ROLE OF SEROTONIN AND NOREPINEPHRINE SYSTEMS IN FUNCTIONAL RECOVERY AFTER STROKE

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Role of Serotonin and Norepinephrine Systems in Functional Recovery After Stroke

by

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Abstract

Stroke is a leading cause of permanent disability worldwide. Physiotherapy facilitates motor recovery after stroke but recovery is often incomplete, highlighting the need to develop other methods (e.g. utilizing more intensive rehabilitation or combining rehabilitation with drug therapy) to augment existing therapies.

Depression is a common occurrence after stroke and consequently a large population of stroke patients are treated with antidepressants. Most antidepressants act to facilitate actions of serotonin, norepinephrine, or both. Despite the prevalence of antidepressant use, little is known about how these drugs might affect the recovery of motor function in these patients. The involvement of serotonin and norepinephrine in motor recovery after focal ischemia in the rat was the focus of this thesis.

Serotonin selective reuptake inhibitors are often the antidepressants of choice in the stroke population. In the first experiment a chronic dose of fluoxetine (a serotonin specific reuptake inhibitor) was combined with rehabilitation to determine if it facilitated motor recovery. No effect of the drug was found suggesting that facilitating the serotonin system has little or no effect on motor recovery.

In the second experiment norepinephrine was depleted using N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine, a neurotoxin specific for terminals of norepinephrine cells of the locus coeruleus. Depleting norepinephrine terminals improved recovery in animals exposed to enriched rehabilitation as well as standard housed animals. This suggests that norepinephrine plays a role in recovery of function but that role may be more complicated than previously thought.

The final experiment compared four different endothelin-1 models of focal ischemia, including the models used in the previous experiments (experiment 1: focal ischemia of the cortex and striatum by localized injection or experiment 2: by injection adjacent to the middle cerebral artery). While all four models produced similar behavioural deficits the model that targeted the middle cerebral artery (the most commonly used model) yielded the lowest success rate (as determined by survival and ability to produce a forelimb reaching deficit). The more successful model combining cortical and striatal injections of endothelin-1 was further characterized using magnetic resonance imaging and found to have blood flow dynamics similar to clinical stroke.

The results of this thesis help to clarify previous findings regarding the anti-depressant, fluoxetine and recovery of function. In addition, the current findings further explore the role of norepinephrine in stroke recovery with indications for future research. Finally, I introduce a new endothelin-1 model of focal ischemia that is a more consistent and clinically relevant alternative to existing models.

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List of Abbreviations

5-HT - serotonin

6-OHDA - 6-hydroxydopamine

ADC - apparent diffusion coefficient

AR - adrenoreceptor

BDNF - brain derived neurotrophic factor

cAMP - cyclic adenosine monophophate

CAST - continuous arterial spin tagging

CBF - cerebral blood flow

CC - contralateral cortex

CH - contralateral hippocampus

CREB - cAMP response element binding protein

c/s - cortical + striatal

DA - dopamine

DβH - dopamine beta hydroxylase

DMSO - dimethyl sulfoxide

DSP-4 - N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine

ELISA - enzyme-linked immunosorbant assay

EPO - erythropoietin

ER - enriched rehabilitation

ET-1 - endothelin-1

fMRI - functional magnetic resonance imaging

GAP-43 - growth associated protein-43

I + ER - ischemic + enriched rehabilitation

I + ER + DSP-4 - ischemic + enriched rehabilitation + DSP-4

I + F + R - ischemic + fluoxetine + rehabilitation

I + R - ischemic + rehabilitation

I + St - ischemic + standard housing

I + St + DSP-4 - ischemic + standard housing + DSP-4

IH - ipsilateral hippocampus

LC - locus coeruleus

MAOI - monoamine oxidase inhibitor

MAP - microtubule associated protein

MCA - middle cerebral artery

MCAo - middle cerebral artery (occlusion)

MRI - magnetic resonance imaging

NE - norepinephrine

NGF - nerve growth factor

PBS - phosphate buffered saline

pCREB - phosphorylated cAMP response element binding protein

PKA - protein kinase A

PSD - post stroke depression

ROI - region of interest

SEM - standard error of the mean

SSRI - serotonin selective reuptake inhibitor

t-PA - tissue plasminogen activator

trkB - tyrosine kinase receptor B

QOL - quality of life

Co-Authorship Statement

Chapter 2

I contributed to the design of the project along with Dr. Dale Corbett. I performed all surgeries, behavioural testing and tissue processing. Shirley Granter-Button performed video analysis of the ladder test but I performed all other video analysis, infarct volume measurement and analysis, as well as all statistical analysis. I wrote the manuscript "Fluoxetine and recovery of motor function after focal ischemia in the rat" published in Brain Research May 2005 with assistance from Dr. Corbett.

Chapter 3

I contributed to the design of the project along with Dr. Dale Corbett. I performed all surgeries and the majority (~90%) of behavioural training, testing and rehabilitation with assistance from Alexandra Power and Sue Evans. I performed all video analysis, processing of tissue, infarct volume measurement, immunohistology and DβH measurement, and statistical analysis. I had assistance with the ELISA from Garry Chernenko. I wrote the manuscript "Norepinephrine depletion facilitates recovery of function after focal ischemia in the rat " submitted to Neuroscience in July 2006 with input from Dr. Corbett as well as Dr. Carolyn Harley.

Chapter 4

I contributed to the design of the behavioural portion of the project along with Dr. Dale Corbett and Aleksandra Szymanska. For the MRI portion I contributed to the design with assistance from Dr. James Peeling and Dr. Corbett. I performed the majority (~80 %) of surgeries, behavioural testing, and tissue processing with the remaining amount performed by Aleksandra Szymanska and Alexandra Power (behaviour only). Video analysis for the forelimb asymmetry task was performed by Aleksandra Szymanska and infarct volume was measured by Shirley Granter-Button. I performed all other video and statistical analysis for the behavioural portion of the study. MR imaging was performed in the laboratory of Dr. James Peeling at the University of Manitoba. I performed all surgeries while imaging was done by Richard Buist and Christopher White. Christopher White calculated infarct volume from T2 images and Richard Buist performed all the calculations to achieve CBF and ADC values. I performed all statistical analysis. I wrote the manuscript "An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat" published in Experimental Neurology 2006 with input from Drs. Corbett and Peeling.

Chapter 1: Introduction

1.1 General rationale

With a mortality rate of approximately 30% stroke is the third leading cause of death worldwide (Dirnagl et al., 1999; Lo et al., 2003). In Canada alone there are 40,000 to 50,000 new stroke victims per year. Of those that survive, approximately 70-80 % will have an upper-extremity impairment (Parker et al., 1986; Nakayama et al., 1994) that greatly affects independence and quality of life, and approximately 30 % will remain severely disabled such that they will be dependant on others for certain activities of daily living, making stroke a leading cause of long-term disability (Muntner et al., 2002; Carmichael, 2005). Given the aging population structure of Canada and the fact that the risk of stroke doubles every 10 years after the age of 55, there is a trend towards increasing stroke incidence (Feigin et al., 2003) and an increasing need for not only preventative and neuroprotective strategies but also therapies aimed at improving function after a stroke.

1.2 Pathology of stroke

Strokes fall into two categories; 1. Hemorrhagic, resulting from a rupture of a blood vessel leaking blood into the brain, and 2. Ischemic, which results from interruption of blood flow to the brain due to an embolus, thrombus or cardiac arrest.

Approximately 80 % of strokes are ischemic in nature and this type of stroke is the focus of this thesis.

Blood supplies oxygen and glucose to cells, which are critical to cell survival. A reduction of blood flow results in energy depletion, which can have a number of devastating effects on a neuron. Without energy neurons are unable to maintain ion gradients, which causes membrane depolarization and release of neurotransmitters, including the excitatory neurotransmitter glutamate, which in turn causes depolarization of neighbouring cells setting up a cycle or wave of depolarizations that spread outward from the most densely ischemic region, the ischemic core (Hossmann, 1994; De Keyser et al., 1999; Dirnagl et al., 1999; Barber et al., 2003). Activation of glutamate receptors results in an influx of Na^+ and Ca^{2+} that further depolarizes the cell as well as initiating other cascades (Hossmann, 1994; De Keyser et al., 1999; Dirnagl et al., 1999; Barber et al., 2003). Protein synthesis is impaired, acidosis occurs causing failure of Na^+-K^+ pumps and Ca^{2+} buffering systems resulting in an even larger elevation of intracellular Ca^{2+} (Hossmann, 1994; De Keyser et al., 1999; Dirnagl et al., 1999; Barber et al., 2003). The end result is the activation of different genes involved in cell death pathways, nitric oxide and free radical formation, inflammation and cytokine release (Hossmann, 1994; De Keyser et al., 1999; Dirnagl et al., 1999; Barber et al., 2003). Following ischemia, cells in the ischemic core die rapidly as a result of necrosis whereas in the border zone of the infarct, the ischemic penumbra, cells die more slowly as a result of gene induction leading to apoptosis (Back et al., 2004; Carmichael, 2005). Many of these mechanisms were established in *in vitro* and *in vivo* models of stroke but are relevant to the

mechanisms of human stroke (Dirnagl et al., 1999). For the majority of human patients the final infarct size is typically established 24 to 36 h after onset but infarcts may evolve over a week (Pantano et al., 1999) with final size depending on the degree and the duration of blood flow reduction (Back et al., 2004). Since some cell death is gradual this suggests that there is a therapeutic time window when treatment could prevent further damage.

