' best latency to locate the visible platform [$t(6) = 2.018$, $p>0.05$]) (Figure 2.15A) or submerged platform trials (acquisition trials

A.




B.

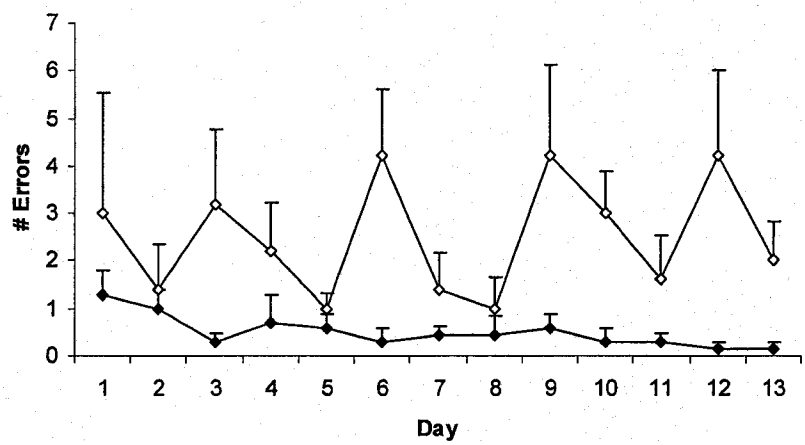



*Figure 2.14.* Performance in the RAM 4 arms baited configuration - Females (means ± SEM). (A) average number correct on the first four selections (HI animals fewer; $p=0.062$). (B) average number of re-entry errors to unbaited arm. HI animals made significantly more re-entry errors to unbaited arms ($p<0.01$).

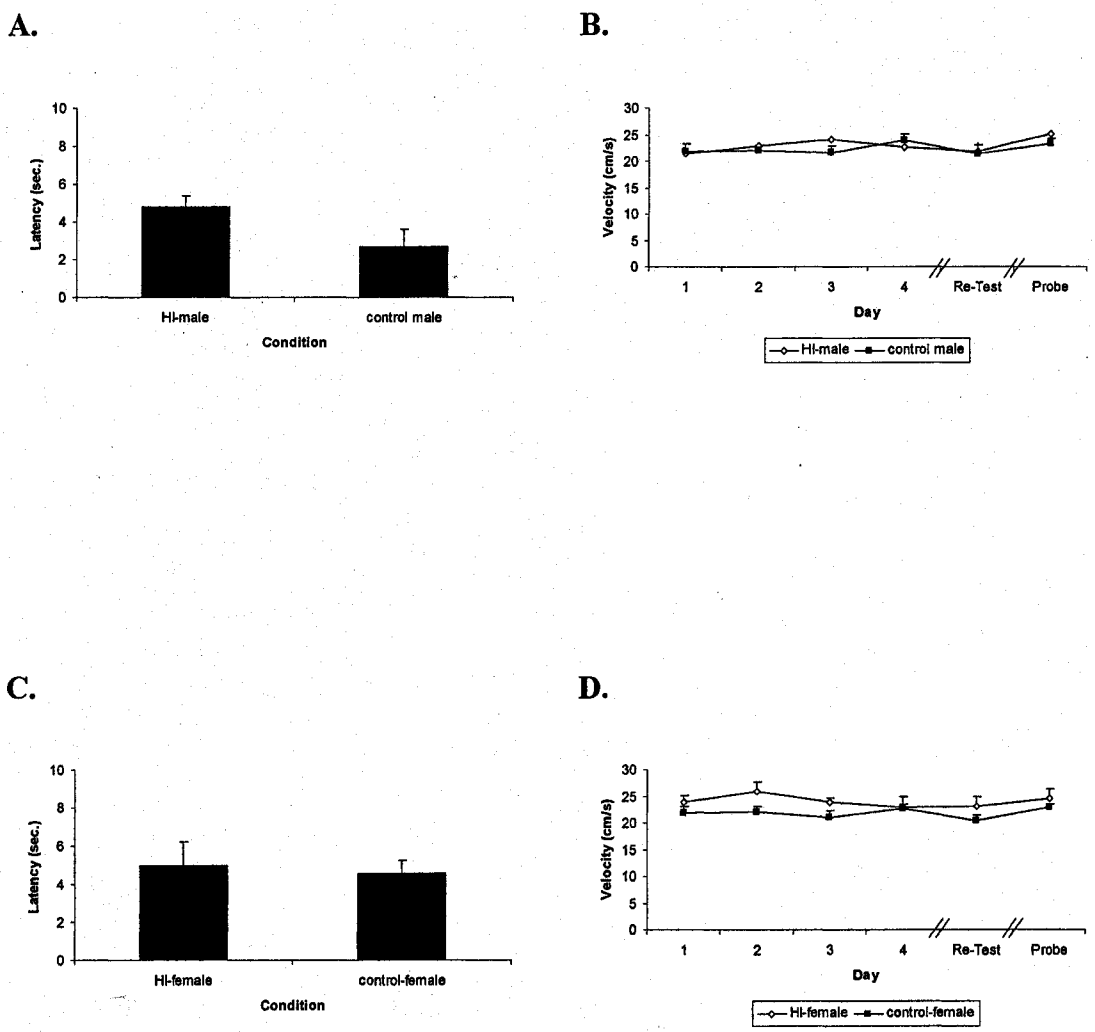



*Figure 2.15.* MWM swim speed data (means ± SEM). (A) males' visible platform best latency and (B) average velocity in hidden platform trials. (C) females' visible platform best latency and (D) average velocity in hidden platform trials. There were no differences between conditions of either males' or females' swim speeds.

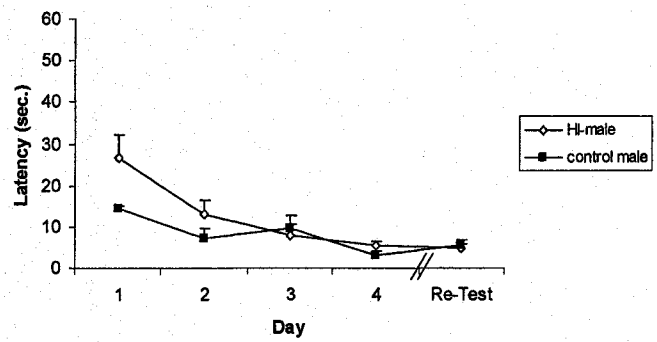
[F(1,6) = 0.119, p>0.05]; re-test [F(1,6) = 0.020, p>0.05]), or the probe trial [F(1,6) = 1.831, p>0.05] (2.15B).

With respect to the latency for males to swim to the hidden platform over four days, two-way repeated measures ANOVA revealed no effect of condition [F(1,6) = 1.473, p>0.05] or interaction [F(3,18) = 3.123, p>0.05], but a significant effect of day [F(3,18) = 16.219, p<0.01], (Figure 2.16A). Similarly, two-way ANOVA with repeated measures on the swimming distances to locate the hidden platform over the four days revealed no effect of condition [F(1,6) = 1.701, p>0.05], but a significant interaction [F(3,18) = 3.398, p<0.05] and a significant effect of day [F(3,18) = 20.499, p<0.01] (Figure 2.16B). There were no differences between the male HI and control conditions during the re-test in latency to locate the platform or the distances swam to locate the platform [t(6) = 0.372, p>0.05; t(6) = 0.264, p>0.05, respectively]. There were no differences between the male HI and control conditions with respect to the number of platform zone crosses [t(6) = 0.360, p>0.05] (Figure 2.16C).

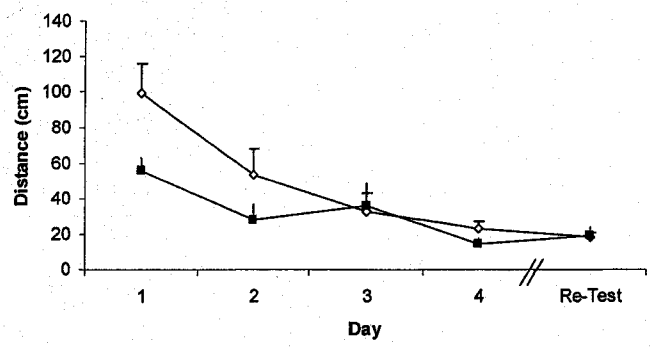
Females: There was no difference in swimming velocity between conditions in either the visible (analyzed using animals' best latency to locate the visible platform [female: t(10) = 0.335, p>0.05]) (Figure2.15C) or submerged platform trials (acquisition trials [F(1,10) = 2.149, p>0.05]; re-test [F(1,10) = 2.167, p>0.05]), nor the probe trial [F(1,10) = 0.789, p>0.05] (Figure 2.15D).

There was a significant difference in both the latency and the distance swam to locate the hidden platform between the female conditions. The repeated measures of two-way ANOVA revealed an effect of condition [F(1,10) = 14.145, p<0.01] and day

A.




B.




C.

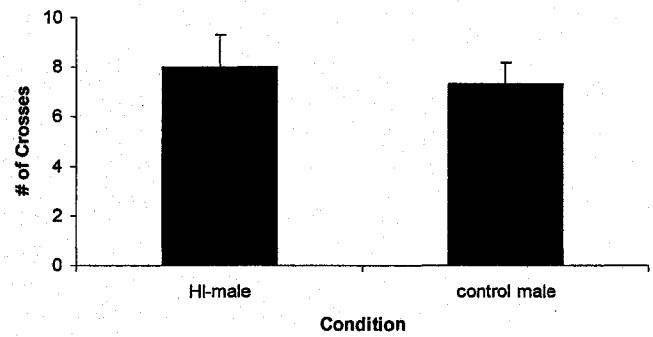



*Figure 2.16.* MWM performance - Males (means ± SEM). (A) latency to locate the hidden platform during acquisition and re-test. (B) swimming distance to locate the hidden platform during the acquisition period and re-test. (C) average number of platform zone crosses during the probe trial. There were no differences between conditions with respect to the above dependent variables.

[F(3,30) = 8.01, p<0.01], but no interaction [F(3,30) = 0.388, p>0.05] with respect to the latency to locate the hidden platform and an effect of condition [F(1,10) = 12.230, p<0.01] and day [F(2.058,20.576) = 6.831, p<0.01], but no interaction [F(2.058,20.576), p>0.05] on the distance swam around the whole maze to locate the hidden platform during the four days. In both cases HI females took significantly longer and swam greater distances in order to locate the submerged platform than control females (Figure 2.17A and B). During the re-test, independent t-test revealed a significant effect of condition in the distance swam around the whole maze to locate the hidden platform [t(10) = 2.373, p<0.05] and a difference (although not statistically significant) between conditions in the latency to locate the platform [t(10) = 2.213, p=0.051], where the HI females swam greater distances had a longer latency.

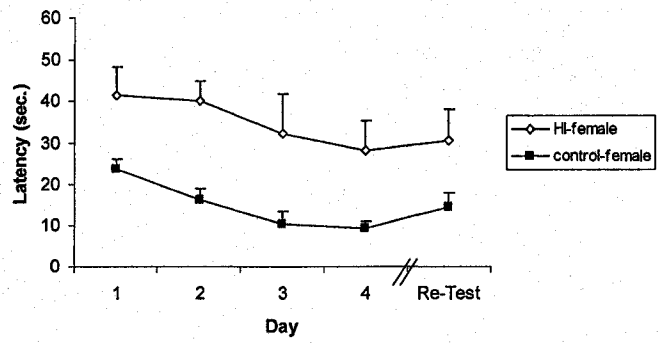
During the probe trial (platform removed), independent samples t-test revealed a significant difference between female HI and control animals in the number of platform zone crosses [t(10) = 3.929, p<0.01], where the HI animals had significantly fewer crosses (Figure 2.17C).

*2.3.4.6 Barnes' Circular Maze:*

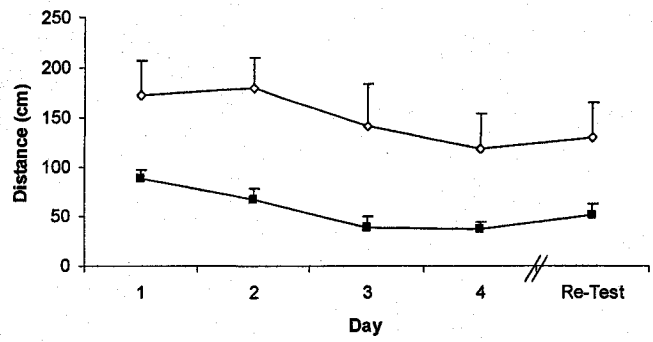
Data from the acquisition trials in the BCM were analyzed using a two-way (day x condition) ANOVA with repeated measures, and data from the re-test were analyzed using an independent t-test.

Males: Analysis of the male latency to enter the escape hole revealed a significant effect of day [F(3,18) = 4.312, p<0.05] but no effect of condition [F(1,6) = 1.576, p>0.05] or interaction [F(3,18) = 0.813, p>0.05] (Figure 2.18A). There was no difference

A.




B.




C.

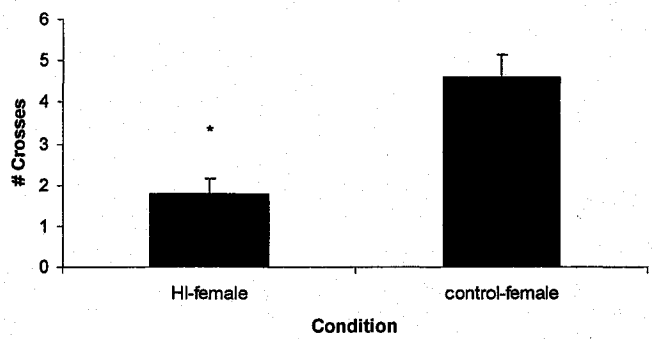
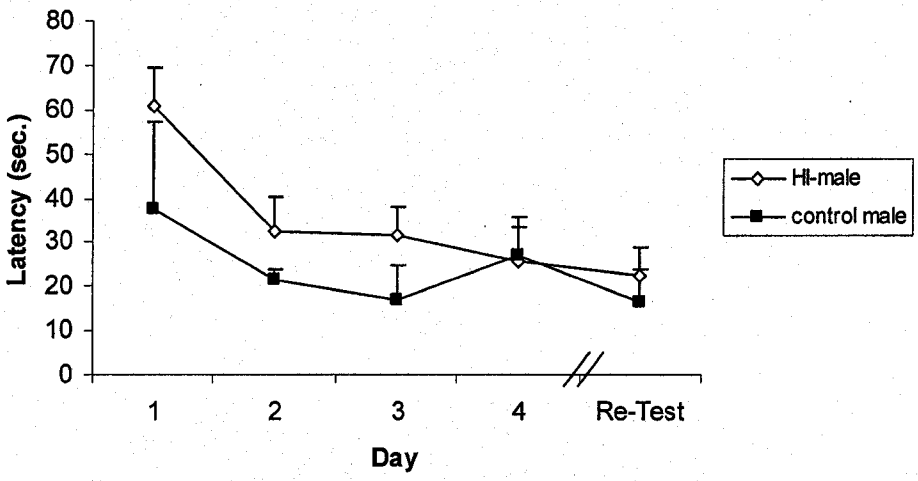



*Figure 2.17.* MWM performance - Females (means ± SEM). (A) latency to locate the hidden platform during the acquisition trials and re-test. HI animals had significantly longer latency to locate the hidden platform during the acquisition trials ($p<0.01$) and during the re-test ($p=0.051$). (B) swimming distance to locate hidden platform during the acquisition period and re-test. HI animals swam significantly greater distances to locate the platform during both the acquisition trials ($p<0.01$) and re-test ($p<0.05$). (C) average number of platform zone crosses during the probe trial. Control animals had significantly more platform crosses than HI animals (* $p<0.01$).

A.




B.

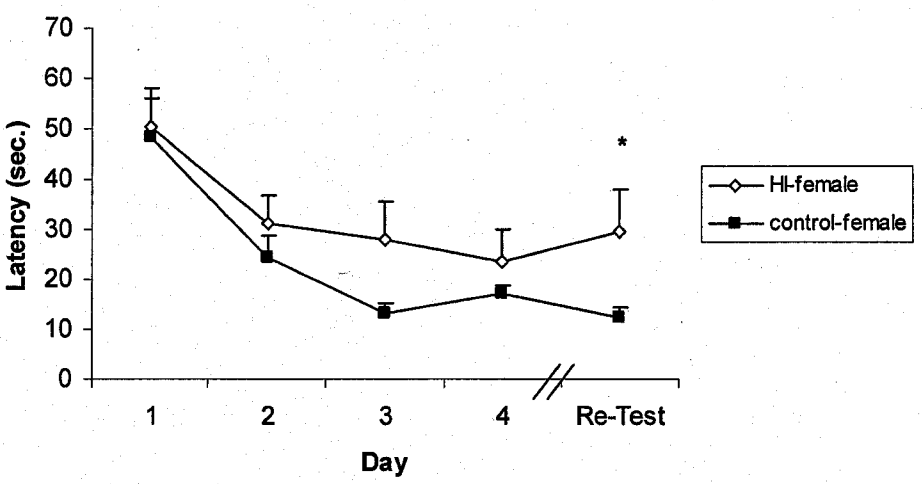



*Figure 2.18.* BCM latency data. (A) males' latency to locate the escape hole. There were no differences between conditions on either the acquisition trials or the re-test. (B) females' latency to locate the escape hole. There were no differences between conditions in latency to locate the escape hole during the acquisition trials, however, HI animals had significantly longer latencies to find the escape hole during the re-test (* $p<0.05$).

between the male conditions in latency to locate the escape hole during the re-test [$t(6) = 0.546$, $p>0.05$]. There were also no statistically significant differences between male conditions with respect to the number of hole errors, re-entry errors, or the total number of errors (see Appendix A).

Females: Analysis of the latency for female animals to enter the escape hole revealed a significant effect of day [$F(1.617,16.170) = 13.660$, $p<0.01$] but no effect of condition [$F(1,10) = 2.885$, $p>0.05$] or interaction [$F(1.617,16.170) = 0.565$, $p>0.05$] (Figure 2.18B). There was, however, a significant difference between female conditions in the latency to locate the escape hole during the re-test [$t(10) = 2.277$, $p<0.05$] where the HI animals had a longer latency to locate the escape hole than control animals. As with the male analysis, there were no statistically significant differences between female conditions with respect to the number of hole errors, re-entry errors, or the total number of errors (see Appendix A).

## 2.4 Discussion

The primary aim of this study was to develop a comprehensive functional test battery that was sensitive to detecting behavioural deficits following neonatal hypoxia-ischemia. From the results obtained, conclusions can be drawn that various functions such as neuromotor and cognitive abilities of Sprague-Dawley rats are affected following a HI episode at seven days of age and can be detected over the long-term.

### 2.4.1 Comparison of Results:

#### *2.4.1.1 Neonatal Tests*:

The neonatal measures that were analyzed in this study included measures of physical and sensory development as well as motor abilities. We did not observe a difference between HI and control animals with respect to weight gain, day to eye opening, incisor eruption, auditory startle or olfactory orientation, similar to that shown by several other researchers (Young *et al.* 1986; Felt *et al.* 2002). Lubics *et al.* (2005) conducted a study in which only neurological reflexes and neonatal motor behaviour were assessed and found that there was a difference in neonatal HI and control animals' weights with HI animals significantly lighter at all days (from pnd 8-20), and the day to which eye opening occurred (HI animals significantly delayed). Neither was found in our study. Similar to the current study, though, Lubics *et al.* did not find a difference in incisor eruption or auditory startle. The disparity between the current study and that of Lubics *et al.* may be due to the number of animals used. In the current study there were

five male and five female HI animals and three male and seven female control animals used. In comparison, Lubics *et al.* used 12 HI and 12 control (sexes combined for analysis) animals which would result in a more powerful analysis than the current study. By increasing the number of animals in the current study, we may have been more likely to find differences between conditions.

