EVALUATION OF NMDA RECEPTOR-MEDIATED NEUROPROTECTION IN THE ENDOTHELIN-1 MODEL OF UPPER EXTREMITY IMPAIRMENT IN STROKE

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ABSTRACT

Upper extremity impairment after focal ischemic stroke represents the most prominent functional deficit for surviving stroke victims. Recently, this condition has been modeled in rats by injecting the vasoconstrictive peptide endothelin-1 (ET-1) to forelimb representation areas of the brain, such as the forelimb sensorimotor cortex and the dorsolateral striatum. This ET-1 model may be a valuable experimental tool for investigating functional neuroprotection after ischemia-induced forelimb impairment in rats. However, there are only a few published studies regarding the use of this model for neuroprotection in stroke research, and compounds that target NMDA receptor-mediated excitotoxicity remain to be tested in this model. Using adult, male Sprague-Dawley rats, initial experiments were performed using different volumes, concentrations, and stereotaxic coordinates of injected ET-1 to the forelimb sensorimotor cortex and dorsolateral striatum. During these preliminary studies, rats were subjected to a behavioural test battery that measured various aspects of forelimb sensorimotor function.

Using the surgical and behavioural protocols developed during preliminary experimentation, the ET-1 model was then evaluated for neuroprotection using a single dose of MK-801 (5mg/kg i.p.) and Ro25-6981 (6mg/kg i.p.). Results revealed that both compounds conferred marked neuroprotection to the cortex and improved forelimb function relative to a vehicle control group when measured over two weeks post-stroke. Using a separate group of rats, an ET-1-induced lesion was placed in the homotopic contralesional hemisphere in order to investigate its functional contribution to forelimb recovery subsequent to the initial lesion. Whether the functional neuroprotection provided by Ro25-6981 was related to diminished contralesional hemispheric involvement during recovery was also examined. Results from this experiment demonstrated that the introduction of a second, homotopic lesion significantly reinstated forelimb impairment when measured 3 hr post-stroke.

In conclusion, the primary finding from this thesis is that the ET-1 model of upper extremity impairment in stroke is suitable for neuroprotection studies using NMDA receptor antagonists as therapeutic compounds. Thus, given its experimental advantages, the ET-1 model may be a useful tool for investigating the therapeutic effect of NMDA receptor antagonists on forelimb recovery after focal cerebral ischemia in the rat.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviation	Term
aCSF	Artificial cerebral spinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ANOVA	Analysis of variance
AP	Anterior/Posterior
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CFL	Caudal forelimb region
Con-G	Conantokin G
CP-101,606	Traxoprodil
DV	Dorsal/Ventral
ET-1	Endothelin-1
FPR	Forelimb postural reflex
hr	Hour
iGluR	Ionotropic glutamate receptor
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
kainate	2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine
mGluR	Metabotropic glutamate receptor

MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MK-801	Dizocilpine
ML	Medial/Lateral
MMP	Matrix metalloproteases
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NMDA	*N*-methyl-D-aspartate
PID	Peri-infarct depolarization
PSD	Post-synaptic density
RFL	Rostral forelimb region
TFP	Tactile-stimulated forelimb placing
TTC	Triphenyl tetrazolium chloride
VACC	Voltage-activated calcium channel
VFP	Vibrissae-stimulated forelimb placing

1.0 General Introduction

1.1 Overview

Stroke, the most common type of cerebrovascular accident, is defined as loss or alteration of bodily function that results from an insufficient supply of blood to part of the brain (Graham et al. 2004). It is a diverse neurological condition with three general types of stroke commonly seen in clinical patients: ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage (American Heart Association 2008). Ischemic stroke results from a thrombotic or embolic occlusion of a major cerebral artery or its branches, leading to a loss of local oxygen and nutrients, which ultimately results in cell death or injury of brain tissue (Durukan and Tatlisumak 2007). Intracerebral hemorrhage, however, is caused by a sudden rupture of an artery within the brain, allowing blood to be released into brain stuctures; whereas subarachnoid hemorrhage is the result of a ruptured artery that leads to blood filling the space surrounding the brain (Flynn et al. 2008). In the United States, 87% of all strokes are ischemic, 10% are due to intracerebral hemorrhage, and the remaining 3% are due to subarachnoid hemorrhage (American Heart Association 2008). In Canada, approximately 80% of all strokes are ischemic (Heart and Stroke Foundation of Canada 2006).

Globally, stroke ranks second to ischemic heart disease as a cause of death and long-term disability (Zaleska et al. 2009). Approximately 76% of people survive their stroke (Carmichael 2005). Of these survivors, 50% are afflicted with hemiparesis, 26% are dependent on others in their activities of daily living, and 26% require nursing home

care (Carmichael 2005). Thus, stroke can be a fatal disease but it disables more frequently than it kills. Estimates indicate that stroke is responsible for half of all patients hospitalized for acute neurological disease, and that 28% of annual stroke victims are under age 65 (Barone and Kilgore 2006). The costs endured by stroke survivors have consequences beyond the realm of the individual; disability resulting from stroke also imparts an enormous socioeconomic burden worldwide (Flynn et al. 2008). In the United States alone, costs for medical care, therapy, lost productivity, and other disability related phenomena exceed $50 billion annually (Graham et al. 2004) (with a yearly cost of roughly $3 billion in Canada) (Heart and Stroke Foundation of Canada 2006).

The magnitude of stroke burden on the individual and the healthcare system necessitates urgent work aimed at developing novel therapies. Over the past several decades, investigations aimed at uncovering the pathophysiological mechanisms of stroke, as well as those seeking to find effective treatments that reduce cell death and enhance functional recovery post-stroke, have formed the foundation of both basic science and clinical stroke research (see reviews by Romero et al. 2006; Young et al. 2007).

During stroke, the excessive release of excitatory neurotransmitters (e.g. glutamate) directly leads to over-activation of various post-synaptic receptors [e.g. *N*-methyl-D-aspartate (NMDA) receptors] which, through a variety of cellular and molecular events, initiate a series of pathological processes ultimately leading to damage or destruction of brain tissue (Lipton, 1999). By disrupting or obliterating crucial brain circuitry, such receptor-mediated pathological events often result in impaired behavioural function for stroke victims (Crafton et al. 2003). It is therefore imperative to implement

therapeutic strategies, such as the pharmacological blockade of excessive receptor activation, that interrupt the damaging consequences of stroke.

Much of the current knowledge concerning stroke pathophysiology, potential stroke treatments, and recovery from stroke can be attributed to scientific discoveries using animal models of stroke (Graham et al. 2004). The clinical diversity of stroke makes it difficult to produce a single animal model in which most aspects of stroke can be studied; hence, many diverse animal stroke models have been developed, each capturing elements of the human condition (Millikan 1992; Muir 2002). Certain animal models are well suited to study the molecular pathophysiology of stroke (Fujimura et al. 1999; Chen et al. 2005), others are more pertinent for measuring cerebral blood flow (CBF) and bioenergetic parameters during ischemia (Dawson et al. 1993; Duverger and MacKenzie 1988), whereas some models are best utilized for research aimed at uncovering the mechanisms of brain reorganization and the effects of rehabilitation therapy (Adkins et al. 2004; Gilmour et al. 2004; Zhengguang et al. 2001). These few examples attest to the nature of basic stroke research to exploit the inherent characteristics of different animal models in order to best address specific research questions. Investigations that examine untapped applications for new animal models of stroke are an imperative component of this systematic process because findings from such projects lead to a more comprehensive understanding of model applicability within the field of experimental stroke research.

At the crux of this thesis is an investigation of the utility of a new stroke model in the rat (i.e. the endothelin-1 model), designed to study upper extremity impairment in

stroke, for use in experiments which aim to reduce brain damage and enhance forelimb functional recovery after stroke via the administration of NMDA receptor antagonists.

1.2 Clinical Consequences of Stroke: Upper Extremity Impairment

Upper extremity sensorimotor impairment is the most prominent functional deficit for surviving stroke victims (Nakayama et al. 1994). Only 25-50% of these individuals are likely to regain efficient use of their affected arm and hand (Feys et al. 2000; Chan et al. 2009). It has been argued that arm and hand function is more important than mobility in achieving independence following stroke (Freeman et al. 2009), yet post-stroke recovery rate from upper extremity hemiparesis is poor compared to independent walking (Chan et al. 2009). Hemiparesis is a collective term for a heterogeneous condition encompassing weakness, motor control abnormalities, and spasticity (Krakauer 2005). Within at least the first few weeks following stroke, individuals with functional impairment of the upper extremity show no evidence of a proximal to distal gradient in sensory or motor capacity – the integrity of each segment, from the shoulder to the hand, is equally impaired (Beebe and Lang 2008; Tyson et al. 2008). The severity of arm paresis in the first month after stroke remains the strongest predictor of functional outcome and is firmly linked to the degree of damage to cortical motor areas, particularly, the corticospinal tract (Beebe and Lang 2008; Krakauer 2005). Lesions to premotor areas and subcortical brain structures such as the internal capsule and basal ganglia also contribute substantially to the magnitude of upper extremity impairment and extent of functional recovery (Feys et al. 2000).

The underlying neuromuscular mechanisms of hemiparesis include alterations in the recruitment order and firing frequency of motor units, or a complete loss of motor unit function (Wagner et al. 2007). These changes in motor unit physiology result in alterations in muscle activation patterns such as a decreased ability to recruit agonist muscles, detrimental co-activation of antagonist muscles, delayed initiation and termination of muscle activity, and loss of selective activation of sets of muscles needed to perform coordinated motor tasks such as reaching (Wagner et al. 2007). In addition to the dysfunctional execution of previously intact motor programs, individuals with upper extremity impairment after stroke typically demonstrate a decreased ability to detect and discriminate objects with the affected arm and hand (Tyson et al. 2008).

Improvements in upper extremity function that may be seen during the stroke recovery process are hypothesized to be driven by spared components of the ipsilesional corticospinal system (Seitz et al. 1999), compensatory control provided by descending motor pathways other than the crossed corticospinal tract (Turton et al. 1996), reorganization of sensorimotor maps in tissue adjacent to the ischemic lesion (Castro-Alamancos and Borrell 1995), as well as contributions from sensorimotor areas in the contralesional hemisphere (Biernaskie et al. 2005; Shanina et al. 2006), although much is still unknown about these processes (Hsu and Jones 2006; Takata et al. 2006).

1.3 Glutamate and Glutamate Receptors in the Brain

During the 1930's high concentrations of glutamate were first recognized in the brain, provoking speculation on a key neurophysiological role of this amino acid

(reviewed in Watkins and Jane 2006). It is understood, today, that chemical transmitters play a primary role in synaptic communication, with glutamate being the principal excitatory neurotransmitter in the mammalian CNS (Matute et al. 2006). Because of the ubiquitous distribution of glutamatergic synapses, glutamate has a critical role in a variety of processes such as development, plasticity, and CNS repair (Barnes and Slevin 2003).

The excitatory responses of this endogenous amino acid are mediated by a number of pharmacologically and functionally distinct cell-membrane receptors (Sattler and Tymianski 2001). These receptors are divided into two primary families: ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs, respectively). There are three major types of iGluRs, which are named after their prototypical agonists; they are, NMDA, α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors (Yamakura and Shimoji 1999). Ionotropic glutamate receptors are believed to typically be composed of 4 distinct subunits, each of which is topically arranged with 3 transmembrane-spanning and 1 pore-lining (hairpin loop) domain (Kew and Kemp 2005). The iGluRs have in common substantial permeability to both Na^+ and K^+, and varying permeability to Ca^{2+} ions; they are responsible for most fast excitatory signaling in the brain (Madden 2002), and play an important role in mediating the synaptic plasticity implicated in learning and memory (Kind and Neumann 2001; Sheng and Kim 2002). Most AMPA and kainate receptors are classified as rapidly activating and desensitizing and have modest Ca^{2+} permeability, whereas NMDA receptors have slower kinetics but their permeability ratio of Ca^{2+} to Na^+ is as high as 10:1 (Mayer and Westbrook 1987). In converse to iGluRs, mGluRs mediate

synaptic transmission via slow excitatory postsynaptic potentials, and the binding of glutamate to mGluRs does not directly activate the opening of an intrinsic channel but rather indirectly regulates synaptic transmission and neuronal excitability through the activation or inhibition of various G protein-coupled effector systems (Simeone et al. 2004). Metabotropic glutamate receptors possess 7 transmembrane domains and a large C-terminus allowing linkage to a large repertoire of signaling pathways (for full review see Kew and Kemp 2005).

The exact subunit distribution of the glutamate receptors is subject to variation, depending on the physiological circumstances and the precise location of the receptor within the CNS; nonetheless, a general subunit taxonomy exists (reviewed in Madden 2002) (see Figure 1.1).

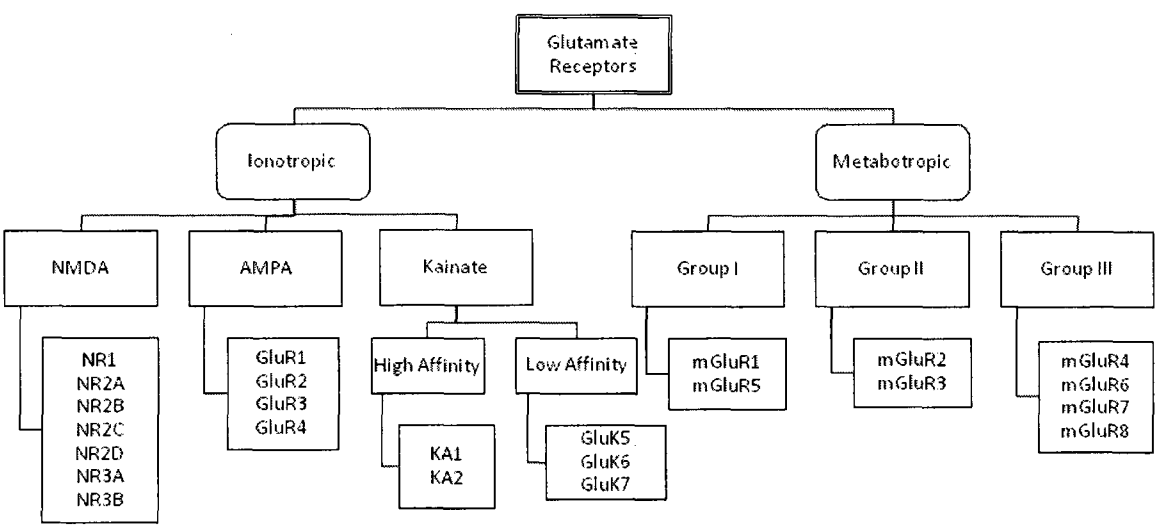

Figure 1.1. Glutamate receptor taxonomy. Adapted from Watkins and Jane 2006.

1.3.1 The NMDA Receptor

Early in the 1980's, Jeffery Watkins and colleagues developed selective competitive antagonists for the NMDA receptor, such as 2-aminophosphonovaleric acid, which was key to the explicit identification of subtypes of excitatory amino acid receptors and the exploration of the specific role of NMDA receptors in brain function (Davies et al. 1981). At present, numerous intrinsic properties of the NMDA receptor including the general domain structure, subunit arrangement, and site-specific pharmacology have been extensively studied.

NMDA receptors are atypical ligand-gated ion channels because activation not only requires the binding of two agonists, glycine and glutamate, but also demands the relief of a Mg^{2+} block within the channel pore (Furukawa et al. 2005). At resting neuronal membrane potential, Mg^{2+} acts as a voltage-dependent antagonist of the NMDA receptor. Membrane depolarization, supplied in many cases by activation of co-localized AMPA receptors, relieves this block because the relatively positive charge in the depolarized cell repels positively charged Mg^{2+} from the channel pore (Lynch and Guttmann 2001). Thus, the opening of the NMDA receptor requires at least 3 distinct events (binding of 2 agonists and membrane depolarization). NMDA receptors are permeable to influx of Na^{+} and, crucially, Ca^{2+}, as well as efflux of K^{+} (Simeone et al. 2004).

As with all multimeric receptors, the subunit composition of the NMDA receptor is suspected to play a prominent role in determining both its physiological and pathological function within the brain (Simeone et al. 2004). Putatively, the majority of NMDA receptors are composed of a heterotetrameric assembly of NR1 subunits and NR2

subunits (McBain and Mayer 1994; Schorge and Colquhoun 2003); that is, 2 glycine-binding NR1 and 2 glutamate-binding NR2 subunits (Kohr 2006). More specifically, native NMDA receptors are believed to be composed of NR1 in combination with one or more NR2 subunit type (e.g. NR1/NR2A, NR1/NR2B, or NR1/NR2A/NR2B) or, less frequently, NR1 in combination with both NR2 and NR3 subunits (e.g. NR1/NR2A/NR3A) (for review see Kew and Kemp 2005). In the adult rodent and human brain, the NR1 subunit is widely distributed, whereas the NR2 subunits are expressed in a spatially distinct manner (Watanabe et al. 1993; Wenzel et al. 1997; Rigby et al. 1996). The NR2A subunit is highly expressed in the neocortex, hippocampus, cerebellum, and several thalamic nuclei, whereas the NR2B subunit is predominately localized to the neocortex, hippocampus, striatum, septum, and thalamic nuclei of the adult rat brain (Laurie et al. 1997; Monyer et al. 1994; Mutel et al. 1998). NMDA receptors containing NR2C subunits are expressed largely in the cerebellum and various select nuclei, and the NR2D containing receptors are largely expressed during early development but restricted to the diencephalon and midbrain in the adult (for reviews see Gill et al. 1999; McBain and Mayer 1994).

Each NMDA receptor subunit contains a channel pore region composed of 3 helices that span the plasma membrane (M1, M3, and M4) and a re-entrant helical M2 loop (Gerber and Vallano 2006) which contributes to the formation of the channel pore. Each subunit also has a large extracellular N-terminus, and an intracellular C-terminus that is large in NR2 subunits (Simeone et al. 2004). Various binding and modulatory sites exist on the NMDA receptor complex that permit regulation of its function (Higgins et al. 2005). Figure 1.2 illustrates the general domain structure of the NMDA receptor

complex along with some key agonist and antagonist binding sites. Finally, beneath the neuronal membrane, within the electron-dense post-synaptic density (PSD), the NMDA receptor is linked, via the C-terminus of NR2 subunits, to a large network of enzymes and other regulatory molecules involved in downstream signal-processing (Aarts et al. 2002; Brenman et al. 1996; Kornau et al. 1995; Kennedy 1997; Muller et al. 1996; Sattler et al. 1999).

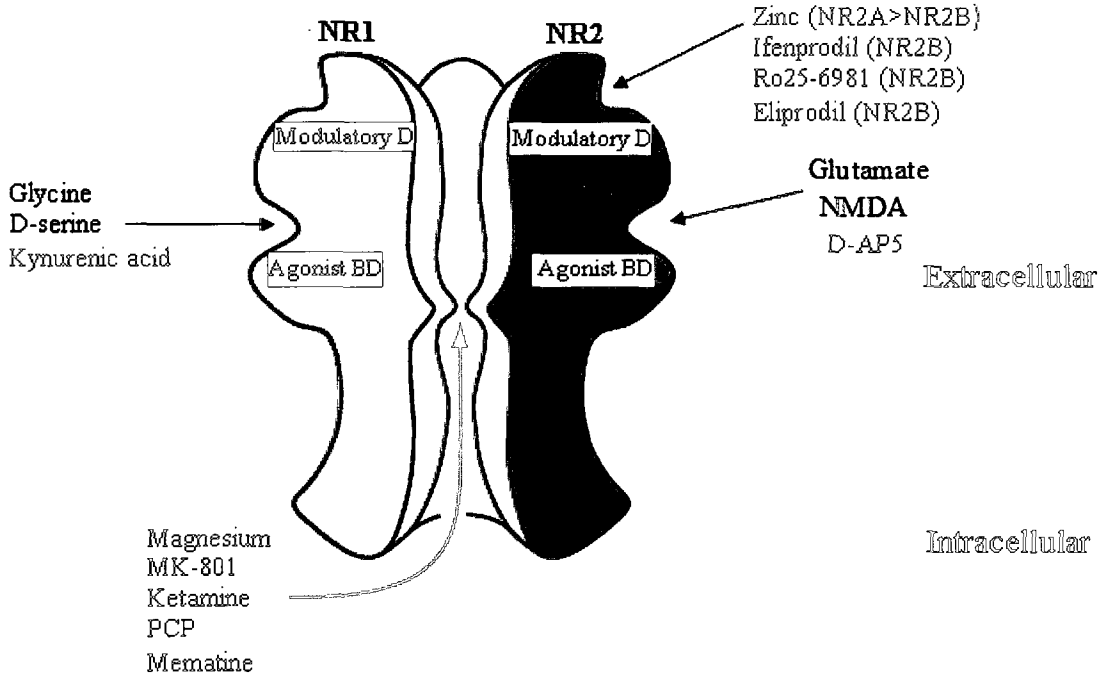

Figure 1.2. NMDA receptor model; showing binding sites for some main agonists and antagonists. Extracellular portions of NR1 and NR2 consist of two domains, the modulatory domain (Modulatory D) and the agonist binding domain (Agonist BD). Binding sites for magnesium, MK-801, ketamine, PCP, and mematine are within the channel pore region (i.e. 'channel blockers'). Antagonists are presented in red; agonists in black. Adapted from Hara and Snyder 2007.

1.4 Mediators of Cell Death: The Cerebral Ischemic Cascade

Cerebral ischemia is defined as a reduction in CBF sufficient to cause metabolic or functional deficits (Liebeskind and Kasner 2001). When blood supply to a brain region is interrupted it triggers a series of complex pathological events that lead to the destruction of brain tissue; this is conventionally referred to as the cerebral ischemic cascade (Neumar 2000). Figure 1.3 depicts a general overview of some of the main pathological events in this cascade.

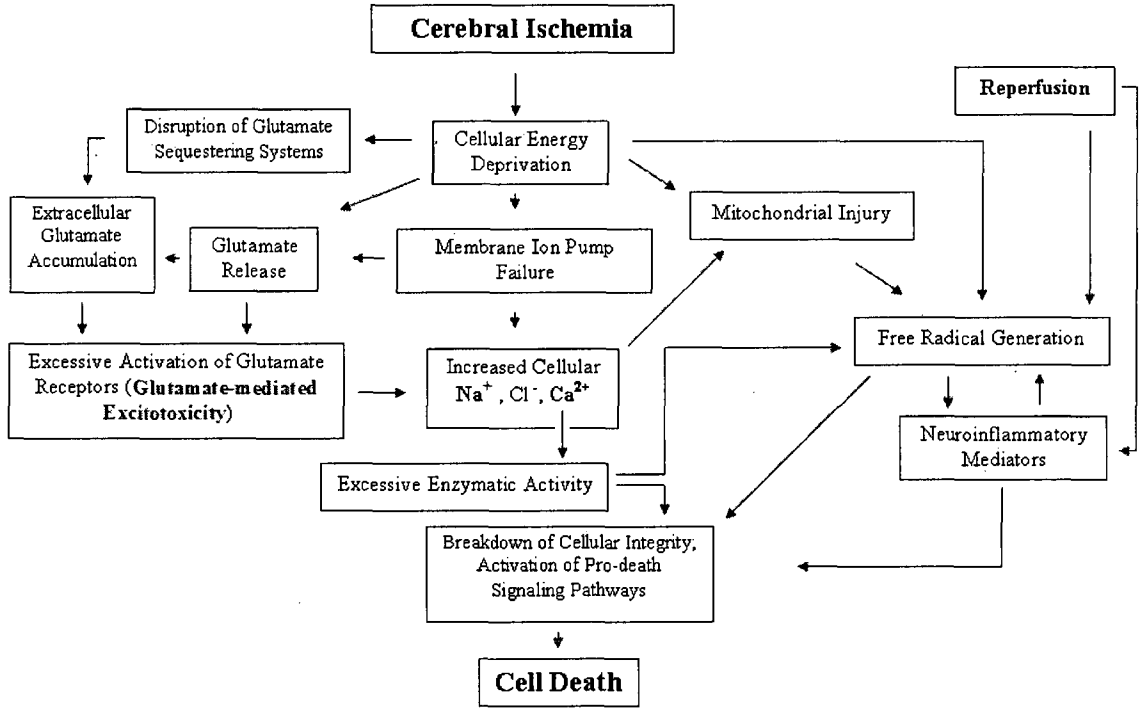

Figure 1.3. General overview of some of the main pathological events within the cerebral ischemic cascade. Figure created by Andrew Hume using Microsoft Word® and Paint®, based on information from several publications cited below in sections 1.4.2 to 1.4.5.

The cerebral ischemic cascade is triggered promptly upon the cessation of blood flow; initial damage to principally affected brain cells occurs almost instantly while damage to less affected cells ensues over a period of hours or even days (Baker et al. 1998). Notably, although individual discussions are detailed below, many of the constituent pathological events in the ischemic cascade are inextricably linked and operate in parallel (Mehta et al. 2007). Following a brief overview of the cerebral ischemic cascade, the crucial role of the NMDA receptor within this cascade is generally discussed. It is essential, however, to first introduce the concept of the core and penumbra since these tissue volumes represent areas of brain that are affected differently by cerebral ischemia.

1.4.1 The Ischemic Core and Penumbra

In the ischemic brain, it is common to distinguish two primary tissue volumes – the core of the infarction and the ischemic penumbra (or peri-infarct zone) (Mergenthaler et al. 2004). The ischemic core, by definition, consists of those portions of the ischemic tissue in which local cerebral blood flow is most severely reduced (Ginsberg 2003; Lipton 1999). The massive reduction of blood flow (i.e. energy) within the core leads to a breakdown of cellular integrity within minutes (Hertz 2008). The penumbral tissue borders the ischemic core and consists of moderately ischemic tissue in which collateral blood vessels provide residual circulation (Ashwal et al. 1998). The penumbra is generally regarded as a tissue volume in which the moderate reduction in blood flow is sufficient to cause the loss of electrical excitability but cells are able to maintain membrane potential and ion gradients for some time (Hertz 2008; Kaufmann et al. 1999). Thus, the disruption of cellular homeostasis in the penumbra is less severe compared to

the core and, as such, has a variable outcome; penumbral tissue includes both ischemic areas that will recover spontaneously and areas that progress to irreversible damage unless effective treatment is implemented (Bandera et al. 2006). Brain cells within the ischemic core undergo rapid necrosis and are essentially unsalvageable, whereas the penumbra is primarily a site of delayed cell death (i.e. apoptosis) due to the increased availability of ATP (reviewed in Mehta et al. 2007; Mergenthaler et al. 2004). Nonetheless, much penumbral tissue eventually becomes irreversibly depolarized, at which point tissue ATP levels are critically reduced, leading to functional and structural deterioration of brain cells and full infarct maturation (Ginsberg 2003; Umegaki et al. 2005).

1.4.2 Cellular Energy Deprivation

The brain requires a steady delivery of blood flow to supply oxygen and glucose in order to generate sufficient adenosine triphosphate (ATP) to maintain various energy-dependent physiological processes (Doyle et al. 2008; Moro et al. 2004). A severe disruption of the blood flow in brain areas affected by vascular occlusion leads to a depletion of oxygen and glucose within minutes, and thus a marked decline in ATP production (Stys 2004). One of the most crucial consequences to brain areas subject to energy failure is the disruption of various adenosine triphosphatases (ATPases) that maintain the neuronal resting membrane potential (Hara and Snyder 2007). Failure of these energy-dependant ion pumps leads to increases in extracellular K^+ as well as an influx of Na^+, Cl^- and Ca^{2+} ions to affected cells, culminating in the loss of neuronal membrane potential (Lipton 1999; Moro et al. 2004). Consequently, depolarization of affected neurons reaches a critical threshold (known as anoxic depolarization) leading to

a frequent and prolonged generation of action potentials (Doble 1999; Lipton 1999). The result is massive release of excitotoxic amino acids (primarily glutamate) into the extracellular compartment (Neumar 2000), initiating a particularly destructive pathological event - glutamate-mediated excitotoxic cell death.

1.4.3 Excitotoxicity

During ischemia, glutamate, which normally undergoes direct active presynaptic or astrocytic reuptake after release, remains in the extracellular compartment and accumulates there since these sequestering systems are disrupted (Krnjevic 2008; Lipton, 1999). Cells of the ischemic core that undergo anoxic depolarization are destined to die due to a severe lack of energy; however, those in the surrounding penumbra can de- and repolarize again (Mehta et al. 2007). Thus, glutamate released from dying neurons in the damaged core spills over to those residing in the penumbra (Kemp and McKernan 2002) instigating a phenomenon known as peri-infarct depolarizations (PIDs). During PIDs, recurrent waves of spreading depolarization start within the ischemic core and extend outward to contiguous, weakened neurons within the penumbral zone (Hartings et al. 2003; Selman et al. 2004). Peri-infarct depolarizations are sporadic and abrupt; lasting between 1-5 min (Nedergaard and Hansen 1993), and their occurrence is not limited to the period of transient brain ischemia. Rather, PIDs recur during a delayed, secondary phase beginning several hours after the onset of reperfusion (May Lu et al. 2005). Each wave of depolarization is associated with large increases in intracellular Ca^{2+}, increased glutamate release, and a gradual reduction in penumbral energy status due to stepwise, progressive ATP depletion (Ginsberg 2003; Umegaki et al. 2005).

Currently, there is general scientific agreement that the molecular basis of

glutamate toxicity is largely dependent on elevations in intracellular Ca^{2+} levels (Arundine and Tymianski 2004; Doyle et al. 2008; Lipton, 1999; Mehta et al. 2007; Sattler and Tymianski 2000). Calcium overload can uncouple mitochondrial electron transfer from ATP synthesis and trigger the activation of enzymes such as calpains and other proteases, protein kinases (e.g. protein kinase C), lipases (e.g. phospholipase A2), nitric oxide synthase (NOS), protein phosphatases (e.g. calcineurins), and endonucleases (Arundine and Tymianski 2004; Doble 1999). Alterations in the activity of these enzymes can lead to increased production of toxic free radicals such as nitric oxide (NO), superoxide, hydrogen peroxide, and peroxynitrite, and ultimately cause cytoskeleton breakdown, lipid peroxidation, DNA fragmentation, general mitochondrial dysfunction, and activation of genetic signals leading to cell death (Arundine and Tymianski 2004; Aarts and Tymianski 2004; Durukan and Tatlisumak 2007; Neumar 2000).

1.4.4 Free Radical Production

Damaging free radicals are not limited to generation via elevated intracellular Ca^{2+}. In transient ischemic stroke, reoxygenation that occurs during reperfusion provides a substrate for numerous enzymatic oxidation reactions (Sugawara et al. 2004) leading to a massive generation of free radicals during this time (Baker et al. 1998). Accordingly, following reperfusion there is an extensive secondary surge in production of free radicals that is particularly pronounced within penumbral tissue (Pacher et al. 2007). Thus, despite the generally favourable outcome associated with reopening a blood vessel, additional injury to brain tissue may result when reperfusion occurs. The formation of free radicals such as NO, superoxide, and peroxynitrite in the vicinity of blood vessels activate matrix metalloproteases (MMPs), which degrade the

integrity of the vascular wall and increase blood brain barrier permeability (Gursoy-Ozdemir et al. 2000). Thus, damage from these radicals during the reperfusion period contributes to 'opening up' the blood-brain barrier, allowing inflammatory cells and toxins to enter the brain (Halliwell 2006).

1.4.5 Neuroinflammation

Eventual recruitment of penumbral tissue into the infarct is believed to involve events related to CNS inflammation that progressively increase cellular injury (Ashwal et al. 1998). The inflammatory response in stroke is a composite process that involves many different cell types, inflammatory mediators, and extracellular responses (Doyle et al. 2008). In general, brain ischemia that leads to pathological events such as Ca^{2+} overload, the generation of free radicals, the presence of necrotic cells, and the production of proinflammatory cytokines and chemokines, spawns a robust inflammatory response (Lucas et al. 2006; Wang et al. 2007). These initiators of inflammation activate resident microglial cells in the brain (Moro et al. 2004). Microglial activation leads to the production of more cytokines and chemokines, causing upregulation of adhesion molecules in the cerebral vasculature and subsequent peripheral leukocyte infiltration (Barone and Kilgore 2006). Recruited leukocytes and the activated resident microglia produce a variety of cytotoxic inflammatory mediators including more cytokines, MMPs, inducible NOS (iNOS), and additional free radicals which can lead to brain edema, hemorrhage, and ultimately cell death (Vexler et al. 2006; Wang et al. 2007).

1.5 NMDA Receptor-Mediated Ischemic Injury

The majority of glutamate receptor subtypes have been implicated to varying degrees in Ca^{2+}-mediated excitotoxicity (Gill et al. 1996; Matucz et al. 2006; O'Neill et al. 2000a). Significant Ca^{2+} currents are observed in some AMPA-gated channels that lack the GluR2 subunit (Mishina et al. 1991) and mGluRs activate second messenger pathways resulting in Ca^{2+} release from intracellular stores (Mehta et al. 2007). However, owing to their high Ca^{2+} permeability (Aarts and Tymianski 2003; Goldberg and Choi 1993; Patel et al. 1992; Weiss et al. 1986) excessive activation of NMDA receptors is crucial for inducing excitotoxic brain injury (Doble 1999; Mehta 2007). In cortical cell cultures, using oxygen-glucose deprivation, at least 80% of total Ca^{2+} entry is prevented by NMDA receptor antagonists (Goldberg and Choi 1993). Sattler and colleagues (1998) demonstrated that Ca^{2+} loading via NMDA receptor channels was toxic, whereas identical Ca^{2+} loads through voltage-activated Ca^{2+} channels (VACCs) was essentially harmless. These results imply that influx pathways distinct to NMDA receptors are of particular importance in determining neuronal vulnerability to high levels of Ca^{2+}. Therefore, the NMDA receptor is not merely a conduit for Ca^{2+} influx.