1.3 Neuroprotection studies

Until recently most stroke research has been focused on cellular events underlying ischemic injury with the goal of developing neuroprotective drug therapies. While cells exposed to near total ischemia die within minutes and cannot be saved (ischemic core), cells in regions with partial ischemia due to collateral blood flow may take hours or days to die (ischemic penumbra) and thus may be salvaged. Several different drugs have been explored as potential neuroprotective agents targeting glutamate receptors, Ca^{2+} channels and free radicals, but to date none has been successful in clinical trials (Lo et al., 2003). Several of these drugs should not have been tested in phase III clinical trials based on the available animal studies, since these studies had several methodological problems including inadequate control of temperature, unrealistic treatment times, inadequate survival time and lack of behavioural endpoints (for review see Corbett and Nurse, 1998; De Keyser et al., 1999; Jonas et al., 2001). In hindsight it is not surprising that the clinical trials failed. The clinical failure of these drugs was primarily due to lack of effect or to severe side effects (De Keyser et al., 1999). One difficulty in treating stroke by

targeting specific receptors or a single step of an intracellular cascade is the heterogeneity of stroke (De Keyser et al., 1999). Different types of neurons and different regions of the brain will respond differently to loss of blood flow and it is likely that a more broad range of therapeutic agents would be required rather than a single agent.

1.4 Current treatment for acute stroke

1.4.1 Hypothermia

Approximately 50 % of human ischemic stroke patients develop a fever after the insult and those that do have worsened outcome (for review see Corbett and Thornhill, 2000). Decreasing body temperature even by a few degrees can have a neuroprotective effect after ischemia. Some of the drugs tested as potential neuroprotective agents actually cause hypothermia and when temperature is controlled the beneficial effects of the drug are lost (Nurse and Corbett, 1996). Several animal studies have shown that decreasing brain temperature reduces cell death, improves electrophysiological properties of cells at risk and improves functional outcome after global and focal ischemia (Colbourne and Corbett, 1994, 1995; Corbett et al., 1997; Colbourne et al., 2000; Dong et al., 2001). Clinical studies have shown that the use of hypothermia after stroke, traumatic brain injury, or cardiac arrest decreases mortality and improves neurological outcome (Schwab et al., 1998; Kammersgaard et al., 2000; Curfman, 2002; Safar and Kochanek, 2002; Kochanek and Safar, 2003; McIntyre et al., 2003). While neuroprotective drugs

targeted a single step in a cascade of cellular events, hypothermia is thought to be successful because it modulates multiple processes involved in cell death. Protective mechanisms may include decreased metabolic rate, increased serum Mg^{2+} which would block glutamate receptors, alteration of Ca^{2+} signaling, restoration of normal protein synthesis, decreased free radicals, interruption of apoptosis, and attenuation of inflammation (Colbourne et al., 1997; Corbett and Thornhill, 2000).

Despite the plethora of animal and human studies supporting hypothermia as beneficial after stroke it is not commonly used in acute stroke treatment. This is likely due to several factors, including the risk of side effects such as pneumonia as well as a lack of emergency room guidelines outlining the extent and duration of a cooling treatment. The animal and clinical studies listed above used various degrees and durations of cooling with differential results. Despite the different methods there does seem to be a consensus that mild hypothermia (~33 °C) for a prolonged period (several hours) seems optimal, but the parameters in humans have yet to be delineated.

1.4.2 Tissue plasminogen activator

Tissue plasminogen activator (t-PA) is a protease that can break up clots and is effective in restoring blood flow if given within 3 hours of stroke onset (Albers et al., 2002). Despite the potential benefits of t-PA it is estimated that only 20-25 % of ischemic stroke patients get to the hospital within the 3 hour time window (Barber et al., 2001). Due to the small time window and the increased risk of hemorrhage with t-PA only about

5 % of ischemic stroke patients are actually treated with the drug (Barber et al., 2001). Ischemic stroke evolves over time and even if given t-PA the lesion will continue to increase in size over 7 days in about 1/3 of the stroke population resulting in a lower degree of clinical recovery (Pantano et al., 1999). Therefore, only a small population of stroke victims will receive this drug and not all of such individuals will have a complete recovery.

1.5 Rehabilitation

For the majority of stroke patients, including many of those treated with hypothermia or t-PA, there will be lasting functional impairments. For these patients physiotherapy, speech therapy and occupational therapy are the only treatment options available.

1.5.1 Physiotherapy and brain plasticity

Rehabilitation has been described as an active form of learning. It is now known that the brain is extremely plastic and that neural circuits are modified by cognitive or motor learning (for review see Nudo et al., 2001; Kolb, 2003). There is strong evidence to suggest that rehabilitation (physiotherapy) is beneficial in many stroke patients, reducing mortality and morbidity (Langhorne and Duncan, 2001; Van Peppen et al., 2004).

Human studies have shown that stroke to the motor cortex resulting in a paretic hand alters the areas of cortex that are activated during movement of the hand (Stephan and Frackowiak, 1997; Marshall et al., 2000; Feydy et al., 2002). Subsequent activation often shifts to surrounding motor areas or to motor areas of the contralateral cortex. As patients regain function of the hand the activation maps change again, reflecting the role that cortical plasticity plays in recovery of function (Stephan and Frackowiak, 1997; Marshall et al., 2000; Carey et al., 2002; Feydy et al., 2002; Cramer, 2004).

1.5.2 Animal studies and brain plasticity

Animal studies have shown that extensive cortical rewiring takes place after brain injury. Areas surrounding the lesion form new connections with other cortical regions as shown by tract tracing studies (Carmichael et al., 2001; Dancause et al., 2005). Changes in dendritic branching and spine density, axonal sprouting, and altered protein levels have also been observed after various types of brain injury (Uryu et al., 2001; Gonzalez and Kolb, 2003; Griesbach et al., 2004). Similar to humans, reorganization of cortical maps occurs in animals after ischemia (Nudo and Milliken, 1996), and cortical plasticity is experience dependent (Nudo et al., 1996a; Nudo et al., 1996b). Specifically, a subtotal lesion to the hand representation of the cortex can result in loss of digit representation in the adjacent undamaged cortex but rehabilitative training can prevent this loss and in some animals even expand digit representation into adjacent areas that were formerly responsible for elbow or shoulder function or motor areas of the contralateral cortex

(Nudo and Milliken, 1996; Nudo et al., 1996b). Some shifts in cortical activation occur immediately after injury, suggesting that connections already exist between these areas and perhaps are unmasked or disinhibited as a result of injury. In other instances changes occur slowly over the rehabilitation process suggesting that cortical rewiring is occurring (Lee and van Donkelaar, 1995; Cramer and Chopp, 2000).

1.5.3 Enhancing functional recovery by environmental manipulation

As mentioned previously, the brain exhibits plasticity and is responsive to changes in behaviour and external stimuli. Simply placing an animal in an enriched environment that encourages exploration, exercise, and socialization results in morphological changes in the brain (Will and Kelche, 1992). This is accomplished by housing rats socially (4-8 rats per cage) in large cages with multiple shelves, and an assortment of objects that are frequently changed. Enriched environments in normal animals increase dendritic branching and spine numbers, neuron size, and induce neurogenesis, synaptogenesis, and up regulate several growth factors associated with plasticity such as neurotrophin-3, nerve growth factor (NGF) (and its receptors), and brain derived neurotrophic factor (BDNF) in the hippocampus, basal forebrain and the cortex (Torasdotter et al., 1998; Pham et al., 1999; Ickes et al., 2000; Mohammed et al., 2002; Briones et al., 2004).