With respect to the neuromotor tests, in the present study we found a significant difference between female HI and control animals in forelimb grip strength whereby HI animals' right (contralateral forepaw) was more likely to slip off of the horizontal wire (Figure 2.8). Lubics *et al.* (2005) had similar findings in that only when the ipsilateral arm was taped to the animals' body, was there a significant difference between HI and control animals in the latency to hold onto the horizontal rope using the contralateral (right) forelimb. The current study was the first to analyze neonates' abilities to traverse the wire mesh at a $75^0$ angle and neonatal pivoting behaviour. We found a significant difference between HI and control animals in the latency to climb to the platform of the wire mesh ascending apparatus where HI animals had a longer latency (Figure 2.9). The longer latency may be attributed to a combination of a deficit in motor strength and skilled motor movements on the wire (Young *et al.* 1986; Lubics *et al.* 2005). With respect to the pivoting behaviour, HI males and females made significantly more right pivots than control animals (Figure 2.7). Since HI animals were exposed to left hemisphere ischemia, the contralateral limbs of these animals should be affected. Our results would suggest this to be true, since a significant difference in the number of left and right pivots would indicate that one side of the animals' body was stronger (or more developed) than the other. Due to the nature of the CCAo, the right side of the body, or

more likely the neural control of the right side, of the HI animals may have been developmentally delayed, thus animals would be more likely to rotate towards the right.

*2.4.1.2 Cognitive Tests*:

In the spontaneous alternation test, we found a significant difference between female HI and control animals in the average number of alternations, whereby the control animals were more likely to alternate. Since there was no difference in the latency to make a choice (motivation) nor in the number of right versus left choices (motor ability) between female conditions, this result is more likely due to an impaired memory ability. Only one additional study (with respect to the HI literature) measured this innate behaviour and also found a difference (although not statistically significant, $p=0.08$), where the control animals were more likely to alternate their choices (although only male animals were tested in the study) (Balduini *et al.* 2000). Further, the results obtained from the current male data show that there were no differences between conditions with respect to the average number of alternations or in the latency to make a choice. There was a difference however, within the male HI condition with respect to the total number of left verses right choices where animals in this condition made significantly more right choices. This result can most likely be attributed to a motor dysfunction.

The results obtained from the current study show similarities with those obtained by other researchers in the RAM and MWM tests. In the eight arms baited configuration of the RAM, our results are similar to those reported in several different studies (Ikeda *et al.* 2001; Ikeda *et al.* 2002; Mishima *et al.* 2005). We found a

significant difference between female HI and control animals in the number of commission errors made with HI animals making significantly more errors. This measure is thought to represent a measure of working memory ability since the commission error score is a score of rats' abilities to remember the variables associated with one particular testing session (arms previously visited / not yet visited) (Olton and Samuelson 1976; Volpe *et al.* 1984; Ikeda *et al.* 2001). Similar to other studies (Ikeda *et al.* 2001; Ikeda *et al.* 2005), there was no difference between conditions in the number of correct choices made on the first eight selections, however, the maze cleaning procedure was changed after the eighth day of testing (see section 2.4.4 for details) whereby the entire maze was cleaned between each run. In this configuration we found a significant difference between female HI and control animals in the number of correct choices in the first eight selections whereby HI animals made significantly fewer correct choices on days nine and 10. The failure to clean the maze may also account for the differences we found between male HI and control animals in the latency to obtain all eight rewards.

Following the eight arms baited configuration of the RAM, animals were tested on a modified version where only four of the eight arms were baited, a version where we could analyze both the animals' working (re-entry errors) and reference memory (number of unbaited arm entry errors) abilities (Huang *et al.* 2004). Similar to the findings with the eight arms baited configuration, there was a difference in the working memory abilities of the HI and control females, whereby HI animals made significantly more re-entry errors to the unbaited arms. However, there was no statistical difference between HI and control animals' reference memory abilities, although as with the 8-arms baited configuration, the number correct on the first four choices approached significance

(p=0.062) wherein control female made more correct choices than HI females, indicating a pattern in reference memory dysfunction exhibited by HI females.

The MWM is thought to assess rats' reference memory abilities and is sensitive to hippocampal damage (Morris *et al.* 1982; Ikeda *et al.* 2002). This test is the most commonly used cognitive test in assessing intervention efficacy in the neonatal HI model of stroke. As in previous studies (Young *et al.* 1986; Ikeda *et al.* 2001; Chou *et al.* 2001; Ikeda *et al.* 2002; Arteni *et al.* 2003; Kumral *et al.* 2004; Ikeda *et al.* 2005), in the current study there was a significant difference between female HI and control animals with HI animals having significantly longer latencies to locate the hidden platform. Distances swam in order to locate the hidden platform were assessed, and revealed that HI females swam significantly greater distances than control females, similar to the findings by Mishima *et al.* (2004). In the current study, animals' reference memory abilities were also assessed in a probe configuration following the acquisition trials in the MWM. There was a significant difference between HI and control females in the number of times the animals crossed the platform zone whereby control animals made significantly more crosses. This finding is similar to those of Arteni and colleagues (2003) who found that control animals spent significantly more time in the platform zone. Both findings indicate a superior memory ability of control animals compared with animals subjected to neonatal HI. Further, during the re-test, HI females swam longer distances and had longer latencies to locate the hidden platform than control animals. Since there were no differences in the swim speeds or in the latency to reach the visible platform between the HI and control animals, the differences in latencies and swimming distances are not due

to confounding factors such as motoric or visual deficits but due to deficits in memory abilities.

2.4.2 Observation of Brain Pathology:

Although there was no systematic analysis of the neuropathology associated with neonatal HI in this study, the brains of the HI animals were removed, fixed and photographed in order to take note of the pattern of neuronal damage that might be affected. From gross inspection of the brains it was found that four of the five HI females had a large area of infarction in the hemisphere ipsilateral to the CCAo (left hemisphere) and one animal had what looked to be a normal brain. The major brain areas affected in these female rats included the cerebral cortex, striatum, hippocampus, and thalamus, consistent with that observed in the experimental literature (Balduini *et al.* 2000; Ikeda *et al.* 2002; Ikeda *et al.* 2005). With respect to gross inspection of the HI males' brains there was one animal with a mildly smaller ipsilateral hemisphere, one animal with an area of white, opaque tissue in the ventral area of the parietal and dorsal area of the temporal lobes, and three animals with what looked to be normal brains. These observations most likely account for the differences found in our analyses of rats' functional abilities following neonatal HI. In most of the analyses, especially with respect to rats' cognitive abilities, it was found that HI females usually exhibited significant memory impairments, but also showed significant neuromotor impairments early in development. Hypoxic-ischemic males, however, showed only transient neuromotor deficits early in development and no deficits in any of the cognitive tests.

These neuropathological findings, however, are consistent with those observed in the experimental literature (Grafe 1994; Nagata *et al.* 2000; Ikeda *et al.* 2001) where 65-75% of animals subjected to HI on pnd 7 have either moderate or severe neuropathologic damage. In the present study, if female and male data were combined, the gross examination of neuropathological damage would result in 60% of animals exposed to HI experiencing moderate to severe damage.

2.4.3 Sex Differences:

Due to the uneven distribution of the neuronal damage, i.e., 80% of females experiencing severe neuronal damage and 40% of males experiencing moderate damage, as well as additional reports of differences in maze solving behaviours (Roof 1993; Ulloa *et al.* 2004), data obtained from male and female animals were analyzed separately. Although it was not the goal of this study to examine sex differences associated with the neonatal HI model of stroke, our results indicate that important differences may occur. To date, only a few studies (Almli *et al.* 2000; Chou *et al.* 2001; Kumral *et al.* 2004) have reported analyzing their data for sex differences in this model of stroke, however, of those reported, sex differences were only analyzed using the MWM latency data as being representative for all behaviours. Further, those who reference literature which states there are no sex differences with this model of stroke, continue to cite the above authors or have also cited experiments that have used mice (Le Roy *et al.* 1999) or only histological measures (Vannucci *et al.* 1997). In the current study we analyzed a representative number of the behaviours and found sex differences in many of these. As

a result, in order to control for possible interactions with the effects of stroke and sex, we separated the sexes and analyzed all data using only condition as the between-subjects factor. We therefore suggest that if additional behavioural measures of stroke are conducted (in addition to the MWM) that sex be considered an additional grouping factor in the analysis.

In the current study, female HI animals showed more consistent functional deficits than male animals. These results are expected however, when the neuropathological observations are taken into consideration. Since more female animals subjected to HI (80%) were considered as having severe damage compared with only 40% of male animals, where these animals had only moderate damage, we expect that female animals' functional abilities would more likely be affected. It can therefore be concluded that the behavioural data obtained are consistent with the neuropathological observations in the current study.

### 2.4.4 Methodological Considerations:

The exact postnatal day of surgery in this study could not be determined due to uncontrollable extraneous factors. Since rat development occurs at such a rapid pace during the neonatal period (Dobbing and Sands 1971; Dobbing and Sands 1979; Ritter *et al.* 2002), the exact day on which the hypoxic-ischemic episode occurs has been shown to affect neuronal injury and may have contributed to some of the variability experienced in the current study (Grafe 1994; Towfighi *et al.* 1997).

With the separation of the sexes for analyzing the behavioural data, we reduced the total number of animals that could be used in the analyses. This was likely more evident with respect to the male data where there were five HI animals and only three control animals, reducing the power with each analysis and thus increasing the likelihood of a type II error. Power analysis of the female data was usually higher, in the range of 0.70-0.90 in tests where there was deemed a significant difference between conditions, whereas power analyses with the male data were lower. The combination of male and female data however, was not appropriate in this case since analysis of the data revealed significant differences between the two sexes. As a result, we separated the sexes in all analyses (for consistency), and are confident that the differences in the behavioural measures can be attributed to the effects of the hypoxic-ischemic episode early in development.

In the eight arms baited configuration of the RAM, the maze was not cleaned between the testing of each animal for the first eight days. The failure to clean the maze added a confounding scent variable to this maze. When the maze was cleaned between each animal, however, we found a significant difference in the number of correct choices made on the first eight selections between HI and control females. Failure to clean the maze may also explain the difference in latency to obtain all eight rewards of male HI and control animals, where the HI animals may have exhibited an increased apprehensive behavioural response to the scent of other animals resulting in a longer latency to solve the maze. This explanation is further supported by the fact that there were no differences between male HI and control animals in the number of errors made in this maze. In

subsequent studies, we suggest that when using the RAM to assess memory abilities in rats, the maze should be thoroughly cleaned between each animal.

2.4.5. Future Directions:

Future studies that use the neonatal HI model of stroke to test various neuroprotective interventions should take into account the results provided by this study. First, since there exists a potential difference between males and females in their maze solving strategies, studies should address this by separating the sexes when analyzing the behavioural effects. Second, since cognition is such a complex process, measuring it in just one maze, such as the MWM, may not reveal the entire potential (or lack thereof) of a neuroprotective intervention. Additional cognitive tests that use different neuronal processes should be used to gain a more complete understanding of protective effects. This study has shown that rats' learning and memory abilities are affected in a number of additional cognitive tests other than the MWM. Each maze is a different assessment of learning and memory abilities, for example, the RAM is considered an appetively motivating task whereas the MWM is considered an aversively motivating task. As a result, differences in neuronal processing are required and may be differentially affected by interventions. Third, in assessing neuroprotection researchers should use a comprehensive set of measurements that not only include cognition, but also physical, sensory, neuromotor, sensorimotor, as well as histological measures to gain a more complete understanding of neuroprotective efficacy.

## 2.5 Conclusion:

Based on the results obtained, we conclude that a test battery consisting of both neonatal and adult tests of function can be constructed for the HI model in rats. Our proposed battery is listed in Table II.

The experiment described in Chapter 3 will use this test battery to measure the putative neuroprotective properties of erythropoietin in this animal model of perinatal stroke.

Table II.

*Functional Assessments Sensitive to Detection of Short- and Long-Term behavioural Deficitis in Rats Following Neonatal HI*

| **Functional Aspect** | **Postnatal Day(s)** | **Behavioural Test** |
|---|---|---|
| *Physical Development* | | |
| | Until weaning | Weights |
| | Until criterion | Eye Opening |
| *Neuromotor* | | |
| | 9-12 | Pivoting |
| | 10-23 | Forelimb Grip Strength |
| | 12-17 | Wire Mesh Ascending |
| *Cognitive* | | |
| | 30-40 | Spontaneous Alternation |
| | 100-140 | Radial Arm Maze |
| | 140-150 | MWM |

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# 3.0 Neuroprotective Assessment of Erythropoietin Administration Following Neonatal Hypoxia Ischemia

## 3.1 Introduction

### 3.1.1 Hypoxic Preconditioning:

Exposing neonatal rats to hypoxic air one day prior to the induction of HI (pnd7), provides significant morphological neuroprotection (Bergeron *et al.* 2000; Jones and Bergeron 2001). An important mediator responsible for this phenomenon is thought to be the hypoxia-inducible factor-1 (HIF-1) transcription factor. Both studies demonstrated that following the preconditioning hypoxia exposure, both HIF-1α and –β were upregulated. The HIF-1 family of transcription factors requires the dimerization of both the α and β subunits for activation (Jelkmann 1992). Once HIF-1 becomes active, it binds to a specific DNA sequence of many of the hypoxia-inducible genes (Krantz 1991). One of these target genes has been shown to encode for the mRNA of the hematopoietic cytokine *erythropoietin* (EPO), and to up-regulate in response to hypoxia (Jelkmann 1992; Jones and Bergeron 2001).

### 3.1.2 Erythropoietin:

As stated, EPO is a hematopoietic cytokine that is responsible for erythropoiesis (the formation of red blood cells) and is primarily produced in the kidney in adults and by

the liver in the fetus (Jelkmann 1992; Jelkmann 1994; Sirén and Ehrenreich 2001). It has been demonstrated that EPO is also produced outside of these two organs, namely within the CNS (primarily by astrocytes in response to tissue hypoxia), which has expanded its role beyond that of hematopoiesis (Marti *et al.* 1996; Baciu *et al.* 2000). Erythropoietin acts on the Janus tyrosine kinase – signal transducers and activators of transduction (JAK-STAT) signaling pathway (Figure 3.1) (Jelkmann 1992; Sirén and Ehrenreich 2001). The binding of EPO to the EPO receptor activates intracellular signaling through a conformational change, leading to the phosphorylation of JAK-2 (Schindler 1999; Sirén and Ehrenreich 2001). JAK-2 phosphorylation then allows the tyrosine phosphorylation of the STAT-5 protein, which then dissociates from the receptor and binds with another phosphorylated STAT-5 protein. This STAT-5 homodimer then phosphorylates IκB, the inhibitor of nuclear factor κ B (NFκB), which in turn, translocates into the nucleus to stimulate gene transcription resulting in a variety of intracellular cascades (Digicaylioglu and Lipton 2001; Kisseleva *et al.* 2002).

### 3.1.3 Neuroprotective Mechanisms of EPO:

There are several proposed mechanisms underlying the protection offered by EPO although none are completely understood. One of the most studied of these mechanisms is the effect that EPO has on apoptosis in response to cell injury. One of these anti-apoptotic mechanisms includes the upregulation of Bcl-2 protein and Bcl-$x_L$ mRNA and protein expression possibly associated with NFκB-dependent transcription (Sirén and Ehrenreich 2001; Digicaylioglu and Lipton 2001; Wen *et al.* 2002). *In vivo*

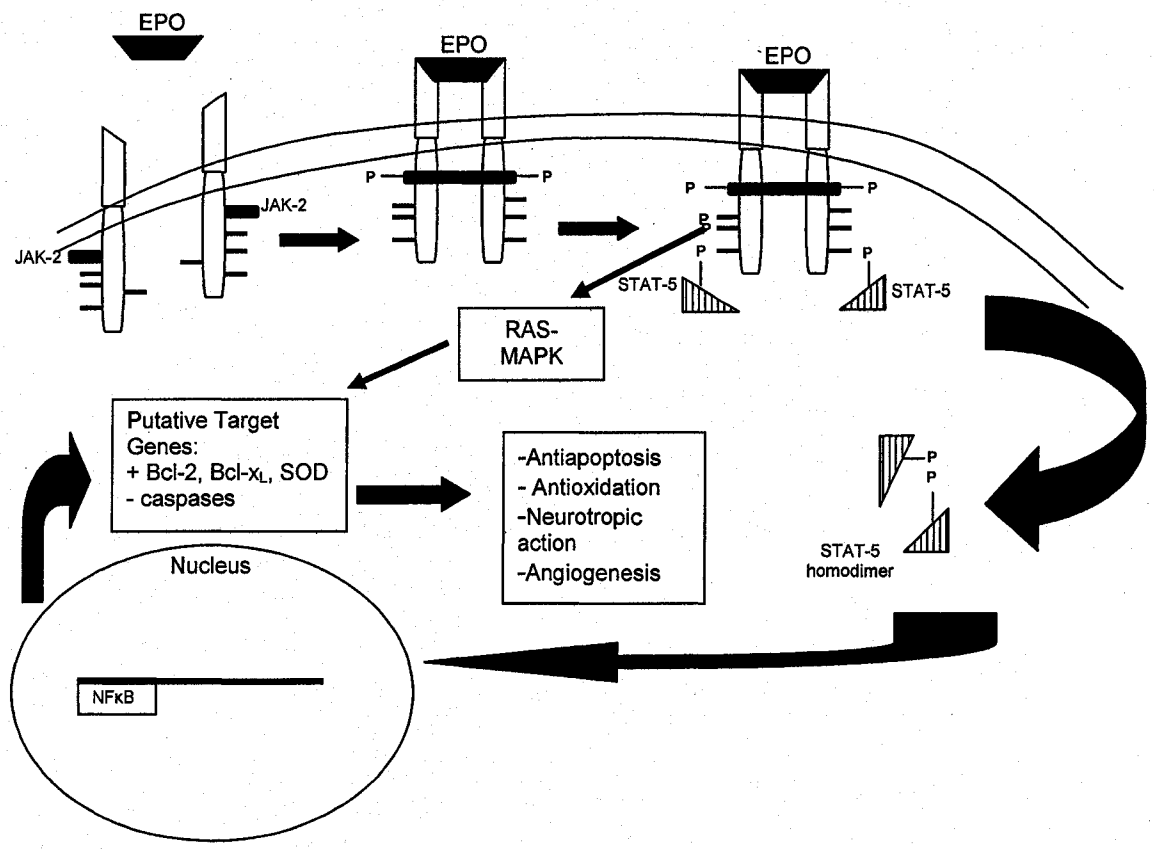



*Figure 3.1.* Intracellular signaling mechanisms of the erythropoietin receptor and proposed neuroprotection mechanisms (adapted from Sirén and Ehrenreich, 2001). See text for details.

morphological evidence of neuroprotection has been shown following neonatal HI, where animals treated with EPO exhibited fewer positively terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) stained cells and had less severe neuropathology up to three weeks post HI (Sakanaka *et al.* 1998; Kumral *et al.* 2003; Spandou *et al.* 2004; Sun *et al.* 2004).