Calcium that enters the cytoplasm via NMDA receptor channels triggers NO production via the activation of neuronal nitric oxide synthase (nNOS) and superoxide production via mitochondrial Ca^{2+} uptake, resulting in the formation of peroxynitrite, a powerful and long acting free radical (Halliwell 2006; Gursoy-Ozdemir et al. 2000; Pacher et al. 2007). Peroxynitrite is more toxic than NO or superoxide individually and causes severe damage to cellular DNA, proteins and lipids (Halliwell 2006; Pacher et al.

2007). Recently, Girouard and colleagues (2009) provided direct evidence that the NOX2 enzyme, a homologue of the nicotinamide adenine dinucleotide phosphate-oxidase family (i.e. NOX family of NADPH oxidases), is the major source of reactive oxygen species (ROS) generation during the over-activation of NMDA receptors. In this study, nNOS derived NO was shown to be the integral upstream signaling molecule linking NMDA receptors to NOX2-dependent ROS generation (Girouard et al. 2009).

Evidence suggests that the activation of NMDA receptors is vital for both the initiation and propagation of PIDs (Menniti et al. 2000; Obrenovitch and Zilkha 1996). In rats subjected to 2 hr of focal ischemic stroke, continuous intrathecal infusion of CGX-1007 (an antagonist selective for NMDA receptors that contain the NR2B subunit) delivered 8-24 hr post occlusion, reduced the incidence of secondary phase PIDs by approximately 50% relative to saline controls if treatment preceded the onset of PIDs (May Lu et al. 2005). Theses authors suggested that a reduced number of PIDs appears to be a salient condition for protection of brain cells (May Lu et al. 2005).

NMDA receptor-mediated excitotoxicity and neuroinflammation have traditionally been considered as two distinct pathological processes within the cerebral ischemic cascade, yet there is evidence for 'cross-talk' between the two. Using an antagonist of the receptor for the proinflammatory cytokine interleukin-1 (IL-1), Relton and Rothwell (1992) reported marked protection against injury elicited by intracerebral administration of NMDA in rodents. Chang and associates (2008) showed that intraperitoneal (i.p.) injection of NMDA upregulates neuroinflammatory markers (glial fibrillary acidic protein, iNOS, as well as IL-1β, and the inflammatory cytokine, tumour necrosis factor alpha) in the rat frontal cortex. Though early IL-1β expression in glial

cells has been implicated to be the consequence of NMDA receptor-mediated excitotoxicity (Pearson et al. 1999), Thornton and colleagues (2006) found that NMDA receptor antagonists only attenuated cell death induced by low doses of IL-1.

Clearly, the NMDA receptor has a large involvement in the ischemic injury process, with its primary role being excitotoxic Ca^{2+}-mediated neuronal injury. It is important to recognize, however, that the large scope of its injury inducing properties is likely due to the fact that Ca^{2+} entry specific to NMDA receptor channels acts as a triggering mechanism for numerous, long-acting pathological reactions that, once triggered, may be self-sustaining (Lipton 1999; Siesjo et al. 1995). Still, their principal role in stroke pathophysiology is undeniable, which makes targeting the NMDA receptor for stroke therapy a primary objective (Albensi 2007; Besancon et al. 2008; Wen et al. 2006).

1.6 Animal Models of Ischemic Stroke

The presence of intact cerebral vasculature is essential to the study of abnormal brain perfusion (Hossman 2008). Thus, because of the complexity of the brain and its response to injury, use of *in vitro* systems alone cannot comprehensively evaluate cerebral ischemia and its consequences (Graham et al. 2004). The vast majority of animal stroke models are based on ischemic stroke, although experimental models of intracerebral and subarachnoid hemorrhage have also been described (MacLellan et al. 2009; Lin et al. 2003). Thus, animal models pertaining to ischemic stroke will form the mainstay of subsequent discussion.

1.6.1 Selection of Species

Animal models of ischemic stroke can first be characterized in terms of the particular species utilized. Models defined in this way can be placed into two general categories – nonrodent (large animal) and rodent (small animal) stroke models (Graham et al. 2004). Although initial scientific knowledge on stroke has largely come from 'higher' species (e.g. dogs, pigs, sheep, cats, and nonhuman primates), now the majority of experiments are carried out in small animals such as the rat and mouse (Durukan and Tatlisumak 2007). The use of rodent models for stroke research offers the clear advantages of lower purchase and maintenance costs and greater acceptability from an ethical perspective compared to the use of larger animals (Graham et al. 2004). Given that the mouse has been deemed the most appropriate animal for genetic modifications, it is predominately used in transgenic technology for studies related to the molecular pathophysiology of stroke (Fujimura et al. 1999; Chen et al. 2005). Currently, the rat is the most commonly used species for modeling human neurological disease (Cenci et al. 2002; Macrae 1992). The rat is especially useful in stroke research for several reasons, including a basic likeness to human cerebrovascular architecture (Yamori et al. 1976) and its moderate size, which allows for easier monitoring of physiological parameters and examining brain specimens (Takizawa et al. 1991). Likewise, using the rat is conducive to reproducible experimentation, and a number of behavioural measures have been well described and standardized for rats which can be applied to stroke paradigms (Kleim et al. 2007; Schallert et al. 2000).

1.6.2 Rodent Models of Ischemic Stroke

Rodent models of ischemic stroke are primarily classified as being either

global or focal in nature (Millikan 1992). Further general classifications pertain to whether blood flow is complete/incomplete or permanent/transient (Hossman 2008). Global cerebral ischemia occurs when cerebral blood flow is reduced throughout most, or all, of the brain (Reempts and Borgers 2000). Clinically, the most dramatic manifestation of global brain ischemia is cardiac arrest (Crumrine et al. 1997; Jackson and Dole 1979). However, to avoid whole body ischemia and potential cardiac interference on the outcome of global brain ischemia, the most commonly used experimental approaches to model global cerebral ischemia involve occlusion of several major cephalic arteries (Li et al. 2000; Spencer et al. 2007; Ulrich et al. 1998). Temporary unilateral carotid artery occlusion in the gerbil, transient bilateral carotid artery ligation combined with systemic hypotension (2-vessel occlusion model) (Eklof and Siesjo 1972), and permanent vertebral artery occlusion with subsequent reversible carotid artery ligation in the rat (4-vessel occlusion model) (Pulsinelli and Brierley 1979) are common examples of such methods. Damage in these models occurs after a maturation period of 1-3 days and is primarily located in selectively vulnerable areas of the brain such as the striatum, parts of the neocortex, and the CA1 pyramidal neurons of the hippocampus (Kirino 1982). Under natural conditions, however, global ischemia in the absence of cardiac failure is a rare circumstance (Hossman 2008).

In contrast to global ischemia, focal ischemia involves a reduction in blood flow to a reasonably distinct portion of the brain (Traystman 2003). Clinically, approximately 65% of ischemic strokes present as lesions in the territory supplied by the middle cerebral artery (MCA) (Bogousslavsky et al. 1988). In experimental stroke research, this situation is reflected by the preferential use of middle cerebral artery occlusion (MCAo) models

(Dawson et al. 1993; Duverger and MacKenzie 1988; O'Neill et al. 2000b; Sharkey et al. 1994). Middle cerebral artery occlusion results in a reduction of CBF in both the striatum and the overlying cortex within the hemisphere ipsilateral to the occlusion, leading to infarction injury of these brain areas (Biernaskie et al. 2004; O'Neill et al. 2000a). At present, the most commonly used MCAo models consist of either permanent or reversible occlusion of the proximal or distal parts of the MCA often combined with carotid artery occlusion to reduce collateral blood flow (Gerriets et al. 2003; Tatlisumak et al. 1998; Veltkamp et al. 2005). Permanent ischemia models are, however, questionable in their representation of human stroke where thrombus disintegration is particularly common, thus allowing reperfusion after ischemia (Hakim et al. 1987).

The intraluminal filament occlusion technique, first described by Koizumi and colleagues (1986; as cited in Traystman 2003), is currently the most widely used procedure for inducing MCAo in rats and mice (reviewed by Hossmann 2008 and Traystman 2003). In this technique, a nylon suture with an acryl-thickened tip is inserted into the common carotid artery and subsequently advanced until the tip passes the origin of the MCA; recirculation can be easily obtained by withdrawal of the filament. The intraluminal filament occlusion technique does not require a craniotomy; it produces focal occlusion of a large cerebral artery leading to defined cellular injury within its territory (reviewed in Durukan and Tatlisumak 2007) and, essentially, is considered of paramount clinical importance (Millikan 1992; Windle et al. 2006). However, several technical complications have been reported. Correct placement of the suture without injury to the vessel wall is difficult to achieve (Rabb 1995), and there is risk of premature reperfusion and inadvertent subarachnoid hemorrhage (Laing et al. 1993). Further,

withdrawal of the suture induces instantaneous reperfusion whereas, in humans, spontaneous reperfusion results in a slow (i.e. hours to days), progressive recirculation (Hakim et al. 1987).

In 1990, Robinson and colleagues introduced an alternative approach to occluding the MCA, accomplished via topical application of the vasoconstrictive peptide, endothelin-1 (ET-1) directly to the exposed MCA. Subsequently, this approach was modified by Sharkey and associates (1993) allowing ET-1 to be stereotaxically microinjected adjacent to the MCA in order to minimize the invasiveness of the surgery. This approach leads to dose-dependent vasoconstriction within the MCA territory (70-93% reduction from baseline) and results in an infarct volume that is similar to, but slightly milder, than the filament MCAo model (Bogaert et al. 2000; Macrae et al. 1993; Nikolova et al. 2009; Riek-Burchardt et al. 2004; Sharkey et al. 1993). Reperfusion in this model has been shown to gradually occur over a period of at least 3-4 hr (Bogaert et al. 2000; Macrae et al. 1993; Nikolova et al. 2009; Sharkey et al. 1993).

1.7 Pharmacological Neuroprotection in Experimental Focal Ischemic Stroke

'Time is brain', a phrase derived from Benjamin Franklin's original aphorism 'time is money', emphasizes that human nervous tissue is rapidly and permanently lost as stroke progresses (Saver 2006). It has been estimated that acute ischemic stroke victims lose a staggering 1.9 million neurons, 14 billion synapses, and 12 kilometers worth of myelinated fibers each minute that stroke is left untreated (Saver 2006). These estimates are sobering, and a testament to the urgency for uncovering pharmacological therapies

that reduce cell death following stroke and lessen the functional consequences that accompany such critical damage to the brain.

Neuroprotection can be defined as any strategy or combination of strategies that antagonizes, interrupts, or slows the sequence of damaging biochemical and molecular events that, if left unrestrained, would result in irreversible structural damage to brain tissue (Ginsberg 2008). Thus, in relation to stroke, neuroprotection is a therapeutic approach that involves dissecting apart the various mechanisms of injury involved in the complicated ischemic cascade in order to arrive at potential target sites for treatment (Young et al. 2007). It is acknowledged that this depiction of neuroprotection excludes strategies that are directed at reversing vascular occlusion and/or improving cerebral blood flow. Such approaches can protect the brain, but they do so primarily via hemodynamic rather than metabolic mechanisms (see review by Meretoja and Tatlisumak 2008). The concept of neuroprotection for stroke essentially revolves around the notion that drugs can be developed to halt otherwise impending cell death within the ischemic penumbra (Green 2008). Currently, the peunumbra is the most clinically relevant target for specific neuroprotective therapies since it represents brain regions which are challenged by ischemia, but temporarily still viable (Bandera et al 2006; Hertz 2008).

Numerous animal studies have demonstrated robust neuroprotective effects via many pharmacological agents in a variety of ischemic models and in a variety of species. That is, anti-excitotoxic strategies targeting NMDA (Gill et al. 1996; Sharkey et al. 1994), AMPA/kainate (Matucz et al. 2006; O'Neill et al. 2000a), and metabotropic (Adamchik and Baskys 2000; Kingston et al. 1999) glutamate receptors, as well as the application of Ca^{2+} and Na^{+} channel blockers (Bielenberg and Beck 1991; Rataud et al.

1994), various free radical reduction strategies (Hall et al. 1996; Marshall et al. 2001), and anti-inflammatory approaches (Goussev et al. 1998; Jiang et al. 2001), to list but a few, have all proved effective for neuroprotection in animal models of stroke. Thus, many of the known pathophysiological events of the ischemic cascade have been successfully targeted for neuroprotection in experimental focal ischemic stroke. Neuroprotection for stroke is, therefore, a well established therapeutic notion, yet it is an increasingly contentious issue. Over the course of approximately the past two decades, when these pharmacological agents have been taken to clinical trials they have been resoundingly unsuccessful (for review see Cheng et al. 2004).

There are myriad logical, albeit speculative, reasons for the clinical failure of neuroprotective agents in stroke (for review on this issue see Cheng et al. 2004, and Young et al. 2007). Nonetheless, spurred by recent, major advances in the understanding of stroke pathophysiology, the advent of new pharmacological agents that target, more specifically, the facets of cerebral ischemia which lead to cell death has substantially increased the level of optimism and degree of experimental growth within the field of neuroprotection for stroke (Gardoni and Luca 2006; Romero et al. 2006; Zaleska et al. 2009). Moreover, investigators have begun to develop more comprehensive, longer-term measures of sensorimotor impairment and recovery and, as such, neuroprotection studies are rightfully expanding beyond simply estimating reductions in cell death at early time points after stroke (Kundrotiene et al. 2004; Sun et al. 2008). The necessity of extending neuroprotection studies for weeks to months in order to uncover a more lasting therapeutic effect, as well as the importance of investigating both structural brain protection and behavioural improvement after pharmacological treatment, is now

acknowledged (Hewlett and Corbett 2006; Kundrotiene et al. 2004; Moyanova et al. 2007a; Sun et al. 2008). At present, neuroprotection for stroke is a concept that necessitates a 'functional' adjective.

1.7.1 Evaluating Functional Neuroprotection in Focal Ischemic Stroke: Experimental Obstacles

Due to their putative relevance to the clinical condition, MCAo models have traditionally been extensively used for studying neuroprotection and recovery of neurological function following focal ischemic stroke (Biernaskie et al. 2001; Bogaert et al. 2000; Macrae et al. 1993; Nikolova et al. 2009; Sharkey et al. 1993; Veltkamp et al. 2005). These models, however, commonly cause very large infarcts that can comprise up to two thirds of the total hemisphere (Garcia et al. 1995; Kanemitsu et al. 2002). Non-fatal human strokes are mostly small in size (4.5-14% of ipsilesional hemisphere) (reviewed in Carmichael 2005) and functional deficits after stroke occur with damage to specific neural circuits, such as sensorimotor cortex maps of the upper and lower limbs (Crafton et al. 2003). Damage to widespread and functionally diverse brain regions characteristic of MCAo models often produces complex and varied behavioural abnormalities in surviving animals which adds ambiguity to studies that aim to investigate functional protection after ischemic brain damage to more relevant sensorimotor circuits (Adkins et al. 2004; Carmichael 2005).

Middle cerebral artery occlusion models have several limitations pertinent to the study of sensorimotor deficits after stroke. The amount and location of injury can be quite variable, particularly in the more clinically relevant transient occlusion models, and they often do not cause significant damage to the primary motor cortex (Biernaskie et al.

2001; Bogaert et al. 2000; Dawson et al. 1993; Duverger and MacKenzie 1988; Macrae et al. 1993; Nikolova et al. 2009; Sharkey et al. 1993). Thus, while MCAo in the rat resembles human stroke in a number of respects, it does not reliably produce upper limb deficits; a common clinical problem (Nakayama et al. 1994; Parker et al. 1986). Moreover, the invasiveness of surgery damages the facial and neck musculature, which frequently results in feeding difficulties and inadvertent deterioration of long-term health in the animals (Sharkey and Butcher, 1995). Issues of high mortality have also been reported when using MCAo models (Lindner et al. 2003; Pan et al. 2005).

The ET-1 MCAo model developed by Sharkey and colleagues (1993) avoids many of the experimental confounds associated with invasive surgery; in this model, animals exhibit no post-operative eating problems and recover their preoperative weight within just a few days (Moyanova et al. 2007b; Sharkey et al. 1993). Still, anatomical variation in the cerebral vasculature across animals often leads to insufficient accuracy in the placement of the injection needle. Thus, some investigators have reported large variability in lesion location and size as well as low model success rates (~50%) when using the ET-1 MCAo model (Biernaskie et al. 2001; Biernaskie and Corbett 2001; Windle et al. 2006). Such disadvantages lead to greater numbers of animals required for experimentation, and make the verification of a legitimate neuroprotective effect difficult to discern from that of an unsuccessful experimental lesion.

In sum, traditional models of focal ischemic stroke (i.e. MCAo and ET-1 MCAo models), despite their acknowledged clinical relevance, appear to provide a rather unreliable and ambiguous approach for investigating brain protection and loss and gain of sensorimotor function after ischemic injury. Hence, other reasonably non-invasive

surgical methods that permit reliable and accurate control over lesion location and extent - thus allowing for reproducible, relevant sensorimotor deficits - may be more suitable for studying the effects of neuroprotective compounds on lesion reduction and functional recovery after stroke.

1.8 Rat Models of Upper Extremity Impairment in Stroke

A key objective in developing experimental stroke models is the achievement of homogenous lesions with minimal variability, in order to maximize reproducibility (Alonso de Lecinana et al. 2005; Durukan and Tatlisumak (2007). Recently, several rat models of focal ischemic stroke have been developed that afford greater precision regarding the size and location of the ischemic lesion, permitting the induction of ischemic injury to brain circuits that directly subserve forelimb function (Adkins et al. 2004; Gilmour et al. 2004; Gonzalez and Kolb 2003; Hewlett and Corbett 2006; Shanina et al. 2006; Zhengguang et al. 2001). By allowing much greater control over lesion location and size, these stroke models have primarily been utilized for studying the effects of various rehabilitation therapies on forelimb recovery after stroke, as well as investigations pertaining to ipsilesional versus contralesional cortex involvement in forelimb recovery (Adkins et al. 2004; Adkins and Jones 2005; Maldonado et al. 2008; Luke et al. 2004; Windle and Corbett 2005; Shanina et al. 2006). It is important to note that various rat models of upper extremity impairment have existed for many years (Barth et al. 1990b; Crowne and Pathria 1982), yet the emergence of several methods that permit modeling of upper extremity impairment in stroke (i.e. via ischemic pathology) are a

rather recent addition to experimental stroke paradigms. The following are the conventional methods used to model upper extremity impairment in stroke by causing ischemic damage to forelimb representation areas in the rat brain.

1.8.1 Devascularization

The devascularization method involves performing a small craniotomy over the forelimb representation areas of the cortex, the removal of dura mater, and subsequent eradication of blood vessels that supply this region either by 'stripping' away the pia mater (Gonzalez and Kolb 2003), removing blood vessels and pia mater with a cotton swab (Erickson et al. 2007) or electrocoagulation (Kleim et al. 2003). All of these techniques produce focal cortical damage underneath the devascularized area that extends from pia down through all layers of the cortex (Erickson et al. 2007; Gonzalez and Kolb 2003; Kleim et al. 2003). The major disadvantages of this method are that it can produce mechanical damage and hemorrhage to the underlying tissue, and it does not allow for reperfusion of blood vessels (Kleim et al. 2007). Additionally, due to the inherent nature of the technique it does not permit damage to subcortical structures that contribute to forelimb functionality. This is a salient drawback because clinical strokes often result in cortical and/or subcortical injury (Cramer 2003) and subcortical brain damage is frequently involved in sensorimotor impairment after ischemic stroke in humans (Feys et al. 2000). Therefore, compared to models that also permit damage to the subcortex, models of pure cortical stroke in the rat may not be as representative of impairment after clinical stroke.

1.8.2 Photothrombosis

The photothrombosis model involves intravenous injection of Rose-bengal or other photosensitive dyes and subsequent production of photooxidation injury via irradiation through the intact skull (Watson et al. 1985). This results in the generation of singlet oxygen, focal endothelial damage, platelet activation, and a simultaneous massive microvascular coagulation of the irradiated tissue (Que et al. 1999). The region of irradiation can be stereotaxically determined so as to place the infarct within functionally distinct cortical areas, such as the forelimb sensorimotor cortex (Shanina et al. 2006). The primary advantage of this model is the production of very reproducible, circumscribed ischemic lesions with minimal surgical invasion (Shanina et al. 2006; Takata et al. 2006). However, the model allows for very little reperfusion or ischemic penumbra to develop and the nature of the vascular occlusion produces significant local vasogenic and cytotoxic edema that more closely resembles traumatic brain injury than ischemic stroke in humans (Kleim et al. 2007). As with the devascularization method of forelimb sensorimotor impairment, photothrombotic lesions are limited to cortical structures (Shanina et al. 2006; Takata et al. 2006).

1.8.3 Chemical Vasoconstriction

An alternative method to physically blocking or removing blood vessels that supply the forelimb region is to chemically constrict them through administration of the potent and long-lasting vasoconstrictor, ET-1 (Fuxe et al. 1997; Hewlett and Corbett 2006). Endothelin-1 reduces local blood flow to produce ischemic injury when either injected directly into the brain (Gilmour et al. 2005) or applied topically to the surface of the brain (Fuxe et al. 1997). This model requires limited surgical invasiveness and

produces a dose-dependent ischemic lesion with minimal tissue edema (Fuxe et al. 1997; Hughes et al. 2003). Both topical and intracerebral microinjection of ET-1 can be stereotaxically directed to forelimb sensorimotor cortex, whereas intracerebral injection of ET-1 also permits ischemic injury to subcortical regions involved in forelimb sensorimotor function such as the lateral striatum (Gilmour et al. 2004; Hewlett and Corbett 2006; Windle et al. 2006). The ET-1 model allows for gradual reperfusion of the circulation over the course of several hours (Fuxe et al. 1997; Nikolova et al. 2009; Windle et al. 2006), a profile that may be more representative of ischemic stroke when compared to the immediate reperfusion that occurs with the use of an intraluminal filament or clip (Hakim et al. 1987; Kleim et al. 2007).

The local application of ET-1 to forelimb representation areas of the brain is, therefore, the only currently available rat model of upper extremity impairment in stroke that allows for adequate reperfusion of brain tissue and permits damage to both cortical and subcortical structures. As such, it may have broader applicability within experimental stroke research. However, receptors for ET-1 are not limited to endothelial and vascular smooth muscle cells; they are also found on both neurons and glia (reviewed by Schinelli 2006). Thus, despite the apparent potential of the ET-1 model, using ET-1 to induce local ischemic lesions raises concern regarding its application in stroke research due to its non-vascular binding within the brain (Blomstrand et al. 1999; Koizumi et al. 1994; MacCumber et al. 1990; Sasaki et al. 1997).

The designation, ET-1 model of upper extremity impairment in stroke, serves to differentiate this variation of ET-1-induced focal ischemic stroke from other applications of this drug in stroke research; particularly ET-1 induced MCAo (Sharkey et al. 1993)

and its use in targeting various other brain areas for ischemia such as white matter or the hippocampus (Frost et al. 2006; Hughes et al. 2003). Henceforth in this thesis, unless otherwise qualified, the ET-1 model of upper extremity impairment in stroke will simply be referred to as the ET-1 model. Figure 1.4 illustrates representative patterns of ischemic brain damage induced by the ET-1 topical, intracortical, and intracortical + striatal models. The typical damage pattern seen in the ET-1 MCAo model is included in Figure 1.4 for comparison. Compared to all ET-l upper extremity impairment models, Figure 1.4 demonstrates how the MCAo method does not typically cause adequate ischemic damage to the forelimb sensorimotor cortex.

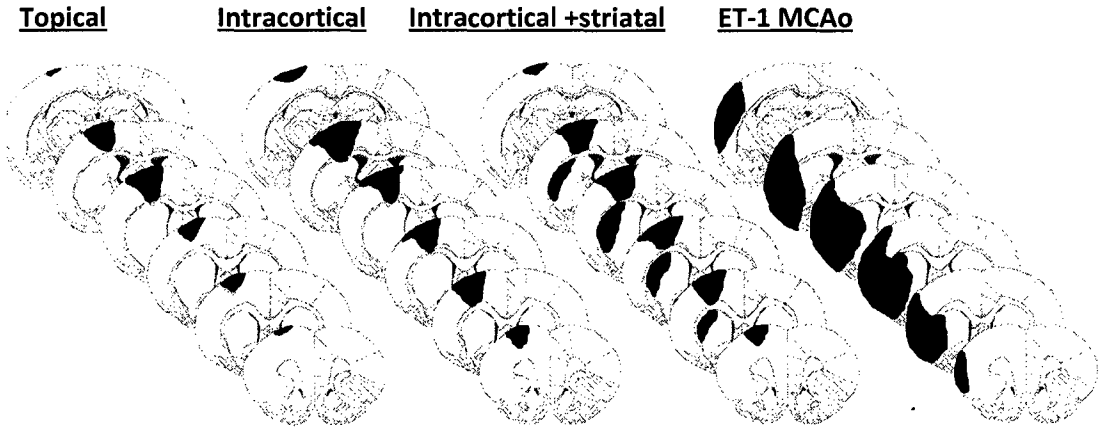

Figure 1.4. Illustration of typical ischemic damage patterns in the ET-1 topical, intracortical, intracortical + striatal, and MCAo models. Patterns of damage are shaded in black. Depicted stereotaxic levels range from approximately +3.8 mm to -2.0 mm (anterior/posterior plane, bottom to top direction) relative to the standard stereotaxic landmark, bregma (intersection of the sagittal and coronal sutures of the skull). (Adapted from Windle et al. 2006).

1.9 Endothelin in the Brain

Endothelin peptides owe their name to the cultured endothelial cells in which they

were first discovered just over 20 years ago (Yanagisawa et al. 1988). Yanagisawa and associates (1988) identified ET as a highly potent and long lasting vasoconstrictive peptide containing 21 amino acid residues. Subsequent analysis by Inoue and colleagues (1989) of the gene encoding ET revealed the existence of two other genes encoding ET-like peptides. The three closely related endogenous isoforms were designated as ET-1, ET-2, and ET-3 (Inoue et al. 1989). The laboratories of Arai and Sakurai demonstrated that the ETs act on two distinct G-protein-coupled receptors, designated as the ET_A and ET_B receptor (Arai et al. 1990; Sakurai et al. 1990).

The stimulation of differing ET receptors elicits opposite effects on the vasculature; the interaction of ETs with ET_B receptors typically mediates vasodilatation and ET_A stimulation largely produces vasoconstriction (Shah 2007). The ET_B receptor binds all three isoforms with approximately equal affinity, whereas the ET_A receptor shows higher selectivity to ET-1 and ET-2 with a 100-fold lower affinity for ET-3 (Kedierski and Yanagisawa 2001). Within the brain, ETs are produced by vascular, neural, and non-neural elements (Kuwaki et al. 1997). ET has been detected in the cerebral cortex, striatum, hippocampus, amygdala, pituitary gland, hypothalamus, Purkinje cells within the cerebellum, medulla oblongata, and dorsal horn of the spinal cord (Lee et al. 1990; Yoshizawa et al. 1990); with the exception of the pituitary gland, ET-1 is the most abundant isoform (Kuwaki et al. 1997). In rat, porcine, and human species, ET_A and ET_B receptors - like the ET peptides - have been shown to be widely dispersed throughout the CNS. They are, however, predominantly located on endothelial and vascular smooth muscle cells (reviewed by Shah 2007).

1.10 Measuring Forelimb Impairment in Rats after Stroke

The development of rat models of upper extremity impairment in stroke has enabled researchers to investigate, with greater efficacy, the value of a variety of therapies designed to enhance sensorimotor recovery after ischemic damage to the brain, as well as elucidate some of the mechanisms within the brain that may mediate this improvement in function (Hsu and Jones 2006; Ramanathan et al. 2006; Shanina et al. 2006). In the rat, sufficient unilateral damage to areas of the brain involved in forelimb function typically renders the contralesional forelimb impaired relative to both its pre-stroke performance and the ability of the ipsilesional (unimpaired) limb after stroke (Adkins et al. 2004; Windle et al. 2006). Accordingly, investigators have developed various sensorimotor tests designed to examine discrepancies in pre- and post-stroke performance as well as the extent of asymmetrical forelimb function after ischemia (Allred and Jones 2008; Barth and Stanfield 1990; Montoya et al. 1991; Schallert et al. 2000).

These tests range from those that measure acquired (i.e. skilled) sensorimotor behaviours (which typically require an extensive amount of training before ischemia) to those that measure preexisting (i.e. unskilled) sensorimotor behaviours (which require very few training sessions) (Kleim et al. 2007). Like all animals, the rat has evolved its own species-specific repertoire of adaptive behaviours (Cenci et al. 2002). Thus, to effectively model human neurological deficits in the rat it is essential to identify functional, rather than physical, similarities in neurological impairments – the question is not whether a rat would show a given neurological impairment, but rather, how that

neurological deficit would manifest itself in a rat (Cenci et al. 2002; Kleim et al. 2007). Some common examples of tests used to measure forelimb motor/somatosensory capacity in the rat are detailed below in Table I. Although it is quite unlikely that any behavioural test taxes an isolated element of forelimb capacity, these tests are suspected to measure, within reason, certain components of forelimb functionality evident in the rat species.

Table I. Some key measures of forelimb impairment in the rat, and the suspected component of forelimb capacity assessed.

Forelimb Measure	*Forelimb component*	*References*
Forelimb placing	Sensorimotor/proprioceptive capacity (unskilled reaching)	Adkins et al. 2004; Barth and Stanfield 1990
Cylinder test	Preference for using non-impaired forelimb for postural support during rearing and weight shifting movements	Schallert et al. 2000; Biernaskie et al. 2004
Balance-beam/Ladder walking	Ipsilesional and contralesional incidence of foot faults during skilled gait	Gilmour et al. 2005; Riek-Burchardt et al. 2004
Adhesive removal (Sticky-tape test)	Preference for responding to stimuli on non-impaired forelimb	Sughrue et al. 2006
Forelimb reaching (Montoya Staircase test and Single pellet reaching test)	Unilateral skilled reaching and grasping of objects	Montoya et al. 1991; Whishaw and Kolb 1988
Sunflower seed test and Vermicelli handling test	Bilateral distal forepaw dexterity	Allred et al. 2008; Gomez et al. 2006
Forelimb postural reflex	Basic neurological function	Bederson et al. 1986; Sun et al. 2008

1.11 Experimental Objectives

The primary objectives for research outlined within this thesis were twofold:

1) To develop an ET-1 rat model of focal ischemic stroke that produced quantifiable tissue damage to forelimb representation areas of the brain and measurable impairments in forelimb sensorimotor function;

2) To determine if the ET-1 model is applicable for neuroprotection research using NMDA receptor antagonists.

A secondary objective was also explored in a small study:

3) To obtain preliminary data on the functional role of the contralesional hemisphere, homotopic to an initial unilateral ET-1 lesion, in the recovery of forelimb placing behaviour subsequent to pre-ischemic treatment with an NR2B subunit-selective antagonist or vehicle control.

2.0 General Methodology

2.1 Animals

Adult, male Sprague-Dawley (SD) rats (Charles River Laboratories; Montreal, Canada) were used for all experiments. Upon arrival all rats weighed between 175g and 200g, and approximately 24 hours later they were housed in individual cages. The animal colony room was kept at 21°C ± 2°C and maintained on a 12 hr light/dark cycle (lights on at 06:00hr). Animals were provided with food (Rodent Chow 5001; Ralston Purina, Mississauga ON) and water *ad libitum*. Aside from routine cage maintenance, animals were left undisturbed when not involved in the experimental procedures described below. All animal experimentation was carried out in accordance with guidelines set by the Canadian Council on Animal Care (CCAC).

2.2 ET-1 Preparation

Approximately 24 hours prior to surgery, lyophilized ET-1 (100μg) (human and porcine; Calbiochem, Germany) was dissolved in sterile distilled H_2O [100μl, to give a concentration of 400pmol/μl for the stereotaxic ET-1 microinjections; 1 group in one experiment (see section 3.2.2.1) received ET-1 at 200pmol/μl]. The ET-1 was then separated into aliquots as needed for each individual experiment, briefly centrifuged, and immediately returned to the -20°C freezer. Each aliquot of ET-1 was left in the freezer

until used for surgery. All aliquots were used within 12 days of reconstitution. See individual experiments for further details.

2.3 Stereotaxic Surgery

Prior to surgery, animals were gently handled then left undisturbed to acclimate to the surgery room for approximately 1 hr. Rats were placed in a small acrylic glass chamber previously filled with 4% isoflurane in oxygen, allowing for rapid induction of general anesthesia with minimal stress on the animal. Surgical anesthesia was produced within 8-10 min using 3.5% isoflurane in oxygen. During surgery, anesthesia was maintained with 2% isoflurane in oxygen. Local anesthetic (Xylocaine Jelly: 2% lidocaine hydrochloride; AstraZeneca Canada Inc. Mississauga, Ont.) was applied to the dorsal surface of the head prior to making a midline scalpel incision, and also to tissue superior to the skull after the incision was made. Anterior/posterior (AP) and medial/lateral (ML) stereotaxic coordinates were taken in relation to bregma, and dorsal/ventral (DV) coordinates were relative to the skull surface at each injection location.

Using a stereotaxically mounted engraving cutter (2.4 mm diameter) (Foredom Motor; Stoelting Co. Illinois, USA) small burr holes, corresponding to appropriate stereotaxic injection coordinates, were drilled through the skull down to dura mater. A 10µl stereotaxically mounted syringe (26 gauge) (Hamilton Co. Nevada, USA) was loaded with ET-1; at each injection site the needle point was lowered to the appropriate depth and left undisturbed in the brain for 1 min. ET-1 was injected at a rate of 1µl/2min,

with a 1 min pause between each µl. At each site, after the final µl was injected, the needle was left undisturbed for 4 min prior to being slowly retracted from the brain. Rectal temperature was closely monitored and maintained between 36°C - 38°C throughout the duration of surgery using a temperature controlled heating pad (Kaz Canada Inc. Milton ON). At the conclusion of surgery, the scalp was sutured, cleaned with 50% ethanol, and the incision site was treated with local anesthetic. Rats were then returned to their home cage and allowed to recover in the surgery room. The ambient temperature in the home cages was maintained at approximately 30°C with the use of a heating pad. When animals had recovered from surgery, the incision site was again treated with local anesthetic and rats were returned to the animal colony room. Weight-matched sham-operated rats received the same surgical procedure up to and including the placement of the needle in the brain, and were anesthetized for the same duration as the ischemic animals.