Environmental enrichment has also been shown to be beneficial after brain injury. A combination of enriched environment and forelimb reaching after a transient middle

cerebral artery occlusion (MCAo) improves forelimb function and motor coordination in rats (Biernaskie and Corbett, 2001). The same study also showed that enriched rehabilitation treatment increased the amount of dendritic branching in the contralateral hemisphere compared to regularly housed animals (Biernaskie and Corbett, 2001). After permanent MCAo, enriched environment increases NGF-induced genes A and B, which correlate with functional recovery on a rotating pole task (Dahlqvist et al., 2003), and also increases spine density in the contralateral cortex (Johansson and Belichenko, 2002). After transient global ischemia, an enriched environment improves performance in odor discrimination, object exploration tasks and spatial memory, and increases BDNF in the hippocampus (Gobbo and O'Mara, 2004), and enriched environment has also been shown to increase synaptogenesis in the hippocampus after global ischemia (Briones et al., 2004).

Many of the plasticity effects of environmental enrichment are the same as those seen after ischemia. Not only does the amount of dendritic branching correlate with the amount of injury but also the amount of recovery seen after environmental enrichment (Biernaskie et al., 2004). A number of the plasticity-associated changes that occur after ischemia occur in the first few weeks (Stroemer et al., 1995; Kawamata et al., 1996; Dahlqvist et al., 1999) and environmental enrichment provides optimal benefit when initiated within the first 2 weeks after ischemia (Biernaskie et al., 2004). Indeed, if the initiation of enrichment/rehabilitation is delayed to 30 days (common clinically) there is little functional recovery and no increase in dendritic branching in the cortex (Biernaskie et al., 2004).

Environmental enrichment studies are good examples of how animal studies can directly impact clinical practices. Indeed, these animal studies relating to stroke rehabilitation have generated clinical studies examining such things as patient time engaged in therapy, socialization and activity (Teasell et al., 2005a). The results of clinical and animal studies related to environmental enrichment are increasingly being used as evidence to support change to current physiotherapy practices for stroke patients (Teasell et al., 2005a; Teasell et al., 2005b).

1.5.3 Enhancing recovery through drug therapy: amphetamine

Just as physiotherapy in humans or environmental enrichment in animals improves recovery of function, so too can certain drugs. Amphetamine, which increases release of monoamines, is one of the most widely studied drugs shown to promote recovery of function after brain injury (for review see Gladstone and Black, 2000). In animal studies it has been shown to increase performance on a beam walking task after cortical injury in cats as well as rats (Feeney et al., 1982; Hovda and Fenney, 1984; Goldstein and Davis, 1990c), and speeds recovery at a maze task (Hurwitz et al., 1991). A more recent study showed that amphetamine after MCAo improved performance on a grid walking task and water maze, and that the drug increased growth associated protein (GAP)-43 and synaptophysin (markers for neural sprouting and synaptogenesis) (Stroemer et al., 1998). It is important to note that amphetamine needs to be paired with a training task in order for benefit to be seen. If animals are restrained to prevent

movement after given amphetamine there is no improvement seen on the beam task (Feeney et al., 1982).

The effect of amphetamine in stroke patients is less clear. Early pilot studies suggested that single or multiple doses of amphetamine paired with traditional physiotherapy improves motor performance in individual sessions and also over the long term (Crisostomo et al., 1988; Walker-Batson et al., 1995). However, these studies only had 8 and 10 patients respectively while two recent studies with more patients (71 and 31) have shown no benefit of amphetamine (Platz et al., 2005; Gladstone et al., 2006). Differences in outcome may be partly due to stroke severity, age of the patients and delay from stroke to treatment time. Perhaps because of the contradictory data, amphetamine is not widely used in stroke patients. Amphetamine is a stimulant, and while some studies report that there are few side effects (Crisostomo et al., 1988; Walker-Batson et al., 1995), there are concerns about giving the drug to an aged population and a population with a high percentage of cardiac complications.

The mechanism by which amphetamine improves recovery is thought to be its ability to increase the release of monoamines. Amphetamine increases dopamine (DA), serotonin (5-HT) and norepinephrine (NE) (Gladstone and Black, 2000) levels in the brain and it has been suggested that other drugs that target these neurotransmitters may also be beneficial. 5-HT and NE action is also increased by antidepressant drugs, which have also been explored in stroke patients due to an increased tendency of stroke patients to develop depression.

1.6 Post stroke depression (PSD)

While the prevalence of depression in the average adult population is approximately 5 %, the prevalence in stroke patients is anywhere from 18 to 79% (Gordon and Hibbard, 1997; Singh et al., 2000; Gillen et al., 2001). The wide range of prevalence is likely due to the difficulty in diagnosing PSD. In the average population, indicators of depression such as fatigue or inability to concentrate are not as reliable in stroke patients for obvious reasons (Gordon and Hibbard, 1997). Nonetheless, most studies conclude that a high percentage of stroke patients suffer PSD and that it greatly affects the patient's recovery. PSD is associated with decreased quality of life (QOL) (Kauhanen et al., 2000), increased disability (Paolucci et al., 2001), and increased mortality (Jorge et al., 2003; Robinson, 2003).

QOL assessment is done by surveying and evaluating patients on several different scales to rate quality of physical, psychological, functional, social, and general health. One study found that minor and major depression significantly decreased QOL scores in all areas measured and that depression is one of the most important determinants of low QOL scores after stroke (Kauhanen et al., 2000). The above study found that depression resulted in significantly lower physical functioning scores (Kauhanen et al., 2000), a finding reported elsewhere (Gillen et al., 2001; Paolucci et al., 2001; Robinson, 2003). Patients with PSD respond similarly to rehabilitation, but even if treated with antidepressants they have greater disabilities in coping with activities of daily living, and lower independence and mobility scores on admission and discharge (Paolucci et al., 2001). One of the more surprising effects of PSD is an increase in mortality. A 10 year

follow up of stroke patients revealed that the mortality rate increased from 41% in non-depressed patients to 70% of those that suffered PSD (Robinson, 2003).

When PSD is suspected, antidepressants are often prescribed and several different types have shown to be effective in improving not only mood but also motor function. Table 1.1 summarizes some of the antidepressants that have been tested after PSD and the outcomes used. Classes of antidepressants include: 1) Tricyclics, drugs that increase NE and 5-HT by blocking the amine transporter pump for NE, 5-HT, or both neurotransmitters, 2) 5-HT selective reuptake inhibitors (SSRI) that increase brain levels of 5-HT by blocking the pump responsible for reuptake of 5-HT, and 3) Monoamine oxidase inhibitors (MAOI) that prevent the oxidation of both NE and 5-HT. There are also NE specific reuptake inhibitors but they have received little testing as yet for PSD. All classes of drugs are associated with side effects, which may limit their use in the stroke population, and depending on the study, different drugs are listed as appropriate for PSD. Despite the high prevalence of PSD there are surprisingly few studies that have looked at the effects of antidepressants on recovery after stroke and many of the studies listed in Table 1 had fewer than 100 subjects, highlighting a need for further investigation.

1.7 Antidepressants and neuroplasticity: rationale for chapter 2

1.7.1 Combined action antidepressants

A number of antidepressants exert their effect by increasing the action of both NE and 5-HT. Nortriptyline is one such drug that has shown to be effective in treating PSD and promoting motor recovery after stroke (Lipsey et al., 1984; Gonzalez-Torrecillas et al., 1995; Robinson et al., 2000).

In animals there have been several studies suggesting that antidepressants targeting both NE and 5-HT may contribute to neural plasticity. For example, imipramine has been shown to increase cAMP response element binding protein (CREB) mRNA (Nibuya et al., 1996), and tranylcypromine (MAOI) restores BDNF levels to normal in rats subjected to stress (Russo-Neustadt et al., 2001). These drugs also increase hippocampal neurogenesis (Malberg et al., 2000). Both CREB and BDNF play a role in synaptic plasticity as well as neurogenesis (Duman et al., 2000; Finkbeiner, 2000) and infusion of BDNF into the dentate gyrus of the hippocampus produces antidepressant effects in behavioural models of depression (Shirayama et al., 2002). Figure 1.1 shows a schematic diagram of how antidepressants might mediate trophic effects through CREB and BDNF.

1.7.2 Serotonergic antidepressants

Although the exact mechanisms of depression are poorly understood, it is known that drugs that increase the action of 5-HT are effective in treating depression in many people including those suffering PSD (see Table 1.1). 5-HT is involved in wakefulness and attention as well as facilitation of motor output (Jacobs and Fornal, 1999), and it has been suggested that in addition to the ability to improve mood, antidepressants that increase 5-HT also improve motor function.