There is also evidence that EPO protects the CNS following ischemic injury by (1) inhibiting the formation of NO (Calapai *et al.* 2000; Kumral *et al.* 2004a), (2) promoting angiogenesis and neurogenesis through upregulation of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor, respectively (Wang *et al.* 2004), and (3) decreasing the formation of free-radicals and lipid peroxidation levels as well as increasing the activity of various anti-oxidant enzymes (Chattopadhyay *et al.* 2000; Sirén and Ehrenreich 2001; Kumral *et al.* 2005). Further, EPO is also thought to have an anti-inflammatory effect, although this may not be a direct effect on the pro-inflammatory cytokines produced in ischemia, but more likely a secondary effect of reducing the amount of cellular injury following an ischemic episode (Villa *et al.* 2003).

The above mentioned studies show that the effects of EPO encompass a wide range of possibilities with respect to the structural neuroprotection mechanisms, however, the majority of these studies only observe the structural neuroprotection over a short period of 24-72 hours post stroke. Further, there have been relatively few studies conducted that assess the long-term functional neuroprotective efficacy of EPO.

3.1.4 Functional Neuroprotection by EPO:

Currently, there is a multicentered clinical study taking place in Germany, 'EPO in stroke', in order to assess the amount of neuroprotection provided by EPO when patients present with an ischemic stroke (Ehrenreich *et al.* 2004). This study is based on a pilot study that found that patients who were admitted to the hospital with an ischemic stroke within the territory of the MCA, and were treated with EPO, showed significantly better recovery than those who were not treated with EPO (Ehrenreich *et al.* 2004). Recovery was assessed by analyzing clinical outcome parameters, the evolution of infarct-size, and the profile of circulating damage markers.

Although there is a clinical trial underway, there have been relatively few experimental studies reported that have evaluated the functional neuroprotective efficacy afforded by EPO following an ischemic episode. There has been one published report of sensorimotor and neuromotor neuroprotection following EPO administration in the neonatal HI model (Spandou *et al.* 2005). Spandou and colleagues administered EPO in one dose (2,000 U/kg) immediately following hypoxia and tested their animals at 42 days of age for a period of six days. Following this, the animals were euthanized and the brains analyzed. These authors report a structural neuroprotective effect in the animals that received the EPO treatment. They also report however, that none of their animals experienced contralateral neuronal damage. Since there is considerable evidence that cell death continues throughout the animals' life (using this model of ischemia), and that this cell death occurs not only in the ipsilateral hemisphere but progresses also to the contralateral hemisphere, a longer-term evaluation of sensorimotor abilities may be

warranted before firm conclusions can be made (Ikeda *et al.* 2001; Ikeda *et al.* 2002; Mishima *et al.* 2005).

With respect to cognitive abilities in rats subjected to neonatal HI, only one study has been published to date that shows a long-term protective effect of EPO on cognition (Kumral *et al.* 2004b). In that study EPO was administered immediately following the hypoxic episode at a dose of 1,000 U/kg. Cognitive abilities were assessed in only the MWM where, although HI EPO treated animals had a significantly shorter latency to locate the platform than HI saline animals, there was still a significant difference between the HI EPO condition and the control condition. That study showed that EPO may have the potential to exhibit moderate protective effects on spatial memory. The authors did not test the animals in a probe or re-test, so there is no way of knowing whether this protective effect lasted over a delay in testing. Further, these authors did not test animals' working memory capabilities. Therefore, a more comprehensive functional test battery must be implemented in order to assess the long-term neuroprotective efficacy of EPO administration following neonatal HI.

### 3.1.5 Study Goals:

The goals of the current study were to address this relative lack of experimental assessments of EPO and its effects on functional capabilities by administering a single high dose of EPO to pnd 7 rats following neonatal HI and also to validate the functional test battery that was developed in Chapter 2 in an experimental paradigm. Since there are no reported detrimental side effects of administration of a single high dose of EPO

(Kumral *et al.* 2003; Kumral *et al.* 2004b; Spandou *et al.* 2005), and EPO has been reported to show only moderate functional neuroprotection at a dose of 1,000 U/kg (Kumral *et al.* 2004b), a higher dose of EPO (5,000 U/kg) was selected for the current study.

To assess the potential protective effects of EPO we tested both male and female rats (data analyzed separately) physical development, sensorimotor, neuromotor and, cognitive abilities (see Table II in chapter 2). Further, we assessed the structural neuroprotection of EPO by analyzing the area of infarction caused by the HI episode. We hypothesize that EPO-treated animals will exhibit fewer deficits in the functional assessments and have a relative structural neuroprotection when compared with animals that do not receive the EPO treatment.

## 3.2 Materials and Methods

### 3.2.1 Animals:

Thirteen untimed, pregnant Sprague Dawley rats (Charles River Laboratories; Montreal, Canada) were housed in individual cages at Dalhousie University (Nova Scotia, Canada) and the day of delivery was considered postnatal day 0 (pnd 0). Litters were culled between 12-24 hours following birth to 10 pups, five males and five females where possible. Animals were raised with the dam in single-housed cages until weaning at pnd 24-25. Animals were kept on a 12:12 hour light:dark (lights on at 07:00) cycle and were provided with *ad libitum* food (except when limited during particular behavioural testing) and water throughout all experimentation. Transportation procedures were the same as in Chapter 2 with the exception that animals were between the ages of pnd 14-23 when transported. Upon arrival at the University of Prince Edward, animals were housed in similar conditions as those reported in Chapter 2.

### 3.2.2 Induction of Hypoxia-Ischemia:

The surgical procedure used in this study is identical to that described in the previous study (Chapter 2). Briefly, under isoflurane anesthesia, pnd 7 rats were subjected to permanent left common carotid occlusion by electrocauterization through a midline incision in the neck. Following the occlusion surgery, pups were returned to the

dam for two hours for recovery and feeding. Next, animals were placed in a chamber that was immersed in $34^0$-$35^0$C water bath and subjected to a humidified gas mixture of 8% $O_2$ and 92% $N_2$ for a period of three hours. The control groups were subjected to the same surgical procedure without the CCAo, and exposed to three hours of normoxia within the chambers in the same water bath.

3.2.3 Experimental Conditions:

Pups from each litter were randomly assigned to one of four experimental conditions: *Group 1* (n=20) underwent HI and were treated immediately following the hypoxic period with EPO (5,000U/kg i.p.; Amgen, Thousand Oaks, CA); *Group 2* (n=20) underwent HI and were treated immediately following hypoxia with an inactivated form of EPO (iEPO; similar volume by body weight); *Group 3* (n=20) underwent the sham surgery and exposed to normoxia were treated immediately following normoxia with EPO (in the same volume as Group 1) and; *Group 4* (n=20) underwent the sham surgery and exposed to normoxia and were treated immediately following three hours of normoxia with the inactivated form of EPO (in the same manner as Group 2). Ten animals in each condition were male and 10 were female, and as with Chapter 2, data obtained were analyzed separately for sexes. The surgical survival rate in the current study was 96.4%.

3.2.4 Drug Preparation:

Mouse recombinant erythropoietin was supplied by Amgen (Thousand Oaks, CA). The vehicle in which EPO was administered to rat pups consisted of a solution of 0.1% bovine serum albumin (BSA) in 1 x phosphate buffered saline (PBS). The inactivated form of EPO was mixed with the same vehicle. Erythropoietin was injected intraperitoneally (i.p.) immediately following either hypoxia or normoxia at a dosage of 5,000U/kg. Inactive EPO was injected in equal volume by body weight.

3.2.5 Behavioural Tests:

In order to measure the neuroprotective efficacy of EPO, we tested all animals in a battery of behavioural tests that were shown to be sensitive to HI induced injury in our first study (Chapter 2; Table II) with several modifications for testing adult animals (see below). These included assessments of *physical development, sensorimotor, neuromotor* and, *cognitive* abilities. All apparatuses used in this study were the same as those described for use in the previous study (Chapter 2). All assessments were made by an experimenter who was blind to the experimental condition of each animal.

*3.2.5.1 Physical Development:*

Weights were recorded from the day of surgery until pnd29. Animals' weights were further recorded during the RAM testing in order to ensure that no animal

experienced significant weight loss during this testing. Further, day on which eye opening occurred was recorded.

*3.2.5.2 Sensorimotor Testing:*

Sensorimotor testing was not conducted in the first study (Chapter 2), however, it was noted that many animals appeared to be exhibiting sensorimotor deficits, so a 14-point bilateral scale was adapted, modified and implemented in the current study (Markgraf *et al.* 1992; Hunter *et al.* 2000). In this testing regiment, limb placement reactions were assessed in response to visual, tactile, and proprioceptive stimuli. Scoring consisted of a 0 and 1 rating scale where 0 corresponded to *no deficit* or *normal* and a score of 1 corresponded to *deficit*. The sensorimotor abilities assessed were as follows: (1) Lateral limb placement: in this test each limb was individually removed from the table's edge. The normal response is to lift the limb and place it back onto the tabletop (maximum score of 4); (2) Vertical forelimb placement: in this measure, animals were cupped in the experimenter's hand with forelimbs hanging free and lowered toward the table. Normal animals will extend both forelimbs toward the table, however, animals with deficits in this behaviour will usually extend only one forelimb toward the table and the other will cross-under the animal's body (maximum score of 2); (3) Horizontal limb placement: animals were held in the same manner as in the vertical forelimb placement with forelimbs hanging free, but in this test, the animals were brought closer to the table edge in a horizontal manner. Normal animals will extend both forelimbs in a controlled fashion and reach for the table's edge, but animals with deficits will only extend one forelimb with the other under the animal's body or extending in an uncontrollable fashion

(maximum score of 2); (4) Somatosensory forelimb placement: for this test, each of an animal's forepaws (top of the forepaw) were pushed against the table edge in order to stimulate the limb muscles. Normal animals will place the forepaw onto the tabletop however, animals with deficits will leave the paw pressed against the table edge (maximum score 2); (5) Pupillary: the lights were turned off in the testing room for 30 seconds to allow the animal's eyes to adjust to the darkness of the room. Following this 30 second period, a light was shone directly into the animal's eye to watch for a constriction of the pupil. Normal animal's pupils will constrict in response to the light but the pupils of animals with a deficit will not constrict (maximum score of 2); (6) Body torsion (tail suspension response (Felt *et al.* 2002)): animals were suspended by the tail above the tabletop and lowered toward the surface. Normal animals will extend both forelimbs toward the table surface and both hindlimbs will also be extended to the sides. Animals with a deficit will exhibit one (or both) of two responses; first they will only extend one forelimb and the other will cross under the animal's body and both hindlimbs will clasp each other, or second, they will rotate or contort their body in such a manner as to 'climb' unto their tail and the experimenter's hand (maximum score of 1); General condition score: animals were assessed on their physical appearance. Animals assigned a score of 1 corresponded to the presence of piloerection, porphyrin and, or lack of muscle tone. Animals without these symptoms were assigned a score of 0 (maximum score of 1). Sensorimotor testing was conducted on two days (pnd91 and 93) and the average score of the two days was calculated and analyzed.

*3.2.5.3 Neuromotor Testing:*

Animals' neuromotor abilities were assessed on two tests: forelimb grip strength and negative geotaxis. Forelimb grip strength assessments included: latency to fall off of wire, recording which forelimb slipped off of wire first (i.e., left or right) and the hindlimb scoring scale used in the previous study (Chapter 2). The negative geotaxis assessments included: latency to rotate $180^0$ and turning direction. Neuromotor abilities were assessed on two separate occasions; once following RAM testing (pnd 119-122), and then again prior to MWM testing (pnd 138-142).

*3.2.5.4 Cognitive Testing:*

Spontaneous Alternation: The same procedures were employed in assessing animal's spontaneous alternation behaviour as in Chapter 2. Animals were tested in this maze on three separate days: pnd 29-32, 34-37 and, 40-43. Latency to choose, number of alternations and total of left and right choices were recorded.

Radial Arm Maze: In this study we used only the eight arm configuration of the RAM because we failed to find a difference in reference memory abilities (assessed in the four arms baited configuration) between male or female HI and sham conditions in the previous study (Chapter 2). All procedures were the same as those described previously with the exception that the maze was thoroughly cleaned between the testing of each animal. Further, testing occurred for a period of 15 days instead of 10 as in the previous study. Measures recorded were: latency to obtain all eight rewards, number correct on first eight choices, number of omission errors and, number of re-entry

errors made (errors of commission, working memory assessment). Animals were food restricted during this testing regime where males received 15-20 g of food per day and females received 10-12 g of food per day. Animals maintained their *ad libitum* body weight throughout testing on this food restriction schedule.

Morris Water Maze: Testing procedures for this maze were identical to those described in Chapter 2. Animals were given four trials per day for four days, tested on a probe trial (platform removed) the following day, and then give a re-test (platform returned to its original location) one week following the probe trial. Following these testing procedures, animals were then tested in a visible platform trial version of the MWM. All trials were video recorded and were analyzed by the computer program Ethovision© (Noldus, Wageningen, Netherlands) (see Acknowledgements). Measures recorded in the hidden platform trials were: latency to locate the platform, distance swam before locating the platform and, swimming velocity. In the probe trial, measures recorded were: number of times animal crossed the platform zone, distance swam around entire maze and, swimming velocity.

3.2.6 Neuropathological Assessments:

Following completion of all functional assessments, rats were euthanized by $CO_2$ exposure and decapitation. Brains were removed and fixed in 10% buffered neutral formalin (Fisher Scientific Co., Ottawa, ON) for 4-6 days. Pictures were taken of the whole brain before sectioning. Brains were then sliced rostrally-caudally at 2.0 mm

sections using a stainless steel rat brain matrix (BSRS01.1 1.0 mm; Zivic Laboratories Inc., Pittsburgh, PA). Slices at 4, 6, 8, 10 and, 12 mm from brain frontal were photographed and used for analysis. Intact (remaining) hemispheric area was calculated and analyzed using National Institutes of Health (USA) computer program ImageJ for Windows (version 1.34n; downloaded in the public domain from http://rsb.info.nih.gov/ij/).

Brains were first categorically scored as appearing either normal (0), moderately damaged (1), or severely damaged (2) (Ikeda *et al.* 2001). Further, absolute area was calculated for both the ipsilateral and contralateral hemispheres for each section.

3.2.7 Statistical Analyses:

Results were expressed as mean ± standard error of the means (SEM). Neonatal weights, day to eye opening, neuromotor, spontaneous alternation, RAM, MWM data were analyzed using two-way (day x condition) analysis of variance (ANOVA) with repeated measures (RM) where applicable. The least significant difference (LSD) was used for post hoc comparisons. In the repeated measures analyses, Mauchly's Test was used to assess the sphericity assumptions and in cases where this assumption was violated, the Huynh-Feldt test was used (Crowder and Hand 1990; Weinfurt 2000). Homogeneity of variance was assessed following both univariate ANOVA and the between-subjects variable in the RM ANOVA using Levene's Test, however, since the Levene's test is considered very sensitive, the alpha value was set at 0.001 (Tabachnick and Fidell 1996). In cases where the Levene's test was to exceed a probability value of

0.001, the Welch correction was used (Dr. Andy Field, personal communication). For all other analyses, the alpha value was set at 0.05. To analyze the grade of neuronal injury caused by the HI episode, Kruskal-Wallis test was used with the Mann-Whitney as a post-hoc test. For all other non-parametric data, chi-square analysis was used. The computer software Statistical Package for the Social Sciences (SPSS for Windows 11.5.1, 2002, SPSS Inc., USA) was used to analyze all of the data obtained.

## 3.3 Results

### 3.3.1 Mortality Rates:

There was no difference in the rate of mortality between male conditions (sham 5/29 [17.2%]; HI+EPO 8/18 [44.4%]; HI+iEPO 3/15 [20.0%]) [$\chi^2(2) = 4.641$, $p>0.05$] or female conditions (sham 2/26 [7.7%]; HI+EPO 2/17 [11.8%]; HI+iEPO 3/17 [17.6%]) [$\chi^2(2) = 0.989$, $p>0.05$]. There were a total of 80 animals used for this study (10 animals in each condition).

Two animals were removed from the statistical analyses in this study (total number of animals used in the statistical analyses were 78). One male animal from the HI + EPO condition was euthanized on the advice of the UPEI laboratory animal veterinarian due to poor health. The animals' health deteriorated after commencement of the RAM and was euthanized at this time. All data preceding the RAM however, was included in the statistical analyses. The second animal, a female animal from the HI + EPO condition, displayed abnormal behaviour during the spontaneous alternation testing (pnd 35), possibly due to a secondary ischemic attack or a seizure. Since this situation was unique and abnormal compared to the remaining animals it was decided to remove this female's data from the spontaneous alternation analyses as well as all subsequent analyses.

The first series of analyses was conducted on both sham conditions (EPO and iEPO) for both males and females. There were no statistical differences between the male sham + EPO and male sham + iEPO animals, nor were there any statistical

differences between the female sham + EPO and female sham + iEPO animals. As a result, both of the male sham and both of the female sham conditions were combined for all comparisons with the two stroke (HI) conditions. Male and female data were analyzed separately.

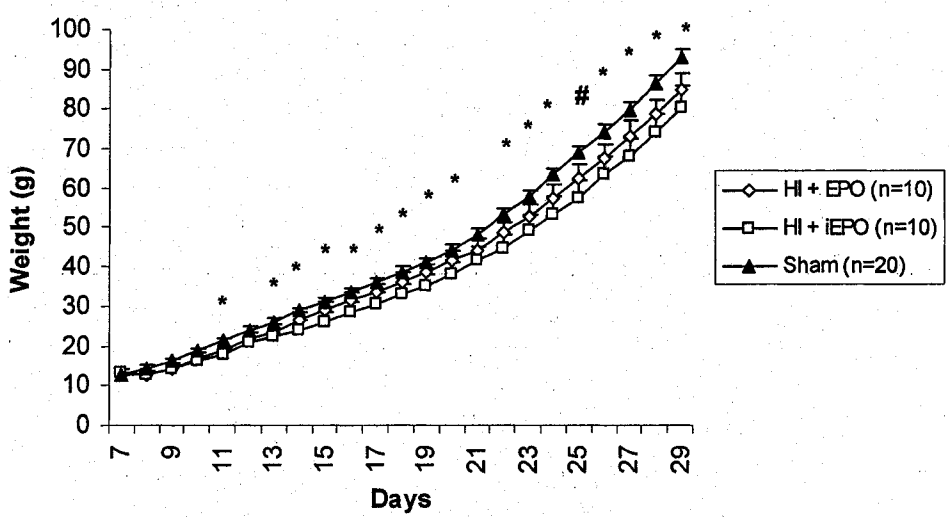
### 3.3.2 Physical Development:

#### *3.3.2.1 Neonatal Weights:*

Male: A repeated measures (day) two-way (day x condition) ANOVA revealed a significant effect of day [$F(1.621,59.983) = 1224.834$, $p<0.01$] and a significant interaction [$F(3.242,59.983) = 3.319$, $p<0.05$] but no effect of condition [$F(2,37) = 2.929$, $p>0.05$] (Figure 3.2A). Further analysis of this interaction revealed that sham animals were significantly heavier than HI+iEPO animals on pnd 13-20 and 22-29.