2.4 Behavioural Tests

All behavioural testing was carried out in a quiet room located in the animal holding facility (Animal Resources, Atlantic Veterinary College). Beginning two days post arrival to the animal colony room, rats were gently handled for approximately 10 min each in order to acclimate the rats to the experimenter and the behavioural testing room. This process was repeated 3 additional times during the 10-14 days prior to the commencement of surgery.

2.4.1 Basic Forelimb Reaching

Two tests of basic (i.e. unskilled) forelimb reaching were employed: tactile-stimulated forelimb placing (TFP) and vibrissae-stimulated forelimb placing (VFP). Rats were held under their torso, limbs dangling freely with the exception of the forelimb contralateral to the forelimb to be tested; this limb was gently restrained by the experimenter. For TFP, the animal was slowly moved laterally towards a table top until the distal portion of the unrestrained forelimb contacted the edge of the table. For VFP, the rat was gently moved in the horizontal plane toward the table edge until the vibrissae ipsilateral to the unrestrained forelimb made contact with the edge of the table.

Accuracy was determined based on the number of correct forelimb placements out of 5 attempts (i.e. a score of '1' for each correct placement). Attempts that missed the table top and/or were delayed for longer than ~ 2 seconds after forelimb or vibrissae contact with the table edge were counted as failed forelimb placements (i.e. a score of '0' was given). Testing was performed during the light phase in order to reduce animal struggling. Sprague-Dawley rats have been shown to have poor visual acuity in general (i.e. estimated at 20/1200) (Prusky et al. 2002); they require approximately 3 hr to visually adapt to dark conditions, and they are unable to visually register colors in the red range (Behn et al. 2003). Thus, rats were tested in the dark (but under red light conditions) in order to abolish, for all practical purposes, the potential influence of visual input on placing. The order of testing was uniform across all animals: ipsilateral VFP, ipsilateral TFP, contralateral VFP, contralateral TFP. Three practice sessions were conducted within 14 days of animal arrival in order to acclimate rats to the manual manipulation imposed by the experimenter. Placing accuracy was first tested

approximately 24 hr prior to surgery. The number of post-surgery testing days for forelimb placing accuracy was dependent on the particular experiment. Statistical analyses were conducted on raw data; however, for simplicity, placing data is graphically presented (and discussed) as percent accuracy for all experiments (e.g. 40% accuracy vs. 2/5).

2.4.2 Forelimb Postural Reflex

Rats were suspended by the base of the tail approximately 50 cm above the floor of their home cage (lid removed) for approximately 2 seconds. In the contralesional forelimb, posture was examined for extension at the shoulder (greater than 90°), adduction to the torso, or combined shoulder extension and elbow flexion (resulting in retraction of the paw to the torso), all indicative of deficits in forelimb postural reflex (FPR) (Bederson et al. 1986). A score of 1 (i.e. deficit) was given if any of the above behaviours were observed, and a score of 0 (i.e. no deficit) if both forelimbs extended out toward the home cage floor. A single score reflecting duration of postural reflex deficit (within the survival period of the experiment) was calculated for each rat during post-preliminary experimentation. Three sessions were conducted within 14 days of animal arrival in order to reduce animal struggling during testing. Approximately 24h prior to surgery, rats were tested for asymmetries in FPR. The number of post surgery testing days was dependent on the particular experiment.

2.4.3 Forelimb Somatosensation

To test for asymmetries in forelimb somatosensory function a modified version of the traditional adhesive removal (i.e. sticky-tape) test was employed (Sughrue et al. 2006). Rats were left undisturbed in their home cage for ~10 min before testing.

Small strips (.75cm x 5cm) of laboratory tape (TimeMed, IL, USA) were clasped around the distal portion of the forelimb. Forelimbs were tested individually on each trial. A stationary timer was used to count down a 30 sec trial while the experimenter used a hand-held, quiet stop watch to record the time spent attending to the tape during the trial. Behaviours such as biting and/or clawing at the tape, as well as vigorous shaking of the taped limb, were considered indicative of paying attention to the foreign stimulus. Following the first trial, a second trial was conducted on the opposite forelimb. Rats were then left alone in their home cage for ~10 min. Subsequently, each limb was individually tested again, but in reverse order. The order of limb testing was balanced within each group.

Asymmetry scores were calculated by averaging time attended to the stimuli for the contralesional limb and dividing this value into the average of time attended to the stimuli for the ipsilesional limb (Sughrue et al. 2006). A score of 1 equates to complete symmetry between ipsilateral and contralateral attendance, and a score of 0 demonstrates complete asymmetry (i.e. no attendance to the stimulus on the contralateral forelimb; full ipsilateral bias). Testing was carried out during the dark phase. Two acclimation sessions were conducted within 7 days prior to surgery, and approximately 24 h prior to surgery rats were first tested for asymmetries on this test. Testing was repeated on day 1, 3, 6, 10, and 14 post-surgery.

2.4.4 Forelimb Postural-Motor Support

Animals were tested for limb preference and their ability to support weight on either forelimb by using the Schallert Cylinder Test (Schallert et al. 2000). Rats were placed inside a clear, open-ended acrylic glass cylinder (30cm x 20cm) and allowed to

rear up and place their forepaws on the cylinder wall 15 times. A Color COMS camera (Strategic Vista Corp. Markham ON) was stationed above the top of the cylinder to record forepaw usage during the initial rear to the cylinder wall and during exploratory behaviour (i.e. wall stepping) between rears. Using a VHS video editor (Panasonic Canada Inc., Mississauga ON) with slow motion capability, the number of forelimb wall contacts (single-limb contacts and bilateral limb contacts) used for postural support were counted for each trial, and the percentage of asymmetry of single limb contacts [(ipsilateral/ipsilateral + contralateral) x 100) as well as the percentage of bilateral limb contacts [(bilateral/(ipsilateral + contralateral + bilateral) x 100] were calculated (Biernaskie et al. 2004). Sliding or swiping motions of the paw along the cylinder wall or brief stabbing motions were not considered as forelimb contacts representative of postural-motor exploratory support, and thus were not counted. Within 7 days prior to surgery, two sessions were conducted in order to acclimate rats to being placed inside the cylinder. Approximately 24 h prior to surgery rats were first tested for asymmetries on this test. Testing was repeated on day 1, 3, 6, 10, and 14 post surgery. Testing was carried out during the dark phase.

2.5 Body Weight

Body weight was taken as a measure of general physical development. All rats were weighed on a standard lab animal scale (Sartorius Canada Inc. Mississauga ON), located in the animal colony room. All weighing of animals occurred during the light phase. Animals were first weighed approximately 70 min prior to surgery and every day

thereafter at roughly 16:00hr, until euthanized.

2.6 Histology

During preliminary experimentation, several histological approaches were utilized. Methods that were generally employed or had a key contribution to protocol development are detailed below.

2.6.1 Brain Removal

Upon completion of each experiment rats were deeply anesthetized with 4.5% isoflurane and rapidly decapitated. Brains were quickly removed; post-mortem histology was contingent on the sectioning and staining method (detailed below).

2.6.2 Fixation, Sectioning, and Staining

Matrix sectioning and triphenyl tetrazolium chloride (TTC) staining

Immediately upon removal, brains were placed in chilled artificial cerebral spinal fluid (aCSF) (composition in mM: NaCl, 145; KCl, 2.5; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 26; $MgCl_2$, 1.3; glucose, 10; pH 7.4.) (pre-existing lab protocol). Brains were then positioned in a chilled matrix (Zivic Instruments. PA, USA) and sliced into ten 1mm coronal sections. Based on results from a small experiment (5 animals) that employed 0.05%, 0.5%, 1.0%, and 2.0% of TTC dissolved in phosphate buffered saline (PBS), individual sections were submerged in 3.5 µl of 1% TTC at room temperature for 45 minutes (i.e. the optimal staining parameters determined from the small study). Sections were subsequently transferred to formalin filled (3.5 µl) well plates and stored at 4°C for approximately 24 hr.

Vibratome sectioning and cresyl violet staining

Whole brains were fixed in 250ml of 10% phosphate buffered neutral formalin for 7-10 days. Between approximately AP +4.0 mm and -2.0 mm (relative to bregma), coronal sections were cut at a thickness of 150μm using a Vibratome 1000 plus (Vibratome Co. St. Louis, USA). Sections were slide mounted and stained using 0.1% cresyl violet (pH 4.07). Every 4^{th} section (600μm apart) was used for estimation of infarct volume (Hsu and Jones 2006).

2.7 Estimation of Infarct

Slides with cresyl violet stained sections were placed on a standard lightbox and photographed with a Canon EOS DS6041 (digital) with 18-55mm lens f/3.5-5.6 using +7 close-up filters (Canon Canada Inc. Mississauga, ON) which was securely stationed on a tripod. Digital photographs of each section were then assessed for infarct damage using Image J software (Image J, National Institutes of Health). On each coronal section, abnormal cellular morphology (i.e. lack of Nissl staining, excessive accumulation of what appeared to be cells containing shrunken, darkened nuclei, or a complete absence of tissue) was delineated as damaged brain tissue. Based on this visual assessment, the lesion area was measured by manually outlining the border of the infarct.

TTC stained sections were scanned using a Canon Canoscan Lide 90 flat-bed scanner (Canon Canada Inc. Mississauga, ON) at 1200dpi. Scanned images were then assessed for infarct damage using Image J software. On each coronal section, the area of

brain tissue in which TTC was not reduced to a deep formazan (excluding non-applicable white matter) was delineated as damaged tissue and measured.

For both staining methods, volume of brain injury was estimated by summing the area of damaged tissue recorded from each slice and multiplying that value by the distance between the measured slices (Swanson et al. 1990). When applicable, area of damage at individual stereotaxic levels was presented in order to illustrate the independence of the rostal-caudal extent of cortical and striatal injury as well as to compare groups and different experimental paradigms on these measures. It was concluded, a priori, that animals with total infarcts beyond 2 standard deviations from the mean of their group would be deemed outliers and excluded from the experiment (Sheskin 2004). See individual experiments for further details.

2.8 Mortality and Success Rates

Mortality rates were calculated for each group in each experiment (given as % mortality) as well as for each entire experiment. For all groups that did not receive neuroprotective drugs, success rates were calculated as follows: (% survival x %survivors with deficit)/100 (Windle et al. 2006). Animals that survived the duration of the experiment and demonstrated at least an 80% reduction in forelimb placing accuracy (combined average for both placing tests) one day post-versus pre-surgery were considered survivors with deficit. Sham controls and animals that were appropriately removed from experimentation were not included in these calculations. Likewise, the number of animals reported to be used (the reported *n*) does not include animals which

died prematurely or those that were removed from the study (or particular tests). See individual experiments for further details.

2.9 Statistical Analysis

Body weight and, when indicated, behavioural data were analyzed using general linear models for two-way repeated measures analysis of variance (ANOVA) for main effects of Group, Day, and Group by Day interactions. The sphericity assumption was measured using Mauchly's Test; when the sphericity assumption was violated, the Huynh-Feldt test was used to correct for this violation (Sheskin 2004). Single scores given for duration of functional impairment on the FPR test were analyzed using one-way ANOVA. Estimations of cortical, striatal, and total infarct volume were analyzed using one-way ANOVA. If a group effect was revealed for cortical and/or striatal infarct volume, then the area of the infarct at individual stereotaxic levels was assessed using one-way ANOVA in order to examine group differences in the pattern of ischemic damage to the brain. Homogeneity of variance was assessed following univariate ANOVA with Levene's Test of Equality of Error Variances. When indicated, Fisher's Least Significant Difference Test for post hoc multiple comparisons was used to elucidate which groups differed from each other.

The parametric statistical treatment of like data is consistent with previous studies (Adkins et al. 2004; Hewlett and Corbett 2006; Hsu and Jones 2006). In cases where parametric tests were not applicable (i.e. preliminary FPR data), the Chi-Square test for independence was used. All data were analyzed using the computer software Statistical

Package for the Social Sciences (SPSS for Windows version 15, SPSS Inc., USA). All results are presented as the mean ± the standard error of the mean (SEM). The a priori α level was set at 0.05 for all comparisons.

3.0 Preliminary Experimentation: Developing an ET-1 Model of Upper Extremity Impairment in Stroke

3.1 Introduction

3.1.1 Endothelin-1: Mechanism of Vasoconstriction

Endothelin-1 is a very potent vasoconstrictor, about ten times more so than other common vasoconstrictors (e.g. sympathomimetics, angiotensin II, and vasopressin) (Yanagisawa et al. 1988). The precise mechanism by which ET-1 induces an intracellular response leading to vasoconstriction remains somewhat unclear (Masaki 2004; Schinelli 2006). However, ET-1 binding to vascular ET_A receptors has been shown to cause a transient increase in the intracellular concentration of free Ca^{2+} ions due to activation of phospholipase C, followed by a sustained increase that results from Ca^{2+} influx from the extracellular space via ET_A receptor-mediated opening of VACCs (Miwa et al. 1999; Takuwa et al. 1990). Calcium elevation within the vascular smooth muscle cells induces vasoconstriction through the binding of free Ca^{2+} to calmodulin in the cytosol, forming a complex that then binds myosin light-chain kinase which, in an energy-dependent manner, ultimately induces tension in the muscle via the initiation of cross-bridge activity (Vander et al. 2001). The result is a continuous contraction of the vascular smooth muscle tissue (Masaki 2004).

3.1.2 Endothelin-1 Injections: Effect of Dosage on Local Cerebral Blood Flow

Endothelin-1 induces contractile responses in the vasculature that are characteristically long lasting and resistant to wash out (Yanagisawa et al. 1988). The reduction in blood flow following ET-1 microinjection is rapid and severe, but not immediate; it takes between 5-20 minutes after injection to initiate (Fuxe et al. 1997; Macrae et al. 1993; Nikolova et al. 2009).

Cerebral blood flow levels within a localized area of brain after direct, multiple microinjections of ET-1 to that specific locale have not been extensively studied. Nonetheless, a few studies have taken such measurements directly within areas of the cortex and/or striatum to be microinjected with ET-1 (Fuxe et al. 1992; Fuxe et al. 1997; Ueki et al. 1993; Windle et al. 2006). These select studies provide some insight into the amount of ET-1 needed to occlude the local cerebral vasculature and maintain this occlusion at ischemic levels for an adequate amount of time (i.e. enough to produce permanent tissue damage).

In halothane anesthetized, male SD rats, Fuxe and colleagues (1992) examined the effects of intrastriatal injection of ET-1 on local striatal blood flow. Results from this study demonstrated that ET-1 (860pmol dissolved in 1μl of aCSF) produced approximately a 60% reduction in striatal blood flow relative to control, which was observed within 20 minutes and largely maintained throughout the 3 hr observation period (40% reduction by 3 hr). This dosage of ET-1 produced consistent striatal lesions (Fuxe et al. 1992). Similar results were generated by Ueki and colleagues (1993) using an almost identical protocol. Microinjections of ET-1 (40pmol/μl and 400pmol/μl,

dissolved in 1μl of aCSF) directly to the fronto-parietal cortex reduced local blood flow in halothane anesthetized, male SD rats (Fuxe et al. 1997); however a concentration of 40pmol/μl was minimally effective compared to a concentration of 400pmol/μl (~ 25% reductions in blood flow for 45-60 min vs ~80% reduction for at least 3 hr.) (Fuxe et al. 1997). In this study, ET-1 delivered at 400pmol/μl resulted in cortical lesions that were approximately 40% larger than those produced with 40pmol/μl (Fuxe et al. 1997), although both concentrations were reported to produce very small lesions (~3 and 5mm^3) (Fuxe et al. 1997).

Using halothane anethetized, male Long-Evans hooded rats, Windle and colleagues (2006) employed a multi-injection application of ET-1 (2 cortical injections of 400pmol/μl, 2μl volume/injection; 1 striatal injection of equal concentration and volume) to the forelimb cortex and lateral striatum. Thus, a total of 2400pmol of ET-1 in 6μl of saline was injected to the brain in this study. Baseline levels of absolute cerebral blood flow were approximately 250ml/100g/min for all measurement points (Windle et al. 2006). Data revealed that this approach reduces blood flow to ≥ 50ml/100g/min in brain surrounding the striatal injection for approximately 3 hr, after which it gradually returns to baseline by 48 hr. Blood flow in brain directly surrounding cortical injection sites (i.e. the suspected core) was also reduced to this level, but for 24 hr and was still below baseline at 48 hr (~80ml/100g/min at 48 hr) (Windle et al. 2006). Lateral cortex surrounding the cortical injection sites exhibited a similar initial reduction but only maintained this decrease for 3 hr; gradual reperfusion to baseline was seen over 48 hr (suggesting the existence of a penumbra). The authors report that this ET-1 protocol consistently resulted in histological damage to the cortex and dorsolateral sector of the

striatum (Windle et al. 2006)

3.1.3 Experimental Literature: Stereotaxic Correlates of Forelimb Representation in the Rat Brain

The rat forelimb cortex, like most other body part representations, is partitioned into motor and sensory regions. Located in the granular cortex, is an area of that contains numerous small receptive fields that represent the forelimb somatosensory area; stereotaxically situated within ~ AP +2.0 mm to -1.0 mm and ML ±3.8 mm to ±5.0 mm (posteriorly) and ML ±4.2 mm to ±5.0 mm (anteriorly) (Chappin and Lin 1984; Hall and Lindholm 1974; Paxinos and Watson 2007; Remple et al. 2003). Medially adjacent to this sensory area lies a sensorimotor overlap region (i.e. each cortical column within this area contains both a granular layer with small cutaneous receptive fields and a large pyramidal/motor layer); located between ~ AP +1.5 mm to 0.0 mm and ML ±2.9 mm to ±4.2 mm (Donoghue and Wise 1982; Hall and Lindholm 1974). Medial and anterior to this overlap zone is a large, predominately motor representation of the forelimb found within ~ AP +2.8 mm to +1.5 mm and ML ±1.8 mm to ±4.2 mm, as well as ~ AP +1.5 mm to 0.0 mm and ML ±1.8 mm to ±2.9 mm (Donoghue and Wise 1982; Gioanni and Lamarche 1985; Hall and Lindholm 1974; Neafsey and Sievert 1982; Neafsey et al. 1986; Ramanathan et al. 2006). Collectively, these 3 sub-regions are referred to as the caudal forelimb region (CFL) (Barth et al. 1990; Neafsey and Sievert 1982). A distinct, entirely motor region - the rostral forelimb region (RFL) - has been shown to exist within the frontal cortex; situated between ~ AP +4.0 mm to +2.8 mm and, at most, ML ±1.5 mm to

±3.5 mm (Neafsey and Sievert 1982; Neafsey et al. 1986; Gioanni and Lamarche 1985; Ramanathan et al. 2006).

It is debatable whether the RFL and CFL are a continuous anatomical structure or simply contiguous to each other anatomically. Moreover, based on the types of forelimb movement evoked subsequent to electrical stimulation within these areas, the CFL mainly elicits gross movements originating from the shoulder and/or elbow, whereas the RFL almost exclusively produces more fine movements of the wrist and digits (Neafsey et al. 1986; Ramanathan et al. 2006). Finally, and importantly, the stereotaxic location of both forelimb areas directly correlates with the anatomical location of very dense patches of retrogradely labeled corticospinal neurons that project to the cervical enlargement (Donoghue and Wise 1982; Neafsey and Sievert 1982). Figure 3.1 illustrates the general stereotaxic coordinates for each of the above described forelimb cortical areas.

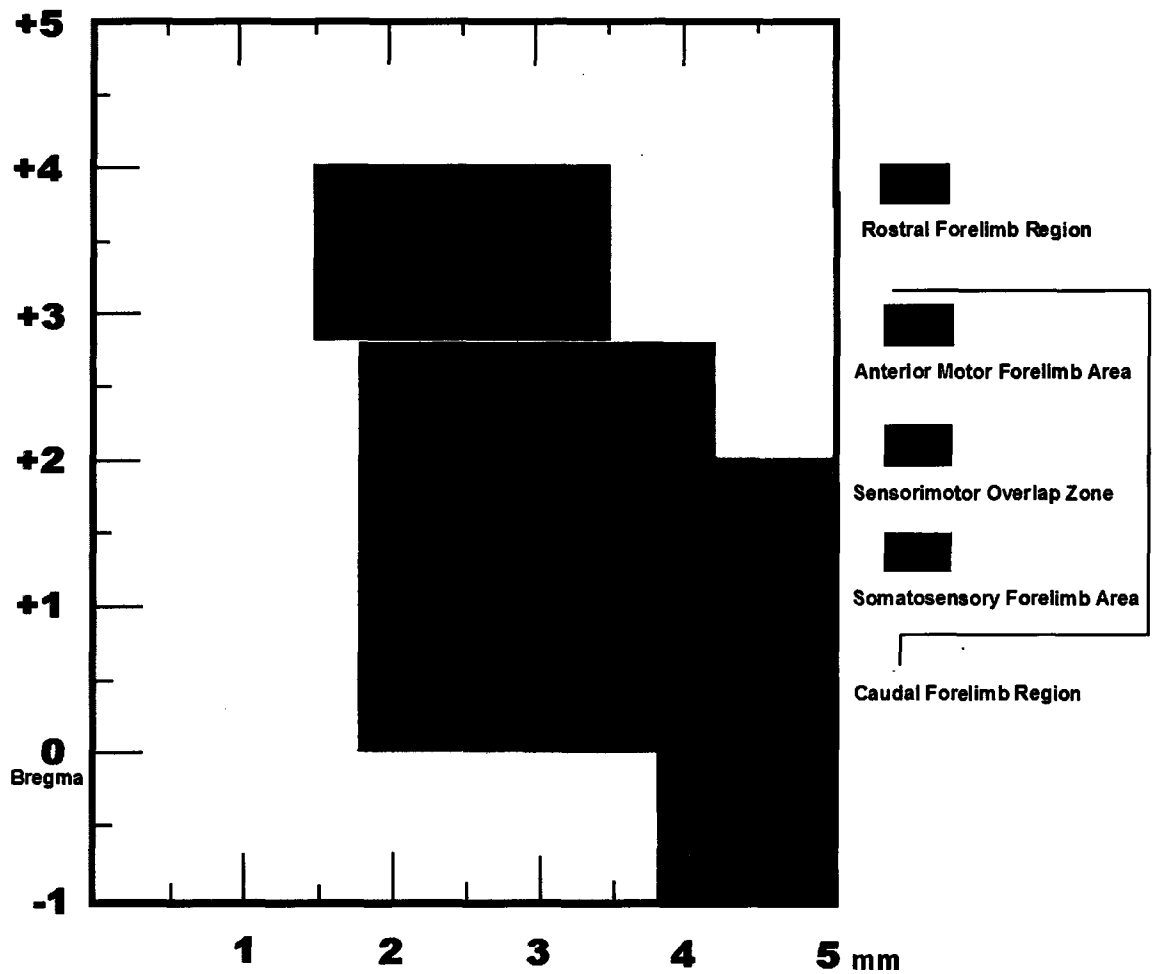

Figure 3.1. General stereotaxic coordinates associated with different forelimb cortical areas. Figure created by Andrew Hume using Microsoft Word® and Paint®. A conservative adaptation based on the collective examination of all publications cited above within this section.

The striatum (or caudoputamen) is believed to have 3 main functional divisions – motor, associative, and limbic (White 2009). This divisional nomenclature is based on the scientific concept that corticostriatal innervation organizes the function of the striatum (Brown et al. 1998; Brown and Sharp 1995; Ebrahimi et al. 1992; Ebrahimi-

Gailard et al. 1994; White 2009). In rats, the motor division is largely anatomically mapped to the dorsolateral sector of the striatum (Brown et al. 1998; Ebrahimi et al. 1992). This aspect of the caudoputamen receives a large amount of organized input from both sensory and motor cortex subserving the limbs and vibrissae (Brown et al. 1998; Brown and Sharp 1995; Ebrahimi et al. 1992; Ebrahimi-Gailard et al. 1994). Regarding stereotaxic placement within the dorsolateral sector of the striatum, it has been documented that forelimb representation is most prominent anterior to bregma, between ~ AP +1.6 mm to 0.0 mm and ML ±3.0 to ±5.0, yet activation patterns are seen throughout much of the rostral-caudal extent of the lateral striatum (Brown et al. 1998; Brown and Sharp 1995; Cho and West 1997; Ebrahimi et al. 1992; Ebrahimi-Gailard et al. 1994). Figure 3.2 depicts the general territory of the dorsolateral striatum, illustrated at AP + 1.0 mm. All together, the literature detailed above serves as a valuable experimental resource for studies which aim to produce ischemic damage to forelimb sensorimotor brain areas in order to generate acceptable levels of behavioural impairment.

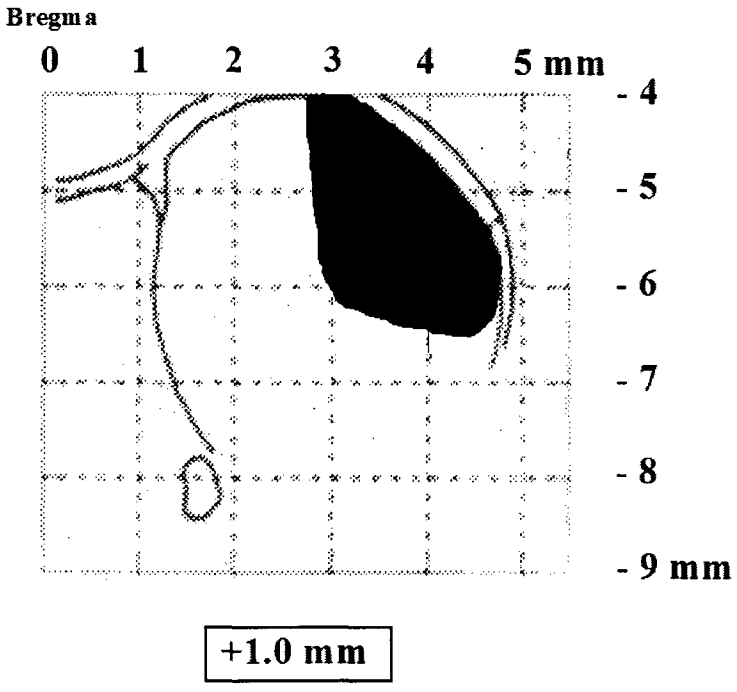

Figure 3.2. General stereotaxic coordinates associated with the territory of the dorsolateral striatum. Coordinates on the y-axis begin at – 4.0 mm because they are relative to skull surface. Figure created by Andrew Hume using Microsoft Word® and Paint®. Adapted from figures presented in Brown and Sharp 1995 and Ebrahimi et al. 1992.

3.1.4 Experimental Use of the ET-1 Model

The ET-1 model, relative to more traditional models of focal ischemia, is an understudied method for inducing experimental stroke. Nonetheless, several investigators have evaluated the ET-1 model on its ability to generate permanent histological damage to forelimb regions of the brain and produce impairment of forelimb function on a number of established sensorimotor tests (Adkins et al. 2004; Gilmour et al. 2004; Giuliani et al. 2007; Hewlett and Corbett 2006; Luke et al. 2004; Maldonado et al.

2008; Windle and Corbett 2005). However, no surgical standard currently exists for the ET-1 model. Several labs have utilized distinct ET-1 injection protocols to produce ischemic damage to forelimb regions of the brain (Adkins et al. 2004; Gilmour et al. 2004; Hewlett and Corbett 2006). Thus, it is an important experimental aim to provide further evidence regarding the appropriate surgical application of this model for stroke research.

3.1.5 Overall Goal of Preliminary Experimentation

The primary goal of preliminary experimentation was to develop an ET-1 model that produced reasonably stable (i.e. size and location) unilateral ischemic lesions to the forelimb sensorimotor cortex and dorsolateral striatum as well as unilateral functional forelimb impairments characterized by a measurable recovery period. Once accomplished, this model was to be employed in the definitive neuroprotection experiment using a conventional approach to the blockade of NMDA receptor-mediated excitotoxicity *in vivo*.

3.1.6 Initial Attempts with a Multi-injection ET-1 Protocol

A multi-injection approach was utilized due to the reported ability of this method to produce ischemic lesions that are well placed to the forelimb sensorimotor cortex and dorsolateral striatum, and generate contralateral forelimb impairment of sufficient duration (Gilmour et al. 2004; Gilmour et al. 2005; Hewlett and Corbett 2006; Windle

and Corbett 2005; Windle et al. 2006). A small experiment (5 rats) using an ET-1 stereotaxic surgical protocol described by Hewlett and Corbett (2006), Windle and Corbett (2005) and Windle and colleagues (2006) was employed. This protocol utilized a 400pmol/μl concentration of ET-1 and consisted of 2 injections (2μl each) to the forelimb sensorimotor cortex (AP +2.3 mm, 0.0 mm ML ± 2.5 mm DV -2.3 mm) and 1 injection (2μl) to the lateral striatum (AP +0.7 mm ML ±3.8 mm DV -7.0 mm). Approximately 24 hr post-surgery, brains were matrix sectioned and subsequently stained with TTC (sectioning and staining details in section 2.6.2). Infarct patterns were visually compared to the Paxinos and Watson stereotaxic atlas (Paxinos and Watson 2007) for assessment of damage to forelimb representation areas of the brain.

Visual inspection of these infarcts revealed unsatisfactory results (i.e. 3 rats with ischemic damage extending well into the parietal cortex as well as substantial damage to the contralesional cingulate cortex and 2 rats with almost unidentifiable cortical damage). Striatal lesions, however, were generally well placed but often produced substantial damage to the adjacent piriform cortex. The multi-injection protocol described by Gilmour and associates (2004, 2005) was not employed due to reports of small cortical lesion volume relative to other intracerebral ET-1 applications (Hewlett and Corbett 2006; Windle and Corbett 2005; Windle et al. 2006), and enduring behavioural deficits only in select tests of skilled forelimb function (Gilmour et al. 2004; Gilmour et al. 2005). Hence, it was considered necessary for the experimenter to establish a multi-injection intracerebral injection method in our lab.

Using the same concentration of ET-1 (400pmol/μl), and injection volumes and stereotaxic coordinates corresponding to cortical 1) AP +2.2, ML +2.8, DV, -2.5 (3μl)

(anterior motor forelimb area); cortical 2) AP +0.8, ML +3.6, DV -2.5 (3μl) (sensorimotor overlap zone); and striatal) AP +0.8, ML +3.6, DV -6.0 (1.5μl), results from several early experiments (24 hr to 3 day survival periods) demonstrated that striatal lesions were typically well placed to the lateral striatum but cortical lesion variability was high with this approach (data not shown). In addition, moderate contralesional hemispheric damage and a substantial degree of bilateral forelimb placing impairment were common results. Preliminary experiment 1 (below) represents the first of 2 controlled studies designed to address these issues.

3.2 Preliminary Experiment 1: Examining the Effect of Different Volumes and Concentrations of ET-1 Injections on the Degree of Brain Injury and Forelimb Impairment

3.2.1 Study Objective

This experiment was designed to test smaller amounts of ET-1 (using the same stereotaxic coordinates previously employed) with the prediction that these lower amounts would yield a pattern of histological damage largely confined to the ipsilesional hemisphere, more stable ischemic lesions across animals in terms of size and location, and a functional deficit profile more amenable to measuring asymmetrical forelimb behaviour (i.e. reduced ipsilesional impairment).

3.2.2 Methods and Materials

3.2.2.1 Surgery and Experimental Conditions

Endothelin-1 (400pmol/μl and 200pmol/μl) was dissolved in sterile distilled H_2O and microinjected to the forelimb sensorimotor cortex and dorsolateral striatum. Two cortical injections and one striatal injection were administered. Stereotaxic injection coordinates were, cortical 1) AP +2.2, ML +2.8, DV, -2.5; cortical 2) AP +0.8, ML +3.6, DV -2.5; striatal AP +0.8, ML +3.6, DV -6.0. In group 1 (n = 8), ET-1 was microinjected at a concentration of 400pmol/μl; volume of ET-1 solution was 2μl, 2μl, and 1μl for cortical 1, cortical 2, and the striatal injection, respectively. For group 2 (n = 8), ET-1 was microinjected at a concentration of 400pmol/μl; the injection volume was 1μl, 1μl, and 1μl for cortical 1, cortical 2, and the striatal injection. Group 3 (n = 8) received ET-1 microinjections at a concentration of 200pmol/μl; administered in a volume of 2μl, 2μl, and 1μl for cortical 1, cortical 2, and the striatal injection. For further details see section 2.3.

3.2.2.2 Histology and Infarct Estimation

At ~72 hr post ET-1 injection, brains were removed and immediately sliced into ten 1mm coronal sections using a brain matrix, and subsequently stained with TTC for infarct analysis (for further details see sections 2.6.1, 2.6.2, and 2.7).

3.2.2.3 Behavioural Testing and Body Weight Measurements

Animals were tested one day prior to ET-1 surgery and at approximately 24, 48, and 72 hr after surgery on tests of vibrissae- and tactile-stimulated forelimb placing as well as the forelimb postural reflex test. Animals were weighed prior to ET-1

injection and for the subsequent 3 days of behavioural testing (for further details see sections 2.4, 2.4.1, 2.4.2, and 2.5).

3.2.3 Results

3.2.3.1 Infarct Data

There was no significant difference between any of the groups on measures of total infarct volume ($F_{(2,21)} = 0.745$, $P = 0.487$), cortical infarct volume ($F_{(2,21)} = 0.678$, $P = 0.518$), or striatal infarct volume ($F_{(2,21)} = 0.910$, $P = 0.418$). Mean cortical, striatal, and total infarct volumes are presented below (Table II) for each of the 3 groups. Figure 3.3 illustrates the area of cortical and striatal damage across the stereotaxic levels of measurement for each ET-1 group.

Table II. Comparison of cortical, striatal, and total infarct volumes (mm^3) among groups receiving differing amounts of endothelin-1 to the cortex and striatum. Data are given as mean ± S.E.M.