Using fMRI, a single dose of paroxetine (a SSRI) was shown to hyperactivate the motor cortex when healthy subjects were performing a hand task (Loubinoux et al., 2002b). This drug also increased motor performance through practice of tests of coordination and dexterity (Loubinoux et al., 2002a). Similar modulation of cortical activation has also been seen after a single dose of fluoxetine (a SSRI) in healthy subjects (Loubinoux et al., 1999). Hyperactivation of the motor cortex was also observed after a single dose of fluoxetine in stroke patients, which correlated with improved grip strength and finger tapping (Pariente et al., 2001). Fluoxetine has been shown to dilate small cerebral arteries and increase cerebral blood flow in rats, which may underlie the increased cortical activation and motor facilitation (Ungvari et al., 1999).

Conversely, chronic doses of paroxetine in healthy subjects resulted in a hypoactivation of the motor cortex when performing a hand task, but this decreased activation correlated with improved motor performance in a finger tapping task

suggesting that paroxetine increased the efficiency of cerebral motor processing (Loubinoux et al., 2005).

While increasing 5-HT results in immediate changes in cortical activation, the mechanisms that promote motor recovery after brain injury may involve other mediators of neural plasticity. Chronic treatment with fluoxetine and sertraline (another SSRI) increases levels of cAMP response element binding protein (CREB) mRNA in the hippocampus (Nibuya et al., 1996). In addition, fluoxetine increases mRNA for BDNF and its receptor, tyrosine receptor kinase (trk)B in the hippocampus (Nibuya et al., 1996; Coppell et al., 2003). Fluoxetine has also been shown to increase hippocampal neurogenesis in normal rats (Malberg et al., 2000) and prevent decreased neurogenesis of the hippocampus in response to inescapable shock (an animal model of depression) (Malberg and Duman, 2003).

Despite the promising results of imaging studies and studies demonstrating that SSRIs can modulate neural plasticity, there are few studies that have explored their potential to improve motor recovery after ischemia. One study that examined fluoxetine given for 10 days after a MCAo in rats found that fluoxetine failed to improve recovery on a motor task or a spatial memory task (Jolkkonen et al., 2000a). While the results of this study are discouraging it is important to note that only one test of motor function was used and it was a test on which there is considerable spontaneous recovery after stroke. Clinically it is not useful to test drugs on deficits that are so transient since it is unlikely that a drug would be given to a patient for an impairment that would resolve in 2 weeks. Because of this we felt that further investigation of fluoxetine was warranted using more

sensitive behavioural tests and longer treatment times that would better approximate the clinical post-stroke scenerio. The main goal of the second chapter of this thesis was to test the hypothesis that fluoxetine would improve motor recovery after focal ischemia in rats.

Citalopram and paroxetine both increase extracellular 5-HT levels in the rat diencephalon more than fluoxetine and have a higher affinity for the 5-HT transporter (Felton et al., 2003), but despite this, fluoxetine was chosen over other SSRIs for study in chapter 2 because of the previous studies showing some promise after PSD (Table 1.1) and the wealth of data linking fluoxetine to neural plasticity.

1.7.3 Noradrenergic antidepressants

The other neurotransmitter targeted by antidepressants is NE. Maprotiline is relatively selective for NE and has been shown to improve symptoms of PSD but does not improve motor function (Dam et al., 1996). Reboxetine, a selective NE reuptake inhibitor has also been found to be effective in treating PSD but has not been explored as a facilitator of motor recovery (Rampello et al., 2005). In animals, reboxetine has been shown to increase neurogenesis in the hippocampus (Malberg et al., 2000) and desipramine (also a selective NE reuptake inhibitor) has been shown to increase CREB mRNA (Nibuya et al., 1996). Despiramine has also been show to facilitate motor recovery after a sensorimotor cortex lesion (Boyeson and Harmon, 1993) but failed to show any benefit after MCAo (Jolkkonen et al., 2000b). Despite the lack of studies using

noradrenergic antidepressants there is additional evidence supporting the idea that increasing NE release with other drugs (i.e. α_2-adrenergic antagonists) enhances neural plasticity and motor recovery after brain injury (Sutton and Feeney, 1992).

1.8 Noradrenergic theory: rationale for chapter 3

Noradrenergic pharmacotherapy is a phrase used to describe the experimental up regulation of NE with various drugs to facilitate recovery of function after brain injury. Much of the support for this theory began when amphetamine was shown to facilitate motor recovery after cortical injury in cats, rats, and in some human studies (see section 1.5.3). This effect can be mimicked by intraventricular infusion of NE (Boyeson and Feeney, 1990) as well as by treating animals with α_2-adrenergic receptor antagonists that act on pre synaptic and somatic receptors to increase release of NE (Sutton and Feeney, 1992). Atipamezole, an α_2-adrenergic antagonist, speeds recovery in limb-placing and beam walking tasks after a transient MCAo (Jolkkonen et al., 2000b; Butovas et al., 2001; Puurunen et al., 2001) but seems to have no effect on spatial learning and memory tasks after focal or global ischemia (Puurunen et al., 1997; Puurunen et al., 2001), suggesting that the effects may be specific to motor tasks.

Not only does increasing NE seem to improve recovery but decreasing NE may also impair recovery. After cortical injury there is a reduction in monoamines, including NE that remains reduced for 40 days, as well as a reduction in α1-adrenergic receptors (Robinson et al., 1975; Dunn-Meynell et al., 1994; Prasad et al., 1994). Prazosin, an α_1-adrenergic antagonist (blocks post-synaptic effects of NE) blocks motor training-

mediated plasticity in cortical activation in healthy humans (Sawaki et al., 2003) and reinstates motor impairment on the beam walking task in rats (Sutton and Feeney, 1992). Similarily, clonidine, an α_2-adrenergic agonist slows the rate of beam walking recovery after a cortical injury in rats (Goldstein and Davis, 1990b).

While these studies do suggest a role for NE in recovery it is important to note that amphetamine increases 5-HT and DA in addition to NE. In addition, α_2-adrenergic antagonists also increase DA and prazosin and clonidine decrease levels of 5-HT and DA in addition to NE (Gobert et al., 1998; Gladstone and Black, 2000; Iwasaki et al., 2006). A more direct approach to determining if NE is required for motor recovery after ischemia would be to selectively deplete NE projections from the locus coeruleus (LC) with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a neurotoxin that leaves 5-HT and DA levels intact. Chapter 3 examines the postulated role of NE in motor recovery.

1.9 Non-ischemic models of injury

Since the goal of this thesis is to contribute to the knowledge of recovery of function after stroke it is evident that a clinically relevant animal model of stroke should be used. However, several studies contributing to the background knowledge of this thesis were not performed using stroke models. Several of the studies used aspiration lesions, which are reproducible but differ in important respects from ischemic injury. For example, comparison of aspiration lesions with permanent ischemic models

(thermocoagulation of pial vessels overlying the cortex or MCAo) reveals that there are differential glucose metabolic responses, differences in gene expression, and different amounts of cortical and subcortical plasticity (both axonal and dendritic) depending on the model used (Napieralski et al., 1996; Uryu et al., 2001; Gonzalez and Kolb, 2003; Mir et al., 2004). Accordingly, in order to best approximate human stroke, ischemic models were chosen for the experiments in this thesis.

1.10 Animal models of stroke: rationale for chapter 4

Human stroke is very heterogeneous in nature and this is reflected by the widespread use of different ischemia models. There are several advantages to using rat models of ischemia: rats have a vasculature that closely resembles higher species; they are capable of being trained at specific tasks for both cognitive and motor function; the size of the brain is well suited for fixation procedures for histological assessment; and they are less costly than higher mammals and more acceptable from both ecological and ethical perspectives (Ginsberg and Busto, 1989).

There are two main ischemic models: 1) Global ischemia that is more representative of cardiac arrest, and 2) Focal ischemia that targets specific vessels or brain regions and is more representative of human stroke due to embolism (blockage by a clot formed elsewhere that has moved to the brain) or thrombosis (blockage caused by a clot formed at that site). Since the focus of this thesis is the recovery of motor function,

focal ischemic models were chosen for the experiments. Several of the most commonly used models are discussed below.