Female: Similar analysis of female data revealed a significant effect of day [$F(1.717,63.532) = 11.58.691$, $p<0.01$] and condition [$F(2,37) = 3.856$, $p<0.01$] but no interaction [$F(3.434,63.532) = 2.482$, $p>0.05$]. Post hoc analysis revealed a significant difference between the sham condition and the HI+EPO condition ($p<0.05$), and a difference between the sham condition and HI+iEPO condition ($p=0.051$) (Figure 3.2B) where, in both cases HI animals were lighter.

A.




B.

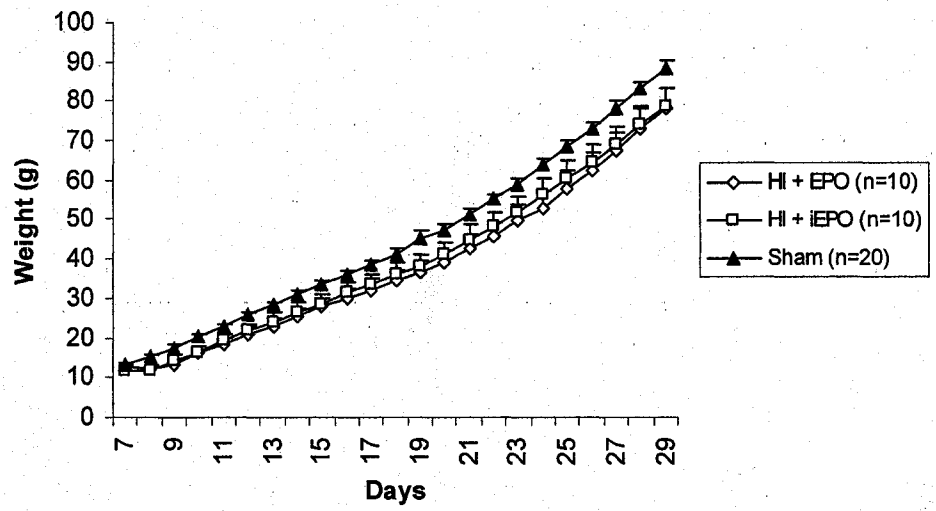



*Figure 3.2.* Neonatal weight gain for (A) males and (B) females (means ± SEM) (*$p<0.05$; #$p<0.01$) analyzed by independent t-test with Bonferroni correction. (B) female: there was a significant difference between the sham and HI+EPO conditions ($p<0.05$) and a difference between the sham and HI+iEPO conditions ($p=0.051$).

*3.3.2.2 Eye Opening:*

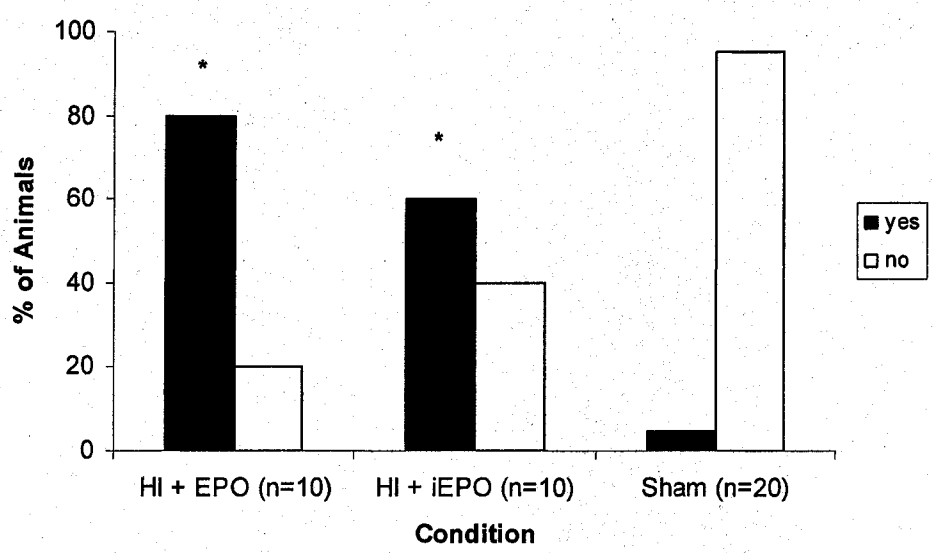
Male: There were no differences between conditions in the day to left (pnd14.9, 14.7 and, 14.3 for HI+EPO, HI+iEPO and, sham conditions, respectively) or right eye opening (pnd14.3, 14.0 and, 14.3). There was a difference, however, between conditions in the percentage of animals with a ptosis of the left eye [$\chi^2(2) = 18.880$, $p<0.01$] (Figure 3.3A). Analyses revealed that there was no difference between the two stroke conditions [$\chi^2(1) = 0.952$, $p>0.05$] but there was a significant difference between the sham condition and the HI+EPO [$\chi^2(1) = 17.857$, $p<0.01$] and HI+iEPO [$\chi^2(1) = 11.273$, $p<0.01$] conditions with sham animals having a lower percentage of ptosis.

Female: There were no differences between conditions in the day to left (pnd14.7, 14.3 and, 14.1 for HI+EPO, HI+iEPO and, sham conditions, respectively) or right (pnd14.3, 13.9 and, 14.1) eye opening. As was the case for male rats, however, there was a difference between conditions in the percentage of animals with ptosis of the left eye [$\chi^2(2) = 18.027$, $p<0.01$] (Figure 3.3B). Analyses revealed that there was no difference between the two stroke conditions [$\chi^2(1) = 0.00$, $p>0.05$] but there was a significant difference between the sham condition and the HI+EPO [$\chi^2(1) = 14.403$, $p<0.01$] and HI+iEPO [$\chi^2(1) = 14.403$, $p<0.01$] conditions with sham animals having a lower percentage of ptosis.

3.3.3 Sensorimotor Testing:

As stated in the Methods section, sensorimotor testing was conducted on two separate days and the average deficit score was calculated and analyzed.

A.




B.

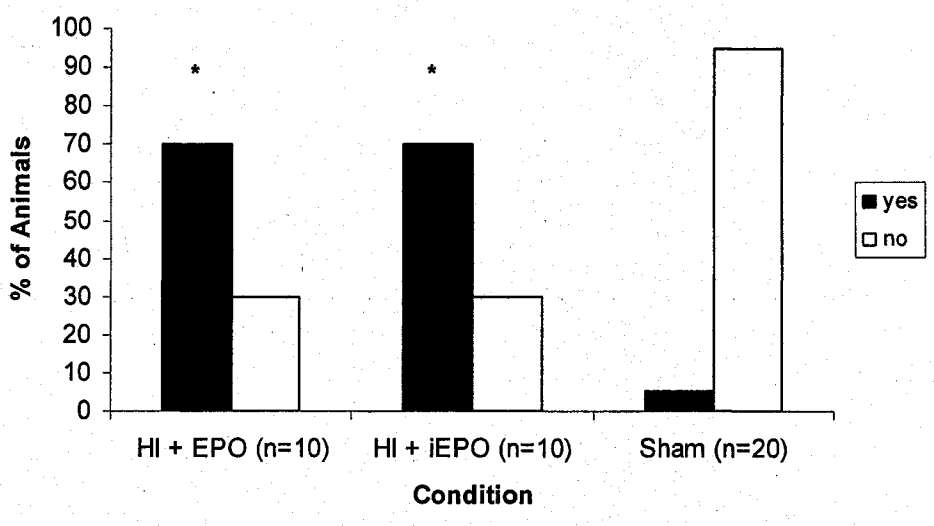



*Figure 3.3.* Left eye ptosis for (A) male and (B) female conditions. * indicates significant difference from sham condition ($p<0.01$).

Male: Univariate ANOVA revealed a statistically significant difference between conditions in the average deficit score [$F(2,37) = 9.073$, $p<0.01$] (Figure 3.4A). LSD post hoc analysis revealed that these differences occurred between the sham condition and HI+EPO ($p<0.05$) and HI+iEPO ($p<0.01$) conditions where both stroke conditions had a significantly higher average deficit score. There was no difference between the two stroke conditions.

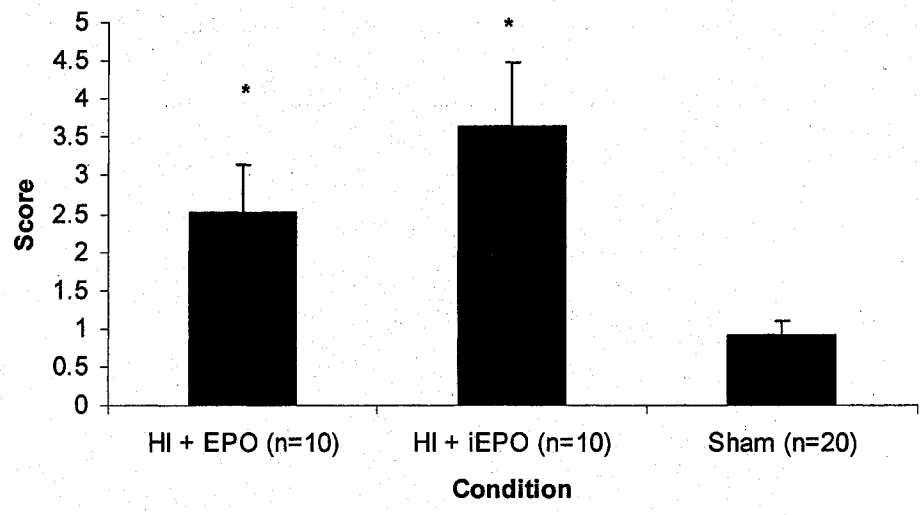
Female: Univariate ANOVA revealed a statistically significant difference between conditions with respect to the average deficit score in sensorimotor testing [$F(2,36) = 4.321$, $p<0.05$] (Figure 3.4B). As with analyses of the male conditions, LSD post hoc analysis revealed that there was a significant difference between the sham condition and HI+EPO ($p<0.05$) and HI+iEPO ($p<0.05$) conditions, with both stroke conditions having a higher deficit score. There was no difference between the two stroke conditions.

### 3.3.4 Neuromotor Testing:

#### *3.3.4.1 Forelimb Grip Strength:*

Male: With respect to the latency to hold on to the wire, RM ANOVA revealed that there was no effect of day [$F(1,36) = 0.795$, $p>0.05$] or condition [$F(2,36) = 1.057$, $p>0.05$] and no interaction [$F(2,36) = 1.534$, $p>0.05$]. Further, there were no differences between conditions on either day in the grip strength score (pnd122 [$\chi^2(4) =$

A.




B.

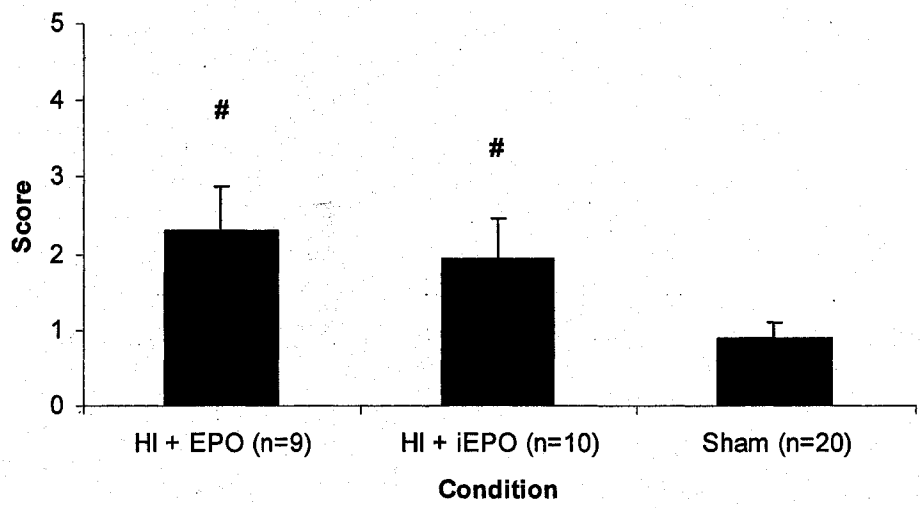



*Figure 3.4.* Sensorimotor deficit score. Average deficit score over two days of sensorimotor testing for (A) male and (B) female conditions. * ($p<0.01$) and # ($p<0.05$) indicates significantly different from sham condition.

6.155, p>0.05]; pnd140 [$\chi^2(4) = 4.241$, p>0.05]) or in the first paw to slip off of the wire before falling (pnd122 [$\chi^2(4) = 1.253$, p>0.05]; pnd140 [$\chi^2(4) = 3.468$, p>0.05]).

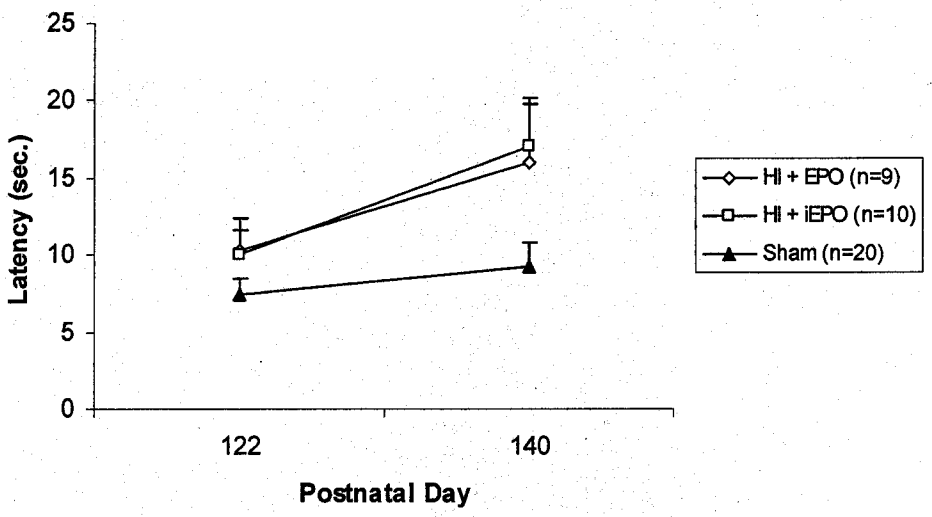
Female: As with analyses of the male data, with respect to the latency to hold on to the wire, RM ANOVA revealed that there was no effect of day [$F(1,36) = 0.250$, p>0.05] or condition [$F(2,36) = 2.643$, p>0.05] and no interaction [$F(2,36) = 0.006$, p>0.05]. There were also no differences between conditions on either day with respect to the grip strength score (pnd122 [$\chi^2(4) = 2.348$, p>0.05]; pnd140 [$\chi^2(4) = 5.175$, p>0.05]) or in the first paw to slip off of the wire before falling off of the apparatus (pnd122 [$\chi^2(6) = 6.167$, p>0.05]; pnd140 [$\chi^2(6) = 6.327$, p>0.05]).

*3.3.4.2 Negative Geotaxis:*

Male: RM of a two-way ANOVA of the latency to turn 180° revealed that there was an effect of day [$F(1,36) = 10.817$, p<0.01] and condition [$F(2,36) = 3.484$, p<0.05] but no interaction [$F(2,36) = 1.430$, p>0.05] (Figure 3.5A). LSD post hoc analysis revealed that there was a significant difference between the sham and HI+iEPO conditions (p<0.05) and a difference between the sham and HI+EPO conditions (p=0.051) where the stroked animals took longer to rotate 180° than sham animals. There were no differences between conditions in the turning direction on either day of testing (pnd122 [$\chi^2(2) = 3.300$, p>0.05]; pnd140 [$\chi^2(2) = 0.282$, p>0.05]).

Female: With respect to latency to turn, RM of a two-way ANOVA revealed that that there was no effect of day [$F(1,35) = 0.191$, p>0.05] or condition [$F(2,35) = 3.065$, p>0.05] but a significant interaction [$F(2,35) = 4.717$, p>0.05] (Figure

A.

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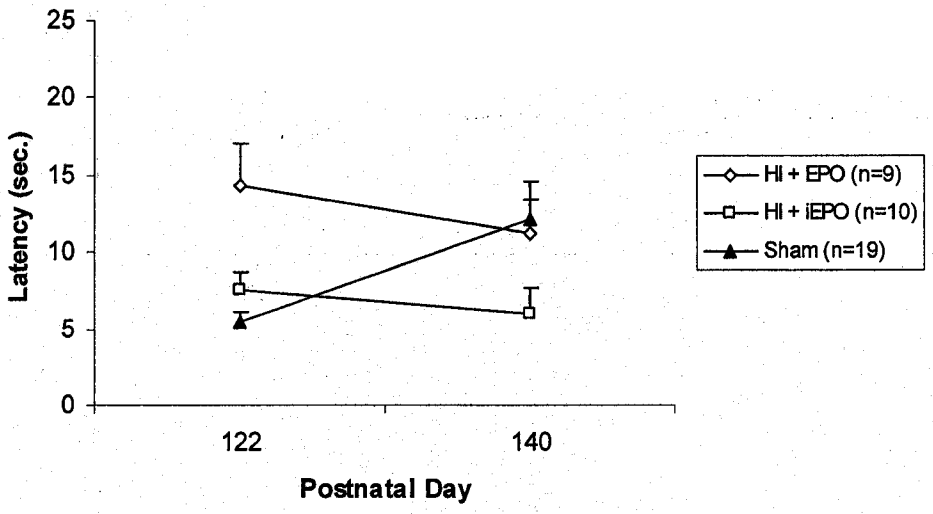



*Figure 3.5.* Performance in the negative geotaxis test. Latency to turn $180^0$ for (A) males' latency: significant difference between the sham and HI+iEPO conditions ($p<0.05$). HI+EPO and sham conditions ($p=0.051$). (B) females' latency: no difference between conditions.

3.5B). There were no differences between conditions in the turning direction on either day of testing (pnd122 [$\chi^2(2) = 3.121$, $p>0.05$]; pnd140 [$\chi^2(2) = 4.232$, $p>0.05$]).

3.3.5 Cognitive Testing:

*3.3.5.1 Spontaneous Alternation:*

Male: Two-way ANOVA with RM of the latency for animals to choose an arm in the T-maze of the spontaneous alternation test revealed a significant effect of day [$F(1.725,63.808) = 21.271$] but no effect of condition [$F(2,37) = 1.036$, $p>0.05$] and no interaction [$F(3.449,63.808) = 2.246$, $p>0.05$]. Similarly, with respect to the average number of alternations on each day, two-way RM ANOVA revealed a significant effect of day [$F(2,74) = 3.264$, $p<0.05$] but no effect of condition [$F(2,37) = 0.970$, $p>0.05$] and no interaction [$F(4,74) = 1.567$, $p>0.05$]. Analyzed further, there were no differences between conditions in the total number of alternations over all days [$F(2,37) = 0.970$, $p>0.05$] nor in the total number of left [$F(2,37) = 2.053$, $p>0.05$] or right [$F(2,37) = 1.766$, $p>0.05$] choices. Additionally, there were no differences within any of the three conditions with respect to the number of left or right choices, that is to say, there was no left or right choice preference within any of the male conditions in the spontaneous alternation test (sham: [$t(19) = 1.636$, $p>0.05$]; HI+EPO: [$t(9) = 0.685$, $p>0.05$] and; HI+iEPO: [$t(9) = 1.524$, $p>0.05$]).