ET-1 Group	Cortical Infarct	Striatal Infarct	**Total Infarct**
400pmol/µl(3µl)	57.8 ± 13.5	9.9 ± 1.5	67.7 ± 12.8
400pmol/µl(5µl)	87.3 ± 18.5	13.7 ± 2.2	101.0 ± 19.2
200pmol/µl(5µl)	80.3 ± 22.8	10.6 ± 2.6	90.9 ± 25.3

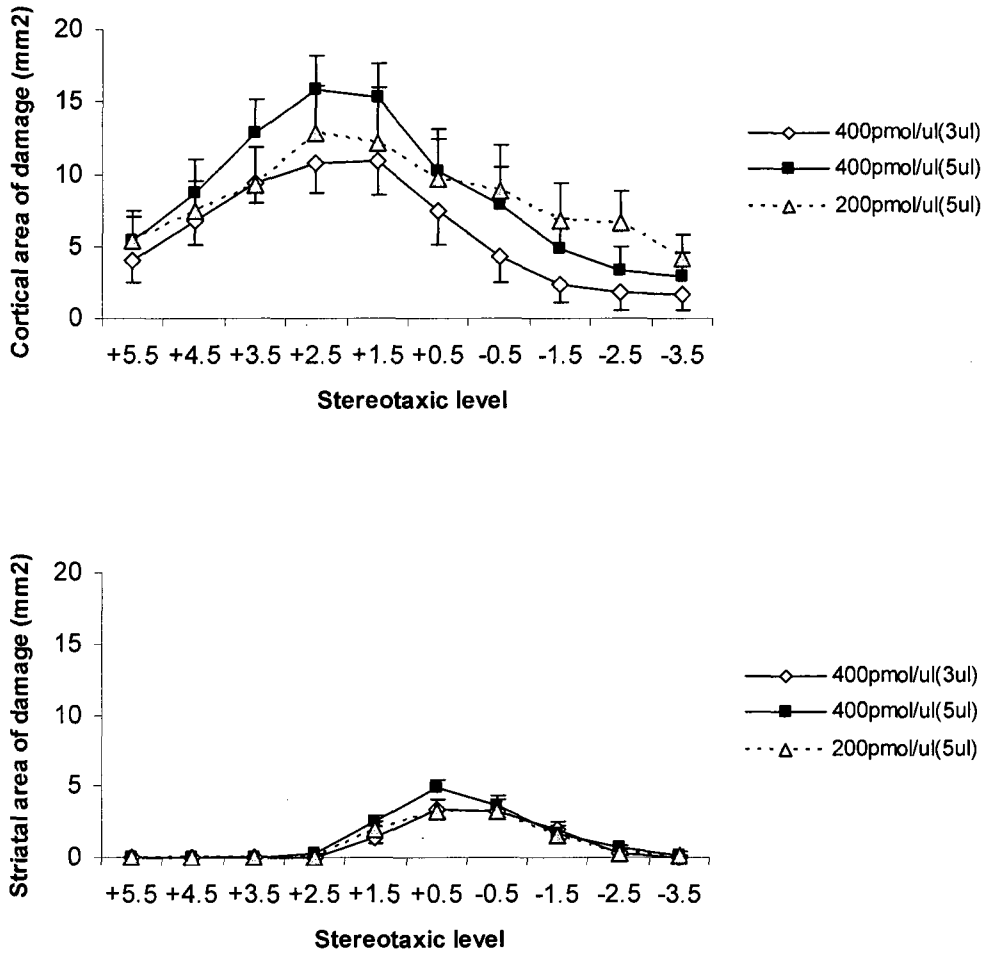

Figure 3.3. A comparison of each ET-1 group on mean (± S.E.M.) area of ischemic brain damage in cortex (top) and striatum (bottom) at 10 stereotaxic levels assessed 3 days following focal ischemic stroke induced by multiple intracerebral injections of ET-1.

Figure 3.4 illustrates TTC stained sections (at the area of most concentrated damage) of the largest, average, and smallest lesion pattern observed in groups receiving differing amounts of ET-1 to the forelimb sensorimotor cortex and dorsolateral striatum.

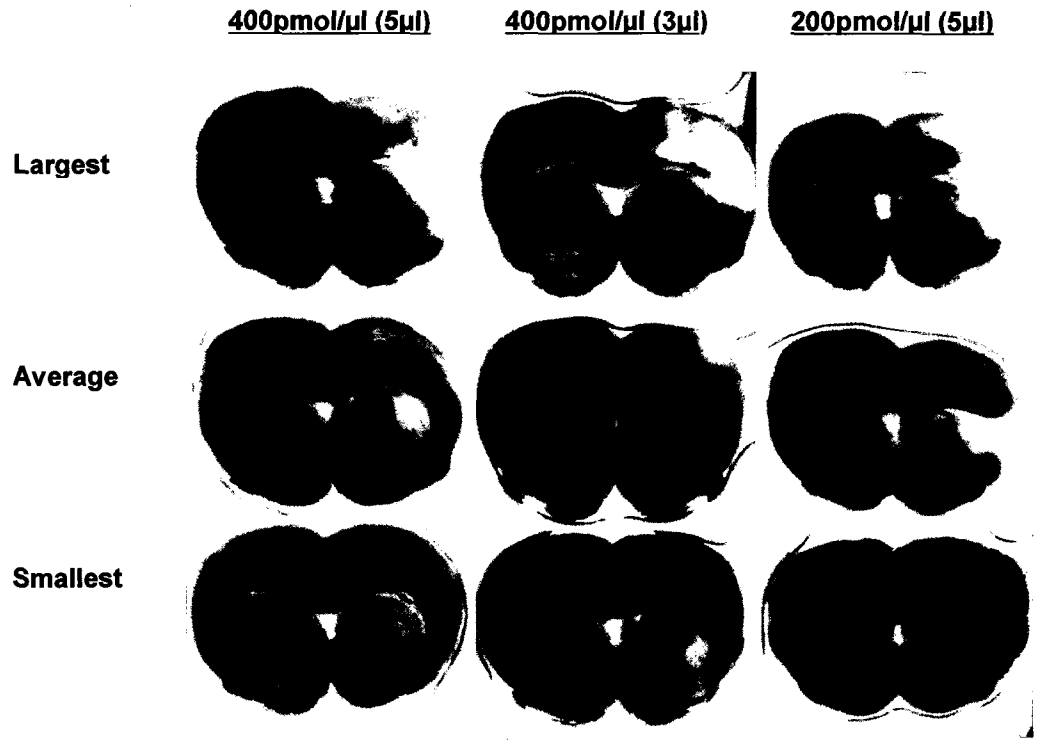

Figure 3.4. TTC stained sections (1 mm) of the largest, average, and smallest lesion pattern observed across ET-1 groups. 1 mm background.

Figure 3.3 demonstrates that all groups had cortical damage that was typically moderate in size and, as desired, most pronounced to the more anterior regions of the cortex (primarily between +3.5 to +0.5 relative to bregma); striatal lesions were relatively mild in size and accurately placed in relation to the desired stereotaxic coordinates. Incidence of ischemic damage to the contralesional hemisphere was rare and typically mild [1 animal at 400pmol/µl (3µl), 2 animals at 400pmol/µl (5µl), and 1 animal at 200pmol/µl (5µl)]. Adequate damage to the forelimb sensorimotor cortex was observed in 88%, 88%, and 75% of the 400pmol/µl (3µl), 400pmol/µl (5µl), and 200pmol/µl (5µl) groups, respectively. In these respective groups, 100%, 100 %, and 75% of rats had sufficient injury to the lateral striatum. Still, 50%, 50%, and 75% of animals in these

respective ET-1 groups also exhibited ample ischemic damage to the lateral sensory cortex (ipsilesional) which was evident, to varying degrees, across most of the rostral-caudal extent of infarct measurement. In contrast, the striatal lesions were typically well contained to the more lateral regions of the striatum for all groups.

3.2.3.2 Behavioural and Body Weight Data

No significant group differences were evident for vibrissae-stimulated forelimb placing ($F_{(2,21)} = 1.174$, $P = 0.329$), tactile-stimulated forelimb placing ($F_{(2,21)} = 0.655$, $P = 0.530$), or FPR [$\chi^2(2) = 2.286$, $P = 0.319$ (post-surgery day 1), $\chi^2(2) = 4.200$, $P = 0.122$ (day 2), $\chi^2(2) = 3.537$, $P = 0.171$ (day 3)]. There was a significant effect of Day for body weight and for all behavioural tests (with the exception of no significant effects found with tactile placing data for the iplsilateral forelimb). All rats showed a drop in body weight relative to pre-surgery levels which approached or exceeded pre-surgery measurements by day 2 post-surgery (see Table III) and, aside from ipsilateral placing performance, most rats demonstrated reduced behavioural performance relative to pre-surgery scores (see Table IV and Figure 3.5).

Table III. Comparison of mean (± S.E.M.) changes from pre-surgery body weight (grams) among ET-1 groups over 3 days of testing.

ET-1 Group	Day1 post-surgery	Day2 post-surgery	Day3 post-surgery
400pmol/μl(3μl)	-3.1 ± 1.7	+5.0 ± 2.0	+10.9 ± 2.1
400pmol/μl(5μl)	-9.3 ± 1.3	-0.9 ± 1.5	+6.4 ± 1.5
200pmol/μl(5μl)	-6.5 ± 2.0	+1.6 ± 2.0	+7.8 ± 2.7

Table IV. Comparison among ET-1 groups on percent incidence of observed deficit in forelimb postural reflex (percentage of impaired animals in each group).

Treatment Group	Pre-surgery	Day1post	Day2post	Day3post
400pmol/μl(3μl)	0%	87.5%	87.5%	75%
400pmol/μl(5μl)	0%	100%	100%	100%
200pmol/μl(5μl)	0%	75%	62.5%	62.5%

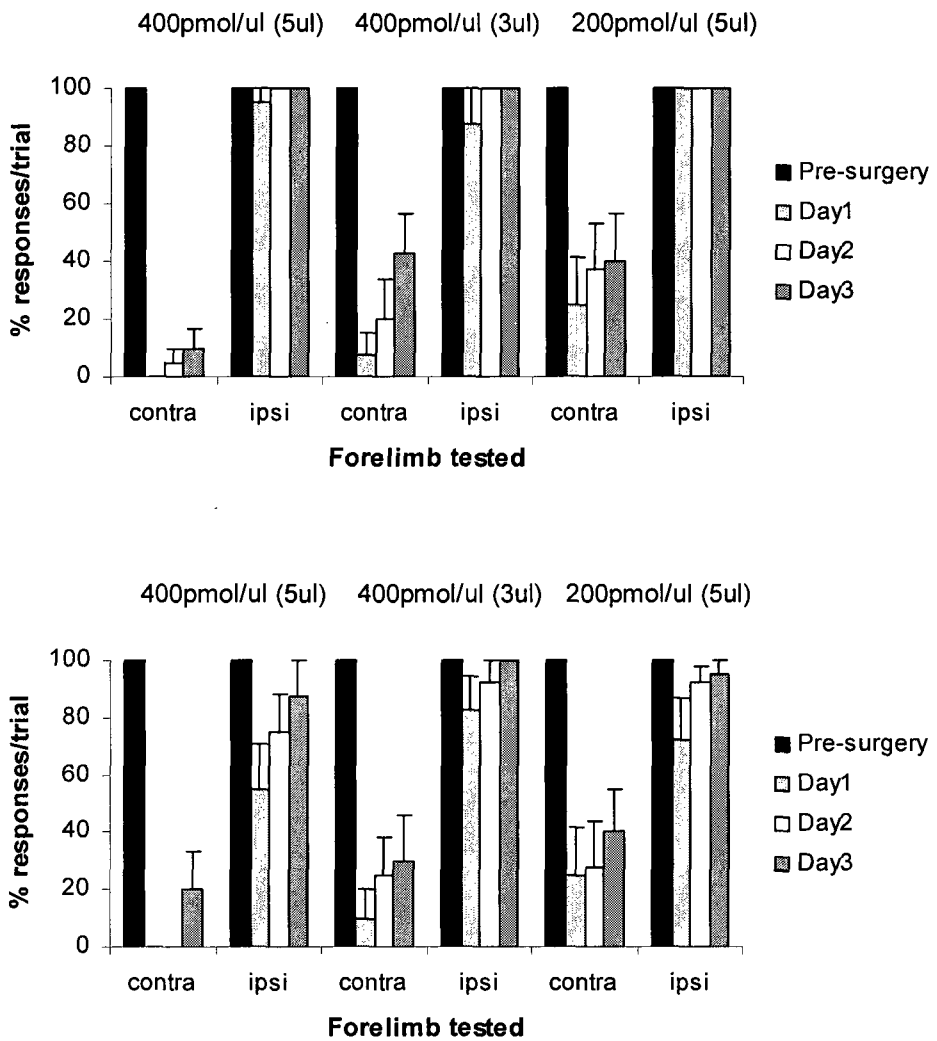

Figure 3.5. Comparison of mean (± S.E.M.) forelimb placing accuracy pre and over 3 days post surgery among ET-1 groups. Contralateral and ipsilateral tactile-stimulated placing (top); Contralateral and ipsilateral vibrissae-stimulated placing (bottom).

Although differences between ET-1 groups were not significant on behavioural measures, some additional observations were noted. The degree of FPR impairment was above 60% (i.e. sufficient deficit) for all groups (Table IV). All groups considered, contralateral forelimb placing accuracy was reduced to approximately ≥ 40% over the 3 days post-surgery (i.e. sufficient deficit) (Figure 3.5). Conversely, ipsilateral impairment

on tactile-stimulated placing behaviour was rare, while ipsilateral impairment on vibrissae-stimulated placing was generally more prominent. Finally, for all groups, ipsilateral forelimb deficits were characterized by a consistent improvement in performance, with a tendency to either reach or approach pre-surgery levels by 3 days post-surgery (Figure 3.5).

3.2.3.3 Mortality Rates and Model Success

The mortality rate for the total experiment was 0%. The model success rates for the respective 400pmol/μl (3μl), 400pmol/μl (5μl), and 200pmol/μl (5μl) groups were 88%, 100%, and 75%. The pooled success rate was 88% (see section 2.8 for details pertaining to mortality and model success rate calculations).

3.2.4 Concluding Remarks

The primary findings of this preliminary experiment were four-fold. First, the smaller volumes of injected ET-1 generally produced only mild levels of bi-hemispheric ischemic brain damage and typically yielded ipsilateral forelimb placing deficits of only mild to moderate magnitude and duration. These are salient observations for 2 main reasons: 1) damage to the contralateral hemisphere may be difficult to quantify (and thus control for) since it appeared to result in optically subtle deviations from normal staining intensity (personal laboratory experience), and 2) additional behavioural tests to be incorporated in subsequent experimentation directly rely on the exemplification of asymmetrical forelimb behaviour for the appropriate interpretation of data (Schallert et al. 2000; Sughrue et al. 2006). Secondly, taking all factors into consideration, these data

suggest that the ET-1 injection protocol of 400pmol/μl (5μl) may be the most ideal method tested up to this point of experimentation, given that this group demonstrated contralateral forelimb impairment that was marked in degree and duration (see Table IV, Figure 3.5), and had a model success rate of 100%. Third, these results imply that a concentration of 400pmol/μl may be required to produce consistent ischemic lesions and forelimb behavioural deficits in this model; thus, ET-1 injected at a concentration of 400pmol/μl was considered an appropriate experimental variable to maintain throughout further experimentation. Finally, and most importantly, this experiment uncovered that the lateral stereotaxic placement of the second cortical injection was likely the primary cause of the inconsistency in lesion location (discussed below).

Although the 400pmol/μl (5μl) and 400pmol/μl (3μl) groups appeared to have improved lesion reproducibility compared to the 200pmol/μl (5μl) group (i.e. see S.E.M. values in Table II), these groups were still marked by considerable variability in cortical lesion extent. This is an important observation because adequate lesion reproducibility is crucial for neuroprotection studies (Sharkey and Butcher 1995). In the present study, despite a discrepancy in mean values for cortical lesion volume of ~ 30% (see Table II), and a reasonable *n* of 8 (Moyanova et al. 2007a; O'Neill et al. 2000a; Sharkey et al. 1994; Sharkey and Butcher 1995), groups were far from being significantly different ($P = 0.518$ for cortical infarct volume) thus suggesting that the model variability was too pronounced to detect any group differences during subsequent experimentation (i.e. post-preliminary studies). With respect to lesion pattern, this variability was characterized by approximately half of all animals tested demonstrating extensive damage along much of the rostral-caudal plane of the lateral sensory cortex, regardless of the volume or

concentration of ET-1 injected (see Figure 3.3, top row, for illustration of extensive damage to the lateral cortex in all groups).

In the rat brain, subsequent to branching from the common carotid artery, the middle cerebral artery extends up the ventral-lateral aspect of the cortex at approximately +1.0 mm anterior from bregma, where it then extends multiple branches of its own across the rostral, middle, and caudal aspects of the lateral cortex, ultimately terminating in myriad end-arterioles that perfuse much of the medial dorsal surface of the brain (Paxinos 2004; numerous personal observations of intact rat brains in the laboratory). Therefore, despite accurately centering the second cortical lesion within the sensorimotor overlap zone of the CFL, it was speculated that the injection coordinates were too lateral, possibly resulting in the injected ET-1 occluding large branches of the MCA to varying degrees in some animals, thus producing lesions that were up to 10 fold larger than those that had more medial damage within the same group. The branching architecture of the MCA is unique among individual rats (Paxinos 2004; personal observations in the laboratory). Thus, if the injection site was indeed the confounding factor, it is not surprising that only about half of the animals showed such extensive ischemic damage to the lateral cortex. In order to correct this suspected issue, a small study was employed using a protocol very similar to the 400pmol/μl (5μl) group.

3.3 Preliminary Experiment 2: Examining the Utility of an ET-1 Protocol for Producing Isolated Damage to Forelimb Brain Regions and Contralateral Forelimb Impairment on a Variety of Sensorimotor Tests

3.3.1 Study Objective

The objective of this preliminary study was to avoid the large discrepancies in infarct size observed in Preliminary experiment 1 (thought to be the result of unintended large arterial occlusion in some animals) by utilizing an ET-1 surgical protocol similar to the method deemed most favorable from Preliminary experiment 1 (0.5µl was added to enhance damage to CFL) but with a second cortical injection that was 0.7 mm more medial. Based on all of the above observations from Preliminary experiment 1 (and earlier lab work), this new protocol was deemed likely to substantially improve upon this final, key experimental barrier. Thus, at this juncture, it was considered appropriate to further characterize our model by initiating a longer survival period that included a more extensive battery of forelimb behavioural tests.

3.3.2 Methods and Materials

3.3.2.1 Surgery and Experimental Conditions

Endothelin-1 (400pmol/µl) was dissolved in sterile H_2O and microinjected to the forelimb sensorimotor cortex and dorsolateral striatum. Two cortical injections and one striatal injection were administered. Stereotaxic injection coordinates were:

cortical 1) AP +2.0, ML -2.9, DV, -2.5; cortical 2) AP +1.0, ML -2.9, DV -2.5; striatal AP +0.9, ML -3.6, DV -6.0. In the Endothelin-1 group (n = 5) ET-1 was microinjected in a volume of 2µl, 2.5µl, and 1µl for cortical 1, cortical 2, and the striatal injection, respectively. Sham-operated rats (n = 3) served as controls. For further details see section 2.3.

3.3.2.2 Behavioural Testing and Body Weight Measurements

Contingent on the existence of an enduring behavioural deficit profile, for this initial longer-term survival experiment it was decided a priori that rats would be tested for a minimum of 2 weeks post-surgery and possibly thereafter until recovery to pre-surgery levels was reinstated or close to reinstated. Behavioural data were tallied throughout the course of the experiment so that the experimenter could make a sound decision regarding the cessation of behavioural testing. Animals were tested one day prior to surgery and every day after surgery for 18 days on tests of vibrissae- and tactile-stimulated forelimb placing as well as the forelimb postural reflex test. Animals were tested one day prior to surgery, and on day 1, 3, 6, 10, and 14 post-surgery for performance on the Schallert cylinder test and a modified version of the adhesive removal test (see sections 2.4 – 2.4.4). Animals were weighed prior to surgery and every day after surgery for the duration of the experiment. For further details see section 2.5.

3.3.2.3 Histology and Infarct Estimation

Nineteen days post-surgery, brains were removed and immediately placed in 100ml of aCSF and, without delay, fresh brain tissue was sectioned into 500µm coronal sections using a Vibratome 1000 Plus. As with previous preliminary experiments coronal sections were to be stained with TTC for infarct analysis. Harvested brains from

animals that underwent sham surgery were sectioned and stained appropriately; however, the initial 3 brains from rats in the ET-1 group were unable to be accurately sectioned (or quantified) due to pronounced tissue disintegration, and technical error. However, by this point in time, the experimenter was adept at identifying the general locale of the forelimb sensorimotor cortex as well as visually recognizing the basic location and extent of the cortical lesions upon brain removal from the skull. In fresh unstained brains, lesioned tissue consistently appears either as a darkened cavity (typically at the center of ischemic damage) and/or an area of pallor relative to intact brain tissue. These visual observations are commented on in section 3.3.3.1 below.

3.3.3 Results

3.3.3.1 Cortical Lesion Observations

Visual estimations suggested that all of the ET-1 rats had sufficient ischemic damage to the forelimb sensorimotor cortex, with 2 of the 5 demonstrating what appeared to be mild-moderate damage of sensory cortex lateral to the forelimb sensory cortex, but still contained within the desired stereotaxic AP range (~ +3.5 to 0.0) (i.e. not spreading to the parietal and occipital cortices as seen in previous preliminary experiments). Overall, the observations appeared to reflect one of the primary aims of preliminary experimentation – reasonably contained and adequate ischemic damage to the forelimb sensorimotor cortex. Figure 3.6 illustrates TTC stained (500μm thickness) sections from one of the initial vibratome-sectioned brains (tissue suffers from some

mechanical tearing) of an ET-1 rat compared to a vibratome-sectioned brain from a sham rat.

ET-1 rat **Sham rat**

Figure 3.6. Comparison of TTC stained coronal sections (500μm) between an ET-1 surgery rat and sham control rat, taken from the area of most concentrated damage seen in the ET-1 rat. Note the ischemic damage isolated to forelimb representation areas. Short (top) arrow points to cortical lesion and longer (bottom) arrow indicates the striatal lesion. 1 mm background.

3.3.3.2 Body Weight Data

There was a significant effect of Day ($F_{(17,102)} = 321.629$, $P = 0.000$) for measures of body weight. Rats undergoing ET-1 surgery demonstrated a non-significant pattern of moderately reduced body weight relative to pre-surgery levels which exceeded pre-surgery measurements by day 3 post-surgery. In contrast, sham-operated rats showed no tendency toward reduced body weight after surgery (see Figure 3.7).

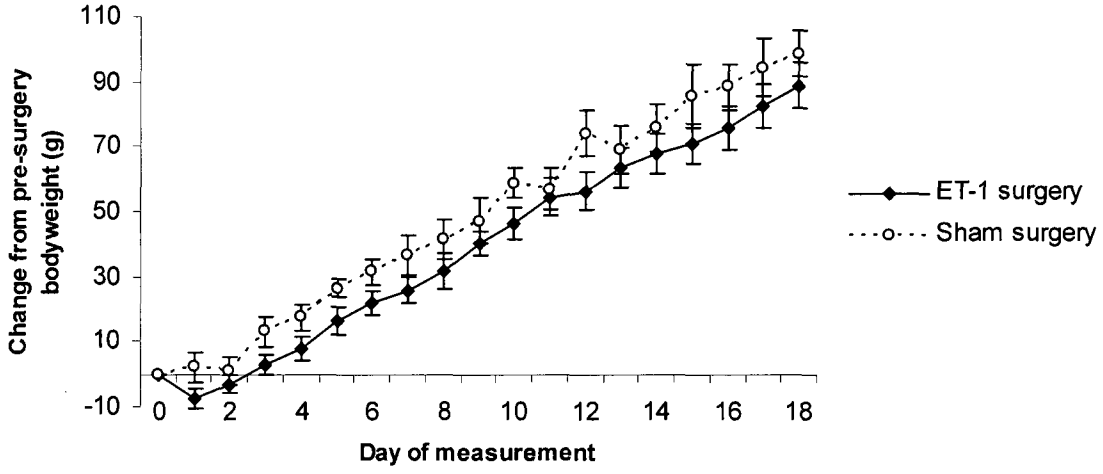

Figure 3.7. Comparison of mean (± S.E.M.) changes from pre-surgery body weight (grams) between ET-1 surgery and Sham surgery groups over 18 days of testing.

3.3.3.3 Forelimb Postural Reflex and Forelimb Placing Data

Pearson Chi-square analysis revealed that ET-1 surgery produced deficits in FPR; the ET-1 surgery group remained significantly impaired relative to sham rats for the first 10 days post-surgery ($P < 0.05$) (individual $\chi 2$ results not shown due to 19 values calculated) . Sham animals did not demonstrate FPR deficits at any time point. Table V illustrates the percent incidence of FPR deficit for the two groups pre-surgery and on days 1, 4, 11, 12, 13 and 17 post-surgery (representing time points in which the percent incidence of FPR altered for the ET-1 surgery group).

Table V. Comparison between ET-1 and sham groups on percent incidence of observed deficit in forelimb postural reflex (percentage of impaired animals in each group) (* indicates a significant difference from Sham control group; $P < 0.05$)

Surgery	*Pre-surgery*	*Day1 post*	*Day4 post*	*Day11 post*	*Day12 post*	*Day13 post*	*Day17 post*
ET-1	0%	100%*	80%*	60%	40%	20%	0%
Sham	0%	0%	0%	0%	0%	0%	0%

There was a main effect of Day ($F_{(18,108)} = 6.626$, $P = 0.000$) and Group ($F_{(1,6)} = 12.956$, $P = 0.011$), as well as a significant interaction of Group by Day ($F_{(18,108)} = 6.626$, $P = 0.000$) for tactile-stimulated forelimb placing behaviour. Rats with ET-1-induced lesions demonstrated significant impairments in tactile-stimulated forelimb placing accuracy over the first 7 days post-surgery ($P < 0.05$) when compared to sham-operated rats (which showed no impairment in placing accuracy at any time point). Similar results were obtained for vibrissae-stimulated placing. There were significant effects for Day ($F_{(18,108)} = 9.607$, $P = 0.000$) Group ($F_{(1,6)} = 20.578$, $P = 0.004$), and Group by Day interaction ($F_{(18,108)} = 9.607$, $P = 0.000$). ET-1 surgery rats, relative to sham rats, were significantly impaired on vibrissae-stimulated forelimb placing accuracy for the first 8 days post-surgery ($P < 0.05$) and also on post-surgery days 10 and 12 ($P < 0.05$). Figure 3.8 depicts both tactile- and vibrissae-stimulated contralateral forelimb placing accuracy for the two surgery groups over the course of behavioural testing. Neither group demonstrated any degree of deficit for ipsilateral tactile or vibrissae-stimulated placing accuracy at any time point. Thus, only contralateral responses are illustrated.

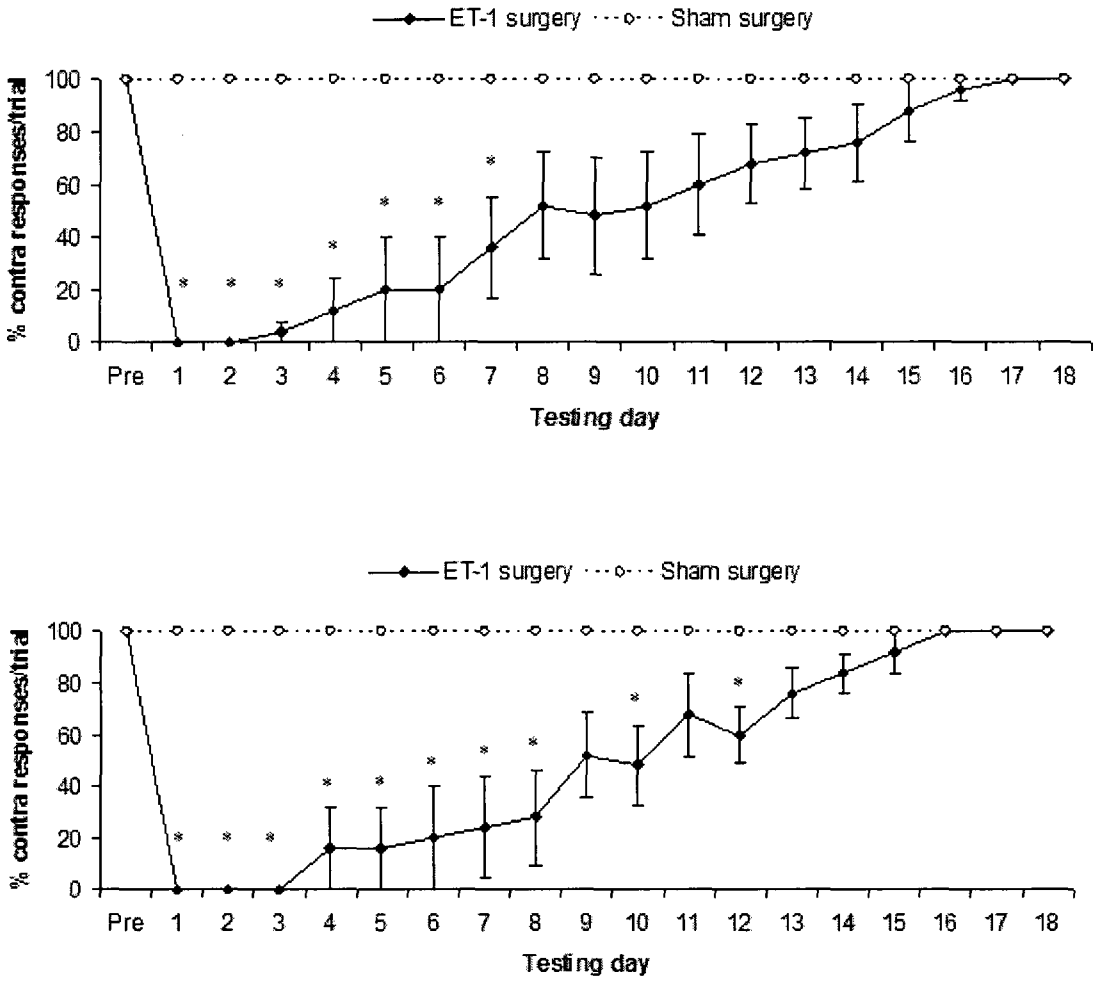

Figure 3.8. Comparison of forelimb placing accuracy pre and over 18 days post surgery between the ET-1 surgery and Sham surgery groups. Contralateral tactile-stimulated placing (top); Contralateral vibrissae-stimulated placing (bottom). Data are mean ± S.E.M. (* indicates a significant difference from sham group; $P < 0.05$).

3.3.3.4 Adhesive Removal and Cylinder Test Data

Testing on the modified adhesive removal test produced significant effects of Day ($F_{(5,30)} = 7.947$, $P = 0.000$), Group ($F_{(1,6)} = 22.236$, $P = 0.003$) and Group by Day interaction ($F_{(5,30)} = 8.289$, $P = 0.000$). Following ET-1 surgery, rats demonstrated a significant ipsilateral bias, exemplified as a reduced forelimb somatosensory

asymmetry ratio on days 1, 3, and 6 compared to shams. Sham-operated rats did not show any significant variation from equal attendance to both forelimbs across all testing days. Figure 3.9 illustrates the degree and duration of forelimb somatosensory impairment for the two surgery groups over the course of behavioural testing.

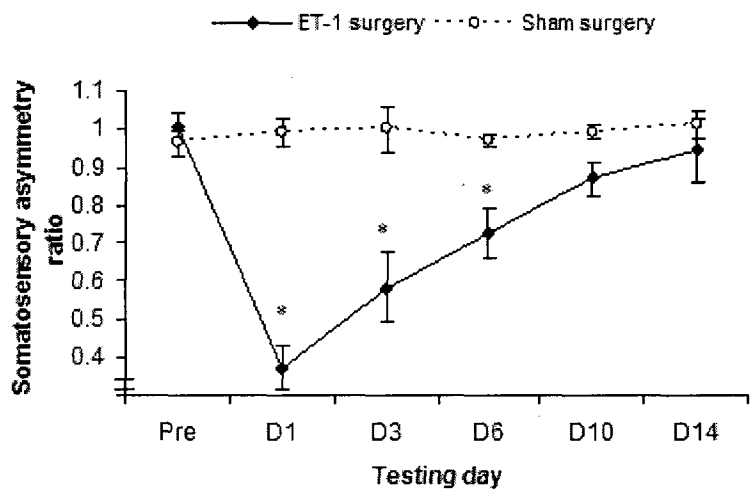

Figure 3.9. Comparison between ET-1 and sham groups on somatosensory asymmetry ratio scores for the modified adhesive removal test. Data are mean ± S.E.M. (* indicates a significant difference from sham group; $P < 0.05$).

For the cylinder test, there was a main effect of Group ($F_{(1,6)} = 14.093$, $P = 0.009$) and a significant interaction of Group by Day ($F_{(5,30)} = 3.464$, $P = 0.014$) for percent simultaneous use of both limbs for postural support. Following ET-1-induced lesions, rats demonstrated significant reductions in bilateral forelimb use on day 1, 3, 6, and 14 relative to sham-operated rats. Regarding the percent usage of the ipsilateral (unimpaired) forelimb compared to the contralateral (impaired) forelimb for postural support in the cylinder, there were significant effects of Day ($F_{(5,30)} = 6.349$, $P = 0.000$) and a significant Group by Day interaction ($F_{(5,30)} = 5.323$, $P = 0.001$), but no main

effect of Group ($F_{(1,6)} = 3.694$, $P = 0.103$). Figure 3.10 illustrates that ischemic animals significantly relied more heavily on the unimpaired limb for postural-motor support during rearing and exploration within the cylinder on days 1 and 6 post-surgery when compared to sham-operated rats ($P < 0.05$).