1.10.1 Middle cerebral artery occlusion (MCAo)

The most commonly used rodent focal ischemia model is transient occlusion of the MCA by the introduction of an intraluminal suture (Ginsberg and Busto, 1989; Longa et al., 1989; Carmichael, 2005). The suture is introduced through the external carotid artery to the internal carotid artery and then into the MCA. This results in immediate reduction of blood flow to brain regions supplied by the MCA. This procedure typically produces injury to the dorsolateral striatum which occurs early and is largely the result of necrosis, and a more delayed infarction of the overlying dorsolateral cortex that is more apoptotic in nature (Carmichael, 2005). In addition to the striatum and cortex, this model variably also results in injury to the thalamus, cervicomedullary junction, substantia nigra, and the hypothalamus, which can cause a variety of deficits (e.g. hyperthermia) that are not typical of human MCAo and complicate the model (Dittmar et al., 2003; McColl et al., 2004; Carmichael, 2005).

To create transient ischemia in the MCAo intraluminal model, the suture is removed, usually after 60, 90 or 120 min after insertion, and blood flow is immediately restored, which may not be typical of most human focal ischemia (i.e. patients not treated with t-PA) where reperfusion occurs more gradually as a result of collateralization or natural clot lysis (Carmichael, 2005). Other disadvantages of the model include risk of

hemorrhage because of vessel wall damage done by the suture, difficulty in feeding after the surgery due to lost blood flow to the muscles of mastication, and fluctuations in temperature (if the hypothalamus is damaged) that would affect infarct size (Carmichael, 2005). While the MCA is commonly a site of occlusion in humans, in the suture model the occlusion duration is often increased (i.e. 10 or 120 min) to reduce variability in damage. Unfortunately, this results in such large areas of cortical and striatal injury that it may only represent the most severe cases of clinical stroke that are viewed as untreatable (Carmichael, 2005).

1.10.2 Photothrombosis

Photothrombosis models use photosensitive dyes, such as rose-bengal, to create highly circumscribed ischemic lesions in the cortex. The dye is injected intravenously and a light source is placed over the exposed skull above the cortical region of interest. The light interacts with the dye creating singlet oxygen, which then reacts with lipid molecules in the vessel to give rise to platelet aggregation and a clot (Ginsberg and Busto, 1989; Carmichael, 2005). Advantages of the model include minimal invasiveness, small lesion size, and ability to place the lesion in modality specific regions of the cortex. Disadvantages include injury limited to only the cortex since the light cannot reach the striatum or other subcortical regions, relatively little penumbra region (potentially salvageable tissue), little to no reperfusion, and early extracellular edema that is not characteristic of human stroke (Carmichael, 2005).

1.10.3 Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide that causes constriction of vessels (Yanagisawa et al., 1988). By stereotaxically injecting ET-1 adjacent to the MCA, vessel occlusion is achieved without exposing the arteries of the neck, and reperfusion is more gradual (taking several hours) and thus more representative of the human condition (Biernaskie et al., 2001). By injecting ET-1 in specific brain regions (i.e. the cortex or the striatum), small infarcts are produced similar to the photothrombosis model but with the advantage of reperfusion. For these reasons, ET-1 induced focal ischemia was chosen for the experiments described in the subsequent chapters. In chapter 2, ET-1 was injected into the cortex and the striatum, effectively producing behavioural deficits in several motor tasks. In chapter 3, ET-1 was injected adjacent to the MCA also producing behavioural deficits. In the course of the experiments for chapters 2 and 3 it was noticed that the success rate (consistency of the surgery to result in a behavioural deficit) of the different ET-1 models was quite different. This lead to the topic of chapter 4, which is concerned with optimizing the ET-1 model to produce focal ischemia and sensorimotor deficits for recovery of function studies.

1.11 Behavioural methods

1.11.1 Tests of forelimb function

Approximately 70-80% of stroke patients have an upper-extremity impairment (Parker et al., 1986; Nakayama et al., 1994) and as such tests of forelimb function are invaluable in studying recovery of function. There are a variety of reaching tests used in rodents that include retrieving pellets from narrow tubes (Pisa, 1988), a food tray located a distance outside the cage (Whishaw et al., 1986), retrieving pasta that is placed in a matrix outside the cage (Ballermann et al., 2001), or shelling sunflower seeds (Whishaw and Coles, 1996). All of these tasks can be accomplished with either paw and as a result require additional training or restraint mechanisms to ensure the animal consistently uses one paw over the other. The staircase test developed by Montoya (Montoya et al., 1991) is shown in Figure 1.2. In this test rats are able to retrieve food pellets with either paw but they can only retrieve pellets on the right set of stairs with the right paw and vice versa, therefore the unimpaired limb cannot be used to compensate for the impaired limb after injury. This test is also useful for determining if animals have bilateral deficits. Apart from providing a quantitative score of the animal's performance, the staircase test also allows detailed observation of reaching dynamics to determine which components of a reach are affected (e.g. limb extension, supination etc.) (Clarke et al., unpublished). It has also been my experience that animals become proficient at the staircase test more

quickly than single pellet reaching and the animals require far less intensive training. For these reasons the staircase test was utilized in all three experiments in this thesis.

Another test of forelimb function used in this thesis is the forelimb asymmetry test shown in Figure 1.3. Following injury to the forelimb motor cortex, rats preferentially use their non-impaired forelimb for support. When animals are placed in a vertical cylinder, which encourages vertical and lateral exploration, instead of using both paws more or less equally animals rely primarily on the unimpaired limb (Schallert et al., 1997). One advantage of this test is that it utilizes innate behaviour and therefore requires no training.

1.11.2 Tests of motor coordination

While it is obvious to focus on the impaired limb to identify motor dysfunction as in the above tests, it is also useful to examine general motor coordination since impairments to one limb will affect overall motor function.

Crossing a narrow elevated beam normally poses little difficulty for rats and they learn the task relatively quickly. Injury to the motor cortex however, results in slips, falls, and other foot placement errors. Several studies of motor recovery previously mentioned have employed the beam walking test and it was used in chapter 4 of the current thesis (Figure 1.4). Previous studies used a rating scale to score the animals and showed deficits which tended to spontaneously recover to pre-injury levels (Feeney et al., 1982; Boyeson and Feeney, 1990; Goldstein and Davis, 1990a). More recently, a more

quantitative measure of reporting the number of foot faults after MCAo showed that ischemic animals do not recover spontaneously to pre-surgery levels (Biernaskie and Corbett, 2001) and this method was employed in this thesis.

A newer adaptation of the beam walking test is the ladder rung walking test (Figure 1.5), in which the rats cross a horizontal ladder with variably spaced rungs. Advantages offered by this task include the ability to more closely evaluate the types of errors being made, and the ability to change the rung spacing to continue to challenge the animal with a new task (Metz and Whishaw, 2002).

1.11.3 Choice of rat species

Both Long-Evans and Sprague-Dawley rats are commonly used for experiments involving motor tasks. There is some evidence to suggest that Sprague-Dawley rats are less skilled in their qualitative reaching movements compared to Long-Evans rats (Whishaw et al., 2003). For this reason Long-Evans hooded rats were originally selected for study in this thesis. It is important to note that in Chapter 3 Sprague-Dawley rats were used due to differential effects of DSP-4 on the two strains. While DSP-4 depletes NE projections from the LC in Sprague-Dawley rats it does not in Long-Evans rats (Schuerger and Balaban, 1995) necessitating the change in species for that portion of the thesis.

1.12 Thesis outline

1.12.1 Fluoxetine and recovery of motor function after focal ischemia in rats

This chapter demonstrates that a chronic dose of fluoxetine (4 weeks) paired with rehabilitative reaching failed to improve motor recovery on a number of behavioural tests following ET-1 induced focal ischemia. This chapter is based on the paper "Fluoxetine and recovery of motor function after focal ischemia in rats", published in *Brain Research* 1044:25-32 (2005) by Windle V and Corbett D.

1.12.2 Norepinephrine depletion facilitates motor recovery after focal ischemia in the rat

This chapter demonstrates that depletion of NE by injection of DSP-4 one week after ET-1 induced MCAo facilitates motor recovery in various behavioural tests. DSP-4 treatment facilitated recovery in both standard housed animals as well as those exposed to environmental enrichment. This chapter is based on the paper titled "Norepinephrine depletion facilitates motor recovery after focal ischemia in the rat", submitted for publication to *European Journal of Neuroscience* (November 2006) By Windle V, Power A and Corbett D.

1.12.3 An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat

This chapter compares four different models of ET-1 induced focal ischemia (including the models used in chapters 2 and 3) to produce consistent infarcts as well as sensorimotor deficits. The models were evaluated histologically and behaviourally, and additionally the cortical + striatal model was evaluated using MR imaging. This chapter is based on the paper titled "An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat", published in *Experimental Neurology* 201(2):324-334 (2006) by Windle V, Szymanska A, Granter-Button S, White C, Buist R, Peeling J and Corbett D.