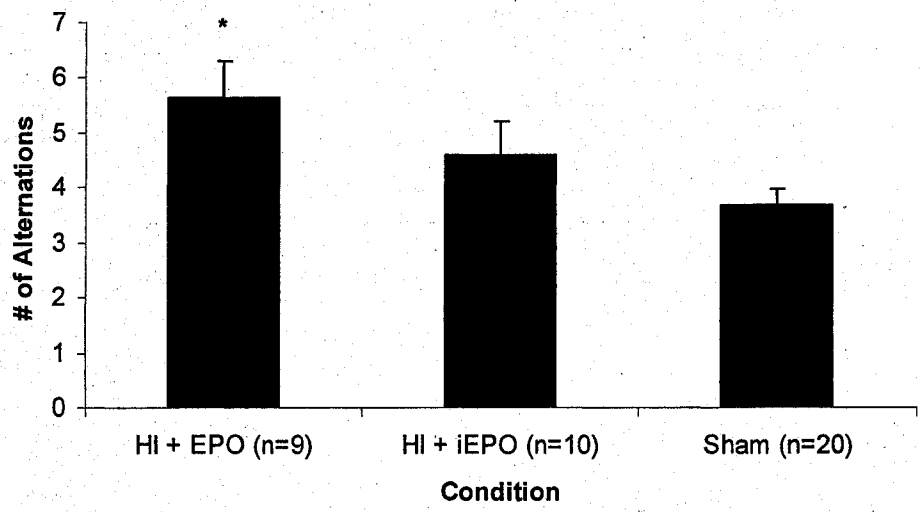
Female: With respect to females' latency to make a choice in the spontaneous alternation test, two-way ANOVA with RM revealed there was no effect of

day [$F(2,72) = 2.706$, $p>0.05$] or condition [$F(2,36) = 2.208$, $p>0.05$] and there was no interaction [$F(4,72) = 0.537$, $p>0.05$]. Further, in analyzing the average number of alternations made each day, RM of a two-way ANOVA revealed that there was no effect of day [$F(2,72) = 0.148$, $p>0.05$] or condition [$F(2,36) = 0.179$, $p>0.05$] and no interaction [$F(4,72) = 1.789$, $p>0.05$]. Univariate ANOVA showed that there was no difference between conditions with respect to the total number of alternations over all days [$F(2,36) = 0.179$, $p>0.05$] or in the total number of right choices [$F(2,36) = 1.666$, $p>0.05$]. There was, however, a significant difference between conditions in the total number of left choices [$F(2,36) = 4.894$, $p<0.05$] where HI+EPO made significantly more left choices than the sham condition (LSD post hoc; $p<0.01$) (Figure 3.6A). There were no differences between the two stroke conditions or between the sham and HI+iEPO conditions. Further analysis revealed that there was a significant difference between the number of left and right choices made by animals in the HI+EPO condition, where these animals made significantly more left choices [$t(8) = 3.028$, $p<0.05$]. There were no corresponding differences between animals in the HI+iEPO [$t(9) = 0.712$, $p>0.05$] or sham [$t(19) = 0.190$, $p>0.05$] conditions (Figure 3.6B).

*3.3.5.2 Radial Arm Maze:*

All analyses conducted on the RAM data were analyzed using a two-way (day x condition) ANOVA with RM. In cases where there was a significant condition effect, post hoc analyses were conducted using the LSD test.

A.




B.

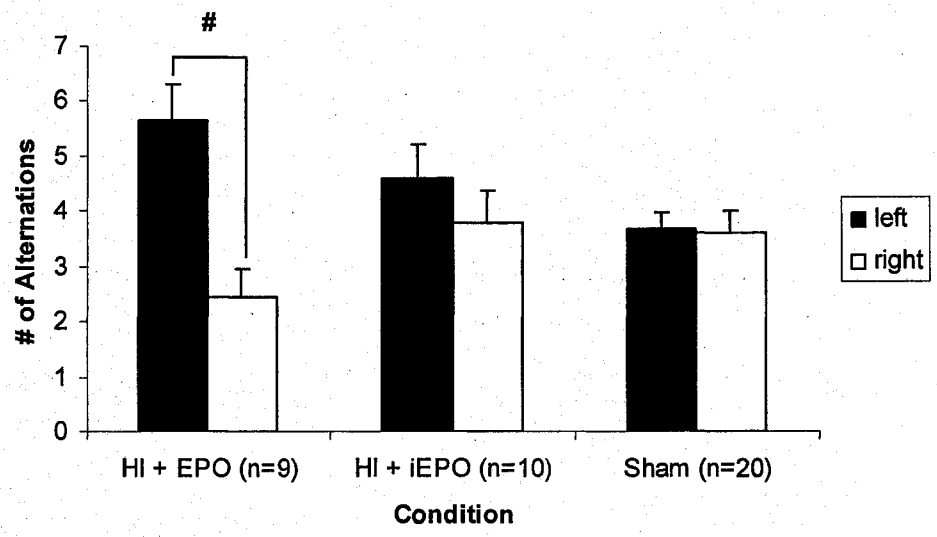
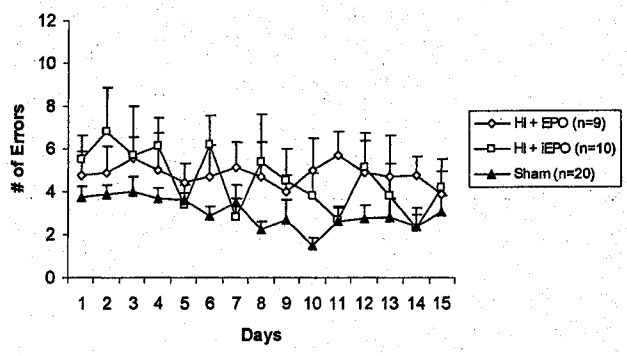



*Figure 3.6.* Spontaneous alternation data. (A) total number of left choices. * indicates significant difference from sham condition ($p<0.01$). (B) total number of left vs. right choices. # indicates significant difference within condition (between number of left and right choices; paired t-test) ($p<0.05$).

Male: There was a significant decrease in males' latency to obtain all eight rewards in the RAM over days [$F(11.372,409.382) = 6.448$, $p<0.01$] but there was no difference between conditions in latency to solve [$F(2,36) = 0.290$, $p>0.05$] and no interaction [$F(22.743,409.382) = 1.150$, $p>0.05$]. In analyzing the number of errors made in the RAM, with respect to the number of re-entry errors, analysis revealed that there were no effects of either day [$F(12.482,449.336) = 1.486$, $p>0.05$] or condition [$F(2,36) = 2.937$, $p>0.05$] or no interaction [$F(24.963,449.336) = 0.915$, $p>0.05$] (Figure 3.7A). With respect to number of omission errors, analysis revealed that there was a significant reduction in omission errors over days [$F(7.321,263.549) = 3.297$, $p<0.01$] but no difference between conditions [$F(2,36) = 0.922$, $p>0.05$] and no interaction [$F(14.642,263.549) = 1.406$, $p>0.05$] (Figure 3.7B). However, when the re-entry and omission errors were combined for a total error score there was a significant decrease over days [$F(12.741,458.666) = 2.127$, $p<0.05$] and a significant difference between conditions [$F(2,36) = 3.779$, $p<0.05$] but no interaction [$F(25.481,458.666) = 1.091$, $p>0.05$] (Figure 3.7C). Post hoc analyses revealed that there was a significant difference between the sham condition and both the HI+EPO and HI+iEPO conditions ($p<0.05$) where animals in the stroke conditions made significantly more errors. There were no differences between the two stroke conditions. In the last measure recorded, number correct on the first eight choices, analysis revealed a significant increase in the number correct over days [$F(14,504) = 2.538$, $p<0.01$] and an effect of condition [$F(2,36) = 4.381$, $p<0.05$] and no interaction [$F(28,504) = 0.861$, $p>0.05$] (Figure 3.8A). Post hoc analysis showed that both the HI+EPO and HI+iEPO conditions made significantly fewer correct choices on the first eight selections ($p<0.05$).

A.

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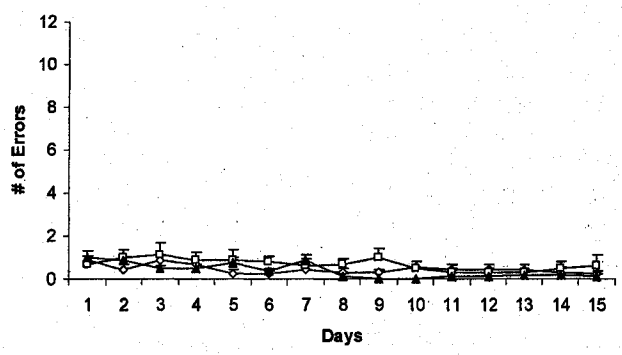


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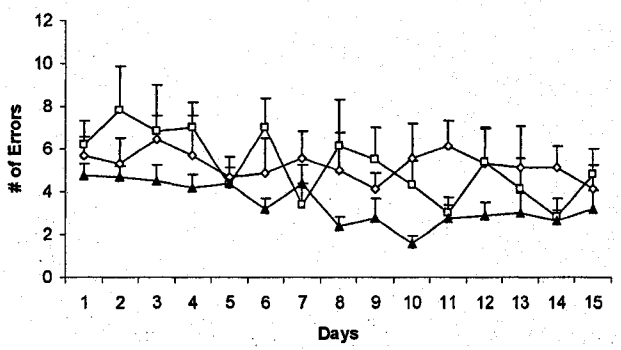
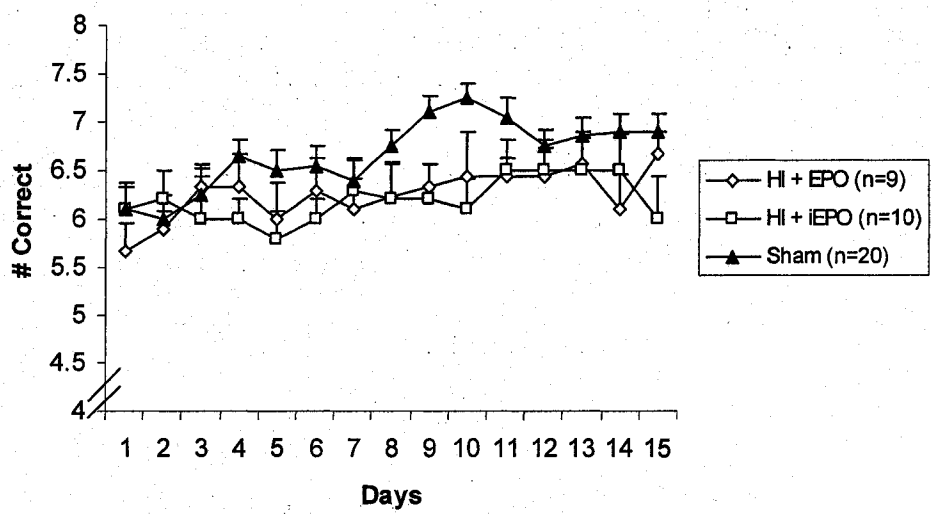



*Figure 3.7.* Performance in the RAM – Males. Number of errors committed by male animals in the RAM. (A) average number of re-entry errors. (B) average number of omission errors. (C) average number of errors (combination of re-entry and omission) made. There was a significant difference ($p<0.05$) between sham and HI animals with respect to the total number of errors made (C).

A.

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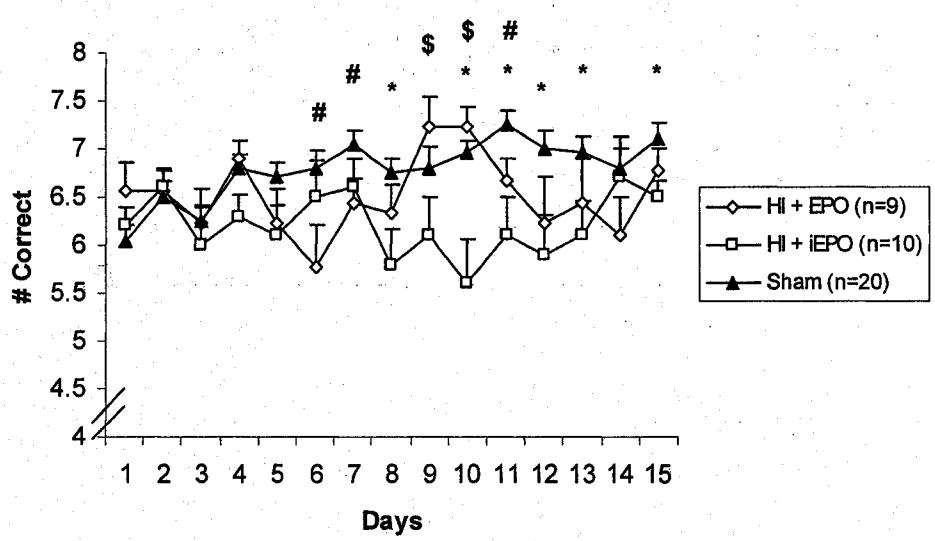
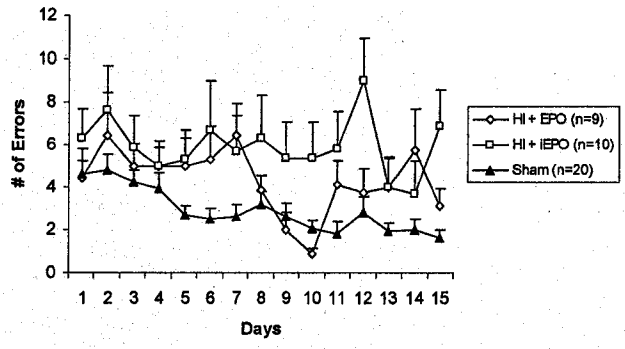


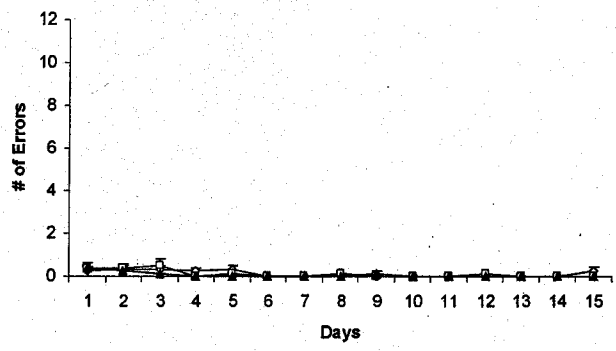

*Figure 3.8.* Fist 8 arm choices in the RAM (number correct on first eight choices; mean ± SEM). (A) males: significant difference between sham condition and both stroke conditions ($p<0.05$). (B) females: * indicates significant difference between HI+iEPO and sham conditions; # indicates significant difference between HI+EPO and sham conditions; $ indicates significant difference between HI+EPO and HI+iEPO conditions.

Female: With respect to females' latency to obtain all eight rewards, there was a significant decrease in latency over days [$F(12.760,459.358) = 13.623$, $p<0.01$] but no difference between condition [$F(2,36) = 1.757$, $p>0.05$] and no interaction [$F(25.520,459.358) = 0.808$, $p>0.05$]. The analysis of the average number of re-entry errors made in the RAM showed that there was a significant improvement (i.e., a reduction in the number of errors) over days [$F(11.482,413.338) = 2.602$, $p<0.01$] and a significant difference between conditions [$F(2,36) = 7.979$, $p<0.01$] but no day x condition interaction [$F(22.963,413.338) = 1.371$, $p>0.05$] (Figure 3.9A). Post hoc analysis showed that the condition effect occurred as a result of HI+iEPO animals making significantly more re-entry errors than the sham condition ($p<0.01$). There was no difference between the HI+EPO condition and either the HI+iEPO or sham conditions. Analysis of the number of omission errors also showed that there was a significant reduction in errors over days [$F(7.525,263.376) = 5.113$, $p<0.01$], but there was no effect of condition [$F(2,36) = 0.899$, $p>0.05$] and no interaction [$F(15.050,263.376) = 0.941$] (Figure 3.9B). Since most of the errors committed were re-entry errors, analysis of the combination of re-entry and omission errors for a total error score revealed similar results as the re-entry analysis where there was a significant reduction in errors over days [$F(11.533,415.199) = 2.887$, $p<0.01$] and a significant effect of condition [$F(2,36) = 8.061$, $p<0.01$] but no interaction [$F(23.067,415.199) = 1.296$, $p>0.05$] (Figure 3.9C), where again, HI+iEPO animals made significantly more total errors than animals in the sham condition ($p<0.01$). There were no differences between the HI+EPO condition and either the HI+iEPO or sham conditions. Analysis of the last measure recorded in the RAM, number correct on first eight choices, showed that there was a significant increase

A.




B.




C.

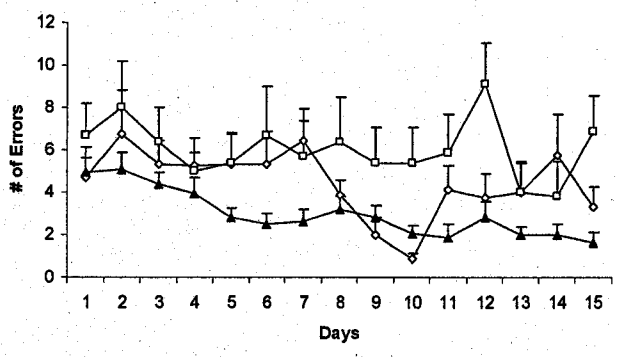



*Figure 3.9.* Performance in the RAM – Females. Number of errors committed by female animals in the RAM. (A) average number of re-entry errors. (B) average number of omission errors. (C) average number of errors (combination of re-entry and omission errors) made. There was a significant difference between HI+iEPO and sham conditions with respect to the average number of re-entry errors (A) and in the total number of errors made (C) ($p<0.01$).

in the number correct over days [$F(14,504) = 2.038$, $p<0.05$], a significant effect of condition [$F(2,36) = 5.190$, $p<0.05$] and a significant interaction [$F(28,504) = 2.295$, $p<0.01$] (Figure 3.8B). Further analysis of this interaction by independent t-tests with a Bonferroni correction showed that there was a complex interaction between the three conditions (Figure 3.8B). On days 6 and 7, the HI+EPO condition made significantly fewer correct choices than the sham condition, but on day 11 the reverse was true. Further, on days 9 and 10 the HI+EPO condition made significantly more correct choices than the HI+iEPO condition. There was a significant difference between the sham and HI+iEPO conditions on days 8, 10-13 and 15.

*3.3.5.3 Morris Water Maze:*

Male: Two-way ANOVA with RM on animals' swimming velocity during the acquisition trials revealed a significant effect of day where animals swam faster over days [$F(3,108) = 11.571$, $p<0.01$], but there were no differences in swimming velocity between conditions [$F(2,36) = 1.607$, $p>0.05$] and no interaction [$F(6,108) = 1.113$, $p>0.05$]. One-way ANOVA showed that there were also no differences in swimming velocity between conditions during the re-test [$F(2,36) = 1.701$, $p>0.05$] or the probe trial [$F(2,36) = 1.302$, $p>0.05$]. Analysis of animals' best (shortest) latency to locate the visible platform following completion of the MWM revealed a significant difference between conditions [$F(2,36) = 4.857$, $p<0.05$]. LSD post hoc analysis showed that there was a significant difference between the sham and HI+iEPO conditions ($p<0.01$) in the latency to locate the visible platform. There was also a difference, albeit not statistically

significant at $p<0.05$, between the sham and HI+EPO conditions ($p=0.052$) where, in both cases, stroke animals took longer to locate the visible platform.