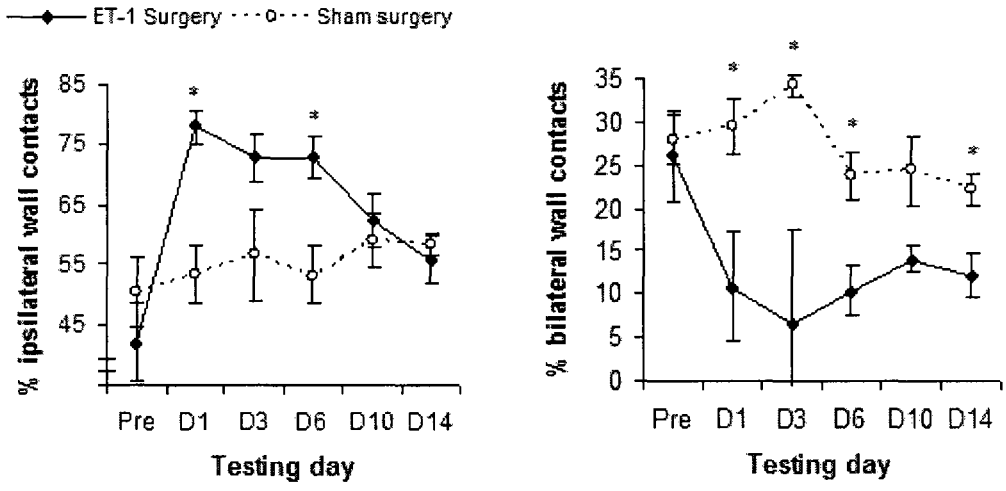

Figure 3.10. Comparison between ET-1 and sham groups on the cylinder test of forelimb use during upright postural support. Percent usage of the ipsilateral forelimb relative to total single forelimb contacts (left); percent usage of both limbs simualtaneously for postural support relative to total limb contacts. Data are mean ± S.E.M. (* indicates a significant difference from sham group; $P < 0.05$).

3.3.3.5 Mortality Rates and Model Success

No animals in the Sham surgery group died during this experiment. The mortality rate for the ET-1 surgery group was 17%. The model success rate was determined to be 83% (see section 2.8 for details pertaining to mortality and model success rate calculations).

3.3.4 Concluding Remarks

Though lesion data was unable to be properly quantified, results from visual inspection of fresh brains by an experimenter experienced with this model suggested that the application of a second cortical injection 0.7 mm more medial than previous preliminary experiments produced adequate ischemic injury to the forelimb sensorimotor cortex that was void of extensive injury to the ventral-lateral cortex. Moreover, being 0.4 mm more lateral than injections described by Hewlett and Corbett (2006), Windle and Corbett (2005), and Windle and colleagues (2006), the previous issue of extensive damage to the contralateral cingulate cortex with these more medial injections also appeared to be avoided (see section 3.1.6). While it was impossible to visualize lesions of the striatum in 3 of the 5 ET-1 surgery rats, satisfactory observations of the initial 2 ET-1 brains plus the reproducibility of damage to the dorsolateral striatum seen with all previous experiments most likely attests to preserved consistency in the present study relative to previous striatal damage patterns.

The current multi-injection approach completely abolished the presentation of bilateral forelimb placing deficits and gave no indication of noteworthy ipsilateral forelimb impairment on either the adhesive removal test of forelimb somatosensory function or Schallert cylinder test of upright postural-motor support. This result suggests that the presence of bilateral forelimb impairment is more contingent on the extent of ipsilesional ischemic damage compared to either the presence of mild-moderate injury to the contralateral cortex or the volume of ET-1 employed. All behavioural tests were characterized by significant impairment in lesioned rats for at least the initial week of

testing relative to control rats, and the general pattern of this impairment continued for approximately two weeks on most forelimb measures. Thus, based on the above observations, an identical multi-injection ET-1 surgical protocol utilizing a two-week post-lesion survival period was employed in the definitive neuroprotection experiment.

4.0 Evaluation of NMDA Receptor-Mediated Neuroprotection In the Endothelin-1 Model

4.1 Introduction

4.1.1 Targeting the NMDA Receptor for Neuroprotection in Stroke

In 1957, Lucas and Newhouse found that systemic injections of L-glutamate could destroy the inner neural layers of the mouse retina, thus implicating glutamate as a putative neurotoxin (Lucas and Newhouse 1957; as cited in Arundine and Tymianski 2004). A decade later, John Olney confirmed the retinotoxicity of glutamate and further showed that this vulnerability to glutamate is shared by all central neurons bearing excitatory amino acid receptors (Olney 1969). In 1969 Olney coined the term 'excitotoxicity', to represent excitatory amino acid mediated degeneration of neurons (Olney 1969). It is currently believed that glutamate receptor-mediated excitotoxicity is one of the primary mechanisms leading to cell death in many acute CNS diseases, including ischemic stroke (Aarts and Tymianski 2004; Matute et al. 2006).

Excessive NMDA receptor activation has been described as the principal vehicle of excitotoxic damage (Doble 1999; Mehta 2007), due to high Ca^{2+} permeability (Aarts and Tymianski 2003; Goldberg and Choi 1993; Patel et al. 1992; Weiss et al. 1986) and known intracellular linkages to pathways that contribute to cell demise when activated in excess (Cui et al. 2007; Hardingham and Badling 2003). Accordingly, NMDA receptors have a long history as therapeutic targets for the treatment of stroke (reviewed by Kemp

and McKernan 2002). The contribution of NMDA receptors to stroke-induced brain injury has been extensively studied since the seminal finding by Simon and colleagues (1984) that blockade of NMDA receptors reduces ischemic damage to the brain. The application of NMDA receptor antagonists for neuroprotection studies using animal models of global ischemia remains controversial (Hicks et al. 1999; Olsson et al. 2003; Radenovic et al. 2008). However, with a few published exceptions (e.g. Auer et al. 1996; Yao et al. 1993), NMDA receptor antagonists of diverse pharmacological characteristics have been consistently neuroprotective in experimental focal ischemic stroke studies conducted with rats, mice, cats, rabbits, and nonhuman-primates (reviewed by McCulloch 1992).

In 1986, Wong and colleagues demonstrated that the anticonvulsant drug, dizocilpine (MK-801), acts by blocking NMDA receptors. Subsequent to this publication, this non-competitive, non-selective NMDA receptor antagonist has been frequently employed in experimental neuroprotection studies (Gill et al. 1996; Moyanova 2007b; Park et al. 1988; O'Neill et al. 2000a; Sharkey et al. 1994; Yang et al. 1994) and is considered by many investigators to be the most reproducible and potent neuroprotective agent in experimental focal ischemic stroke (Park et al. 1988; McCulloch 1992; Sharkey and Butcher 1995; Sharkey et al. 1994). However, the side-effect profile (i.e. psychotomimetic, learning and memory impairment) (Morris et al. 1986; Koek et al. 1988) associated with non-selective blockade of the NMDA receptor at doses likely required for any therapeutic effect, plus evidence of vacuole production and select neuronal damage in rat cortical tissue (Olney et al. 1990) led to the abandonment of this drug and other non-selective NMDA receptor antagonists for clinical stroke research

approximately 20 years ago (see review by Ginsberg 2008). Currently, MK-801 is commonly employed as either a reference compound (i.e. positive control) against which other neuroprotective agents are compared or for testing the utility of new stroke models for use in neuroprotection studies (Moyanova et al. 2007a; Moyanova et al. 2007b; Onal et al. 1997; O'Neill et al. 2000a; Sharkey and Butcher 1995). Due to the considerable potential of NMDA receptor antagonists in the clinic, more recent efforts have focused on developing compounds that retain the beneficial effects of non-specific antagonists but with a greatly reduced side effect profile (Mutel et al. 1998; Sattler et al. 1999).

4.1.2 NR2B Subunit-Selective Antagonism in Experimental Focal Stroke

Within the last several years, attempts have been made toward developing NMDA receptor subunit-selective antagonists (Chennard and Menniti 1999; Fischer et al. 1997; Mutel et al. 1998). The justification for such efforts largely comes from original findings that the prototypical drug, ifenprodil, and its derivative, eliprodil, are effective neuroprotective compounds, subsequently found to be specific blockers of NMDA receptors containing the NR2B subunit (Shalaby et al. 1992; Toulmond et al. 1993; Williams et al. 1993). At present, although NR2A subunit 'preferring' antagonists, such as NVP-AAMO77 (Auberson et al. 2002) are currently available for experimental applications, the large majority of these subunit-specific NMDA receptor antagonists are compounds highly selective for NMDA receptors containing the NR2B subunit (Fischer et al 1997; Lekieffre et al. 1997; Williams et al. 2003). NR2B subunit-selective compounds, such as ifenprodil, traxoprodil (CP-101,606), and Ro25-6981 inhibit just 50-

60% of the MK-801 binding to all of the NMDA receptors in the adult rat forebrain (Fischer et al. 1997; Mutel et al. 1998), yet they have been equally efficacious compared to non-selective NMDA receptor antagonists in their ability to reduce ischemic injury and/or improve functional recovery after experimental focal ischemic stroke (Di et al. 1997; Gill et al. 2002; Kundrotienne et al. 2004; Sun et al. 2008).

These NR2B subunit-selective antagonists, which are typically non-competitive in nature [a competitive antagonist at the glutamate binding site, Conantokin G (Con-G) is the only exception] (reviewed by Layton et al. 2006), bind with higher affinity to activated and desensitized states of the receptor relative to the resting state and thus display an activity- or state-dependent mode of action (Kew et al. 1996; Fischer et al. 1997). Accordingly, such compounds may preferentially block NMDA receptors that are continuously activated by sustained high glutamate levels in ischemic brain areas, while leaving those that are physiologically activated in non-ischemic brain areas relatively unaffected (Kew et al. 1996; Gill et al. 2002). NR2B subunit-selective antagonists have a primary advantage over most non-selective NMDA receptor antagonists in that they show much improved tolerability in animals as well as humans (Bullock et al. 1999; Di et al. 1997). The activation of NMDA receptors containing the NR2B subunit has also shown to be crucial in determining the neurotoxic signaling potential of the NMDA receptor, as it is the NR2B subunit-containing, not the NR2A containing, NMDA receptor subpopulation that primarily leads to the triggering of intracellular cell death cascades when exposed to NMDA or ischemia (Chen et al. 2008; Liu et al. 2007).

4.1.3 Suitability of the ET-1 Model for Neuroprotection Studies

Application of the vasoconstrictor peptide, ET-1, has been verified by several investigators as a useful method for producing well-controlled ischemic lesions in desired regions of the brain (particularly forelimb representation areas) that are amenable to quantifiable infarct analysis (Adkins et al. 2004; Fuxe et al. 1997; Gilmour et al. 2004; Hewlett and Corbett 2006; Luke et al. 2004; Windle et al. 2006). Previous reports on this model suggests that it consistently results in forelimb functional impairment of sufficient duration (i.e. at least 2 weeks) (Adkins et al. 2004; Gilmour et al. 2004; Hewlett and Corbett 2006; Luke et al. 2004; Windle et al. 2006). Moreover, topical, intracortical, and intracortical + intrastriatal applications are characterized by a high success rate (~80-85%), thus making it a valuable tool for neuroprotection studies where, in the absence of neuroimaging, it is difficult to identify animals with incomplete ischemia (Windle et al. 2006; Windle and Corbett 2005).

In recent years, a modest corpus of literature has helped substantiate the utility of the ET-1 model for studying rehabilitation therapy and/or neural repair processes after ischemic damage to the sensorimotor system (Adkins et al. 2004; Adkins et al. 2008; Gilmour et al. 2005; Maldonado et al. 2008; O'Bryant et al. 2007; Windle and Corbett 2005). However, the initial publication on localized ET-1 induced cortical ischemia suggested that this model may represent an important new approach for the testing of neuroprotective compounds, given that the lesions were well controlled and associated with a large penumbral region (Fuxe et al. 1997). Yet the number of studies that have used this model to test pharmacological agents designed to preserve brain tissue and

enhance functional recovery after stroke is, in general, remarkably limited, and virtually nonexistent for NMDA receptor antagonists.

Marked neuroprotection via NMDA receptor blockade has previously been demonstrated in the ET-1 MCAo model (Moyanova et al. 2007a; Moyanova et al 2007b; O'Neill et al. 2000a; Sharkey et al. 1994; Sharkey and Butcher 1995). However, this model involves injecting a small amount of ET-1 adjacent to the MCA (i.e. in the piriform cortex) to occlude it at its base, leading to ischemic brain damage within the large territory of this artery (i.e. akin to the damage pattern seen with traditional MCAo) (O'Neill et al. 2000b; Sharkey et al. 1994; Sharkey and Butcher 1995). Thus, the ischemic damage in this model is contingent on large artery occlusion of sufficient magnitude and duration rather than the infiltration of ET-1 to the brain per se. In contrast, based on the correlation between ET-1 immunoreactivity after cerebral microinjection and localized CBF changes/pattern of histological damage (Adkins et al. 2004; Fuxe et al. 1997; Windle et al. 2006), the ET-1 model of upper extremity impairment in stroke relies much more directly on diffusion of ET-1 throughout specific brain areas for the production of localized lesions.

4.1.4 Use of ET-1 for the Production of Localized Cerebral Ischemia

Endothelin-1 putatively induces cellular injury via its vasocontrictive properties, without any direct neurotoxic effects of the peptide (Fuxe et al. 1992; Lustig 1992; Nikolov et al. 1993). Endothelin-1 does, however, have explicit cellular effects on neurons and glia (as demonstrated *in vitro*) which raises some uncertainty regarding its

value for use in neuroprotection for stroke studies (Blomstrand et al. 1999; MacCumber et al. 1990; Leonova et al. 2001; Sasaki et al. 1997), particularly for anti-excitotoxic approaches. Endothelin-1 has been shown to inhibit astroglial glutamate uptake (Leonova et al. 2001) and enhance glutamate release from cultured astrocytes (Sasaki et al. 1997). Moreover, ET-1 may facilitate synaptic glutamatergic transmission (Shihara et al. 1998) and enhance glutamate-induced neuronal cell death (Kobayashi et al. 2005). Several studies have also demonstrated increased intracellular Ca^{2+} levels in neural cell lines, cultured neurons, and hippocampal slices as a direct result of ET-1 application (Koizumi et al. 1994; MacCumber et al. 1990; Reiser and Donie 1990; Yue et al. 1990). Considering these reported findings, the ET-1 peptide may produce cellular effects that exacerbate, or alter in some fashion, glutamate-induced excitotoxicity relative to other focal ischemic stroke models. Such events may or may not render the ET-1 model inapplicable for *in vivo* neuroprotection studies that aim to block NMDA receptor-mediated excitotoxicity.

4.1.5 Neuroprotection Studies Using the ET-1 Model

In the few studies that have tested neuroprotective compounds in the ET-1 model, results pertaining to infarct reduction have been non-significant (fluoxetine trial), protection has been robust only within the striatum (minocycline trial), or the resultant protection was only relevant to assess within the striatum (melanocortins trial; γ-hydroxybutyrate trial; modafinil trial) (Giuliani et al. 2007; Hewlett and Corbett 2006; Ottani et al. 2003; Windle and Corbett 2005; Ueki et al. 1993). Likewise, the

achievement of functional neuroprotection in this model also remains somewhat ambiguous, as it appears that only three studies have shown improved functional outcome following neuroprotective drug treatment (Giuliani et al. 2007; Hewlett and Corbett 2006; Ottani et al. 2003), all as a result of primary protection to subcortical tissue using rather unconventional pharmacological agents. Thus, in general, successful neuroprotection in the ET-1 model is far from clear, and it remains to be determined if this model represents a viable alternative approach for studying functional neuroprotection via compounds that block NMDA receptor-mediated excitotoxicity.

4.1.6 Study Rationale and Objective

Given the apparent advantages of the ET-1 model for studying functional neuroprotection and the primary role of NMDA receptor-mediated excitotoxicity in the ischemic injury process, it was of interest to evaluate whether antagonism of NMDA receptors is an achievable therapeutic approach for reducing brain damage and improving functional recovery in the ET-1 model. Such an investigation would assist in characterizing the ischemic injury process in the ET-1 model and further clarify its applicability in experimental stroke research. For this purpose, the non-specific, non-competitive NMDA receptor antagonist, MK-801 was chosen because of its well established ability to reduce brain damage in rat models of focal ischemic stroke (O'Neill et al. 2000a; Park et al. 1988; Sharkey and Butcher 1995). Thus, MK-801 administration serves to test the general relevance of the ET-1 model for neuroprotection via NMDA receptor blockade. However, non-specific NMDA receptor antagonists are not clinically

useful due to the undesirable side effects associated with administration of these drugs (Morris et al. 1986; Koek et al. 1988). The NR2B subunit-selective compounds have potential as a foundation approach for the development of effective yet tolerable NMDA receptor antagonists for the treatment of focal ischemic stroke (Chen et al. 2008; Layton et al. 2006; Liu et al. 2007). Consequently, the non-competitive NR2B subunit-selective antagonist, Ro25-6981, was also evaluated since it represents one of the most selective drugs within this category of compounds (Fischer et al. 1997; Mutel et al. 1998) and has proven efficacy against excitotoxicity *in vitro* and focal ischemic stroke *in vivo* (Fischer et al. 1997; Gill et al. 2002; Liu et al. 2007; Sun et al. 2008).

4.2 Methods and Materials

4.2.1 Surgery

Endothelin-1 (400pmol/μl) was dissolved in sterile distilled H_2O and microinjected to the forelimb sensorimotor cortex and dorsolateral striatum. Two cortical injections and one striatal injection were administered. Stereotaxic injection coordinates were, cortical 1) AP +2.0, ML -2.9, DV, -2.5; cortical 2) AP +1.0, ML -2.9, DV -2.5; striatal AP +0.9, ML -3.6, DV -6.0. Endothelin-1 was microinjected in a volume of 2μl, 2.5μl, and 1μl for cortical 1, cortical 2, and the striatal injection, respectively. For further details see section 2.3.

4.2.2 Drug Treatment/Experimental Conditions

Approximately 24 hr after arrival to the animal colony room, rats were pseudo-randomly assigned (i.e. assigned to groups without any predetermined criteria) to

one of 4 groups (ET-1/MK-801, ET-1/Ro25-981, ET-1/Saline, or Sham surgery). Approximately 15 min prior to the initiation of the ET-1 injections, rats were given i.p. injections of either MK-801 (5mg/kg) (n = 8) [Ischemia + MK-801 (I + M)], Ro25-6981 (6mg/kg) (n = 8) [Ischemia + Ro25-6981 (I + R)], or equal volumes of saline vehicle (n = 9) [Ischemia + Vehicle (I + V)]. Dosing regimens were chosen based on positive results from previous neuroprotection studies using these compounds in models of transient focal ischemic stroke (Liu et al. 2007; Moyanova et al. 2007b; O'Neill et al. 2000a; Sharkey et al. 1994; Sharkey and Butcher 1995; Sun et al. 2008). Groups receiving drug treatment were coded by a lab technician for the duration of the experiment through to data analysis in order to blind the experimenter to condition. Rats receiving sham surgery served as behavioural controls (n = 7). Over a period of approximately 6 months, rats in the above groups were tested in 4 separate cohorts, with total cohort size ranging from 7 to 12 animals. Within cohorts, I + V, I + R, and I + M treated rats were equally represented, to the extent possible. Sham-operated rats were tested at a subsequent time point to fulfill the behavioural control condition.

Previous reports using the ET-1 MCAo model have shown that an *n* of 7-14 is sufficient to detect an approximate 30% - 50% reduction in total infarct volume (with at least 80% power, and at α level 0.05) using a single i.p. injection of MK-801 at a dose range of 2.5mg/kg to 5mg/kg (Moyanova et al. 2007b; O'Neill et al. 2000a; Sharkey et al. 1994; Sharkey and Butcher 1995). Considering that the ET-1 MCAo model is possibly the most similar method of transient focal stroke compared to the current ET-1 model and that the aforementioned reports detailed levels of lesion variability similar to those seen with preliminary experiments herein, group *n* for the current neuroprotection study was

based on these aforementioned reports.

4.2.3 Histology and Infarct Estimation

Rats were euthanized fifteen days post-surgery. Brains were vibratome sectioned and stained with cresyl violet for infarct analysis (for further details see sections 2.6.1, 2.6.2, and 2.7). Sham operates were assessed for damage to brain tissue but were not included in infarct volume analyses in order to avoid inappropriate bias.

4.2.4 Behavioural Testing and Body Weight Measurements

Animals were tested one day prior to surgery and every day after surgery for 14 days on tests of vibrissae- and tactile-stimulated forelimb placing as well as the forelimb postural reflex test. Animals were tested one day prior to surgery, and on day 1, 3, 6, 10, and 14 post-surgery for performance on the Schallert cylinder test and a modified version of the adhesive removal test. Rats that demonstrated a severe lack of attendance to stimuli placed on either forelimb during acclimation and pre-testing procedures were not tested on the adhesive removal measure after surgery ($n = 1$). Animals were weighed prior to surgery and every day after surgery for the duration of the experiment. Table VI illustrates the general schedule for the behavioural test battery employed. For further details see sections 2.4 – 2.4.4 and 2.5. Approximately 3 hr post surgery, all animals were tested for placing accuracy as an approximate check for completeness of ischemia (Zhao et al. 1997). Sham animals were tested to rule out any effect of surgery/anesthetic (not accounted for in Table VI).

Table VI. Testing schedule for each forelimb measure employed.

Forelimb Measure	*Acclimation Period*	*Pre-surgery Testing*	*Post-surgery Testing*	*Light/Dark Cycle*
Forelimb Placing	3, 5-trial sessions within ~ 14 days	1, 5-trial session ~ 24 hr prior to surgery	14, 5-trial sessions over PSD 1-14	Light
Forelimb Postural Reflex	3 sessions within ~ 14 days	1 session ~ 24 hr prior to surgery	14 sessions over PSD 1-14	Light
Schallert Cylinder Test	2, 1-trial sessions within 7 days prior to surgery	1 trial ~ 24 hr prior to surgery	5, 1-trial sessions over PSD 1, 3, 6, 10, 14	Dark
Mod-Adhesive Removal Test	2, 2-trial sessions within 7 days prior to surgery	2 trials ~ 24 hr prior to surgery	5, 2-trial sessions over PSD 1, 3, 6, 10, 14	Dark

4.3 Results

4.3.1 Infarct Data

Statistical analysis revealed a significant difference between groups on the measure of total infarct volume ($F_{(2, 22)} = 5.735$, $P = 0.010$). Observed power was 81.4% for this effect. Groups receiving pre-ischemic i.p. injections of Ro25-6981 and MK-801 demonstrated reductions in total infarct volume of 49% (19.1 ± 2.4 mm^3) ($P = 0.005$) and 44% (21.9 ± 4.0 mm^3) ($P = 0.014$) compared to the group receiving time-matched equal volumes of the saline vehicle (39.1 ± 6.1 mm^3), respectively. Cortical infarct volume was also significantly different between groups ($F_{(2, 22)} = 6.787$, $P =$

0.005). Rats in the I + R and I + M groups had respective cortical infarct volumes 66% (8.6 ± 1.8 mm^3) ($P = 0.003$) and 62% (9.8 ± 2.4 mm^3) ($P = 0.006$) smaller than the I + V treated rats (25.5 ± 5.3 mm^3). Observed power for this effect was 87.7%. No effect of drug treatment was found for striatal infarct volume ($F_{(2,22)} = 0.523$, $P = 0.600$). Sham surgery produced negligible injury to the cortex (0.95 ± 0.11 mm^3) and striatum (0.13 ± 0.07 mm^3). Mean total, cortical, and striatal, infarct volumes are presented below in Figure 4.1 for each of the drug treatment groups. Figure 4.2 gives the area of cortical and striatal damage across the stereotaxic levels of measurement for the I + V, I + R, and I + M groups.

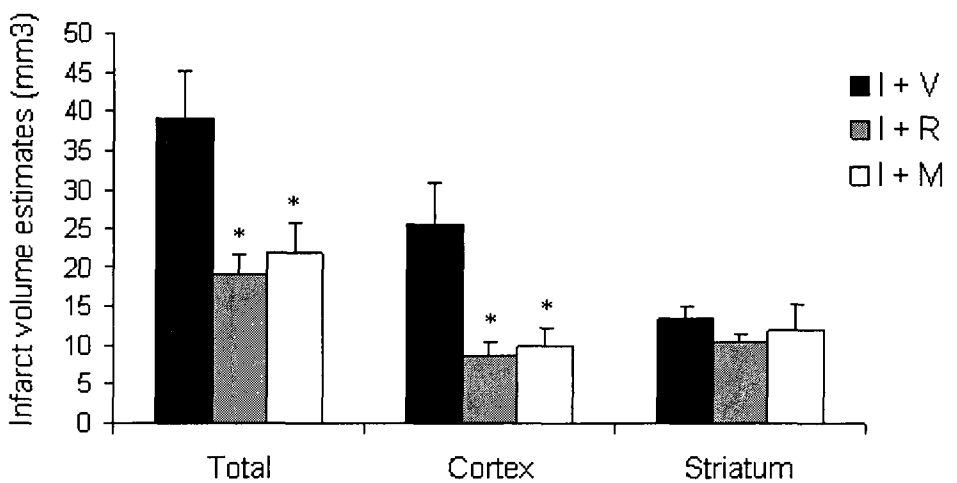

Figure 4.1. Comparison of mean (± S.E.M.) total, cortical, and striatal infarct volume estimates among I + V, I + R, and I + M treated rats (* indicates significant difference from I + V group, $P < 0.05$).

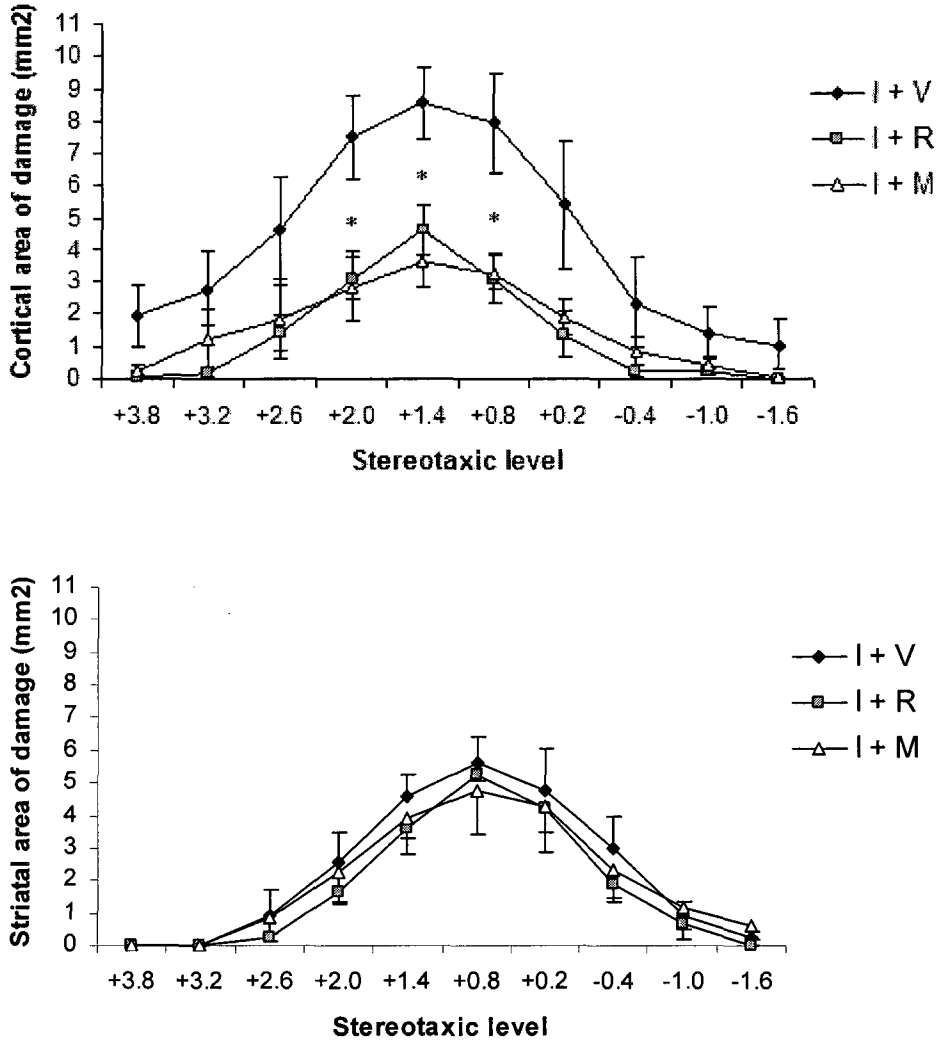

Figure 4.2. Comparison of I + V, I + R, and I + M treated rats on mean (± S.E.M.) area of ischemic brain damage in cortex (top) and striatum (bottom) at 10 stereotaxic levels assessed 15 days following focal ischemic stroke induced by multiple intracerebral injections of endothelin-1. (* indicates a significant difference of both I + R and I + M from I + V, $P < 0.05$).

Figure 4.2 illustrates that, for all groups, cortical injury was largely between AP +2.6 mm and + 0.2 mm. The I + R and I + M treated rats had a similar pattern of ischemic damage to the cortex, and these groups differed from the I + V group in relation to both the magnitude of injury and spread of injury from the injection core. However,

the I + V group only demonstrated significantly more ischemic damage to the cortex within the area of most concentrated damage common to all groups (AP +2.0 mm to +0.8 mm), when compared to both the I + R and I + M groups ($P < 0.05$). For all groups, striatal damage was most prominent within AP +2.0 mm and -0.4 mm, and each condition showed a similar degree and extent of ischemic injury to the striatum. Figure 4.3 illustrates cresyl violet stained sections representative of the average cortical lesion pattern observed within these groups.

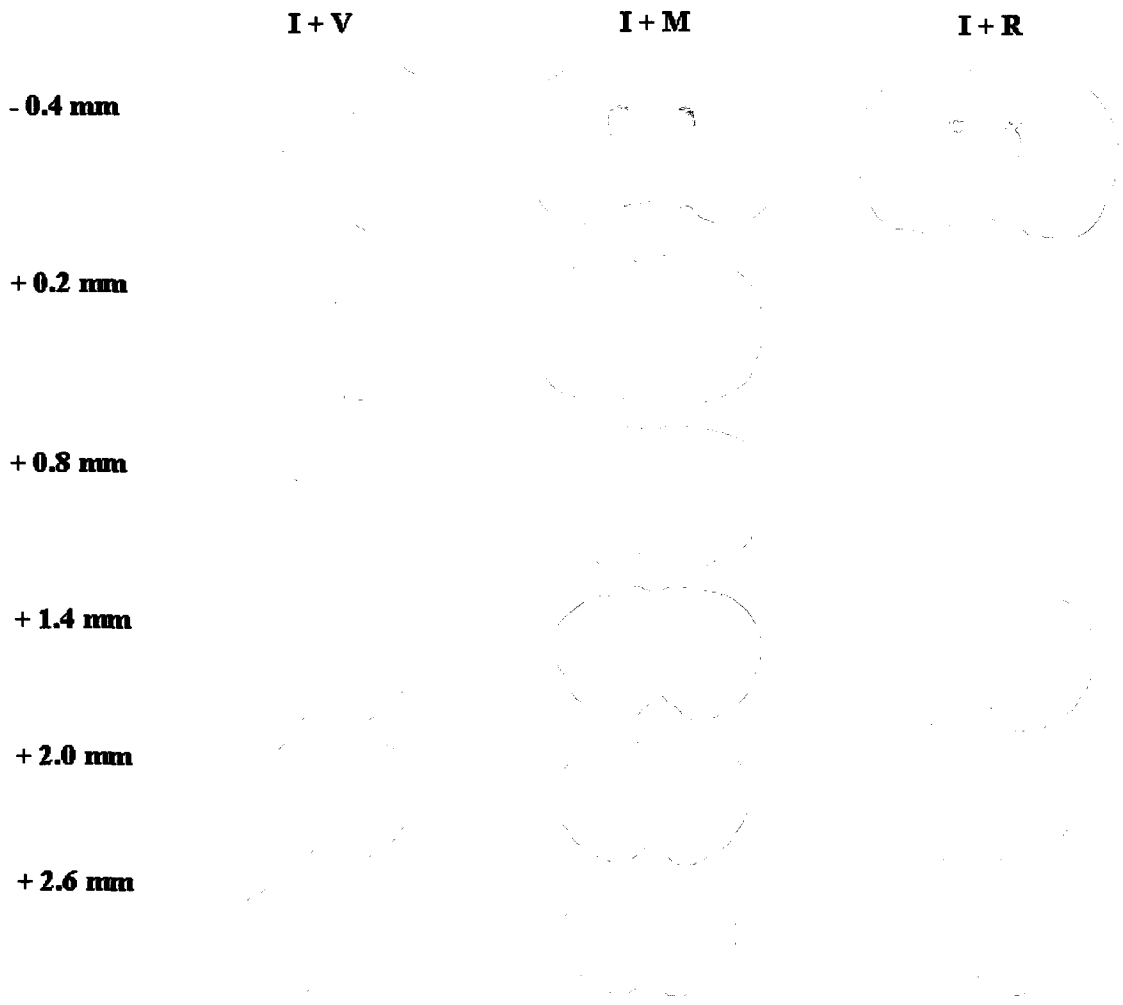

Figure 4.3. Cresyl violet stained sections (150μm) representative of the average cortical lesion pattern observed across the I + V, I + M, and I + R treated groups. Shown from stereotaxic level AP: +2.6mm to – 0.4mm.

4.3.2 Initial Behavioural Assessment

At ~ 3 hr post-surgery all ET-1 treated rats had complete or near complete impairment in contralateral forelimb placing behaviour (i.e. at least 80% impairment/no more than 1/5 paw placements). Two I + V rats also demonstrated mild ipsilateral impairment (i.e. 20% impairment). Sham surgery rats placed with 100% accuracy. However, all MK-801 treated animals were completely unable to place either forelimb at

this 3 hr mark. Although not directly measured in the current study, it is important to note that all MK-801 treated animals were largely paralyzed for, on average, approximately 7 hr after surgery, and they typically presented with slowed movements and limp body posture (i.e. when handled) for approximately the first 24 hr after surgery. Beyond the appropriate contralateral forelimb impairment induced after ET-1 surgery, the initial behavioural disturbances noted in MK-801 rats were not observed in any Ro25-6981 or saline vehicle treated animals.