Table 1.1. Antidepressants used to treat post stroke depression

Drug	Investigators	Outcome
Increase NE		
Maprotiline	(Dam et al., 1996)	reduced depression but did not improve motor recovery
Reboxetine	(Rampello et al., 2005)	reduced depression
Increase 5-HT		
Trazodone	(Reding et al., 1986)	improved motor recovery but did not reduce depression
Citalopram	(Andersen et al., 1994)	reduced depression
Fluoxetine	(Robinson et al., 2000)	did not reduce depression
	(Wiart et al., 2000)	reduced depression
	(Gainotti et al., 2001)	reduced depression and improved motor recovery
	(Dam et al., 1996)	reduced depression and improved motor recovery
	(Fruehwald et al., 2003)	reduced depression
Increase NE and 5-HT		
Nortriptyline	(Lipsey et al., 1984)	reduced depression
	(Robinson et al., 2000)	reduced depression
	(Gonzalez-Torrecillas et al., 1995)	reduced depression and improved motor recovery

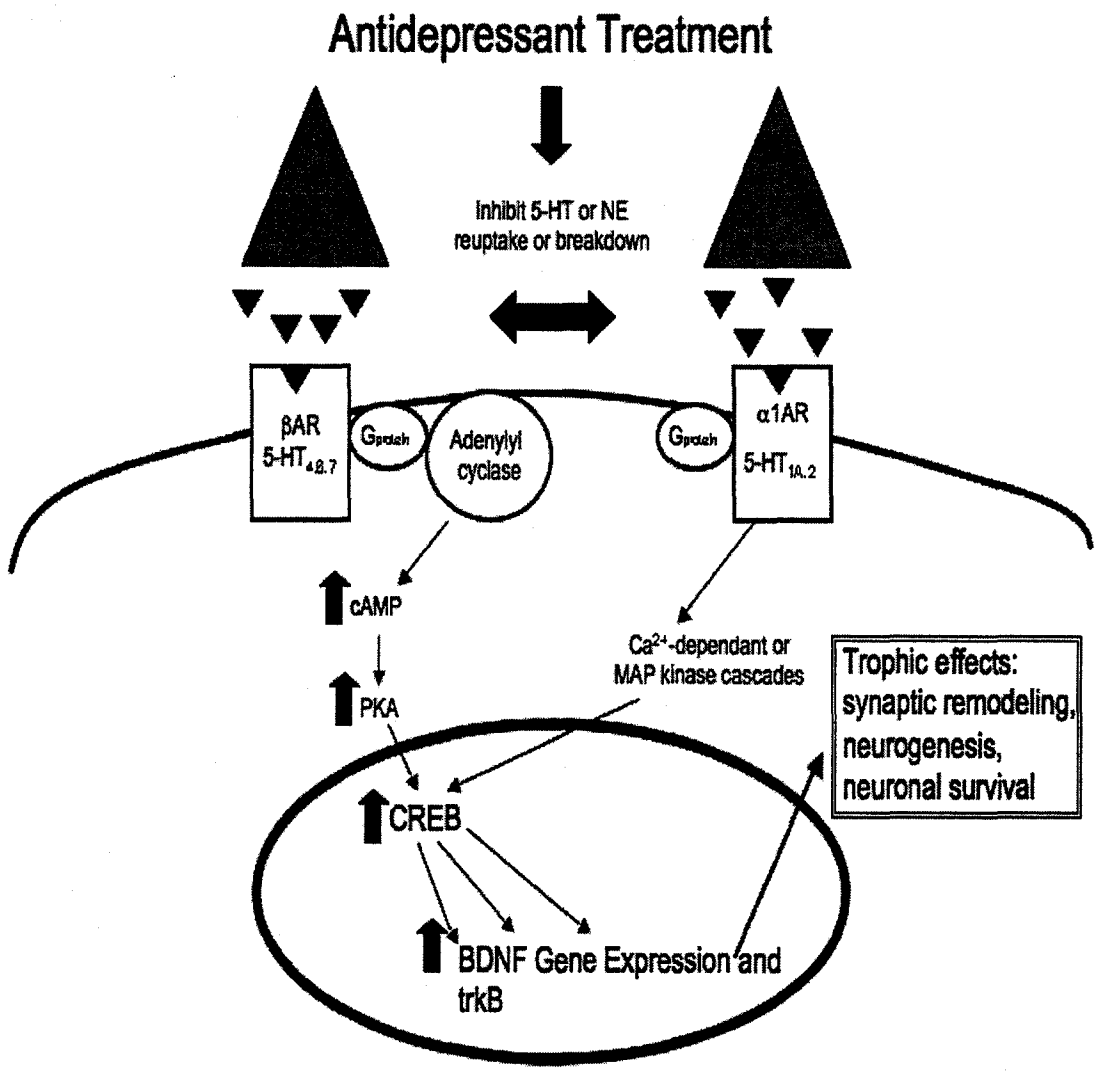

Figure 1.1. Schematic of possible action of chronic antidepressant treatment within the brain. Binding of serotonin (5-HT) or norepinephrine (NE) to their various receptors may differentially activate different pathways that both result in increased cAMP response element binding protein (CREB) and brain derived neurotrophic factor (BDNF). AR = adrenoreceptor, PKA = protein kinase A, MAP = microtubule associated protein. Adapted from Duman et al., 2001 and Nibuya et al., 1996.

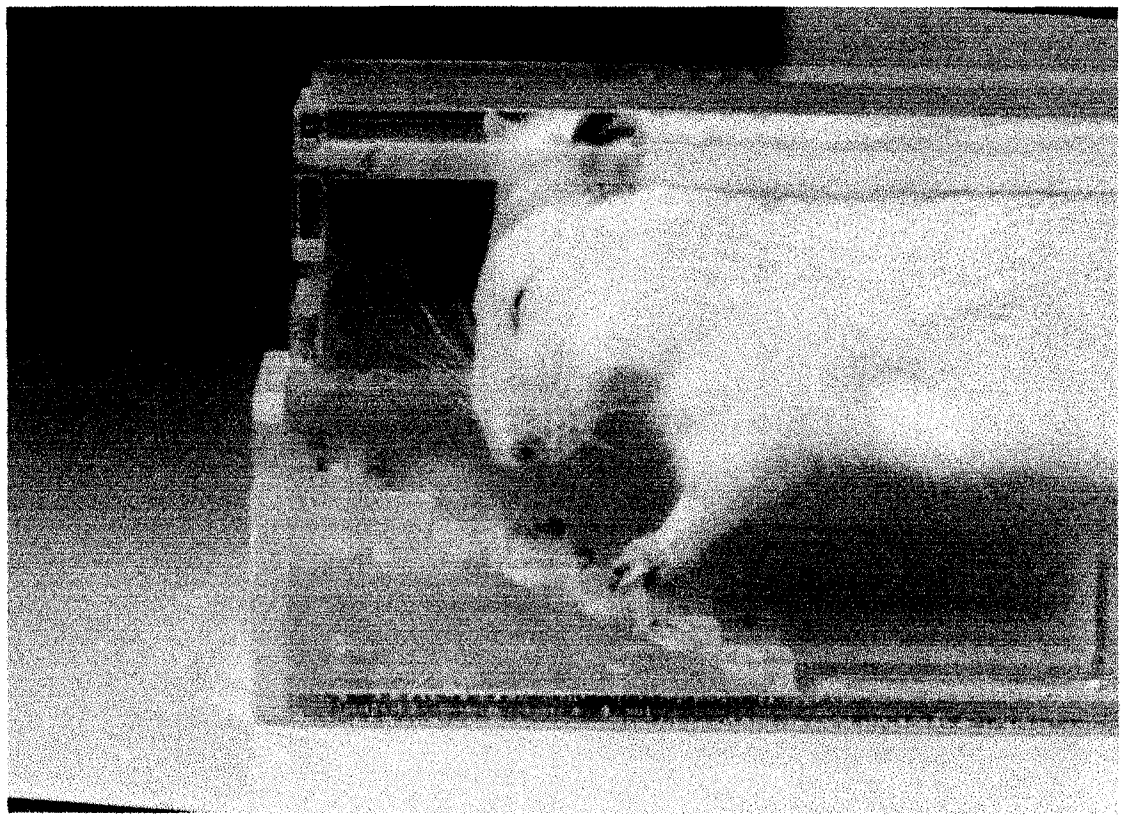

Figure 1.2. Staircase reaching test. Animals are placed in a box with a set of staircases on either side. Each step holds three food pellets that the animal can only retrieve with the paw on the same side as the steps.