Two-way ANOVA with RM of the average latency to locate the hidden platform during the acquisition trials revealed a significant decrease in latency across days [$F(2.483,89.401) = 60.451$, $p<0.01$] and a significant effect of condition [$F(2,36) = 10.230$, $p<0.01$] but no interaction [$F(4.967,89.401) = 1.176$, $p>0.05$] (Figure 3.10A). LSD post hoc analysis showed that the difference between conditions occurred as a result of a significant difference between the sham condition and both stroke conditions ($p<0.01$) with the stroke animals having longer latencies to locate the platform. Additionally, analysis of the average distances swam revealed similar results. There was a significant reduction in the distance swam to locate the platform across days [$F(2.448,88.133) = 54.906$, $p<0.01$] and a significant effect of condition [$F(2,36) = 8.633$, $p<0.01$] but no interaction [$F(4.896,88.133) = 1.096$, $p>0.05$] (Figure 3.10B). LSD post hoc analysis showed that the difference between conditions resulted from a significantly greater swimming distance of both the HI+EPO and HI+iEPO conditions compared to the sham condition ($p<0.01$). We also analyzed the latency to locate the platform during only the first trial of each day (started from the same location each day) as a measure of reference memory ability. Two-way ANOVA with RM showed that there was a significant decrease in latency over days [$F(2.614,94.118) = 34.470$, $p<0.01$] and a significant effect of condition [$F(2,36) = 3.829$, $p<0.05$] but no interaction [$F(5.229,94.118) = 0.525$, $p>0.05$] (Figure 10C). LSD post hoc analysis showed that there was a significant difference between the sham and HI+iEPO conditions ($p<0.01$) where the HI+iEPO condition had a significantly longer latency to locate the platform on

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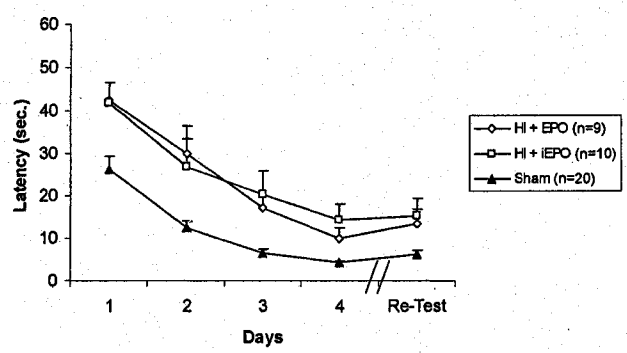


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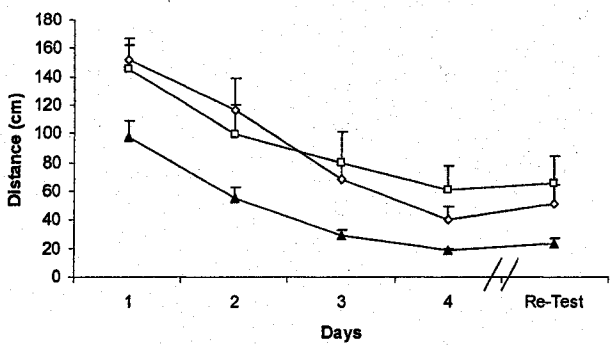


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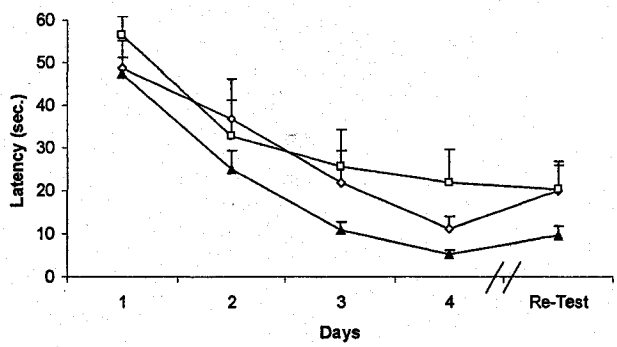


*Figure 3.10.* Performance in the MWM - Males (A) latency to locate the hidden platform. There was a significant difference between the sham and both stroke conditions during the acquisition trials ($p<0.01$). During the re-test there was a significant difference between the sham condition and the HI+EPO ($p<0.05$) and HI+iEPO ($p<0.01$) conditions. (B) distance swam around the whole maze. There was a significant difference between the sham condition and both stroke conditions during the acquisition trials ($p<0.01$). During the re-test however, there was a significant difference between only the sham and HI+iEPO conditions ($p<0.01$). (C) trial 1 latency data. There was a significant difference between sham and both stroke conditions during the acquisition trials ($p<0.01$). There were no differences between conditions during the re-test.

trial 1 during the acquisition period. There were no corresponding differences between the sham and HI+EPO conditions. There was also no difference between conditions on the trial 1 latency during the re-test trial [$F(2,36) = 2.211$, $p>0.05$].

In the one-way ANOVA of the average latency and distance to locate the platform during the re-test, the Welch F-ratio is reported due to violation of the homogeneity of variance assumptions in both cases. These analyses revealed that there was a significant difference between conditions during the re-test with respect to latency to locate the platform [$F(2,12.742) = 4.068$, $p>0.05$] (Figure 3.10A) and distance swam to locate the platform [$F(2,12.640) = 4.128$, $p<0.05$] (Figure 3.10B). LSD post hoc analysis showed that there was a significant difference between the sham condition and both the HI+EPO ($p<0.05$) and the HI+iEPO ($p<0.01$) conditions with respect to the latency data where the sham condition had a shorter latency to locate the platform. The distance data was somewhat different however, where post hoc analysis revealed that there was a significant difference between the sham and HI+iEPO conditions ($p<0.01$) where the HI+iEPO condition swam significantly greater distances to locate the platform, but there were no corresponding differences between the sham and HI+EPO condition (nor was there a difference between the two stroke conditions).

During the probe trial there were several measures recorded: distance swam throughout maze, number of times animals crossed through the platform zone, time spent in the platform zone and, distance swam around the platform zone. One-way ANOVA of these measures revealed a significant difference between conditions on the number of platform zone crosses [$F(2,36) = 3.616$, $p<0.05$] (Figure 3.11). LSD post hoc analysis showed that there was a significant difference between the sham condition and both of

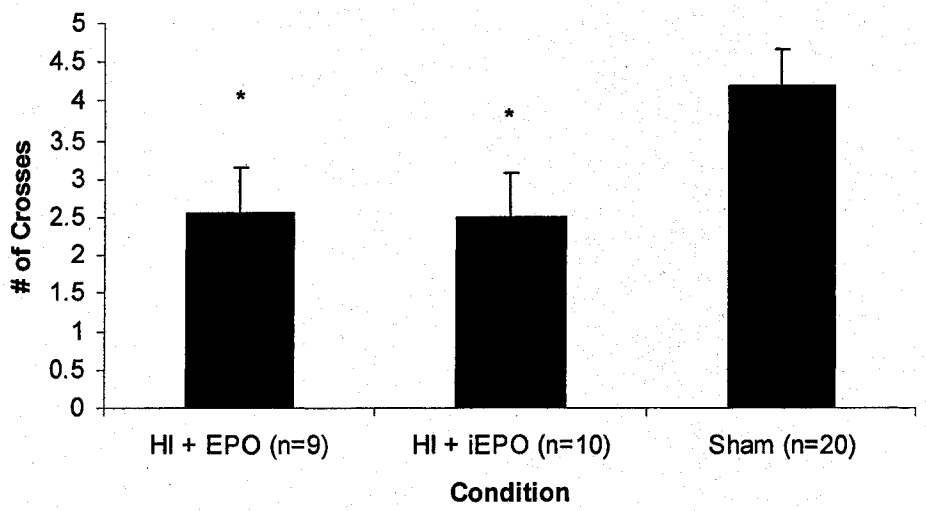


*Figure 3.11.* Probe trial data in the MWM - Males. The average number of times males' crossed through the platform zone. * indicates a significant difference from sham condition ($p<0.05$).

the stroke conditions ($p<0.05$) where sham animals made significantly more platform zone crosses than the stroke animals.

Female: Two-way ANOVA with RM on females' swimming velocity during the acquisition trials showed that there was no effect of day [$F(3,108) = 2.642$, $p>0.05$], or condition [$F(2,36) = 1.138$, $p>0.05$] and there was no interaction [$F(6,108) = 0.482$, $p>0.05$]. Further, one-way ANOVA of the swimming velocities during the probe trial and re-test as well as females' best latency during the visible platform trial showed that there were no differences between conditions on either of these measures ([$F(2,36) = 1.544$, $p>0.05$]; [$F(2,36) = 0.646$, $p>0.05$] and; [$F(2,36) = 1.033$, $p>0.05$], respectively).

Two-way ANOVA with RM on the average latency to locate the platform during the acquisition trials showed that there was a significant learning effect as the latencies to locate the platform decreased across days [$F(2.795,100.619) = 38.657$, $p<0.01$]. There was also a significant difference between conditions [$F(2,36) = 8.438$, $p<0.01$] but no interaction [$F(5.590,100.619) = 0.543$, $p<0.05$] (Figure 3.12A). LSD post hoc analysis revealed that there was a significant difference between the sham condition and both the HI+EPO ($p<0.01$) and HI+iEPO ($p<0.05$) conditions where, in both cases, sham animals had significantly lower latencies to locate the platform. Analysis of the average distances swam during these same trials revealed similar results. There was a significant decrease in swimming distances across days [$F(3,108) = 40.023$, $p<0.01$], a significant effect of condition [$F(2,36) = 8.121$, $p<0.01$] but no interaction [$F(6,108) = 0.528$, $p>0.05$] (Figure 3.12B). LSD post hoc analysis of this measure also showed that the HI+EPO ($p<0.01$) and HI+iEPO ($p<0.05$) conditions had longer swimming distances than sham animals. In the reference memory assessment (i.e., trial 1 latencies), there was a significant decrease

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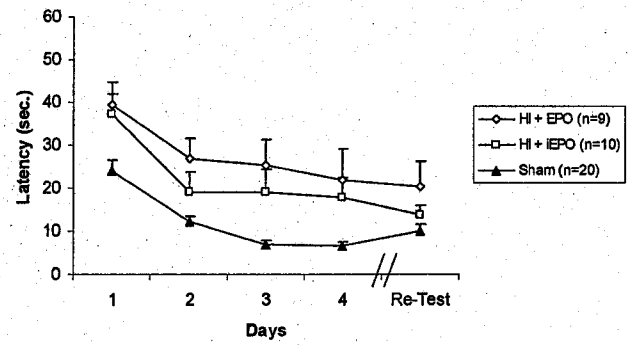


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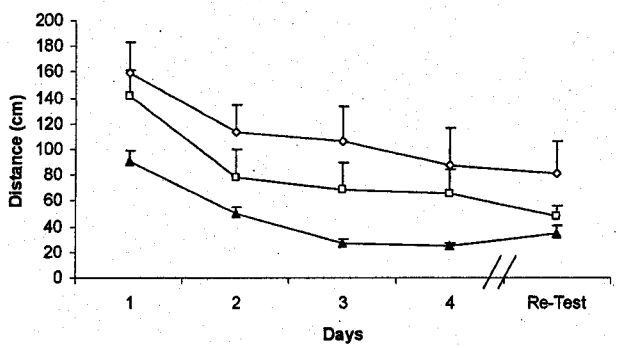


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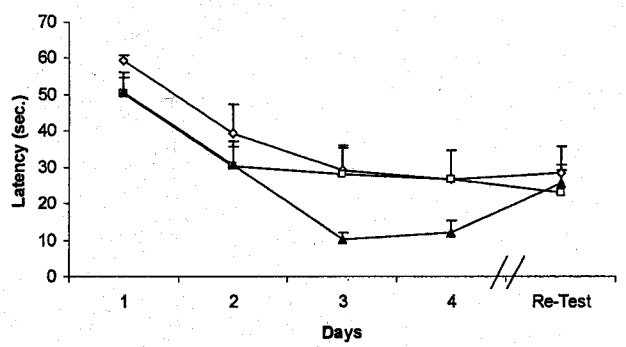



*Figure 3.12.* Performance in the MWM - Females. (A) latency to locate the hidden platform. There was a significant difference between the sham and the HI+EPO ($p<0.01$) and HI+iEPO ($p<0.05$) conditions during the acquisitions trials. (B) distance swam around the whole maze. There was a significant difference between the sham and the HI+EPO ($p<0.01$) and HI+iEPO ($p<0.05$) conditions during the acquisitions trials. (C) trial 1 latency data. There was a significant difference between the sham and HI+EPO conditions ($p<0.05$) during the acquisitions trials. There were no differences between conditions during the re-test in the above dependent measures.

in trial 1 latencies across days [$F(3,108) = 26.001$, $p<0.01$] and a significant effect of condition [$F(2,36) = 3.897$, $p<0.05$], but no interaction [$F(6,108) = 1.138$, $p>0.05$] (Figure 3.12C). Post hoc analysis revealed that there was a significant difference between the sham condition and the HI+EPO condition ($p<0.05$) where the HI+EPO condition had higher swim latencies.

Females' re-test data were analyzed using a one-way ANOVA. The homogeneity of variance assumption was violated for the average latency and distance measures; therefore, the Welch test correction F-ratio is reported. There were no significant differences between conditions in the measures recorded: average latency [$F(2,15.237) = 2.077$, $p>0.05$], average distance swam to reach platform [$F(2,15.021) = 1.940$, $p>0.05$] and, the trial 1 latency [$F(2,36)$, 0.147, $p>0.05$].

Data from the probe trial included measures of total distance swam throughout whole maze, number of platform zone crosses, time spent in platform zone and, distance swam in platform zone. One-way ANOVA revealed no significant differences between conditions on any of the measures.

### 3.3.6 Neuropathological Analysis:

#### *3.3.6.1 Grade of Injury:*

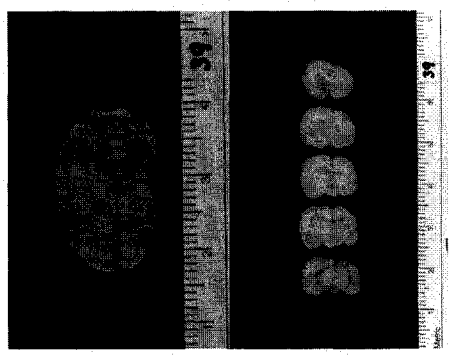
Scores from the gross examination of the brains were categorized into three groups: 0, no injury; 1, moderate injury and; 2, severe injury (Figure 3.13).

**A.**

Normal Brain

**B.**

Moderate Injury



**C.**

Severe Injury

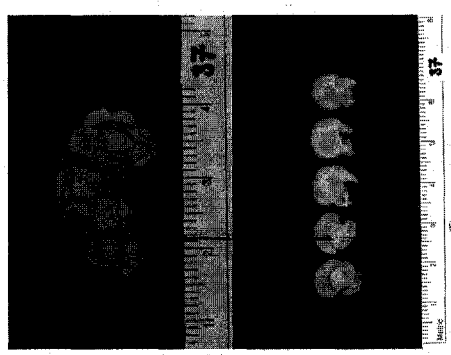


*Figure 3.13.* Representative images illustrating grades of injury: (A) normal brain; (B) brain with moderate injury; (C) brain with severe injury.

Male: Only one of the sham animals reported with an abnormality (score of 1), the remaining 19 animals' brains from this condition were considered normal. In the HI+EPO condition, four animals were considered normal; three animals were scored as having moderate injury and; two animals were scored as having severe injury. In the HI+iEPO condition, five animals were scored as normal; one animal was scored as having moderate injury and; four animals were scored as having severe injury (Table III). Analysis of the grade of injury using the Kruskal-Wallis test revealed that there was a significant difference between the conditions [$H(2) = 11.050$, $p<0.01$]. Mann-Whitney post hoc analysis showed that there was a significant difference between the sham condition and both the HI+EPO [$W = 253.500$, $p<0.01$] and HI+iEPO [$W = 263.000$, $p<0.01$] conditions, where animals in either stroke condition were more likely to have neuronal damage. There were no differences between the two stroke conditions [$U = 87.500$, $p>0.05$].

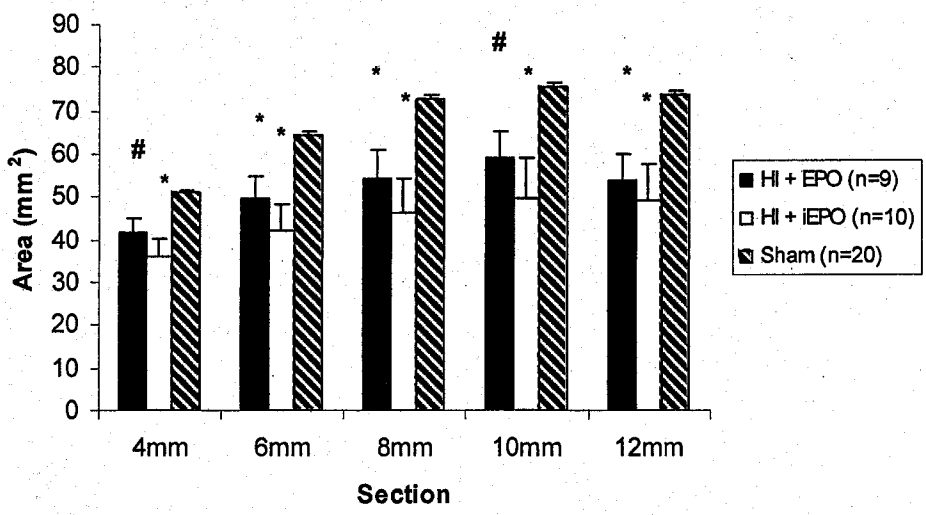
Brains were then sliced into five 2.0 mm sections corresponding to sections at 4, 6, 8, 10 and, 12mm from brain frontal (Figure 3.13). Total hemispheric area of all brains was calculated for each the ipsilateral (left) and contralateral (right) hemispheres. One-way ANVOA was used to analyze the intact hemispheric area. With respect to the ipsilateral hemisphere, there was a significant difference between conditions at all sections ($p<0.01$) (Figure 3.14A). LSD post hoc analysis showed that in all cases both of the stroke conditions had significantly smaller ipsilateral hemispheres. There was a trend, however, that the HI+EPO condition had a larger hemispheric area than the HI+iEPO condition at each brain section (Figure 3.14A). With respect to the analysis of the contralateral hemisphere, there was a significant difference between conditions at

Table III.

*Neuropathology Grade of Injury Score Results*

| | | **Grade of Injury Score: Total # of Animals (%)** | | |
|---|---|---|---|---|
| **Sex** | **Condition** | **0: No Injury** | **1: Moderate Injury** | **2: Severe Injury** |
| Male | Sham | 19 (95%) | 1 (5%) | 0 (0%) |
| Male | HI+EPO | 4 (44.4%) | 3 (33.3%) | 2 (22.2%) |
| Male | HI+iEPO | 5 (50%) | 1 (10%) | 4 (40%) |
| Female | Sham | 20 (100%) | 0 (0%) | 0 (0%) |
| Female | HI+EPO | 5 (55.6%) | 0 (0%) | 4 (44.4%) |
| Female | HI+iEPO | 5 (50%) | 1 (10%) | 4 (40%) |

A.