4.3.3 Body Weight Data

For measures of body weight there were significant effects of Day ($F_{(3.021, 84.584)} = 735.700$, $P = 0.000$) and Group ($F_{(3, 28)} = 4.965$, $P = 0.007$). The I + V, I + R, I+ M, and Sham rats did not differ from each other in weight loss after surgery or in subsequent weight gain, and all of these groups reached or approached pre-surgery body weight by post-surgery day 2. In contrast, I + M treated rats did not regain pre-surgery weight until post-surgery day 5. The I + M treated rats lost significantly more weight relative to all other groups after surgery and this reduction in body weight remained significant until post-surgery day 9 ($P < 0.05$) (Figure 4.4).

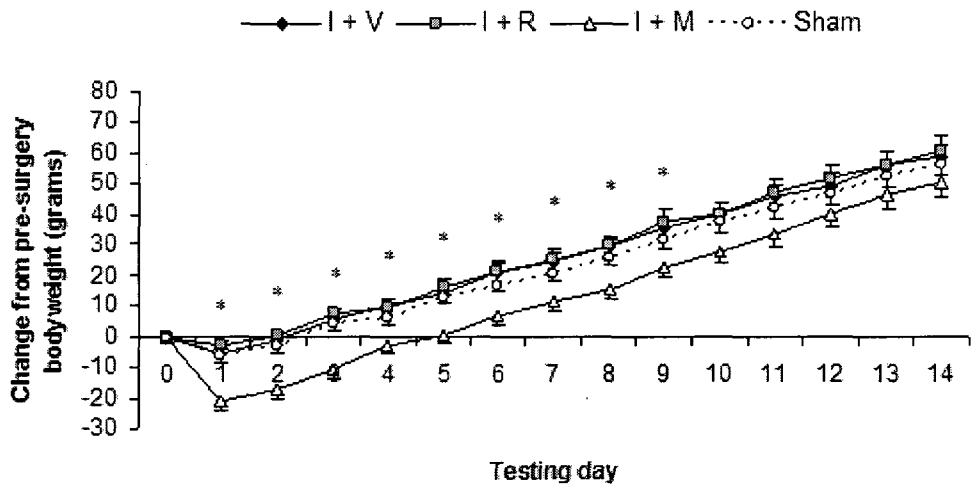

Figure 4.4. Comparison of mean (± S.E.M.) changes from pre-surgery body weight (grams) among I + V, I + R, I + M and Sham groups over 14 days of testing. (* indicates a significant difference of all other groups from I + M, $P < 0.05$).

4.3.4 Forelimb Postural Reflex and Forelimb Placing Data

There was a significant Group effect ($F_{(3,28)} = 3.838$, $P = 0.020$) for the duration of functional impairment on the FPR test. Post hoc analysis revealed that the I + V group had a significantly longer mean duration of impairment compared to the I + R ($P = 0.025$), I + M ($P = 0.037$), and Sham ($P = 0.004$) groups. In contrast, the I + R and I + M groups did not differ from each other, or sham controls. Figure 4.5 demonstrates that rats receiving I + V treatment did not, as a group, recover from FPR deficits until ~ the end of the first week of testing, while the I + R and I + M treated groups recovered by ~ day 2 post surgery. Sham rats showed no indication of FPR deficit over the course of testing.

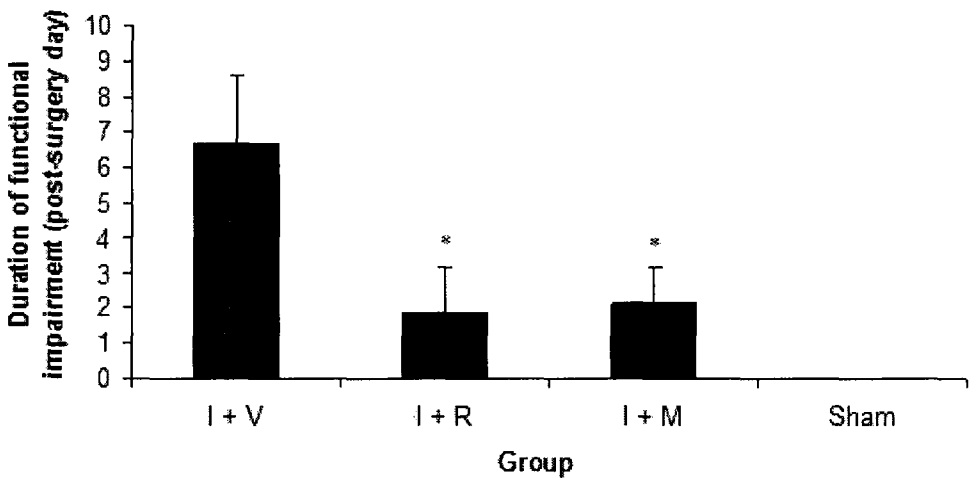

Figure 4.5. Comparison on mean (± S.E.M.) duration of impairment for the FPR test over 14 days post surgery among the I + V, I + R, I + M and Sham surgery groups. (* indicates a significant difference from I + V group, $P > 0.05$).

Analysis of the data obtained for tactile-stimulated forelimb placing revealed a main effect of Day ($F_{(14,392)} = 15.690$, $P = 0.000$) and Group ($F_{(3,28)} = 5.795$, $P = 0.003$), as well as a significant interaction of Group by Day ($F_{(42,392)} = 2.349$, $P = 0.000$). Following surgery, the I + V treated rats were significantly impaired relative to sham controls for the first week, and again on days 9, 10, 11, 12 ($P < 0.05$). I + V rats had significantly reduced tactile-stimulated placing accuracy relative to I + R rats from day 2 to 7 post surgery, as well as on days 9, 10, 11, and 12 ($P < 0.05$). In comparison, I + V treated rats showed significantly reduced tactile-stimulated placing accuracy on post-surgery days 7, 9, 10, 11, and 12 compared to I + M treated rats ($P < 0.05$). I + R rats only differed significantly from sham rats on post-surgery day 1 ($P < 0.05$). I + M rats showed significantly reduced performance relative to sham rats for the first 2 days post-surgery ($P < 0.05$). I + R and I + M rats did not significantly differ from one another at any time point. Sham animals placed with 100% accuracy throughout the course of

testing (see Figure 4.6).

For tests of vibrissae-stimulated placing accuracy, there were significant effects for Day ($F_{(14,392)} = 17.400$, $P = 0.000$) Group ($F_{(3,28)} = 4.869$, $P = 0.008$), and Group by Day interaction ($F_{(42,392)} = 2.308$, $P = 0.000$). After surgery, the I + V treated rats were significantly impaired relative to sham controls for the first 6 days ($P < 0.05$). I + V rats had significantly reduced vibrissae-stimulated placing performance relative to I + R rats on days 3, 5, and 6 post surgery ($P < 0.05$). In contrast, I + V treated rats only showed significantly reduced vibrissae-stimulated placing accuracy on post-surgery day 5, compared to I + M treated rats ($P < 0.05$). I + R rats significantly differed from sham controls for the first 2 post-surgery days ($P < 0.05$), whereas I + M rats significantly differed from sham controls for the first 3 days post-surgery ($P < 0.05$). I + R and I + M rats did not significantly differ from one another at any time point. Sham animals placed with 100% accuracy throughout the course of testing (see Figure 4.6).

Figure 4.6 depicts both tactile- and vibrissae-stimulated contralateral forelimb placing accuracy for all groups over the course of behavioural testing. On post-surgery day 1, MK-801 treated rats typically presented with slowed motor responses for both tactile- and vibrissae-stimulated placing and one animal from this group was unable to place the ipsilateral limb for both tests. All other rats showed no indication of impairment for ipsilateral tactile- or vibrissae-stimulated placing accuracy at any time point. Thus, only contralateral responses are presented. Despite an inconsistent pattern of significant group by day differences, Figure 4.6 shows a clear tendency for groups receiving NMDA receptor antagonists to be less impaired throughout testing compared to the I + V group (with some significant group by day differences) . Also, the general

pattern of impairment depicted in Figure 4.6 demonstrates that these groups were largely recovered within approximately one week after surgery. In contrast, Figure 4.6 illustrates that I + V treated rats had a tendency to retain a certain degree of impairment for the duration of the experiment. These observed patterns were similar for both tactile- and vibrissae-stimulated placing accuracy.

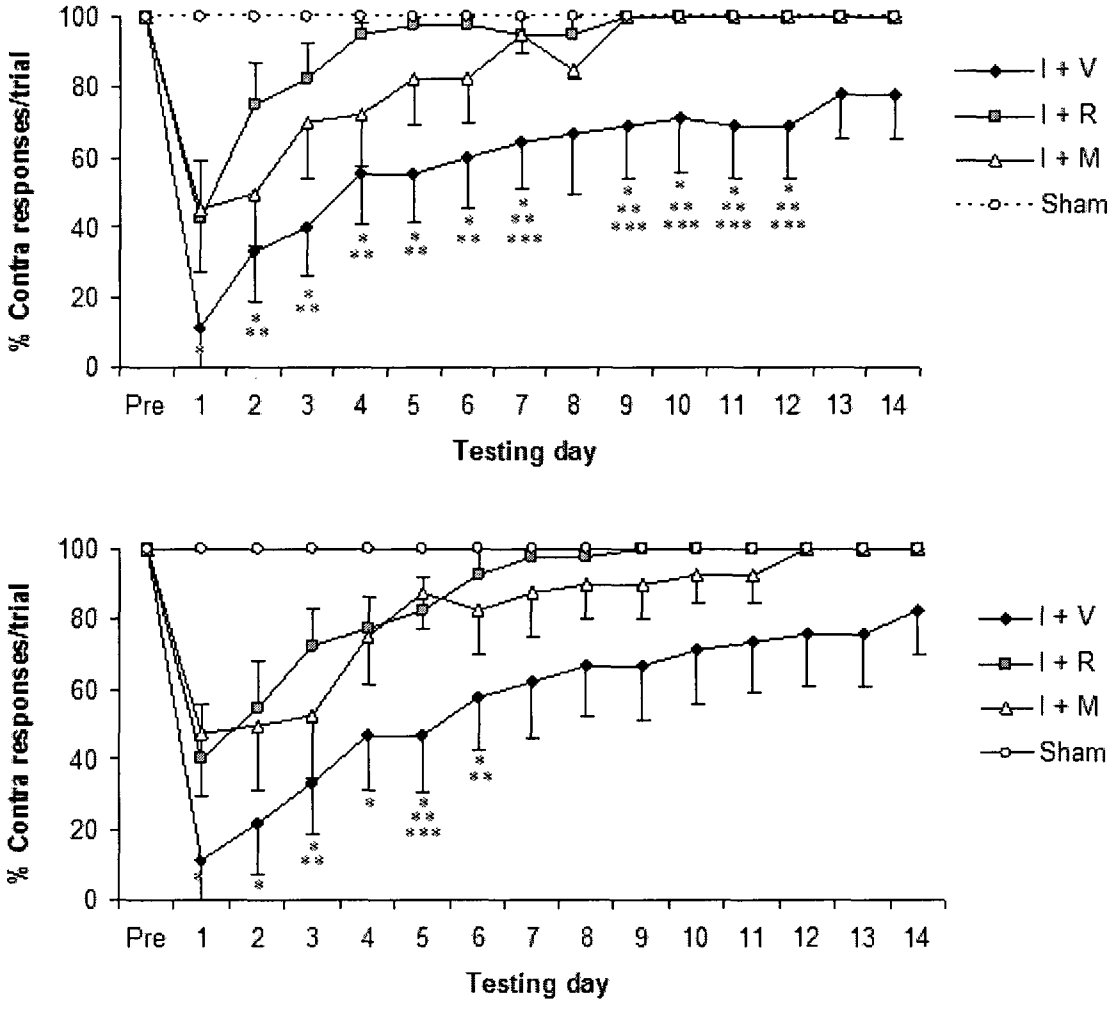

Figure 4.6. Comparison of forelimb placing accuracy pre and over 14 days post surgery among the I + V, I + R, I + M and Sham groups. Contralesional tactile-stimulated placing (top); Contralesional vibrissae-stimulated placing (bottom). Data are mean ± S.E.M. (* indicates a significant difference of I + V from Sham group; ** indicates a significant difference from I + R; *** indicates a significant difference from I + M, $P < 0.05$).

4.3.5 Adhesive Removal and Cylinder Test Data

Testing on the modified adhesive removal test produced significant effects of Day ($F_{(3.181,85.887)} = 9.591$, $P = 0.000$) and Group ($F_{(3,27)} = 4.887$, $P = 0.008$), and are presented in Figure 4.7. The I + V group demonstrated a significant ipsilateral bias (i.e. reduced forelimb somatosensory asymmetry ratio) compared to sham control rats on post-surgery days 3 and 10 ($P < 0.05$). Both I + R and I + M treated rats had significantly improved asymmetry ratios compared to I + V rats on post-surgery day 10 ($P < 0.05$). Both I + R and I + M treated animals did not differ significantly from sham controls, or from each other. Sham operated rats showed mild fluctuations from equal attendance to both forelimbs across all testing days. Figure 4.7 illustrates the degree and duration of forelimb somatosensory impairment for all groups over the course of behavioural testing.

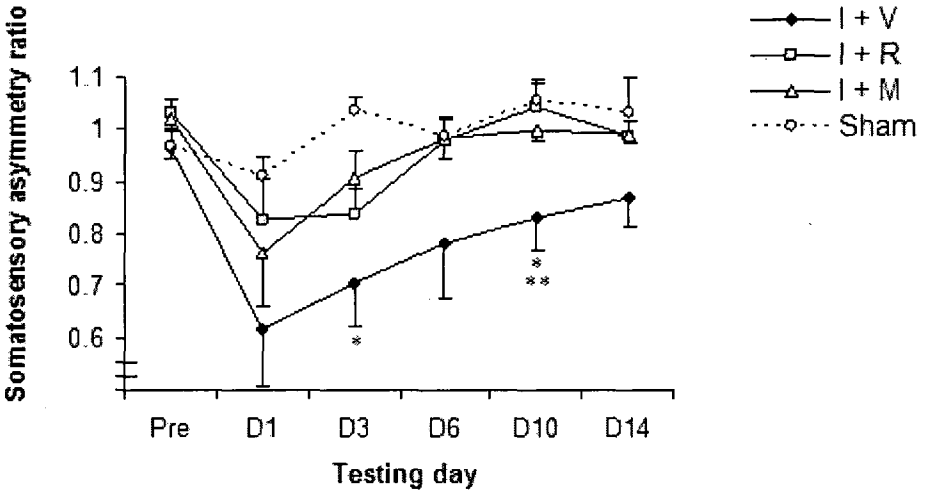

Figure 4.7. Comparison among I + V, I + R, I + M, and Sham surgery groups on somatosensory asymmetry ratio scores for the modified adhesive removal test. Data are mean ± S.E.M. (* indicates a significant difference from Sham group; ** indicates a significant difference from both I + R and I + M, $P < 0.05$).

For the cylinder test, there was a main effect of Day ($F_{(5,140)} = 6.617$, $P =$

0.000) but not for Group ($F_{(3,28)} = 2.289$, $P = 0.100$) for percent simultaneous use of both limbs for postural-motor support. However, Figure 4.8 clearly shows that sham controls were the only group to retain a consistent pattern on this measure over testing days. All other groups demonstrated a pattern of reduced bilateral forelimb use after surgery. The mean pre-test value for the sham controls was negatively shifted relative to the other groups leading to similar post-surgery values compared to ischemic animals, despite no drop in bilateral forepaw usage for this group (Figure 4.8).

Regarding the percent usage of the ipsilateral (unimpaired) forelimb compared to the contralateral (impaired) forelimb for postural support in the cylinder there were significant effects of Day ($F_{(4.918,137.706)} = 9.154$, $P = 0.000$), Group ($F_{(3,28)} = 6.505$, $P = 0.002$) and Group by Day interaction ($F_{(14.754,137.706)} = 1.967$, $P = 0.022$). Data are presented in Figure 4.8. After surgery, I + V treated rats relied more heavily on the unimpaired limb for postural-motor support during rearing and exploration within the cylinder until post-surgery day 10, compared to sham controls ($P < 0.05$). I + V rats showed more reliance on the ipsilateral limb on post-surgery day 1 compared to I + R rats ($P < 0.05$), but did not significantly differ from I + M rats on any testing day. I + R treated rats had elevated usage of the ipsilateral paw relative to control rats on post-surgery days 3 and 10 ($P < 0.05$), whereas ipsilateral usage in the I + M treated rats was increased relative to controls on post-surgery days 1, 3, and 10 ($P < 0.05$).

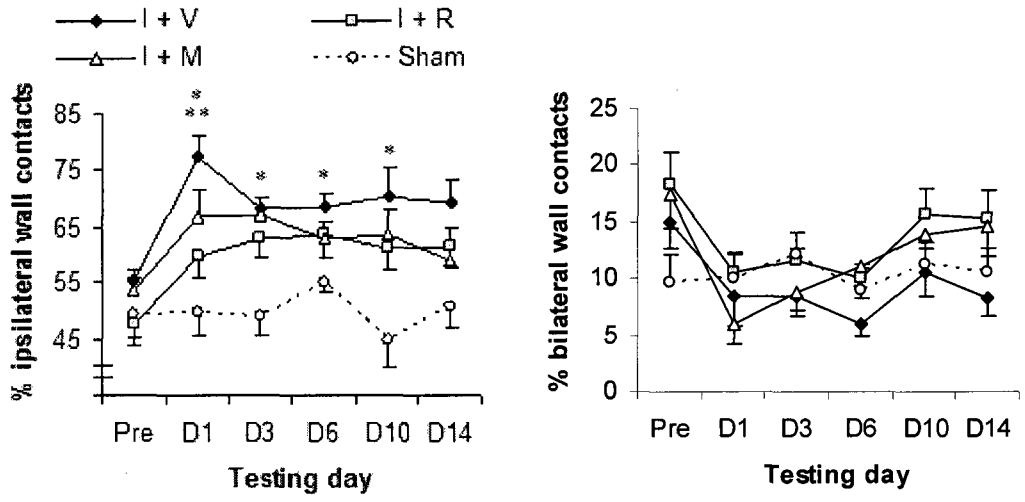

Figure 4.8. Comparison among I + V, I + R, I + M, and Sham groups on the cylinder test of forelimb use during upright postural support. Percent usage of the ipsilateral forelimb relative to total single forelimb contacts (left); percent usage of both limbs simualtaneously for postural support relative to total limb contacts. Data are mean ± S.E.M. (* indicates a significant differerence of I + V from Sham group, ** indicates a significant difference from I + R, $P < 0.05$).

4.3.6 Mortality Rates and Model Success

The overall mortality rate for this experiment was 17%. Group mortality rates were: 18% (I + V), 11% (I + R), and 20% (I + M). Model success rate was calculated to be 73% for the current study (see section 2.8 for details pertaining to mortality and model success rate calculations).

4.4 Discussion

The current study was designed to investigate whether NMDA receptor-mediated neuroprotection is feasible in the ET-1 model. Pre-ischemic treatment with MK-801 (non-selective, non-competitive NMDA receptor antagonist) as well as the non-

competitive NR2B subunit-selective antagonist, Ro25-6981, was employed to test this experimental aim. Utilizing a 2-week survival period, it was of interest to determine if administration of these compounds would reduce both infarct and forelimb functional impairment as measured using a variety of established sensorimotor tests.

The principal findings of this experiment were two-fold. First, pre-ischemic administration (single i.p. injection) of both MK-801 (5mg/kg) and Ro25-6981 (6mg/kg) significantly reduced the volume of cortical (62%, 66%) and total (44%, 49%) hemispheric brain infarct (respectively) compared to vehicle control when measured at 15 days post ischemia (Figure 4.1). Compared to I + V controls, the volume of brain damage within the striatum was not affected by administration of NMDA receptor antagonists (Figure 4.1). Second, administration of each NMDA receptor antagonist generally produced a clear tendency toward improved forelimb functional outcome compared to vehicle control on the forelimb measures employed, although the incidence of group by day statistical significance as well as the general magnitude/duration of this pattern varied between behavioural tests.

4.4.1 Infarct Reduction via NMDA Receptor Antagonists in the ET-1 Model

The neuroprotection demonstrated herein for the ET-1 model is consistent with previous published results using MK-801 and Ro25-6981 in other rat models of focal ischemic stroke (Liu et al. 2007; O'Neill et al. 2000a; Sharkey et al. 1994; Sun et al. 2008). In particular, the degree of neuroprotection provided by MK-801 in the current study is comparable to the level of total hemispheric protection conferred by pre-ischemic

i.p. administration of this compound in the ET-1 MCAo model (~ 30% to 50%) (Moyanova et al 2007b; O'Neill et al. 2000a; Sharkey et al. 1994; Sharkey and Butcher 1995). To the best of the experimenter's knowledge, Ro25-6981 has not been evaluated in any ET-1 model; however, the magnitude of protection provided by this compound in other models of focal stroke has been reported to range from approximately 25% (Gill et al. 2002) to 70% (Liu et al. 2007; Sun et al. 2008). Thus, the level of neuroprotection provided by Ro25-6981 in the present study (49%) is within the published range for this compound, as well as that provided by other NR2B subunit-selective antagonists such as ifenprodil, eliprodil, and CP-101,606 (Dogan et al. 1997; Kundrotiene et al. 2004; Lekieffre et al. 1997).

The lack of significant protection in the striatum (Figure 4.1) is also consistent with published reports using NMDA receptor antagonists in the MCAo model. Other authors report no protective effect within this structure or only slight protection compared to that within the cortex (Moyanova et al. 2007b; Sharkey et al. 1994; Yang et al. 1994). There are several possible explanations for the common lack of significant neuroprotection within the striatum. For MCAo models (particularly permanent MCAo), it may be directly related to a lack of sufficient generation of penumbral tissue within this structure, since the striatum generally represents the core of ischemic infarction in different applications of this model (Tatlisumak et al. 1998; Veltkamp et al. 2005). With particular regard to ET-1-induced ischemia, the importance of non-glutamatergic neurotoxic transmitters within the striatum (i.e. dopamine) has also been demonstrated (Bogaert et al. 2000). This notion coincides with results from Tayag and associates (1996) showing that the rat striatum primarily contains the ET_B, not the ET_A, endothelin

receptor. The ET_B receptor is believed to mediate the release of dopamine (Kataoka et al. 1995). Thus, the cytotoxic events within the striatum subsequent to ET-1 injection may be initially mediated via the excessive release of dopamine, possibly rendering compounds that primarily target glutamate-mediated excitotoxicity ineffectual for achieving neuroprotection within the striatum when using the ET-1 model.

The close similarity in infarct volume reduction between Ro25-6981 and MK-801 treated rats is of considerable interest (Figure 4.1). For Ro25-6981 treated animals, both total and cortical infarct volumes were only about 13% smaller compared to rats receiving MK-801. Thus, the data imply that almost all of the neuroprotection afforded by MK-801 (non-selective) was due to antagonism of NR2B-containing NMDA receptors and provide further evidence that NR2B antagonists are equally efficacious to non-selective blockers. Furthermore, the similar level of protection provided by Ro25-6981 compared to MK-801 in the present study is consistent with, and may be explained by, several recent studies that suggest the subunit arrangement and/or synaptic location of the NMDA receptor is paramount in determining their potential for inducing neurotoxicity (Chen et al. 2008; Hardingham et al. 2002; Liu et al. 2007). The NR2B-containing NMDA receptors are believed to be specifically linked to downstream signaling cascades that lead to the initiation of apoptosis under ischemic conditions. In contrast, the NR2A containing receptors appear to mediate cell survival signaling both *in vitro* and *in vivo* (Chen et al. 2008; Liu et al. 2007). Moreover, Ca^{2+} entry through synaptic NMDA receptors has been shown to trigger anti-apoptotic activity and promote pro-survival events, whereas Ca^{2+} entry via extrasynaptic NMDA receptors (believed to be primarily NR1/NR2B containing NMDA receptors) (Tovar and Westbrook 1999) is coupled to cell

death pathways (Hardingham et al. 2002; Vanhoutte and Bading 2003). However, the specific contribution of each major NMDA receptor subtype, and the importance of NMDA receptor location, to excitotoxic-induced cell death remains controversial (von Engelhardt et al. 2007; Sattler et al. 2000).

There are additional factors to consider when interpreting the infarct data presented in this chapter. In addition to the non-competitive, non-selective blockade of NMDA receptors, MK-801 is also reported to block VACCs (possibly of the L-type) that are activated under stressful conditions such as ischemia (Schurr et al. 1995). In the present study it can not be determined whether MK-801 had an effect on VACCs after the induction of ischemia, but this is still a particularly salient point considering that the mechanism of ET-1 vasoconstriction is suspected to be largely mediated via influx of Ca^{2+} through VACCs (Miwa et al. 1999; Takuwa et al. 1990). Hence, despite the fact that various concentrations of MK-801 appear to have no direct effect on the binding of ET-1 to vascular endothelin receptors (Sharkey et al. 1994) it is theoretically possible that the neuroprotection provided by MK-801 herein is at least partially related to reduced vasoconstriction caused by an interruption of Ca^{2+} influx to the endothelial and vascular smooth muscle cells. However, data obtained using Ro25-6981 provide an argument against a significant role for VACCs. Ro25-6981 is highly selective for NMDA receptors containing the NR2B subunit. The selectivity of Ro25-6981 for NR2B over NR2A is ~ 5000-fold and it lacks significant activity at AMPA/kainate receptors, as well as Na^{+} and Ca^{2+} channels even when applied at concentrations that show maximal protection against *in vitro* excitotoxicity (Fischer et al. 1997). Hence, the high selectivity of Ro25-6981 suggests that the significant reductions in infarct volume observed in this study are likely

more related to alterations in NMDA receptor-mediated excitotoxicity as opposed to any significant, direct hemodynamic effects.

4.4.2 Functional Neuroprotection via NMDA Receptor Antagonists in the ET-1 Model

The observation of functional neuroprotection against forelimb sensorimotor impairment in the current study (see Figures 4.5-4.6 for significant effects, and 4.7-4.8 primarily for patterns) is in agreement with earlier studies using MK-801, Ro25-6981, and other NR2B subunit-selective antagonists for treatment of stroke-induced behavioural deficits (Hoane et al. 1997; Liu et al. 2007; Moyanova et al. 2007b), and forelimb impairment in particular (Barth et al. 1990a; Kundrotiene et al. 2004; Moyanova et al. 2007a). In the present study, rats that were given an i.p. injection of saline vehicle prior to ischemia did not, as a group, tend to approach control (or pre-ischemia) levels of performance until the cessation of behavioural testing (i.e. 2 weeks) (Figures 4.5-4.7). Also, the degree and rate of behavioural improvement for these I + V treated animals within the second week of behavioural testing was marginal. In contrast, animals treated prior to ischemia with either Ro25-6981 or MK-801 showed either complete, or near complete, functional recovery within the first week post-ischemia on most behavioural measures (Figures 4.5-4.7). Indeed, aside from testing in the cylinder, it was rare for any I + R or I + M treated rats to demonstrate measurable forelimb impairment after the first week post-ischemia. Considering the magnitude of neuroprotection provided to the cortex within these two groups (section 4.3.1, and Figures 4.1-4.2), and that ischemic damage was largely restricted to the forelimb sensorimotor cortex in this model, these

observations are consistent with the notion that the degree of damage to the forelimb sensorimotor cortex is likely related to the level of impairment in forelimb function on most tests.

Similar to the results for infarct volume, administration of either MK-801 or Ro25-6981 resulted in similar levels and rates of forelimb functional recovery, a pattern that was consistent across all behavioural measures. However, there was a slight tendency for Ro25-6981 treated rats to have an improved behavioural outcome relative to MK-801 treated rats during the initial days of post-surgical testing on several behavioural measures (see Figures 4.6-4.8). Nevertheless, this observation may also be related to the dramatic weight loss initially experienced by MK-801 treated rats (Figure 4.4) and/or lingering behavioural disturbances induced by the administration of MK-801.

Administration of MK-801 in rats has been shown to induce weight loss, as well as stereotypies of the head and forepaws, head weaving, ataxia, or even a complete loss of movement (Andine et al. 1999; Moyanova et al. 2007a). These behavioural abnormalities induced by MK-801 are most pronounced during the initial hours after administration, and largely diminish within 24 hr (Moyanova et al. 2007a). It is acknowledged, therefore, that the existence of early behavioural disturbances may have hampered forelimb ability in the MK-801 treated animals for at least the first day of testing. In addition, although core body temperature was maintained between 36°C and 38°C throughout recovery, it is possible that the severe lack of movement evident in MK-801 rats for several hours after ET-1-induced ischemia could have afforded an additional means of brain protection to these rats. Neurons in peri-infarct tissue have been shown to be vulnerable to use-dependent excitotoxicity after the initiation of ischemia (Woodlee

and Schallert 2004).

Using the ET-1 model, numerous studies have shown that vehicle injection to the cortex and/or striatum does not result in behavioural abnormalities and only minor or no visible histological injury (Frost et al. 2006; Fuxe et al. 1997; Giuliani et al. 2007; Hughes et al. 2003; Windle et al. 2006). In the present study it was imperative for the sham group to serve as an intact behavioural control; thus, despite reports concerning the inertness of vehicle injection, sham surgery was deemed the most undisruptive and appropriate control group for behavioural testing (Adkins et al. 2004; Gilmour et al. 2004; Hsu and Jones 2006; Windle and Corbett 2005). As outlined in section 4.2.3, rats receiving ET-1 surgery and i.p. vehicle injection were deemed to represent the most appropriate control group for infarct analyses. Thus, the current experiment did not employ the use of stereotaxically injected vehicle in the brain as an additional control.

For the current study, it is acknowledged that the degree of histological injury and behavioural impairment due to injection of the ET-1 peptide can not be partitioned from any potential effects of stereotaxically injected vehicle in the brain. However, the reader is directed to Appendix B for an illustration of data pertaining to a very small group (n = 2) of rats that were given stereotaxic microinjections of vehicle (sterile distilled H_2O) control substance (equal volumes and surgical procedure as described in section 4.2.1) and subjected to behavioural testing procedures as previously described (see section 4.2.3-4.2.4). Data from this group imply that injection of vehicle in the brain causes negligible damage within the cortex but that at least part of the injury within the striatum may be accounted for by the vehicle substance (see Appendix B, Figure B-1). For the present study, this observation does offer a potential rationale for lack of significant

neuroprotection within the striatum (Figure 4.1), as well as a possible relationship between lack of striatal protection and the observation of no prominent preservation of behavioural function on the Schallert cylinder test (Figure 4.8). The small group that was given stereotaxic microinjection of vehicle only demonstrated pronounced forelimb impairment as measured on the cylinder test (see Appendix B, Figure B-4). It is likely that the cylinder test taxes other brain functions such as stimulus-response-associations, motivation, and spatial orientation much more heavily than other forelimb measures utilized in this study. This notion is consistent with numerous previous reports demonstrating a prominent role of the striatum in such 'higher' functions (see White 2009 for a comprehensive review on this matter).

In summary, compared to rats receiving saline vehicle prior to ischemia, the general observation of reduced forelimb deficits (in both magnitude and duration) for rats treated with NMDA receptor antagonists complements the marked reduction in infarct volume seen in these groups, and provides evidence that the ET-1 model is a suitable experimental method for studying structural neuroprotection as well as functional neuroprotection against forelimb sensorimotor impairment. These findings mark the first time that NMDA receptor antagonists have been shown to be neuroprotective in the ET-1 model, and further suggests that this new model of localized ischemic stroke may be a useful tool for *in vivo* experimental investigations which aim to dissect apart both the structural and functional protective properties of selective NR2B subunit antagonists compared to non-selective blockers of the NMDA receptor.

5.0 Pre-ischemic Treatment with Ro25-6981: Role of the Contralesional Hemisphere in Recovery from ET-1 induced lesions of the Forelimb Sensorimotor Cortex and Dorsolateral Striatum

5.1 Introduction

5.1.1 Contralesional Hemispheric Involvement in Recovery from Unilateral Stroke

The brain is currently viewed as a functionally and structurally dynamic entity (Nudo 2006). Many recent studies show reorganization within the brain following an ischemic lesion (Frost et al. 2003; Ramanathan et al. 2006; Schaechter et al. 2006; Xerri et al. 2003). An area of ongoing attention in stroke research is the role of the contralesional hemisphere in recovery from unilateral cerebral infarction injury (Biernaskie et al. 2005; Hsu and Jones 2006). The cortex contralateral and homotopic to unilateral cortical lesions in rats has been found to undergo structural neuronal plasticity, such as axonal sprouting, dendrite arborization, and synaptogenesis (Adkins et al. 2004; Biernaskie and Corbett 2001; Hsu and Jones 2006; Luke et al. 2004). In humans, increased excitability and enhanced cerebral blood flow have been found within contralesional premotor and sensorimotor cortical areas during recovery from unilateral ischemic stroke (Cramer et al. 1997; Johansen-Berg et al. 2002; Marshall et al. 2000).

However, whether or not these alterations contralateral to the damaged hemisphere actually facilitate recovery from unilateral stroke remains controversial.

Results from some publications have shown (or implied) that the contralesional cortex takes over some function of the impaired limb during the recovery process (Abo et al. 2001; Biernaskie et al. 2005; Zhengguang et al. 2001; Emerick et al. 2003); however, most findings from animal studies utilizing a 2-stage lesion or cortical inactivation procedure directly indicate that the homotopic contralesional hemisphere does not have a functional role in recovery from unilateral brain damage (Barth and Stanfield 1990; Barth et al. 1990b; Liu and Rouiller 1999; Shanina et al. 2006; Takata et al. 2006). The extent to which the contralesional hemisphere contributes to functional recovery is further debatable since numerous experimental and clinical studies have demonstrated that significant adaptive changes also take place in the direct vicinity of the infarct – within the ipsilesional hemisphere. (e.g. Castro-Alamancos and Borrell 1995; Frost et al. 2003; Loubinoux et al. 2003).