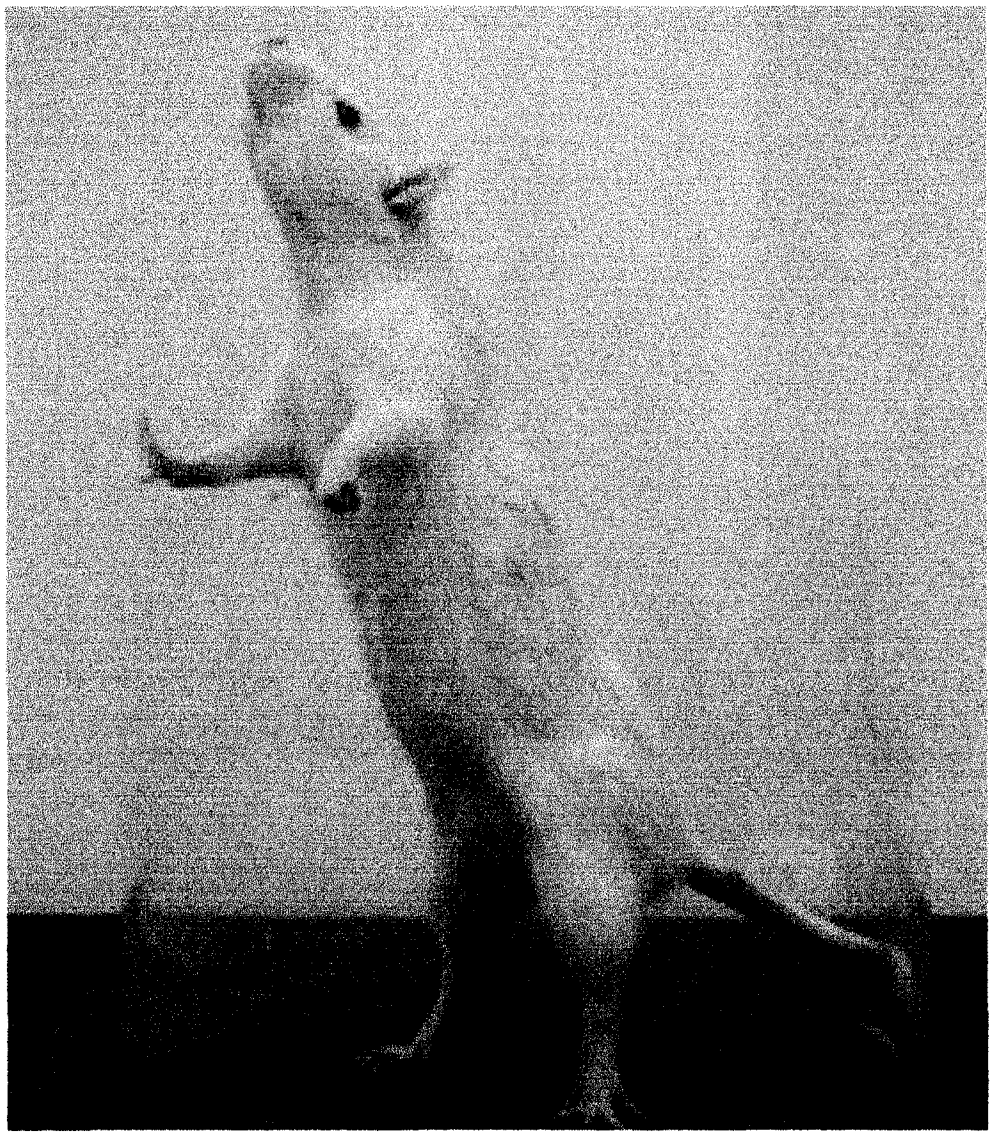

Figure 1.3. Forelimb asymmetry test. This is an example of an animal with an ischemic lesion in the right hemisphere resulting in a deficit of the left forepaw.

Figure 1.4. Balance beam test. An impairment of one limb (left forepaw) can result in motor coordination deficits seen as errors made with any of the four limbs.

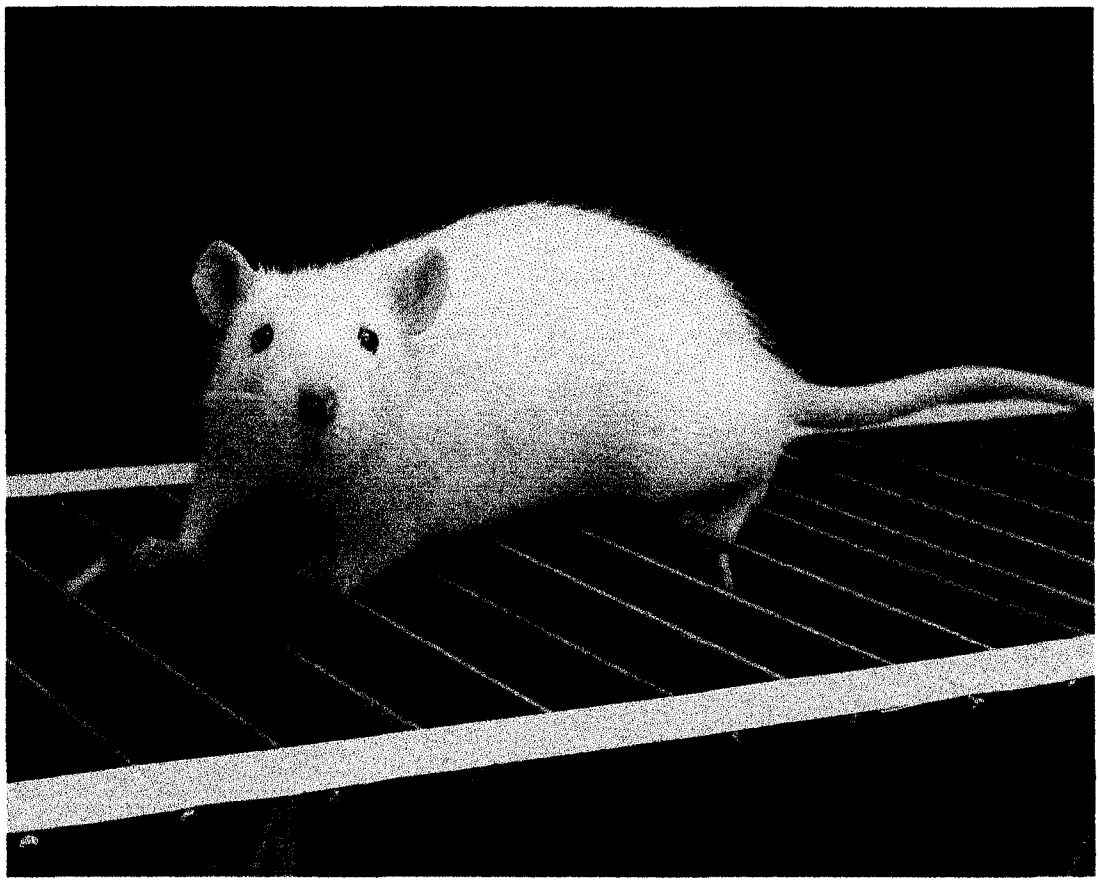

Figure 1.5. Ladder-rung walking test. This test requires less training than the beam walking test and also allows for observation of more subtle deficits.

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Chapter 2: Fluoxetine and Recovery of Motor Function After Focal Ischemia in Rats

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2.1. Introduction

Stroke is one of the leading causes of permanent disability in the world. The focus of stroke research has been on drug treatments that would limit brain damage once stroke has occurred. Unfortunately, neuroprotective drug therapy has not been successful in reducing injury (De Keyser et al., 1999). Restoration of blood flow with t-PA (tissue plasminogen activator) is most beneficial if given within 3 h of stroke onset, however only 20-25% of ischemic stroke patients arrive at hospital within this time frame and fewer still are eligible for this treatment (Barber et al., 2001). Another problem is the heterogeneity of the stroke population, which often limits the effectiveness of a treatment to a small subpopulation of patients (De Keyser et al., 1999). Thus, for the majority of patients the only option is to rely upon rehabilitation to improve functional recovery and quality of life after stroke. Recent experimental evidence suggests that following brain injury environmental factors (e.g. environmental enrichment) as well as pharmacological and rehabilitative treatment (e.g. amphetamine in combination with training) can markedly alter neuronal plasticity and behavioral recovery even when delayed by several weeks after the insult in rats (Gladstone and Black, 2000; Biernaskie and Corbett, 2001; Biernaskie et al., 2004).