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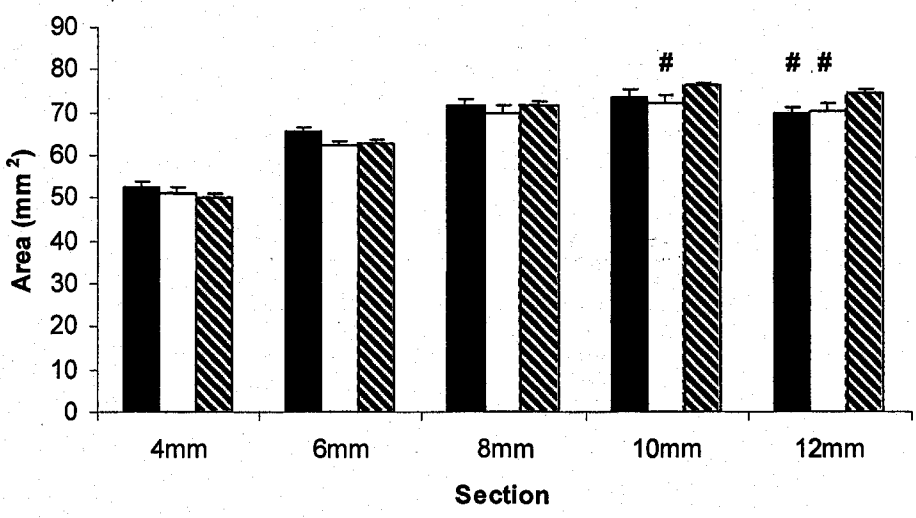



*Figure 3.14.* Neuropathology analysis – Males. Analysis of intact hemispheric area of male brains. (A) ipsilateral hemisphere: # and * indicates significantly different from sham condition at $p<0.05$ and $p<0.01$, respectively. (B) contralateral hemisphere: # indicates significantly different from sham condition $p<0.05$.

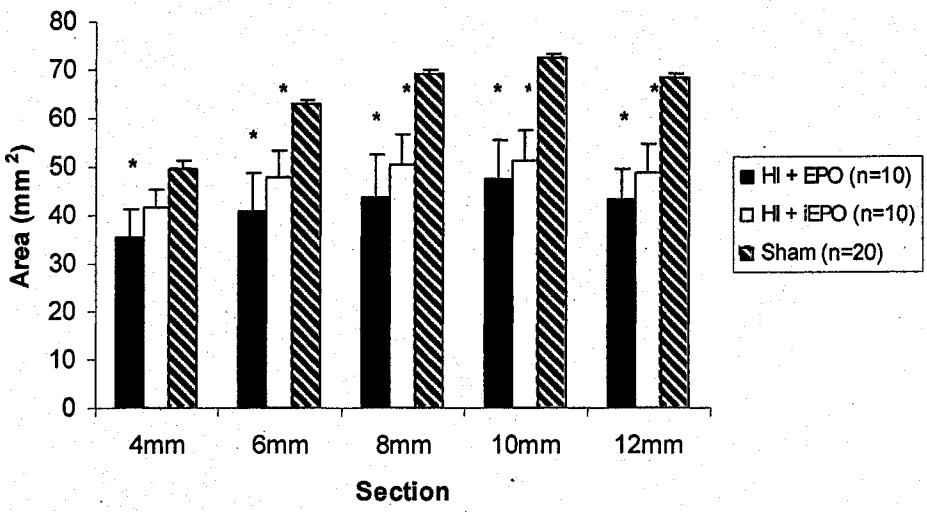
sections 10 [$F(2,36) = 3.526$, $p<0.05$] and 12mm [$F(2,35) = 3.897$, $p<0.05$] from frontal (Figure 3.14B). LSD post hoc analysis showed that at the 10mm section there was a significant difference between the sham and HI+iEPO conditions ($p<0.05$) and at the 12mm section there was a significant difference between the sham condition and both of the stroke conditions ($p<0.05$), in each case the stroke condition having a larger area of neuronal tissue remaining. There were no differences between the stroke conditions with respect to either the ipsilateral or contralateral hemisphere.

Female: All of the animals (n=20) in the sham condition were observed to have no gross abnormalities. In contrast, in the HI+EPO condition, five animals were scored as normal and the remaining four animals were scored as having severe injury. Further, in the HI+iEPO condition, five animals were scored as normal; one animal was scored as having moderate injury and; four animals were scored as having severe injury (Table III). Analysis of the grade of injury using the Kruskal-Wallis test revealed a significant difference between conditions [$H(2) = 11.948$, $p<0.01$]. Mann-Whitney post hoc analysis showed that there was a significant difference between the sham condition and both the HI+EPO [$W = 260.000$, $p<0.01$] and HI+iEPO [$W = 260.000$, $p<0.01$] conditions where, as with the male animals, stroke animals were more likely to experience brain injury than animals in the sham condition. There was no difference between the stroke conditions [$W = 89.500$, $p>0.05$].

As with the male brains, the female brains were then cut into five 2.0 mm sections corresponding to sections at 4, 6, 8, 10 and, 12mm from brain frontal (Figure 3.13). Total hemispheric area of all brains was calculated for each the ipsilateral (left) and

contralateral (right) hemispheres. One-way ANVOA was used to analyze the hemispheric area. With respect to the ipsilateral hemisphere, there was a significant difference between conditions at all sections analyzed. LSD post hoc analysis showed that at all sections except the 4mm section, there was a significant difference between the sham condition and both the HI+EPO and HI+iEPO conditions ($p<0.01$) where the stroke conditions always had smaller hemispheres (Figure 3.15A). At the 4mm section, there was only a significant difference between the sham and HI+EPO condition ($p<0.01$) with the HI+EPO condition having a smaller hemisphere. There was a difference (although not statistically) between the sham and HI+iEPO conditions ($p=0.060$), with the HI+iEPO also having a smaller hemisphere. With respect the contralateral hemisphere analysis, there was no significant difference between conditions at any section analyzed (Figure 3.15B). The F-statistic did however, approach the significance level at $\alpha = 0.05$ in several cases (at sections 6 [$p=0.076$], 8 [$p=0.053$], 12mm [$p=0.082$] from brain frontal).

A.




B.

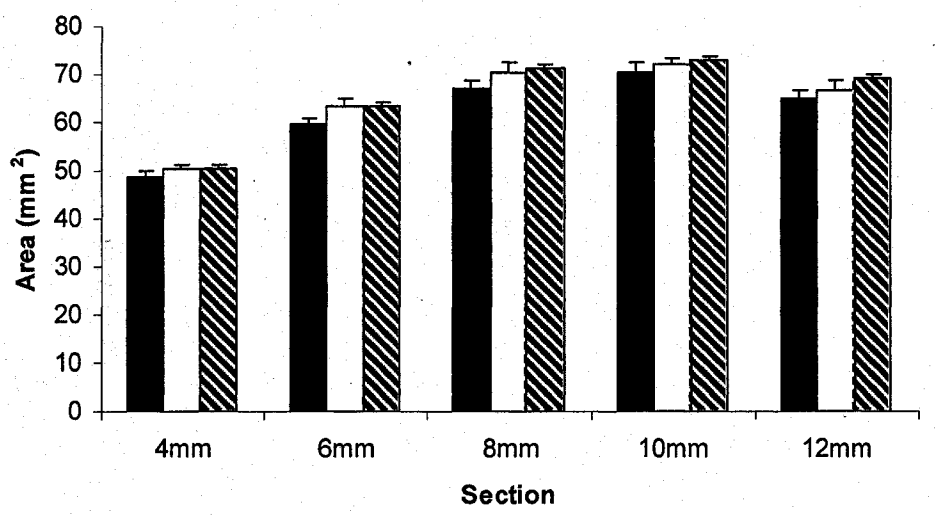



*Figure 3.15.* Neuropathology analysis – Females. Analysis of intact hemispheric area of female brains. (A) ipsilateral hemisphere: * indicates significantly different from sham condition ($p<0.01$). (B) contralateral hemisphere

## 3.4 Discussion

### 3.4.1 General Considerations:

The aim of the present study was to evaluate the neuroprotective efficacy afforded by the administration of a single dose of erythropoietin immediately following neonatal HI. Neuroprotection was evaluated by measuring various functions of animals subjected to HI such as physical development, sensorimotor, neuromotor and cognition. Further, following these assessments we analyzed the structural neuroprotection by calculating the remaining neuronal area throughout the brain of each animal. We found that in the male condition, EPO somewhat attenuated brain injury when compared with animals administered iEPO, but EPO males still exhibited significantly larger areas of infarct than sham animals. We did not see the same structural protection with respect to females administered EPO. Approximately 50% of both male and female animals subjected to HI displayed at least some brain injury (Table III).

With respect to our functional measures, it appeared that the administration of EPO had very little, if any, improvement on physical development or on the functional abilities of animals subjected to HI. With respect to the analysis of males' data, HI+iEPO animals' weights were significantly different from sham animals from pnd11 onward (with the exception of a couple of days), but there were no corresponding differences between the HI+EPO and sham conditions on any of these days. However, with respect to the male eye opening data, there was no effect of EPO in protecting animals from ptosis of the left eye. Further, there was no protective effect of EPO on males'

sensorimotor or neuromotor abilities. In the spatial learning and memory tasks we found a significant effect of stroke in many of the measures recorded, whereby animals subjected to HI exhibited significant memory impairments. Although 'stroked' animals still showed significant learning capabilities (as assessed from a decrease in latencies, or a reduction in the number of errors made), these capabilities were deficient when compared to sham animals. This was particularly evident in the working memory assessments in the RAM. With respect to reference memory abilities as assessed in the MWM, HI+EPO males' latency and swimming distance were somewhat lower than HI+iEPO animals, but were still significantly different than the sham animals. This effect has been shown in rats subjected to the same model of stroke used in this study (Kumral *et al.* 2004b). In the study conducted by Kumral and colleagues, reference memory abilities were assessed in the MWM and HI+EPO animal were significantly impaired compared to control animals, but also showed significant improvements compared to HI animals treated with saline.

With respect to our results obtained from female animals, the animals that were treated with EPO did not show a protective effect with respect to neonatal weight gain or in left eye ptosis. Further, there was no sensorimotor or neuromotor protection offered by EPO. In the cognitive assessments, we found a protective effect of EPO with respect to working memory abilities as assessed in the RAM. HI+EPO females performed as well as the sham condition with respect to the number of re-entry errors made as well as in the number of correct choices made on the first eight selections, where there was a significant difference between the HI+iEPO and sham conditions on these measures. This protection did not extend to reference memory abilities however, since HI+EPO

animals performed significantly poorer than sham animals in measures in the MWM and often poorer than the HI + iEPO animals (although there was never a statistically significant difference between these two conditions on any of these measures). One possible explanation for this difference in protection may be a result of the time of testing. Animals were first tested on the RAM beginning around pnd 100 and were later tested in the MWM at approximately pnd 135. During this five-week differential, more neuronal cellular death may have been occurring which may have resulted in an increase in neuronal injury and a decreased memory (reference) capabilities. If animals were tested in the MWM at an earlier time point, reference memory abilities may have been preserved.

The results, taken together, show that the dosage of EPO that we used (5,000U/kg) in this study using the neonatal HI model of stroke was ineffective in many tests, but may provide some selective neuroprotection and that this neuroprotection may be different depending on the sex of the animal. Since the studies that have analyzed the functional neuroprotective effects of EPO have either failed to find a difference between sexes or did not analyze this factor (Kumral *et al.* 2004b; Spandou *et al.* 2005), the effects of EPO administration on stroke may have been skewed.

### 3.4.2 Functional Neuroprotection:

Sensorimotor and neuromotor abilities were shown to be protected by EPO administration following neonatal HI in one study (Spandou *et al.* 2005). In our study we tested sensorimotor abilities around pnd 90 and neuromotor function at pnd 120 and 140.

This difference of six and 12 weeks post-stroke of testing compared to Spandou *et al.* may explain the differential findings. For example, as the animals in the current study continued to age, the cell death process may have also continued which would result in more neuronal damage possibly the loss of any sensorimotor and neuromotor protection that may have been offered earlier in development. The protection observed by Spandou *et al.* (2005) may be a result of delayed cell death instead of a permanent functional neuroprotection.

Our results were similar with respect to male animals in the MWM, wherein HI+EPO treated animals had a tendency to have lower latencies to locate the hidden platform, but this effect was not observed in working memory abilities. Male HI+EPO and HI+iEPO animals performed similarly in the RAM, both of which had significantly poorer performance than control animals. With respect to the female animals however, the opposite was true. There was no tendency toward a lower latency to locate the hidden platform in the MWM of HI+EPO animals compared with HI+iEPO animals but, in the RAM, HI+EPO treated animals had fewer errors than HI+iEPO animals and on several days made significantly more correct choices on the first eight selections. It seemed that EPO may have had a more positive effect on females' working memory capabilities.

Since Kumral et al. (2004) combined males and females in their analyses, there is no way of knowing whether one particular sex contributed more to their findings. In that study, each condition consisted of seven animals with sexes combined in the analyses. Consequently, it is difficult to directly compare those results with that obtained in our study since we did find a difference between male and female animal performance in

these tasks and accordingly analyzed the data from the male and female animals separately.

3.4.3 Methodological Considerations and Future Directions:

In the current study we administered recombinant murine EPO (rmEPO) whereas others who have found neuroprotective effects using this cytokine mainly used recombinant human EPO (rhEPO). The results in our study may differ somewhat from others as a result of using EPO from a different species. It has been reported however, that the amino acid sequence of murine EPO shows 80% homology with the rhEPO and that there is an 82% homology between the murine and human EPO receptor (Jelkmann 1992). Since we used a rat model of stroke in the current study, one might conclude that the rmEPO should at least be as potent as the rhEPO in protecting the rat brain following an HI episode.

The dosage used in this current study also differed from that used in either of the studies that assessed the functional protection provided by EPO, where we administered 5,000 U/kg, and Kumral *et al.* (2004b) and Spandou *et al.* (2005) administered 1,000 and 2,000 U/kg, respectively. Ehrenreich and colleagues (2004) also administered EPO at a dose of 5,000 U/kg, but implemented a different dosing regime. In that study, it was shown that administering EPO at three different intervals, immediately prior, 24 and 48 hours following hypoxia, resulted in a reduction of apoptotic cells and a lesser degree of grade of injury (the time at which these assessments were recorded was not indicated). This multiple dosing regiment may prove more efficacious since it is more likely that the apoptotic response is delayed possibly for a long enough period to provide long-term

morphological neuroprotection and consequently, be more likely to result in the protection of functional capabilities.

Pilot results released from a clinical trial in Germany have shown that patients who suffered from occlusion of the MCA and were treated with EPO i.v. within 8-hours of presentation, and then again 24 and 48 hours later showed significantly better recovery than patients who did not receive this treatment (Ehrenreich *et al.* 2004). Recovery consisted of evaluation of clinical outcome (assessed with NIH Stroke Scale, Scandinavian Stroke Scale, Barthel-Index and Rankin Scale), the evolution of infarct size and the profile of circulating damage markers. Results from this pilot study are currently being evaluated in a multicenter "EPO in stroke" study (Ehrenreich *et al.* 2004).

It would be interesting to test the efficacy of this dosing regime in the long-term functional assessments implemented in this study. Others have tested a somewhat similar theory where EPO was administered via an osmotic minipump implanted directly into the animals' brain and was given over a period of 28 or 7 days, respectively (Sadamoto *et al.* 1998; Sakanaka *et al.* 1998). Administration of EPO in this manner resulted in both short-term morphological (Sakanaka *et al.* 1998) and long-term functional (Sadamoto *et al.* 1998) neuroprotection following ischemia. Future stroke-related studies should take these results into consideration and test whether the administration of multiple doses of EPO provides more and longer-lasting neuroprotection.

Further, the possibility that EPO does not provide complete neuroprotection following neonatal HI still exists. In the current study the protection provided by EPO was transient and differed with respect to male and female abilities on various tasks. Although it has been shown that EPO provides various beneficial effects (see above),

these effects may only last for a short period, thus potentially only delaying the cell death process instead of completely preventing it. If this were true however, it would be of clinical interest since EPO could be administered upon presentation to the hospital with stroke symptoms, delaying the cell death for a long enough period in order to initiate another treatment, thus making a combination-like therapy more likely to combat the effects of a stroke.

In conclusion, the results obtained from this study have shown that the administration of 5,000 U/kg of EPO following neonatal HI did not result in generalized structural and functional neuroprotection. We have shown that the potential for functional neuroprotection exists in some circumstances (such as in males' reference memory abilities and females' working memory abilities). There is also evidence that EPO may have provided a slight structural neuroprotective effect in the male animals as observed in Figure 13 where HI+EPO animals had a tendency to have larger areas of their brains remaining when compared with HI+iEPO animals. The same was not observed however, with female animals, thus further supporting our rationale to analyze male and female animals' data separately. Researchers who plan to further evaluate EPO as a neuroprotective compound following stroke should take into consideration the limitations and suggestions stated in this and the previous chapter as well as the beneficial potential that a different dosing or compound regime (combination therapy) may have on outcome measures following neonatal HI.

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# 4.0 GENERAL DISCUSSION AND OVERALL CONCLUSIONS

## 4.1 Behavioural Test Battery

Chapter 2 of this thesis describes a study that was conducted to assess rats' abilities in various areas of functioning following exposure to an episode of hypoxia-ischemia during the neonatal period. Although there are a few published experimental studies to date that describe different aspects of functioning that are affected following neonatal HI [such as sensorimotor and neuromotor abilities (Lubics *et al.* 2005; Spandou *et al.* 2005) or cognitive abilities (Ikeda *et al.* 2001; Kumral *et al.* 2004)], there has yet to be published a single study that assesses animals' abilities encompassing multiple areas of functioning throughout both the neonatal and adult period. In the study described in Chapter 2, animals were tested in a number of behavioural tests that measured physical development, sensory, neuromotor, and cognitive function. Results revealed that rats subjected to HI on pnd 7-8 were significantly impaired in many of these functions when compared with age-matched control animals. Further, we found a difference between male and female animals with respect to the severity of functional deficits, displayed as well as in the observed severity of neuropathology, which has not been reported in the experimental literature (Young *et al.* 1986; Ikeda *et al.* 2001; Lubics *et al.* 2005). From these results a behavioural test battery that is sensitive to detecting both short- and long-term functional deficits following neonatal HI was proposed (Table II).

## 4.2 Evaluation of EPO Neuroprotection

From the results obtained in Chapter 2, we used the proposed behavioural test battery to assess the putative functional and structural neuroprotection offered by the hematopoietic cytokine, erythropoietin (EPO). Others have reported that administration of EPO post-HI results in sensori- and neuromotor (Spandou *et al.* 2005), cognitive (Kumral *et al.* 2004) and structural (Aydin *et al.* 2003; Spandou *et al.* 2004) neuroprotection. A limitation to the above studies however, is that neuroprotection was either not assessed over a long-term period, or was only assessed on one aspect of behaviour. Chapter 3 describes a study in which neonatal rats received an injection of EPO (5,000 U/kg, i.p.) following HI and assessments of physical development, sensorimotor, neuromotor and cognitive abilities as well as structural neuroprotection were recorded.