It is, however, of interest that most investigations have specifically examined the functional role of the contralesional cortex in these processes, neglecting the functional involvement of the homotopic subcortex after unilateral cortical and subcortical ischemic damage (Barth and Stanfield 1990; Barth et al. 1990b; Liu and Rouiller 1999; Shanina et al. 2006; Takata et al. 2006). Results from neuroimaging studies in humans demonstrate that if subcortical structures subserving motor functions are also damaged, activation within the undamaged hemisphere is significantly augmented during the recovery process relative to individuals with purely cortical lesions (Feydy et al. 2002; Luft et al. 2004). As previously outlined, subcortical infarction is common in clinical ischemic stroke (Cramer 2003), and damage to these structures is strongly linked with impairment in upper extremity sensorimotor function following stroke (Feys et al. 2000). Therefore,

animal studies that aim to impede neuronal function in the contralesional hemisphere in order to examine its functional role in recovery from upper extremity impairment after unilateral ischemic stroke may be better served by also incorporating ischemic damage to the subcortex in such experiments.

5.1.2 NR2B Subunit-Selective Antagonism and Functional Neuroprotection within the Ipsilesional Hemisphere

Alterations in functional plasticity after brain injury can be further affected by a variety of experimental conditions, including electrical stimulation, behavioural experience, and pharmacologic treatment (Nilsson et al. 1999; Uy et al. 2003; Xerri et al. 2003). Indeed, the administration of anti-ischemic agents and/or exposure to an enriched environment following stroke has been shown to interact with injury-induced remodeling processes in a manner that better preserves somatotopic organization and neural responsiveness in ipsilesional sensorimotor regions (Pariente et al. 2001; Xerri et al. 2003). Potentially, such manipulations may limit an otherwise necessary involvement of the contralesional hemisphere for reinstatement of pre-ischemic behavioural function.

Results from previous work outlined within this thesis (i.e. Chapter 4) demonstrated that a single, pre-ischemic i.p. administration (6mg/kg) of the NR2B-subunit-selective antagonist, Ro25-6981 substantially improved recovery from unilateral deficits in both vibrissae- and tactile-stimulated forelimb placing tests and significantly reduced infarct volume relative to rats receiving time-matched saline injections. If Ro25-6981 does indeed confer authentic functional neuroprotection (i.e. behavioural

preservation is due to an actual sparing of ipsilesional neuronal circuitry) then a greater amount of residual ipsilesional sensorimotor circuits should be left functionally intact. Thus, this compound could limit the unfavorable involvement of the undamaged hemisphere for reinstatement of behavioural function.

5.1.3 Study Goals

The primary aim of this study was to obtain preliminary data on the functional role of the contralesional hemisphere, homotopic to an initial unilateral ET-1 lesion, in the recovery of forelimb placing behaviour subsequent to pre-ischemic treatment with an NR2B subunit-selective antagonist or vehicle control. For this experiment, it was of interest to determine if a second ischemic lesion to the previously non-lesioned hemisphere, introduced subsequent to recovery of placing behaviour with the forelimb contralateral to the initial lesion, would produce a reinstatement of placing inaccuracy with the contralateral forelimb. It was of further interest to examine the possible effect of pre-ischemic treatment with the NR2B subunit-selective antagonist Ro25-6981 on this phenomenon.

5.2 Methods and Materials

5.2.1 Surgery

Endothelin-1 (400pmol/μl) was dissolved in sterile distilled H_2O and microinjected to the forelimb sensorimotor cortex and dorsolateral striatum. Two cortical

injections and one striatal injection were administered to the right hemisphere. Stereotaxic injection coordinates were: cortical 1) AP +2.0, ML -2.9, DV, -2.5; cortical 2) AP +1.0, ML -2.9, DV -2.5; striatal AP +0.9, ML -3.6, DV -6.0. Endothelin-1 was microinjected in a volume of 2µl, 2.5µl, and 1µl for cortical 1, cortical 2, and the striatal injection, respectively. For further details see section 2.3. At 15 days post-surgery rats received unilateral ischemic lesions to the homotopic contralesional hemisphere (i.e. forelimb sensorimotor cortex and dorsolateral striatum of the left hemisphere) using the same stereotaxic surgical method (modified only to target the opposite hemisphere) and dosage of ET-1 as outlined for the initial ischemic insult. Rats receiving time-matched 2-stage sham surgeries served as intact controls (n = 4).

5.2.2 Drug Treatment/Experimental Conditions

Approximately 24 hr after arrival in the animal colony room, rats were pseudo-randomly put into one of 3 groups (ET-1/Ro25-6981, ET-1/Saline, or Sham surgery). During the initial surgery, approximately 15 min prior to the initiation of the ET-1 injections rats were given i.p. injections of either Ro25-6981 (6mg/kg) (n = 5) [Ischemia + Ro25-6981 (I + R)], or equal volumes of saline vehicle (n = 5) [Ischemia + Vehicle (I + V)]. Groups receiving drug treatment were coded by a lab technician for the duration of the experiment through to data analysis in order to blind the experimenter to condition. All rats in the above groups were represented within one cohort. Given that appropriate investigation of the study objective was conditional upon some degree of initial behavioural impairment, animals that did not meet the a priori criteria of 'survivors with deficit' (detailed in section 2.8) were removed from the experiment ($n = 2$).

5.2.3 Behavioural Testing and Body Weight Measurements

Animals were tested for forelimb placing accuracy (as conducted previously) one day prior, 3 hr post, and every day over the next 14 days after the first surgery. Following the second surgery (day 15), rats were again tested for forelimb placing accuracy at 3 hr post-surgery, and on days 1 and 2 post-second surgery. Both ipsilateral and contralateral placing responses were examined. During the initial 2 weeks post-surgery, all rats in this experiment were exposed to a behavioural testing regimen identical to that described in chapter 4 of this thesis (i.e. FPR, cylinder, and adhesive removal testing) in order to combine this data with previous I + V and I + R groups if warranted. Animals were weighed every day of experimentation.

5.2.4 Histology and Infarct Estimation

Seventeen days post-surgery rats were euthanized. Brains were vibratome sectioned and stained with cresyl violet for infarct analysis (for further details see sections 2.6.1, 2.6.2, and 2.7). In order to examine the effect of NR2B subunit-selective antagonism on infarct volume within a larger group of rats, infarct measurements (i.e. volume and area) for these groups were combined with previous I + V and I + R groups, contingent on no significant difference between their respective patterns of ischemic damage or estimated volumes of brain injury. Combined data are presented in Appendix A. The degree of ischemic damage caused by the second ET-1 surgery was not quantified in this study, though the experimenter did verify that contralesional histological damage was adequate and properly placed.

5.3 Results

5.3.1 Infarct Data

Pre-ischemic treatment with Ro25-6981 significantly reduced total infarct volume by ~ 32% relative to saline vehicle control rats (23.8 ± 3.1 mm^3 vs 34.7 ± 3.3 mm^3, respectively) ($F_{(1, 8)} = 5.952$, $P = 0.041$) (see Figure 5.1 for group infarct volume comparisons). Figure 5.2 illustrates the cortical and striatal areas of ischemic brain damage at measured stereotaxic levels produced by the initial ET-1 injections.

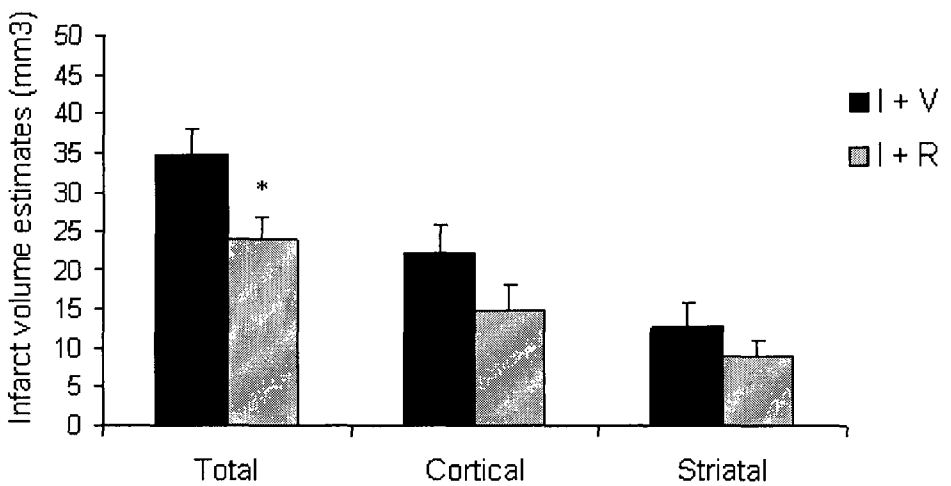

Figure 5.1 Comparison of total, cortical, and striatal infarct volume estimates between the I + V and I + R groups. Data represent mean ± S.E.M. ($P < 0.05$).

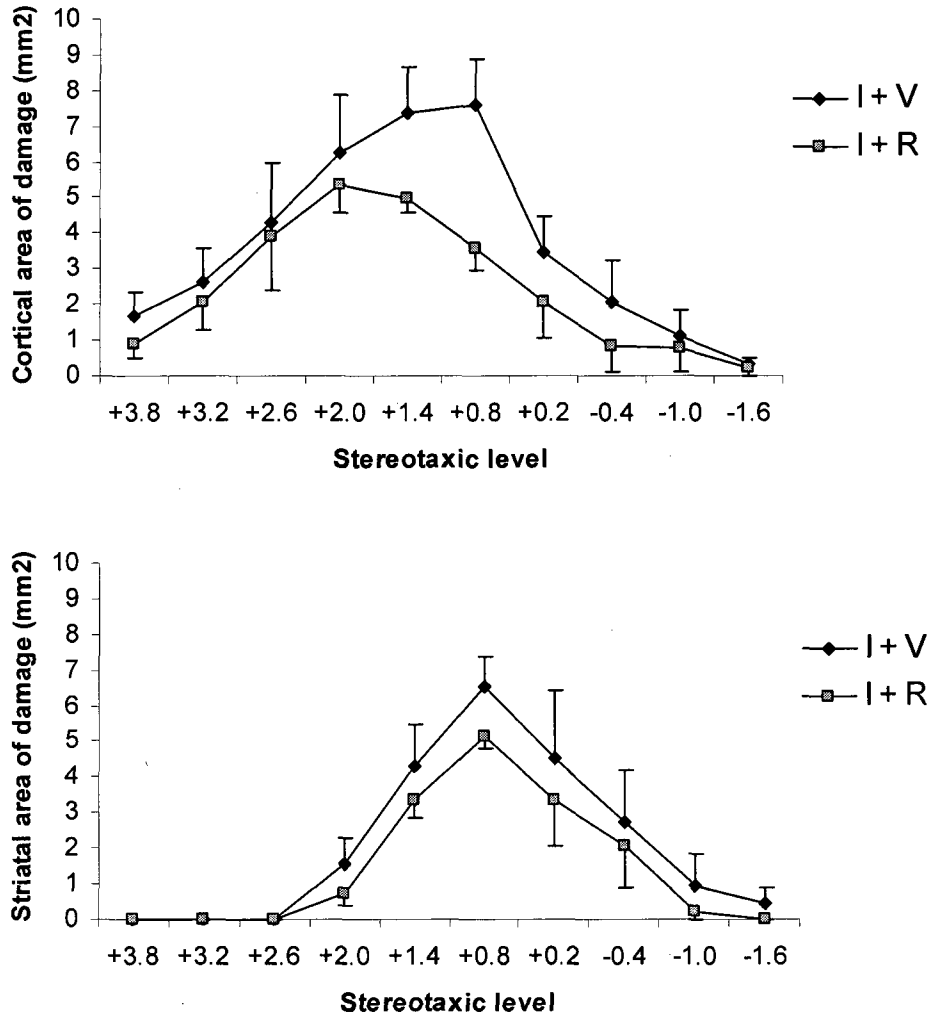

Figure 5.2. Comparison of I + V and I + R treated rats on area of ischemic brain damage in cortex (top) and striatum (bottom) at 10 stereotaxic levels assessed 17 days following focal ischemic stroke induced by multiple intracerebral injections of ET-1. Data represent mean ± S.E.M.

Figure 5.3 depicts representative cresyl violet stained coronal sections from the area of most concentrated ischemic damage for the I + V and I + R groups, with comparison to a typical sham brain. The brain sections shown in Figure 5.3 also illustrate the damage caused to the contralesional forelimb sensorimotor cortex and dorsolateral striatum after the second ET-1 surgery. Regarding the application of a second ischemic

lesion, all rats had ample damage to the homotopic forelimb sensorimotor cortex and lateral striatum.

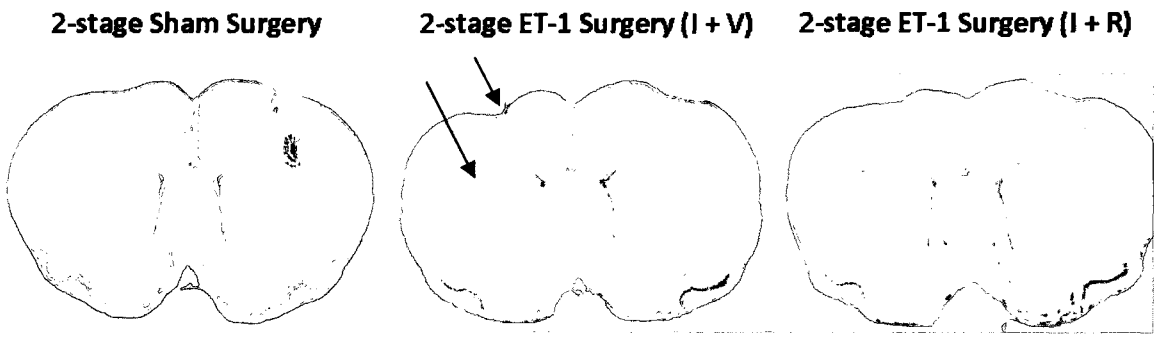

Figure 5.3. Representative cresyl violet stained sections (150μm) from the I + V and I + R groups showing ischemic damage to both the ipsilesional (first surgery) and contralesional (second surgery) hemispheres. Sham surgery control is shown on far left. Arrows for I + V indicate damage from initial surgery (applies to all three sections). Short (top) arrow points to cortical lesion and longer (bottom) arrow indicates the striatal lesion.

5.3.2 Behavioural and Body Weight Data

Prior to surgery, all rats placed with 100% accuracy. Contralateral placing accuracy was reduced to 0% for both I + V and I + R conditions when measured at 3 hr after the initial ET-1 surgery. Ipsilateral placing accuracy was largely unaffected at this time point; one rat in the I + V condition had ipsilateral forelimb placing accuracy reduced to 60% and 80% for vibrissae- and tactile stimulated tests, respectively. Subsequent to the initial testing at 3 hr post-surgery, but prior to the second surgery, ipsilateral placing accuracy was unaffected for all animals. Appropriately, the forelimb contralateral to the second ET-1 induced ischemic lesion was impaired to a substantial degree in all animals for the 2 days post-second surgery. This 2-stage surgical experiment did not reduce placing accuracy in sham controls to any notable degree throughout the experiment (i.e. for one sham rat, vibrissae-stimulated placing accuracy

was reduced to 80% for the contralateral forelimb at 3 hr after the first surgery). In consideration of all the above listed events, only placing performance of the forelimb contralateral to the initial ischemic lesion is detailed below.

There were significant effects of Day ($F_{(18, 198)} = 18.684$, $P = 0.000$), Group ($F_{(2, 11)} = 20.480$, $P = 0.000$), and Group by Day interaction ($F_{(36, 198)} = 5.735$, $P = 0.000$) for tactile-stimulated placing accuracy with the forelimb contralateral to the initial lesion (see Figure 5.4). Rats in the I + V condition were significantly impaired relative to sham controls until day 10 after the initial ischemic lesion ($P < 0.05$), whereas rats in the I + R condition were significantly impaired until day 4 ($P < 0.05$) (see Figure 5.4). There was no group effect for contralateral tactile-stimulated placing accuracy subsequent to the second lesion ($P = 0.586$) (Figure 5.4). Although no significant difference was noted between I + V and I + R rats after the second lesion, I + R rats showed significantly improved contralateral tactile-stimulated placing performance relative to I + V rats on days 7, 8, 9, and 10 during recovery from the first ET-1 surgery ($P < 0.05$).

There were significant effects of Day ($F_{(18, 198)} = 17.191$, $P = 0.000$), Group ($F_{(2, 11)} = 20.557$, $P = 0.000$), and Group by Day interaction ($F_{(36, 198)} = 5.049$, $P = 0.000$) for vibrissae-stimulated placing accuracy with the forelimb contralateral to the initial lesion (Figure 5.4). Rats in the I + V condition were significantly impaired relative to sham controls until day 10 after the initial ischemic lesion ($P < 0.05$), whereas the I + R rats showed significant impairment until day 5 ($P < 0.05$). Rats in the I + R condition had significantly improved placing accuracy relative to I + V rats on days 7, 8, 9, and 10 of testing. Subsequent to the second lesion, the I + V rats demonstrated a significant (72%) reduction in contralateral vibrissae-stimulated placing accuracy when measured 3 hr post-

surgery relative to sham rats (28% accuracy vs. 100% accuracy, respectively) ($P < 0.05$) (Figure 5.4). In contrast, contralateral placing accuracy in the I + R group was not significantly impaired relative to sham controls, when measured at 3 hr post-surgery ($P = 0.166$). The ~ 40% difference in placing accuracy between the I + V and I + R groups approached significance (28% accuracy vs. 68% accuracy, respectively) ($P = 0.075$) (Figure 5.4). Vibrissae-stimulated placing accuracy gradually returned or approached control levels over the subsequent 2 days of testing for both drug treated groups.

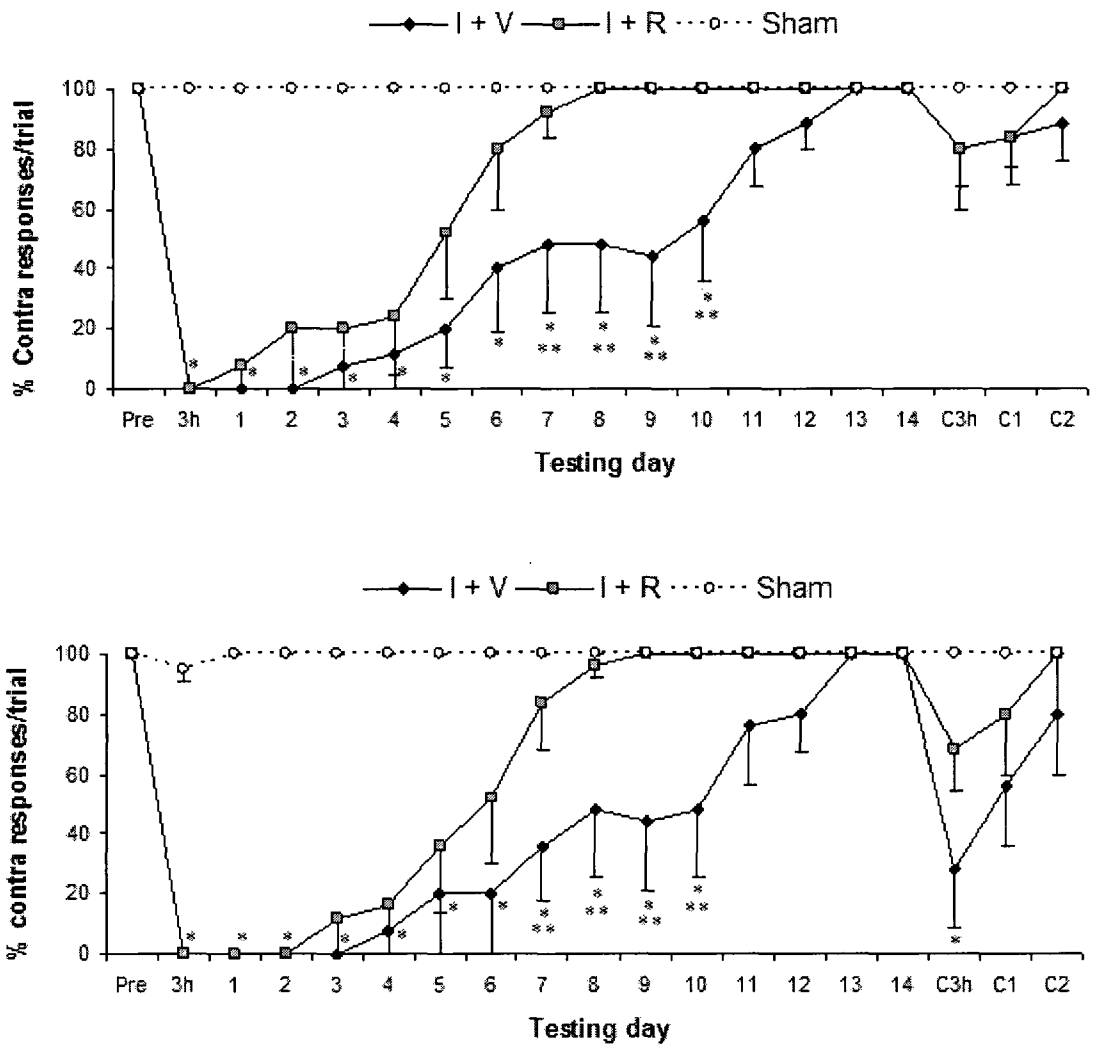

Figure 5.4. Comparison between I + V, I + R, and sham surgery rats on contralateral forelimb placing accuracy for 14 days after an initial ischemic lesion to the right hemisphere, followed by placing accuracy with the contralateral limb for 2 days subsequent to a 2nd lesion (day 15) in the homotopic left hemisphere. Contralateral tactile-stimulated placing (top); Contralateral vibrissae-stimulated placing (bottom). C3h, C1, C2 = testing at 3 hr, 1 day, and 2 days after the second surgery, respectively. Data represent mean ± S.E.M. (* indicates a significant difference of I + V from Sham group, ** indicates a significant difference from I + R, $P < 0.05$).

For body weight measures there was a significant effect of Day ($F_{(16, 176)} =$ 231.304, $P = 0.000$). All groups dropped in body weight after the initial surgery which

returned to pre-surgery levels in just a few days. Figure 5.5 illustrates the mean change from pre-surgery body weight for all groups subsequent to the initial surgery.

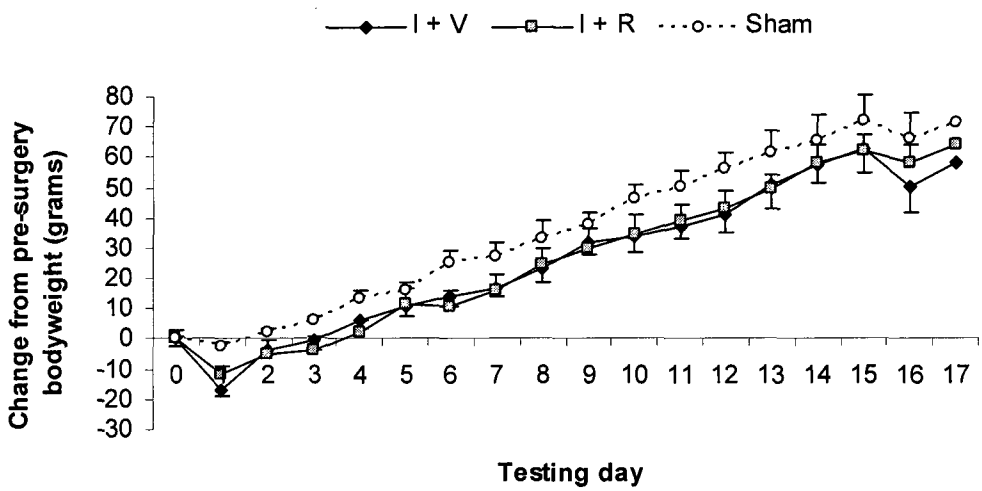

Figure 5.5. Comparison of changes from pre initial surgery body weight (grams) among I + V, I + R, and Sham groups over 17 days of testing. Second stereotaxic surgery was conducted on day 15. Data are mean ± S.E.M.

5.3.3 Mortality Rates and Model Success

The overall mortality rate for this experiment was 0%. Model success rate was calculated to be 83% for this study (see section 2.8 for details pertaining to mortality and model success rate calculations). .

5.4 Discussion

The forelimb placing tests represent a very straightforward method for measuring forelimb functionality and previous work in this thesis demonstrated a sufficient degree

of initial impairment (that was almost exclusively contralateral to a unilateral ischemic lesion) followed by a recovery profile that gradually returned to control levels. Accordingly, the vibrissae- and tactile-stimulated forelimb placing tasks were utilized to examine the effect of a 2-stage bilateral ischemic lesion paradigm on contralateral forelimb capacity.

The primary finding of this study was that the introduction of a second, homotopic ischemic lesion in the left hemisphere significantly disrupted the recovered function of the forelimb contralateral to an initial right hemisphere lesion for the I + V control group. This disruption was transient in nature (i.e. only significantly disrupted at 3 hr post-second surgery), and only significant for contralateral vibrissae-stimulated placing. Thus, recovered tactile-stimulated placing was unaffected after the second surgery (Figure 5.4). Functional imaging studies of recovering stroke patients have reported on the recruitment of bilateral sensorimotor areas during movement of the impaired limb, compared to primarily contralateral recruitment in control participants (Feydy et al. 2002; Johansen-Berg et al. 2002), yet such studies can not directly determine whether these alterations in contralesional activity actually contribute to functional recovery of the upper limb. Though the current study used a small number of animals, it does provide preliminary evidence in support of a functional role for the undamaged, contralesional hemisphere in the recovery of vibrissae-stimulated forelimb placing behaviour in rats.

This finding is in agreement to previous results produced by Biernaskie and colleagues (2005). After ET-1 induced MCAo, and 4 weeks of rehabilitation, these authors showed that contralateral forelimb reaching impairment was significantly

reinstated by blocking neuronal activity in the contralesional forelimb motor cortex via stereotaxic microinjection of lidocaine to this area; results implied that this effect was not due to a confounding general reduction in reaching attempts (Biernaskie et al. 2005). In contrast, using 2-stage photothrombotic infarcts to the forelimb sensorimotor cortex, Shanina and coworkers (2006) found that damage to the homotopic contralesional cortex only affected the forelimb opposite the second lesion, and thus did not reinstate original deficits observed (i.e. forelimb sliding in the cylinder and forelimb misplacement during grid walking) (Shanina et al. 2006).

With specific regard to forelimb placing behaviour, after a 2-stage cortical ablation lesion paradigm designed to examine the role of the undamaged sensorimotor cortex, Barth and Stanfield (1990) showed that a second cortical lesion in the undamaged sensorimotor cortex of adult rats only produced vibrissae-stimulated placing deficits in the forelimb contralateral to the second lesion when tested over 3 days after the second lesion. The limb contralateral to the initial lesion was completely unaffected (Barth and Stanfield 1990). The lack of functional involvement in the homotopic contralesional hemisphere for recovery from forelimb placing deficits has not been contested since this experiment by Barth and Stanfield almost 20 years ago. If the results observed in the study herein legitimately reflect an actual functional role of the homotopic hemisphere, an unanswered question is what specific structure(s) or pathway(s) mediate these functional contralesional effects. Since the homotopic cortex and subcortex were both subjected to ET-1 induced lesions, it is impossible to know whether the functional contribution came from alterations within the cortex, striatum, or, perhaps more likely, from both structures – due to a disruption in newly contrived cortico-striatal and

interhemispheric cortico-cortico neuronal connections (reviewed in Carmichael 2003).

In opposition, another possibility is that unilateral ET-1 induced ischemic lesions cause bi-hemispheric neurodegeneration that creates functional abnormalities in both forelimbs when using a 2-stage lesion approach. The likelihood of such an event seems small, given that previous lab work with this particular ET-1 model showed negligible bi-hemispheric tissue damage and failed to disrupt ipsilateral forelimb placing performance to any notable degree at any time point. Nonetheless, in the present experiment, despite no clear histological evidence against the notion that both surgeries generated ischemic lesions only to the intended hemisphere (plus an almost exclusive generation of contralateral forelimb deficits after the initial surgery) such an effect can not be ruled out due to the already compromised state of the ipsilesional (right) hemisphere prior to creating ischemic lesions in the contralesional (left) hemisphere.

Finally, using a very similar ET-1 surgical protocol, Windle and associates (2006) demonstrated that, in addition to profound reductions in ipsilesional hemispheric blood flow after ET-1 injections ($\geq$ 50ml/100g/min vs. baseline of ~250ml/100g/min), local CBF is also moderately reduced in homotopic brain areas (~100ml/100g/min vs. baseline of ~250ml/100g/min) for several hours after surgery. As with the possibility of bi-hemispheric neuronal damage subsequent to the second lesion, a reinstatement of contralateral forelimb placing deficits due to reduced CBF in an already compromised area of brain can not be directly refuted in the present study. In this report by Windle and colleagues (2006), the only region in the hemisphere opposite the ET-1 injections to sustain reductions in CBF comparable to those in the ipsilesional hemisphere was an area of brain that directly corresponds to representation of the vibrissae (Hall and Lindholm

1974; Windle et al. 2006). Thus, an effect of reduced CBF in the opposite hemisphere may partly explain the significant reinstatement of vibrissae-stimulated placing deficits compared to the negligible reinstatement for tactile-stimulated placing observed herein. In large, however, the enhanced reinstatement of contralateral vibrissae-stimulated placing compared to tactile-stimulated placing is due to unknown factors.

In the current study, pre-ischemic treatment with NR2B-subunit-selective antagonist, Ro25-6981 (6mg/kg) significantly reduced the total volume of the initial infarct by ~ 32% relative to the vehicle control condition. It was of interest to explore whether treatment with Ro25-6981 would protect against the required involvement of the contralesional hemisphere during recovery, possibly due to a legitimate neuroprotective effect within the ipsilesional hemisphere (i.e. a drug-induced sparing of the ipsilesional sensorimotor circuitry). However, findings from this study do not support a significant effect of pre-ischemic treatment with Ro25-6981 on reduced contralesional hemispheric involvement in recovery from forelimb placing deficits, despite significant neuroprotection from the initial ischemic insult. It is important, though, to consider the small group sizes employed. Further investigation with larger group sizes may therefore be warranted, especially since the protective effect of Ro25-6981 against reinstatement of injury approached significance compared to the group receiving the saline vehicle (see section 5.3.2).

Taking all of the data into consideration, the experimental factor most likely contributing to the occurrence of reinstated contralateral forelimb injury may be duration of recovered placing performance prior to the introduction of a second, homotopic lesion. Within the I + V group, the 3 rats which had vibrissae-stimulated impairments lasting

beyond 10 days after the initial lesion showed substantial reinstatement of injury up until at least day 1 after the second lesion. In contrast, the other 2 rats fully recovered on day 5 and day 8; interestingly, the day-5-recovered rat showed no evidence of re-injury and the day-8-recovered rat only exhibited moderate re-injury when tested at 3 hr post-second surgery. All rats in the I + R group fully recovered within approximately 1 week, and indeed this may be the primary reason for their more modest reinstatement of forelimb impairment. Such a pattern corresponds well with clinical imaging data demonstrating that contralesional activation patterns are often temporally limited (Cramer et al. 1997; Feydy et al. 2002) but may persist for patients that do not make a good functional recovery (Serrien et al. 2004). Thus, it would be sound to investigate such an effect in a much larger group of animals by either customizing the time of the second, homotopic lesion to the day of recovery for each rat, or by splitting a larger data set into early and late recovery conditions with a uniform second surgery time.

In sum, results obtained from the present experiment provide preliminary evidence that the contralesional forelimb sensorimotor cortex and/or dorsolateral striatum may have a functional role in the recovery of forelimb placing behaviour in the rat following ET-1-induced focal ischemia. The above findings, however, do not directly support an effect of pre-ischemic treatment with Ro25-6981 for reducing the putative involvement of the undamaged homotopic hemisphere during the recovery process, despite a significant reduction in initial infarct volume. Nonetheless, given the small number of animals utilized, and possible confounding effects of the second lesion, further investigation is required to draw more firm conclusions on the above observations.

6.0 Overall Conclusions and Future Directions

6.1 Evaluation of the ET-1 Model used for this Thesis

Pertinent modifications pertaining to the volume and concentration of injected ET-1, as well as the stereotaxic location of the needle injection site, were performed in order to produce a model of localized ischemic stroke that resulted in consistent injury to forelimb representation areas of the brain (i.e. forelimb sensorimotor cortex and dorsolateral striatum) and measurable unilateral impairment of the contralateral forelimb. Thus, this primary experimental objective was accomplished.

The forelimb deficit and recovery profile demonstrated in chapter 4 and 5 (see Figures 4.5-4.8; 5.4) using the ET-1 model was characterized by reductions in forelimb function that typically did not recover or approach recovery until approximately 2 weeks post-surgery. Although the observed reductions in forelimb performance (i.e. I + V compared to sham control) only reached significance on some days post-ischemia (Figure 4.6-4.8), data analysis of amalgamated groups (see Appendix A) demonstrates that employing an *n* of 14 is effective in correcting this issue. Likewise, results obtained by combining groups in chapter 4 and 5 suggest that the model is reproducible regarding infarct volume and pattern of ischemic damage within both the cortex and striatum (compare data for I + V groups in Figures 4.1-4.2 with Figures 5.1-5.2, as well as Figure A-1 and A-2 in Appendix A). Similar reproducibility was observed for the behavioural data, given that the forelimb impairment/recovery profile was largely unchanged across all forelimb measures subsequent to combining these groups (compare data for I + V

groups in Figures 4.5-4.8 with A-3 - A-6 in Appendix A). The magnitude of functional forelimb impairment observed herein is consistent with published reports using similar forelimb measures in the ET-1 model (i.e. forelimb placing, traditional 'sticky-tape', and cylinder testing) (Adkins et al. 2004; Allred and Jones 2008; Hewlett and Corbett 2006; Windle and Corbett 2005; Windle et al 2006), though the duration of functional impairment in the cylinder has been reported by others to last for at least one month post-surgery (Hewlett and Corbett 2006; Windle and Corbett 2005; Windle et al 2006).

One of the most attractive features of the ET-1 model for functional neuroprotection studies is its high success rate (Windle et al. 2006; Windle and Corbett 2005). Favourable model success rates detailed herein (~ 84 % averaged across all experiments) are consistent with this notion (see sections 3.2.3.3, 3.3.3.5, 4.3.6, and 5.3.3). Thus, the large majority of rats survived the duration of the experiment, and of these survivors, almost all presented with forelimb functional impairment relative to control rats. Indeed, this result directly reflects the quintessential feature of the ET-1 model: functional loss to the contralateral forelimb subsequent to direct damage within forelimb regions of the brain. Therefore, although it is important to recognize that almost any behaviour is the result of complex interconnections among various adjacent and remote sub-regions within the brain, based on results within this thesis it appears that the ET-1 model, due to its high level of precision, produces forelimb impairment in a high throughput manner after experimentally induced ischemia.

6.2 Neuroprotection in the ET-1 Model

The ET-1 model has clear advantages for functional neuroprotection studies (outlined in sections1.7.3 and 4.1.3), yet the attainment of neuroprotection in this model is far from clear (outlined in section 4.1.5). Previously, unconventional pharmacological agents have been investigated in the ET-1 model with either no success (Windle and Corbett 2005), moderate success (Hewlett and Corbett 2006), or the significant neuroprotective effects were only relevant within the striatum (Giuliani et al. 2007; Ottani et al. 2003; Ueki et al. 1993). Thus, neuroprotection studies using more reliable compounds, such as NMDA receptor antagonists, have not been conducted in the ET-1 model. Data generated within this thesis demonstrate that the conventional NMDA receptor antagonists, MK-801 (5mg/kg) and Ro25-6981 (6mg/kg) significantly reduce the volume of both total hemispheric brain infarct (44%, 49%; respectively) and cortical infarct (62%, 66% reductions, respectively) compared to vehicle control rats (see Figure 4.1).

Notably, the neuroprotection conferred to groups receiving either MK-801 (non-selective antagonist) or Ro25-6981 (NR2B subunit-selective antagonist) was of the same degree (see Figures 4.1-4.2). Further, it was interesting to find that the degree of functional protection between these two groups mirrored their similarities in infarct reduction. Thus, results herein demonstrate that NR2B subunit-selective antagonists are as effective as non-selective NMDA receptor antagonists for reducing both structural damage to the brain and preserving forelimb function subsequent to ischemia, without the undesirable side-effects associated with non-selective blockade of NMDA receptors.

Data from chapter 5, however, suggest that the functional protection provided by the NR2B subunit-selective antagonist, Ro25-6981, was not directly associated with a reduced involvement of the contralesional hemisphere during the recovery process (Figure 5.4).

Forelimb performance was generally improved across most behavioural tests for groups receiving NMDA receptor antagonists, but the incidence of statistical significance for this effect varied between measures and across testing days (see Figures 4.5-4.8; 5.4). It is of note, however, that the amalgamation of data from chapter 4 and 5 afforded a more consistent pattern of significant group by day differences between rats receiving Ro25-6981 and rats serving as ischemic control. This result suggests that a larger n (~ 12-15) may be necessary to consistently detect significant protective effects to forelimb function in the ET-1 model. Further, using Ro25-6981, the observation of significant neuroprotection against total hemispheric damage (~ 32%) (Figure 5.1), coupled with a hastened recovery of forelimb function (Figure 5.4), when using just a small group of rats (n = 4-5) is particularly encouraging. Such results, plus the high level of protection seen subsequent to the amalgamation of groups, suggest that the neuroprotection provided by Ro25-6981 is robust and reproducible in the ET-1 model.

In conclusion, these results strongly suggest that the ET-1 model of upper extremity impairment in stroke is suitable for neuroprotection studies using NMDA receptor antagonists as therapeutic compounds, but that an n of ~ 14 may be required to further stabilize significant group by day effects. Thus, given the advantages associated with using the ET-1 model, such as the production of well-defined, reproducible ischemic lesions and a high model success rate, this model may prove highly valuable for

experimental stroke research projects that aim to use NMDA receptor antagonists for studying protection to the brain and the enhancement of forelimb functional recovery after focal ischemia.

6.3 Future Directions

Building on the results obtained in these experiments, additional investigations should first attempt to clarify the potentially injurious effect of stereotaxically injected vehicle control substance in a larger experiment, with particular regard to its influence on cell viability within the striatum. Endothelin-1 injections do produce ischemia within the brain (Fuxe et al. 1997); however, further bench-work is required to more clearly elucidate other potential mechanisms of brain injury associated with use of this model. Second, although the data within this thesis provide evidence that neuroprotection with NMDA receptor antagonists is possible in the ET-1 model, it does not specifically address the question of whether or not this model represents a clinically relevant experimental tool for such research endeavors. Thus, other NMDA receptor antagonists that hold clinical potential should be tested in the ET-1 model at various time points post-ischemia.

Third, the neuroprotective effect conferred by NMDA receptor blockade detailed in this thesis may not, necessarily, be evident *in spite* of the known glutamatergic and Ca^{2+}-related effects of ET-1 (Blomstrand et al. 1999; Koizumi et al. 1994; MacCumber et al. 1990; Sasaki et al. 1997), but rather as a direct consequence of such events. In other words, given that ET-1 may augment glutamate excitotoxicity, it is conceivable that anti-

excitotoxic agents may actually be the most efficacious neuroprotective compounds in this model. Thus other neuroprotective compounds that do not primarily act by perturbing glutamate and Ca^{2+}- mediated excitotoxicity should also be more thoroughly examined in the ET-1 model.

Finally, an important next step next step to further clarifying the applicability of the ET-1 model would be to implement tests of skilled forelimb function, such as reaching and grasping for objects, skilled gait (i.e. ladder or beam walking), and distal forepaw dexterity (i.e. sunflower seed opening), since forelimb impairment on such measures has been shown to persist for up to 12 weeks post-surgery using the ET-1 model, as determined by an additional group of researchers (Gilmour et al. 2004, 2005). Favourable results in this area could lead to a highly relevant application of this model for use in stroke research studies. Specifically, investigators could examine the therapeutic effect of clinically relevant NMDA receptor antagonists (and other compounds) on forelimb recovery into the more chronic stages of stroke when applied in conjunction with various neurorehabilitation strategies. Although such an application is first contingent on the demonstration of post-ischemic neuroprotection in this model, results outlined herein indicate the potential for this valuable, and currently largely vacant, experimental niche to be occupied by the ET-1 model.

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Appendix A – Amalgamated Infarct and Behavioural Data for Groups Represented in Chapter 4 and 5.

Objective: To examine the effect of pre-ischemic administration of the NR2B subunit-selective antagonist, Ro25-6981 (compared to controls), on infarct volume and forelimb behavioural capacity, when using an increased experimental *n*.

Methods: Infarct and behavioural data from experiments in chapter 4 and 5 were combined in the current analysis. All statistical methods utilized herein were consistent with previous applications (see section 2.9). Rats excluded from previous studies (chapter 4 and 5) (i.e. one I + V, and two I + R rats) were not included in the amalgamation (see sections 4.2.2 and 5.2.2). The remaining thirty-eight rats [I + V (n = 14), I + R (n = 13), and Sham (n = 11)] were analyzed herein. However, one Sham rat (from chapter 5 study) and one I + R rat (from chapter 4 study) were removed from the modified adhesive removal test due to an almost complete lack of attendance to the 'sticky-tape' prior to surgery. Thus, specifically for the 'sticky-tape' test, groups presented here are: I + V (n = 14), I + R (n = 12), and Sham (n = 10). See sections 2.2-2.4; 2.6-2.7; 2.9; 4.2.1-4.2.4 for all other methodological details.

Results: For infarct data, results are shown for infarct volumes, and cortical and striatal areas of ischemic damage. For behavioural data, results are presented for the FPR test, forelimb placing, "sticky-tape" test, and cylinder test.

Figure A-1 demonstrates a significant reduction in both total and cortical infarct volume for I + R treated rats compared to vehicle control.

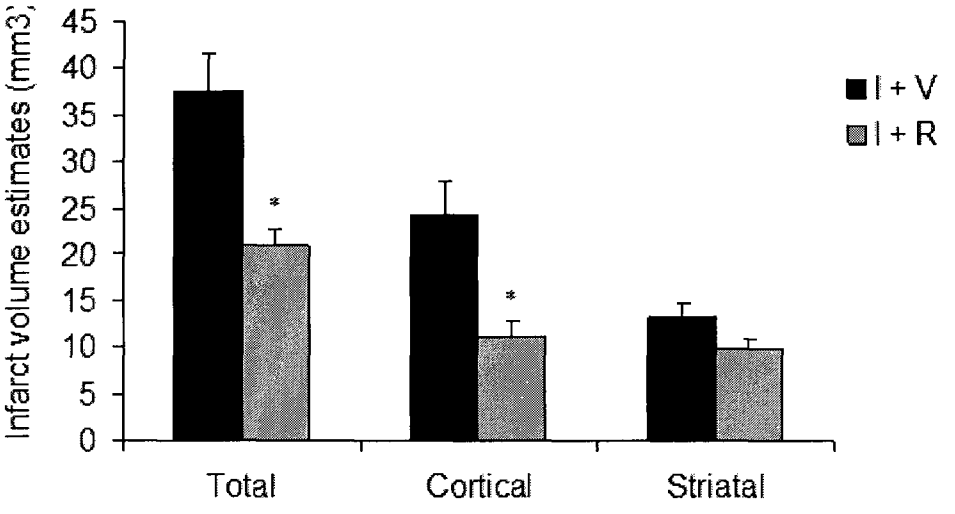

Figure A-1. Comparison of mean (± S.E.M.) total, cortical, and striatal infarct volume estimates among I + V and I + R treated rats (* indicates a significant difference from I + V group, $P < 0.05$).

Figure A-2 illustrates the areas of ischemic damage to cortical and striatal regions across the measured stereotaxic levels. I + R treated rats had significantly less damage to the cortex at 5 stereotaxic levels, compared to control.

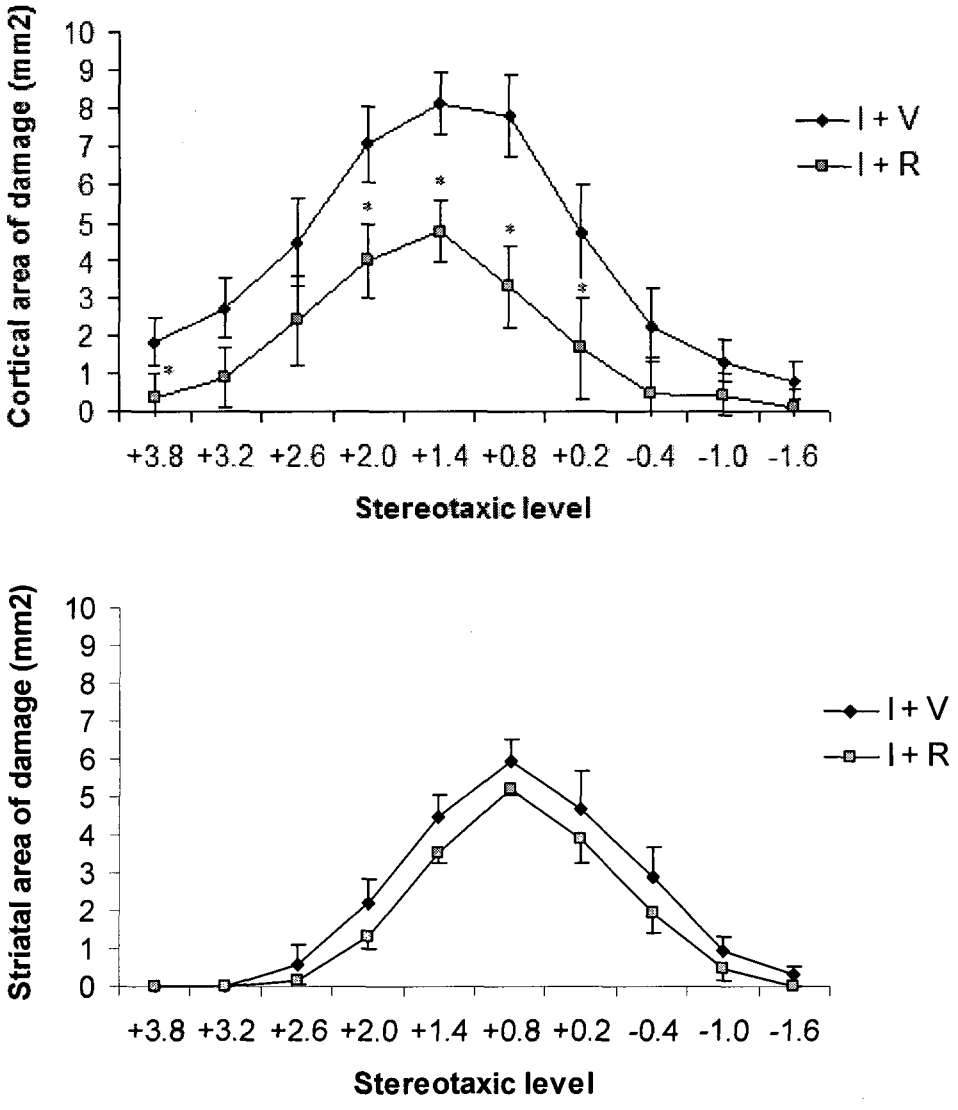

Figure A-2. Comparison between I + V and I + R treated rats on mean (± S.E.M.) area of ischemic brain damage in cortex (top) and striatum (bottom) at 10 stereotaxic levels assessed 15-17 days following focal ischemic stroke induced by multiple intracerebral injections of endothelin-1. (* indicates a significant difference from I + V group, $P < 0.05$).

Figure A-3 shows that the duration of FPR deficit was significantly shorter in the I + R group compared to vehicle control. As a group, control rats did not recover from FPR deficits until 1 week post-surgery, whereas rats treated with Ro25-6981 recovered in just a few days.

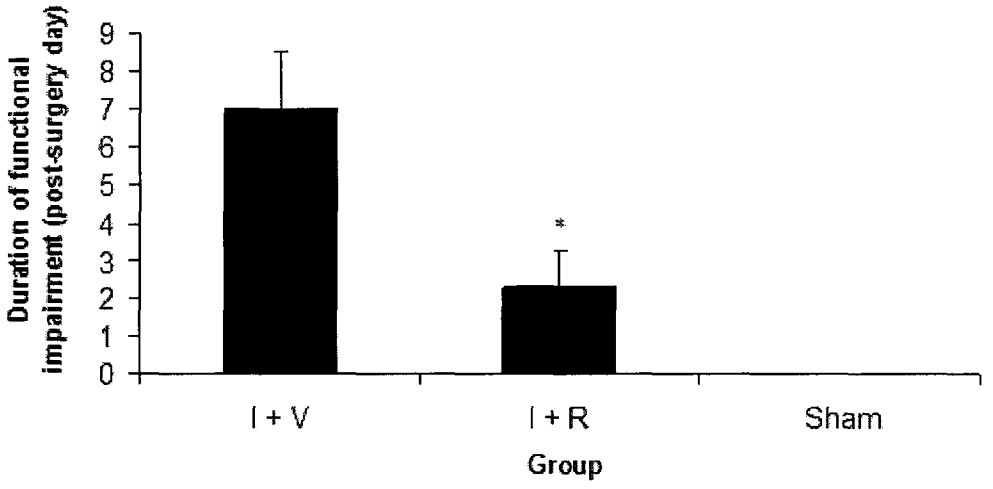

Figure A-3. Comparison on mean (± S.E.M.) duration of impairment for the FPR test over 14 days post surgery among the I + V, I + R, and Sham groups. (* indicates a significant difference from I + V group, $P < 0.05$).

Figure A-4 illustrates both TFP and VFP performance for the contralateral forelimb. For both TFP and VFP, results show significantly reduced placing accuracy for ~ 2 weeks in I + V rats relative to sham group; in contrast, the I + R group recovered forelimb placing ability within ~ 1 week compared to sham group. I + R treated rats had significantly better placing accuracy compared to I + V controls across several days of testing.

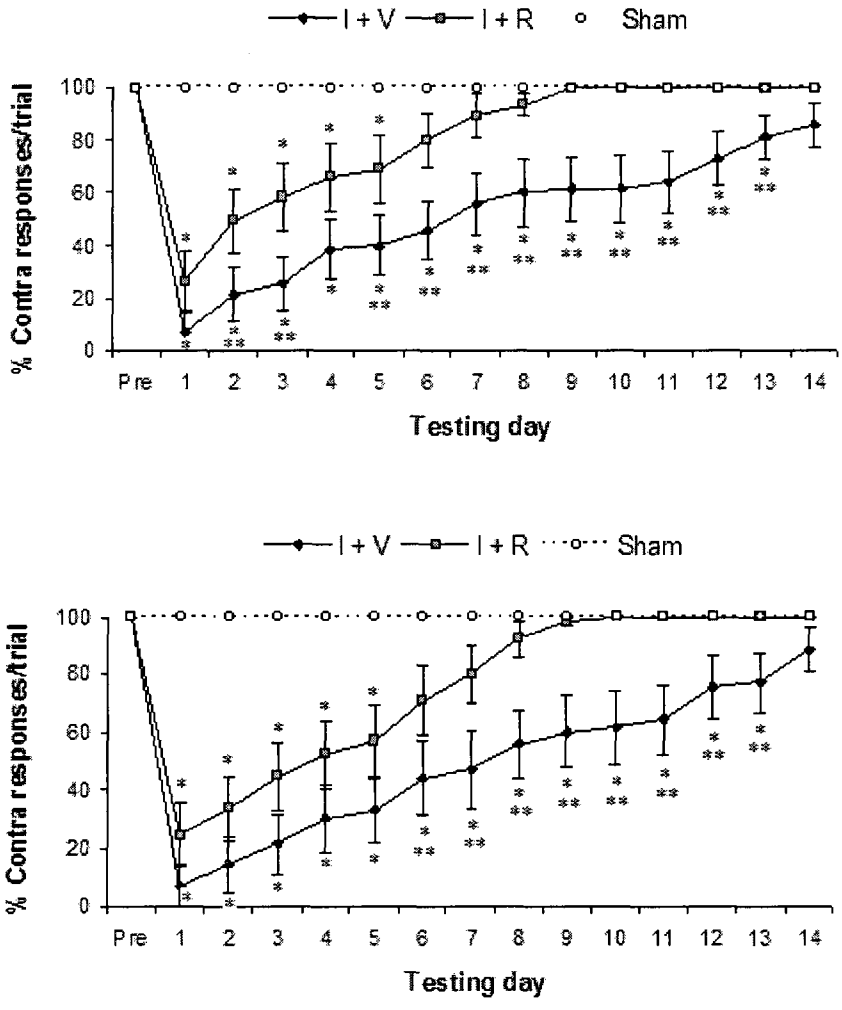

Figure A-4. Comparison of forelimb placing accuracy pre and over 14 days post surgery among the I + V, I + R, and Sham groups. Contralesional TFP (top); Contralesional VFP (bottom). Data are mean ± S.E.M. (* indicates a significant difference from Sham; ** indicates a significant difference from I + R, $P < 0.05$).

Figure A-5 shows results obtained for the modified adhesive removal test. Ischemic control animals showed a significantly enhanced ipsilateral bias (i.e. spent significantly more time attending to the 'sticky-tape' on the non-impaired limb relative to the tape on the impaired limb) compared to shams over the entire 2 weeks of post surgery testing. I + R treated rats did not differ from sham controls at any time point, and showed significantly reduced ipsilateral bias compared to I + V treated rats across most testing days.

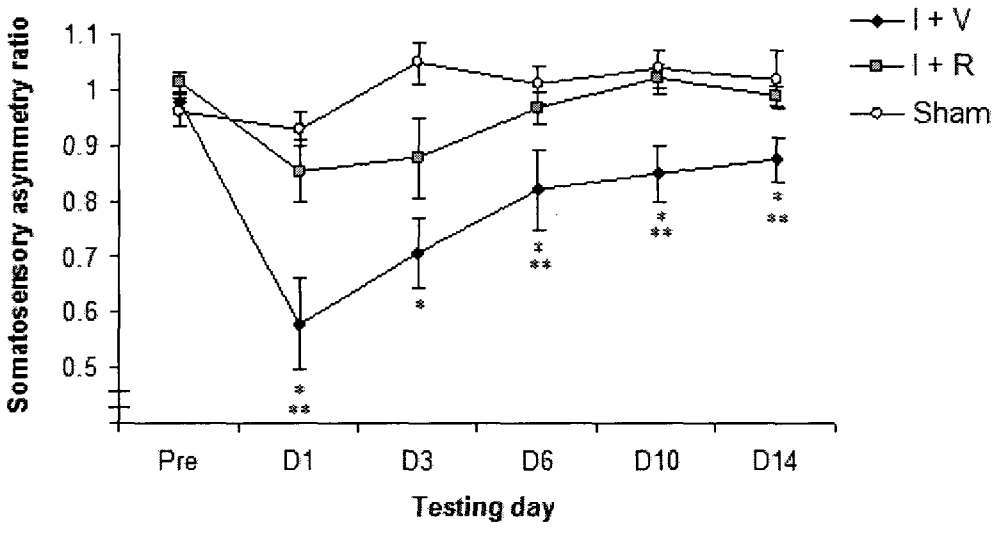

Figure A-5. Comparison among I + V, I + R, and Sham groups on somatosensory asymmetry ratio scores for the modified adhesive removal test. Data are mean ± S.E.M. (* indicates a significant difference from Sham group; ** indicates a significant difference from I + R group, $P < 0.05$).

Figure A-6 demonstrates results for the cylinder test of postural-motor support. Both Ro25-6981 treated rats and ischemic control rats showed significantly enhanced usage of the ipsilateral forelimb while rearing and 'wall-stepping' within the cylinder compared to sham group. For I + V rats, this significant effect continued for the duration of testing, but ceased to be significant for the I + R group on day 14 of testing. I + R treated rats showed significantly reduced usage of the ipsilateral forelimb compared to I + V treated rats on day 1 post-surgery.

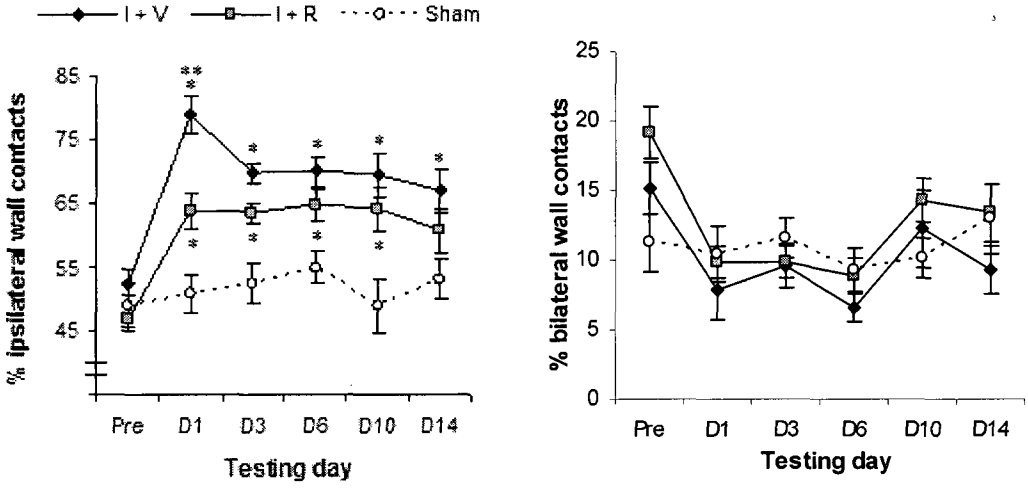

Figure A-6. Comparison among I + V, I + R, and Sham groups on the cylinder test of forelimb use during upright postural support. Percent usage of the ipsilateral forelimb relative to total single forelimb contacts (left); percent usage of both limbs simualtaneously for postural support relative to total limb contacts. Data are mean ± S.E.M. (* indicates a significant differerence from Sham group, ** indicates a significant difference from I + R group, $P < 0.05$).

Conclusion: Compared to the original I + V, I + R, and Sham groups (n = 9, 8, and 7, respectively), the addition of 4-5 more animals per group was constructive in that it largely maintained the respective differences in infarct volume (and damage pattern) as well as the behavioural impairment/recovery profile across most forelimb measures. This observation attests to the reproducibility of the model. A notable exception was a profound shift in the level of forelimb placing impairment during the initial days post-surgery for rats treated with Ro25-6981 (Figure A-4), compared to results obtained with just the original group from the chapter 4 study (Figure 4.6). The amalgamation also afforded a more favourable pattern of significant group by day differences.

Appendix B – Surgical Vehicle Control Data

Objective: To examine whether stereotaxic microinjection of vehicle (sterile distilled H_2O) causes measurable histological injury to the brain.

Methods: Two rats received stereotaxic microinjection of vehicle to the forelimb sensorimotor cortex and dorsolateral striatum using equivalent volumes and stereotaxic coordinates described previously in this thesis. These rats were also tested on the same forelimb behavioural battery (2-week survival) as described previously. See sections 2.2-2.4; 2.6-2.7; 2.9; 4.2.1-4.2.4 for all methodological details.

Results: Forelimb placing behaviour and FPR function was unaltered from pre-surgery values subsequent to vehicle injection in the brain. Thus, data is only presented for the modified adhesive removal test and cylinder test. Figure B-1 shows cresyl violet stained images depicting minor damage to the forelimb sensorimotor cortex, but substantial damage to the underlying striatum. This observation is further detailed in Figure B-2, illustrating the mean cortical and striatal infarct volumes for these two animals. Figure B-3 shows that performance on the 'sticky-tape' test was unaffected by vehicle injection in the brain since the somatosensory scores were maintained at ~ a value of 1 (no forelimb bias) across all testing days. Figure B-4, however, demonstrates that these 2 surgical vehicle control rats presented with impairments on the cylinder test. These animals showed a large reliance on the ipsilateral (unimpaired) forelimb for postural-

motor support post-surgery, as well as consistent reductions in bilateral forelimb usage in the cylinder.

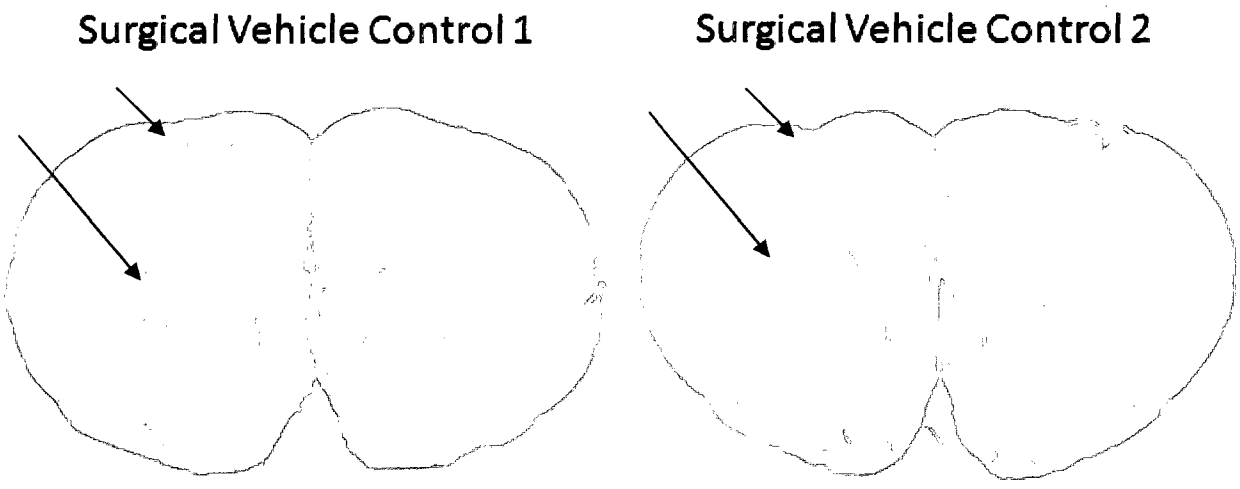

Figure B-1. Illustration of damage to the cortex and striatum as seen in 2 rats receiving stereotaxic microinjection of vehicle (sterile distilled H_2O). Arrows indicate the minimal damage produced within the cortex and the sizeable damage sustained within the striatum. Short (top) arrow points to cortical lesion and longer (bottom) arrow indicates the striatal lesion.

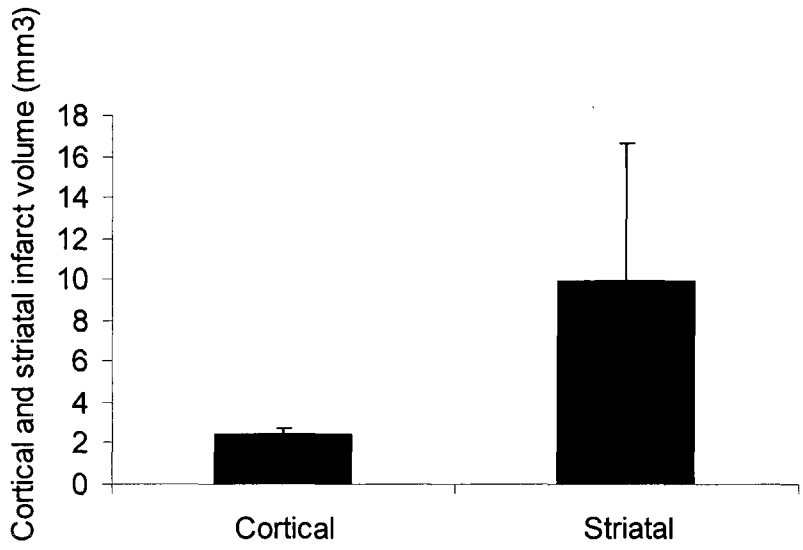

Figure B-2. Mean (± S.E.M.) cortical and striatal infarct volumes for surgical vehicle control rats.

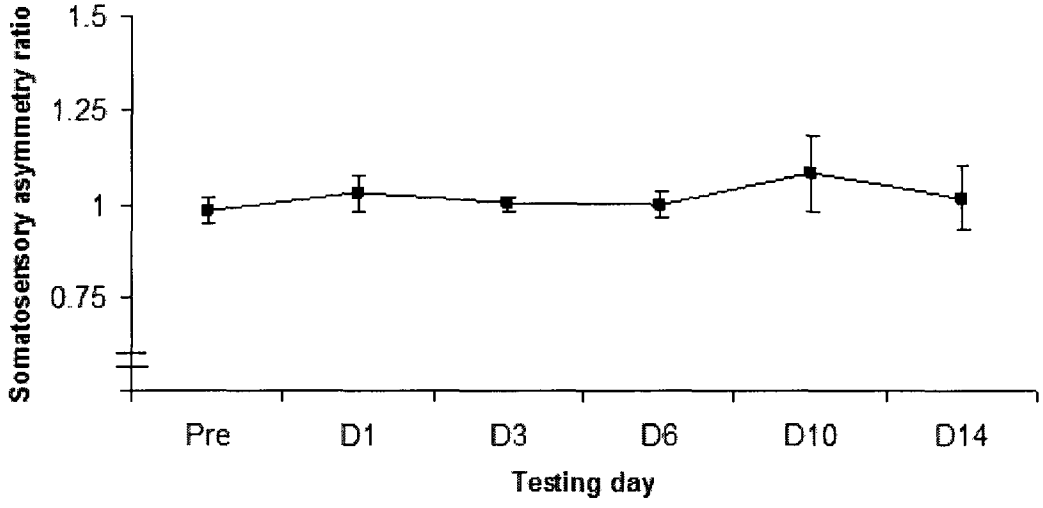

Figure B-3. Illustration of mean (± S.E.M.) somatosensory asymmetry ratio scores on the modified adhesive removal test for surgical vehicle control rats. A score of 1 equates to balanced attendance to stimuli for both forelimbs.

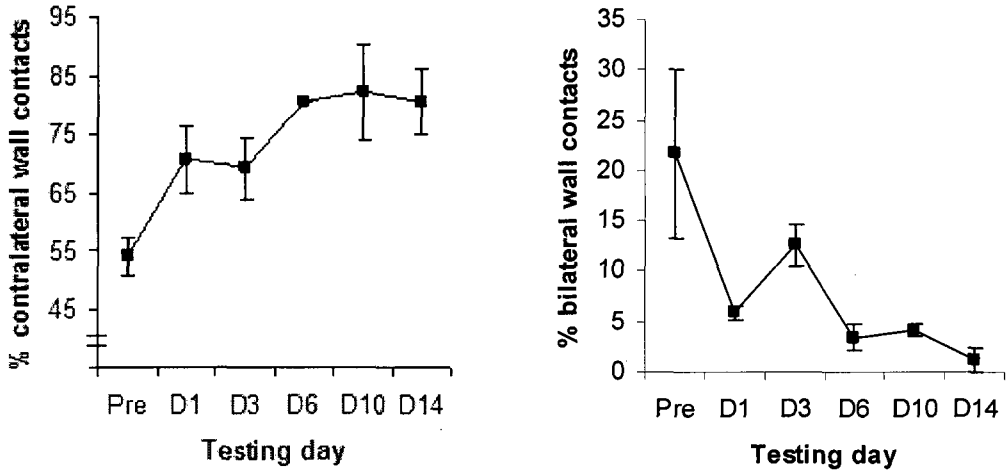

Figure B-4. Behaviour on the cylinder test of forelimb use during upright postural support for surgical vehicle control rats. Percent usage of the ipsilateral forelimb relative to total single forelimb contacts (left) (for comparision, ~50% usage across all days equates to typical behaviour for sham surgery control rats); percent usage of both limbs simualtaneously for postural support relative to total limb contacts (right). Data are presented as mean ± S.E.M.

Conclusion: The stereotaxic microinjection of vehicle in the brain appears to cause sizable damage to the striatum, but minimal damage to the cortex; it also only impaired forelimb function on the cylinder test. These observations should be examined further using a larger number of animals in order to verify if these findings represent a legitimate barrier for using sterile distilled H_2O as the vehicle for ET-1 injections within the rat striatum. These findings are commented on further in section 4.4.2.