Results from Chapter 3 validate the usefulness of the proposed behavioural test battery. Functional deficits of animals exposed to HI were identified and were consistent with those in Chapter 2. With respect to the neuroprotection offered by EPO, there were differential findings. There was no protection offered in sensorimotor or neuromotor abilities (Figure 3.5 and 3.6), since both male and female HI+EPO and HI+iEPO animals performed equally on the tasks assessed. There was possible protection offered for male animals with respect to weight gain during the neonatal period because HI+iEPO male animals were significant lighter than control animals (Figure 3.2A), but there was no corresponding difference between male HI+EPO and control animals. With respect to

the female data however, both stroke conditions were significantly lighter than the sham condition during the neonatal period (Figure 3.2B).

In the assessment of cognition, results showed that there was no protection in male animals' working memory abilities as assessed in the RAM (Figure 3.7 and 3.8A). There was however, a protective effect of EPO in females' working memory abilities as assessed in the RAM, where HI+iEPO animals made significantly more working memory errors than sham animals but this difference was not observed between HI+EPO and sham conditions (Figure 3.9).

With respect to reference memory abilities as assessed in the MWM, male HI+EPO animals appeared to have a moderately superior ability. Male HI+EPO animals had a lower latency to locate the hidden platform and swam shorter distances than HI+iEPO animals (although not statistically different at $p<0.05$ in either case) to locate the platform (Figure 3.10). During the re-test however, when animals were tested one week following the probe trial, HI+iEPO animals were shown to have significantly longer swimming patterns to locate the hidden platform than sham animals and there was no corresponding difference between male HI+EPO and sham conditions. Female stroke conditions, however, showed a significant reference memory deficit in all dependent measures when compared to the sham controls (Figure 3.12).

In assessments of neuropathology, male HI+EPO animals showed a trend toward a larger intact ipsilateral (left) and contralateral (right) hemispheric area than HI+iEPO animals, although there was no statistically significant difference between these two conditions (Figure 3.14). With respect to female animals, both stroke conditions experienced significant loss of neuronal tissue, with a trend (although not statistically

significant) towards HI+EPO females experiencing more neuronal loss than HI+iEPO animals (Figure 3.15).

### 4.3 Conclusions

From the results obtained in the studies conducted for this thesis it can be concluded that rats subjected to neonatal HI at pnd 7 will experience deficits in various aspects of functioning and will suffer significant neuronal damage. Administration of a single high dosage of EPO (5,000 U/kg) may offer minimal functional and structural neuroprotection, and the protective outcomes may have a differential effect in male and female animals, but in general, the data do not support a significant neuroprotective effect of EPO at this dose in this model.

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# APPENDIX A: ANALYSIS OF ALL BEHAVIOURAL TESTS IN 'THE DEVELOPMENT OF A FUNCTIONAL TEST BATTERY' STUDY

| Test | Measure(s) | Description | Significance |
|---|---|---|---|
| **Weights:** | | pnd 8-30, 37, 44, 125, 177 | ♂: No dif b/w cond. on any day (p=0.558; 8-30)(p=0.924, 0.641, 0.090, 0.180; 37, 44, 125 & 177, respectively)<br><br>♀: No dif b/w cond on any day (p=0.401; 8-30)(p=0.218, 0.560, 0.739, 0.422; 37, 44, 125, 177, respectively) |
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| **Physical Development** | *Auditory Startle* | Day on which the auditory startle reflex developed | ♂: No dif b/w cond (p=0.062)<br><br>♀: No dif b/w cond (p=0.723) |
| | *Incisor Eruption: Top* | Day on which top incisors erupted | ♂: No dif b/w cond (p=0.482)<br><br>♀: No dif b/w cond (p=0.167) |
| | *Incisor Eruption: Bottom* | Day on which bottom incisors erupted | ♂: No dif b/w cond (p=0.119)<br><br>♀: No dif b/w cond (p=0.450) |
| | *Eye Opening: Right* | Day on which Right eye suture was broken | ♂: No dif b/w cond (p=0.623)<br><br>♀: No dif b/w cond (p=0.734) |
| | *Eye Opening: Left* | Day on which Left eye suture was broken | ♂: No dif b/w cond (p=0.260)<br><br>♀: No dif b/w cond (p=0.888) |
| | *Left Eye Squinting* | Number of animals displaying a left eye squint | ♂: No dif b/w cond (p=0.464)<br><br>♀: No dif b/w cond (p=1.000) |
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| **Neuromotor:** | *Surface Righting (pnd 9 & 10)* | Average latency for animal to turn over from a supine position | ♂: No dif b/w cond (p=0.413)<br><br>♀: No dif b/w cond (p=0.151) |

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| | *Negative Geotaxis (pnd 9-14)* | Latency for animal to turn 180° when placed facing downward on an inclined surface (45°) | ♂: No dif b/w cond (p=0.275)<br>♀: No dif b/w cond (p=0.641) |
| | | Scoring system:<br>0 pup falls off or remains head down for entire time<br>1 pup turns > 40s<br>2 pup turns > 20s<br>3 pup turns ≤ 19s | ♂: No dif b/w cond (p=0.256)<br>♀: No dif b/w cond (p=0.836) |
| *** | *Pivoting (pnd 9-12)* | # of Right pivots (90°) during a 60s bin | ♂: H-I sig. more right pivots than controls (p=0.044)<br>♀: H-I sig. more right pivots than controls (p=0.041) |
| | | # of Left pivots (90°) during a 60s bin | ♂: No dif b/w cond (p=0.104)<br>♀: No dif b/w cond (p=0.488) |
| | | # of Total pivots (90°) during a 60s bin | ♂: No dif b/w cond (p=0.811)<br>♀: No dif b/w cond (p=0.283) |
| | | H-I ♀ Right vs. Left | ♀: No dif on any day (p=0.374, 0.154, 0.239 & 0.120) |
| *** | | H-I ♂ Right vs. Left | ♂: Sig more right pivots on pnd 12 (p=0.035)<br>No dif on any other days (p=0.717, 0.103 & 0.250) |
| | | Control ♀ Right vs. Left | ♀: No dif on any day (p=0.941, 0.684, 0.638 & 0.154) |
| | | Control ♂ Right vs. Left | ♂: No dif on any day (p=0.289, 0.686, 0.624, 0.377) |
| | *Forelimb Grip Strength (pnd 10-17, 19 & 23)* | Average latency to fall off of wire | ♂: No dif b/w cond (p=0.657)<br>♀: No dif b/w cond (p=0.729) |

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| | | Scoring System:<br>0 falls off <30s<br>1 holds on for 30-59s<br>2 holds on > 60s<br>3 places one hindlimb on bar<br>4 places both hindlimbs on bar | ♂: No dif b/w cond (p=0.904)<br><br>♀: No dif b/w cond (p=0.460) |
| *** | | First paw to slip off of wire<br><br>(pnd 19 & 23) | ♂: No dif b/w cond on either day (p=0.688 & 0.315)<br><br>♀: No dif b/w cond on pnd 19 (p=0.180)<br>H-I animals sig more Right forepaw slips on pnd 23 (p=0.014) |
| *** | *Wire Mesh Ascending (pnd 12-17)* | Average latency for animal to ascend a 70° inclined wire mesh to reach littermates | ♂: No dif b/w cond (p=0.330)<br><br>♀: H-I animals took longer to reach littermates than controls (p=0.036) |
| *** | | Scoring System:<br>0 pup remains at the bottom or fails to reach platform<br>1 pup reaches platform >60s<br>2 pup reaches platform 30-59s<br>3 pup reaches platform <30s | ♂: No dif b/w cond (p=0.330)<br><br>♀: H-I animals obtained a lower score than controls (p=0.025) |
| ***Open Field (pnd 18, 20, 22, 27 & 29*** | *Total Grid Crosses* | The *total* number of grid crosses | ♂: No dif b/w cond (p=0.352)<br>♀: No dif b/w cond (p=0.844) |
| | *Inner* | The number of inner grid crosses | ♂: No dif b/w cond (0.770)<br>♀: No dif b/w cond (0.322) |
| | *Outer* | The number of outer grid crosses | ♂: No dif b/w cond (0.284)<br>♀: No dif b/w cond (0.664) |
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| **Sensory:**<br><br>**pnd 9-12** | *Olfactory Orientation* | Latency for animal to orient 90° toward either clean or home cage bedding | ♂: No dif b/w cond (p=0.807)<br><br>♀: No dif b/w cond (p=0.325) |

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| | | Choice orientation:<br>measured difference between animal's initial orientation (i.e., b/w soiled or clean bedding) | ♂: No dif b/w cond on any day<br><br>♀: No dif b/w cond on any day |
| | | Latency for animal to come in contact with choice bedding | ♂: No dif b/w cond (p=0.254)<br><br>♀: No dif b/w cond (p=0.176) |
| | | Choice:<br>measured difference between animal's choice to contact either soiled or clean bedding | ♂: No dif b/w cond on any day (p=1.000, -, 0.464 & 1.000)<br><br>♀: No dif b/w cond on any day (p=0.081, 1.000, 0.205 & 0.081) |
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| **Swimming**<br><br>**pnd 16-19, 21, 23 & 31** | *Swimming Ontogeny* | Scoring System:<br>*Direction:*<br>0 pup sank<br>1 pup floated<br>2 swam in a circle<br>3 swam in a straight line<br>*Nose angle:*<br>0 nose submerged<br>1 nose at water surface<br>2 nose and top of head at/above surface<br>3 nose and head elevated so that water level at mid-ear or below<br>*Limb usage:*<br>0 no limb paddling<br>1 paddling with front limbs only<br>2 paddling with all 4 limbs<br>3 paddling with hind limbs only | ♂: No dif b/w cond (p=0.336)<br>No dif on pnd 31 (p=0.482)<br><br>♀: No dif b/w cond (p=0.197)<br>No dif on pnd 31 (p= -) |
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| **Spontaneous Alternation**<br><br>**pnd 20, 25, 30, & 35**<br>*** | *Total Alternations Over Days* | The cumulative total of alternations over all days | ♂: No dif b/w cond (p=0.284)<br><br>♀: H-I animals have sig. fewer cumulative alternations (p=0.010) |
| | *Total Left Choices* | The cumulative total of Left alternations over all days | ♂: No dif b/w cond (p=0.297)<br><br>♀: No dif b/w cond (p=0.785) |
| | *Total Right Choices* | The cumulative total of Right alternations over all days | ♂: No dif b/w cond (p=0.297)<br><br>♀: No dif b/w cond (p=0.785) |
| *** | *Left vs. Right Choices* | Total Left alternations vs. total Right alternations over all days (paired t-test) | ♂: H-I animals sig more Right alternations (p=0.035)<br>No dif for control animals (p=0.808)<br><br>♀: No dif for H-I females (p=0.729)<br>No dif for control females (p=0.253) |
| *** | *Alternations over days* | # of alternations over each day | ♂: No dif b/w cond (p=0.284)<br><br>♀: H-I animals have less alternations (p=0.010) (pnd30) |
| | *Alternation Latency* | Latency for the animal to alternate | ♂: No dif b/w cond (p=0.563)<br><br>♀: No dif b/w cond (p=0.827) |
| | *Total Left Choices over each day* | | ♂: No dif b/w cond on any day<br><br>♀: No dif b/w cond on any day |
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| **T-Maze**<br><br>**15 days** | *Correct Choices* | # of correct choices over days<br>- forced choice<br>- 8 trials / session<br>- 1 session / day | ♂: No dif b/w cond (p=0.094)<br><br>♀: No dif b/w cond (p=0.866) |

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| | *Latency to Choose* | | ♂: No dif b/w cond (p=0.207)<br><br>♀: No dif b/w cond (p=0.401) |
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| **Radial Arm Maze**<br><br>**8-Arms Baited**<br><br>**10 days**<br><br>*** | *Latency* | Latency for animals to obtain all 8 rewards | ♂: H-I animals took longer to obtain all rewards (p=0.005)<br><br>♀: No dif b/w cond (p=0.168) |
| *** | *Total Errors* | The total number of errors made in the maze (working & reference memory errors) | ♂: No dif b/w cond (p=0.186)<br><br>♀: H-I animals make more errors than controls (p=0.011) |
| *** | *Commission Errors* | Total number of commission errors made<br>Commission errors (re-entry errors) indicate a working memory errors | ♂: No dif b/w cond (p=0.259)<br><br>♀: H-I animals make more commission errors than controls (p=0.011) |
| | *Omission Errors* | Total number of omission errors made<br>Omission errors (failure to obtain a reward) indicates reference memory errors | ♂: No dif b/w cond (p=0.178)<br><br>♀: No dif b/w cond (p=0.207) |
| *** | *First 8 Choices* | The number of rewards (absence of errors) received on the first 8 choices<br>(days 9 & 10) | ♂: No dif b/w cond (p=0.127)<br><br>♀: H-I animals receive less rewards (i.e., more likely to make commission errors) than controls (p=0.014) |
| **4-Arms Baited**<br><br>**13 Days** | *Latency* | Latency for animals to obtain all 4 rewards | ♂: No dif b/w cond (p=0.239)<br><br>♀: No dif b/w cond (p=0.321) |
| | *Unbaited Arm Errors* | The number of times animal entered an unbaited arm (Reference Memory) | ♂: No dif b/w cond (p=0.593)<br><br>♀: No dif b/w cond (p=0.457) |

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| *** | *Unbaited Arm Re-Entry Errors* | The number of times animal re-entered an unbaited arm (Working Memory) | ♂: No dif b/w cond (p=0.896)<br><br>♀: H-I animals made more re-entry errors than control (p=0.004) |
| * | *Omission Errors* | The number of times animal failed to enter a baited arm (Reference Memory) | ♂: No dif b/w cond (p=0.913)<br><br>♀: No dif b/w cond (p=0.055) |
| *** | *Total Errors* | The total number of errors (unbaited arm, unbaited arm re-entry & omission errors) | ♂: No dif b/w cond (p=0.826)<br><br>♀: H-I animal make more errors than control animals (p=0.005) |
| * | *First 4 Choices* | The number of rewards (absence of errors) received on the first 4 choices | ♂: No dif b/w cond (p=0.151)<br><br>♀: No dif b/w cond (p=0.062) |
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| **Morris Water Maze**<br><br>**-4 days testing**<br>**-1 day probe**<br>**-1 day re-test**<br>**-1 day visible platform** | *Average Velocity* | The average swimming velocity of animals over all trials (measured by Ethovision) | ♂: No dif b/w cond (p=0.752)<br><br>♀: No dif b/w cond (p=0.138) |
| *** | *Average Latency* | The average latency for animal to locate hidden platform | ♂: No dif b/w cond (p=0.318)<br><br>♀: H-I animals take longer than controls to locate platform (p=0.006) |
| *** | *Average Distance Swam* | The average distance swam around entire maze | ♂: No dif b/w cond (p=0.268)<br><br>♀: H-I animals swam longer distances around maze than controls (p=0.008) |
| | *Average Distance Swam in Probe Trial* | The average distance swam around entire maze when platform was removed (60 sec. trial) | ♂: No dif b/w cond (p=0.238)<br><br>♀: No dif b/w cond (p=0.392) |

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| | *% of Time Spent in NE Quadrant* | The percentage of time spent in the northeast quadrant (platform location quadrant) over trials | ♂: No dif b/w cond (p=0.164)<br><br>♀: No dif b/w cond (p=0.158) |
| * | *% of Time Spent in NE Quadrant in Probe Trial* | The percentage of time spent in the northeast quadrant (platform location quadrant) during the probe trial (when the platform was removed) [memory for location of platform] | ♂: No dif b/w cond (p=0.489)<br><br>♀: No dif b/w cond (p=0.054) [H-I animals less] |
| | *% of Distance Swam in NE Quadrant* | The percentage of distance swam around the northeast quadrant over trials (measured by Ethovision) | ♂: No dif b/w cond (p=0.179)<br><br>♀: No dif b/w cond (p=0.592) |
| *** | *% of Distance Swam in NE Quadrant in Probe Trial* | The percentage of distance swam around the northeast quadrant during the probe trial (memory for location of platform) | ♂: No dif b/w cond (p=0.919)<br><br>♀: H-I animals swam less in NE quad than controls (p=0.018) |
| *** | *Time Spent Around Platform Zone* | The total time animal spent around the platform zone during probe trial<br>- platform zone was outlined and measured using Ethovision | ♂: No dif b/w cond (p=0.141)<br><br>♀: H-I animals spent less time around platform zone than controls (p=0.019) |
| *** | *Distance Swam Around Platform Zone* | The total distance swam around the platform zone during probe trial | ♂: No dif b/w cond (p=0.967)<br><br>♀: H-I animal swam less around platform zone than controls (p=0.004) |
| *** | *Platform Crosses* | The total number of times the animal crossed into the platform zone during probe trial | ♂: No dif b/w cond (p=0.731)<br><br>♀: H-I animals had fewer platform crosses than controls (p=0.003) |
| *** | *% of Time Spent in Inner Zone* | The percentage of time animal spent swimming around inner zone of maze over trials (H-I ♀'s animals showing thigmotaxis) | ♂: No dif b/w cond (p=0.467)<br><br>♀: H-I animals spent less time in inner zone than controls (p=0.018) |

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| *** | *Time Spent in Inner Zone in Probe Trial* | The total time animal spent swimming around inner zone during probe trial | ♂: No dif b/w cond (p=0.276)<br><br>♀: H-I animals spent less time in inner zone during probe trial than controls (p=0.004) |
| *** | *% of Distance Swam Around Inner Zone* | The percentage of distance swam around inner zone over trials | ♂: No dif b/w cond (p=0.502)<br><br>♀: H-I animals swam less in inner zone than controls (p=0.012) |
| *** | *Total Distance Swam Around Inner Zone in Probe Trial* | The total distance swam around inner zone during probe trial | ♂: No dif b/w cond (p=0.833)<br><br>♀: H-I animals swam less in inner zone during probe trial than controls (p=0.003) |
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| **Barnes' Circular Maze**<br><br>**-4 days testing**<br>**-1 day re-test** | *Latency to Enter Hole* | Average latency for animal to escape from lights and noise by locating escape hole<br>-Maze:<br>Circular maze with 18 holes around perimeter with only 1 hole representing correct choice | ♂: No dif b/w cond (p=0.256)<br><br>♀: No dif b/w cond (p=0.120) [H-I longer]<br>HI animals longer latency during re-test (p=0.046). |
| | *Average # of Hole Errors* | Average number of incorrect choices animal made over trials (Reference Memory Error) | ♂: No dif b/w cond (p=0.546)<br><br>♀: No dif b/w cond (p=0.363) |
| | *Re-Entry Errors* | Average number of times animal re-visited an incorrect hole over trials (Working Memory Error) | ♂: No dif b/w cond (p=0.226)<br><br>♀: No dif b/w cond (p=0.444) |
| | *Total # of Errors* | The total number of errors (reference & working memory errors) over trials | ♂: No dif b/w cond (p=0.466)<br><br>♀: No dif b/w cond (p=0.351) |

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| | *Total # of Errors Re-Test* | The total number of errors (reference & working memory errors) during Re-Test | ♂: No dif b/w cond (p=0.285)<br>♀: No dif b/w cond (p=0.210) |