Many stroke patients suffer from post stroke depression (PSD), a disability that affects from 18 to 78 percent of stroke patients (Singh et al., 2000). Several clinical

studies have shown PSD correlates with increased disability (Paolucci et al., 2001) and hospital stay after stroke (Gillen et al., 2001). Accordingly, many stroke patients are given antidepressants that have some actions in common with amphetamine and yet little is known as to how these drugs alter recovery of function. Potential side effects limit tricyclic antidepressant use in elderly patients and they are not generally prescribed for patients with cardiac complications (Robinson, 2003). Thus, fluoxetine (Prozac), a selective serotonin reuptake inhibitor, is often prescribed for treating PSD (Paolucci et al., 2001; Gupta et al., 2002). Pariente *et al.* (Pariente et al., 2001) used functional magnetic resonance imaging (fMRI) to show that a single dose of fluoxetine alters brain activity and modulates motor performance in stroke patients in a use-dependent fashion. This was seen as increased activation of the cortex ipsilateral to the injured limb when moving the injured index finger, as well as improved performance in a finger tapping test and increased grip strength.

Antidepressants may also improve recovery of function in stroke patients through improved mood and motivation to perform rehabilitative tasks. However, a more interesting possibility is that antidepressants could have direct effects on brain plasticity processes. Several antidepressants, including fluoxetine increase growth factors and other proteins associated with plasticity such as brain derived neurotrophic factor (BDNF) (Russo-Neustadt et al., 2000; Russo-Neustadt et al., 2001; Coppell et al., 2003) and phosphorylated cAMP response element binding (pCREB) protein (Nibuya et al., 1996), as well as neurogenesis (Malberg et al., 2000; Duman et al., 2001). Amphetamine (which has some mechanisms in common with fluoxetine) improves recovery of function

in rats if it is given in unison with training (Hovda and Fenney, 1984; Stroemer et al., 1998) and provides benefit in clinical trials when combined with physical therapy or motor training (Crisostomo et al., 1988; Walker-Batson et al., 1995; Butefisch et al., 2002). Based on these amphetamine studies it is likely that training is also required for fluoxetine to have a benefit. Stroke patients are given rehabilitation after a stroke, therefore in order to mimic the clinical setting the goal of this study was to examine the combination of fluoxetine with a rehabilitative reaching task on functional recovery after focal ischemia in rats using a battery of sensitive neurobehavioral tests.

2.2 Materials and methods

2.2.1 Subjects

Twenty-nine male Long-Evans rats (Charles River, Montreal, QC, Canada) weighing 300-320g at time of initial surgery were used. Subjects were housed in pairs in standard Plexiglas cages on a reverse day-night cycle (12 h). Behavioral testing was performed during the dark phase. Food and water were provided *ad libitum* except during behavioral testing periods when food was restricted to 12-15 g/day. All procedures were in accordance with guidelines set by the Canadian Council on Animal Care and approved by the Memorial University Animal Care Committee.

2.2.2 Surgery

Subjects were anesthetized initially with 2 % isoflurane in 30 % oxygen and 70 % nitrous oxide and maintained with 1.5 % isoflurane. Animals were placed in a stereotaxic frame and a midline skull incision was made. Three small burr holes were made in the bone overlying the forelimb and hind limb sensory-motor cortex, and the dorsolateral striatum. The vasoconstrictive peptide endothelin-1 (ET-1; 400 pmol/μl in H_2O) (Calbiochem, Germany) was injected at the following stereotaxic coordinates as determined from the Paxinos and Watson Atlas (Paxinos and Watson, 1986): 1. anteroposterior (AP) +2.3 mm, mediolateral (ML) +/–2.5 mm, dorsoventral (DV) -2.3 mm (2.0 μl), 2. AP 0.0 mm, ML +/–2.5 mm, DV –2.3 mm (2.0 μl), 3. AP +0.7 mm, ML +/–3.8 mm, DV –7.0 mm (1.0 μl) relative to Bregma (all DV measurements were taken from the skull surface). This method results in a similar but more restricted lesion than the more traditional method of injecting ET-1 adjacent to the middle cerebral artery (Sharkey et al., 1994). In the past we have found that due to anatomical variation of the middle cerebral artery (MCA) in individual rats and accuracy in placing the needle, the success rate (percentage of animals that survive the surgery and have a significant deficit) with this method is only about 50-60 % (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). In the current study the success rate was 85 %. The lesion was made on the right side unless the animal showed a pronounced preference in reaching ability with the right forelimb, in which case the animal was lesioned on the left (n = 4). Sham animals

received the same surgical procedures up to and including drilling of the burr holes and were anesthetized for the same duration as the ischemic animals. Injection of the vehicle was not performed in shams because previous studies from our laboratory have shown that vehicle injection into the territory of the MCA does not result in behavioural abnormalities (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). Temperature was monitored and maintained between 36.5 and 37.5 ^{0}C using a feedback regulated heating blanket (Harvard Apparatus, Holliston, MA) throughout the surgery.

2.2.3 Treatment conditions

Behavioral assessments (see below) were made on days 5 and 6 after surgery to give the animals sufficient time to recover from surgery and begin to regain weight. Animals with similar behavioral impairments in reaching were randomly assigned to one of four treatment groups. Treatment began on day 7 after ischemia once animals had been placed into the various groups. Ischemic + fluoxetine + rehabilitation (I+F+R) animals received fluoxetine for 4 weeks as well as 6 h a day of rehabilitation for 4 weeks (excluding behavioral testing days) (n = 5). Ischemic + rehabilitation (I+R) animals received the same rehabilitation as the previous group but received only vehicle for 2 weeks (n = 6). Ischemic (I) animals received only vehicle for 2 weeks (n = 5). Shams + fluoxetine + rehabilitation (Sham) received the same treatment as the ischemic + fluoxetine + rehabilitation animals (n = 7).

2.2.3.1 Drug treatment

A maximum dose of 10 mg/kg/day fluoxetine (determined by animal weight at time of pump implantation) was delivered via 2-week mini osmotic pumps (2ML2, Alzet, Cupertino, CA) subcutaneously implanted between the shoulders while animals were aneaesthetized with isoflurane (1.5 - 2 %). Implantation occurred on day 7 after ischemia. After 2 weeks the pumps were removed and replaced with fresh pumps, so that animals received 4 weeks of drug treatment. At the end of the first 2-week treatment period animals that received vehicle did not have a second pump implanted in order to determine if the pumps or the vehicle were having a negative effect on behaviour. Fluoxetine (donated by Eli Lilly, Indianapolis, IN) was dissolved in 50 % DMSO (Sigma, St. Louis, MO)/H_2O, and this vehicle was used for animals not receiving fluoxetine. After the pumps were removed the remaining fluid was extracted and the volume recorded as an approximate measure of pump effectiveness.

2.2.3.2 Rehabilitation

Procedures for rehabilitation were similar to those described previously (Biernaskie and Corbett, 2001). Briefly, beginning on day 7 post ischemia animals were exposed for 6 h per day during the week and 3 h per day on the weekends for 4 weeks (except on testing days), to a Plexiglas reaching apparatus filled with sugar-rich Noyes precision pellets (45 mg, Research Diets Inc, New Brunswick, NJ). The design of the apparatus was modified from the staircase apparatus with a central platform and two wells on either side that could be filled with pellets. Only the well on the side of the impaired limb was filled and the design prevented retrieval of pellets with the non-

impaired limb and thus encouraged the use of the impaired forelimb. Animals that did not receive rehabilitation were fed the average daily intake of the sugar pellets consumed by all animals that received rehabilitation (~16 mg/day).

2.2.4 Behavioral assessment

Functional recovery was assessed using 3 different behavioral tests. Subjects were trained before ischemia on the staircase task for 2 weeks and the ladder-rung test for 4 days. Animals were retested on all tasks on days 5 and 6 after ischemia and then 2, 4, and 6 weeks after the onset of treatment (Figure 2.1).

2.2.4.1 Staircase reaching test (Montoya et al., 1991)

This test consists of a chamber with a central platform for the rat to climb onto and a set of seven steps on each side. Each step holds three Noyes precision pellets (45 mg, Research Diets Inc, New Brunswick, NJ). The rats remained in the staircase for 15 min and the total number of pellets eaten on each side was recorded. This test provides a sensitive measure of skilled reaching ability of the forepaw, and also of sensory neglect. The animals were pre-trained twice per day over a 14-day period. Animals that failed to consistently retrieve over 55% of the total available pellets were excluded from the study. Animals were retested for two trials per day on days 5 and 6 after ischemia, and any animals that showed a mild deficit ($\geq$ 80 % of original score) were excluded from the study (n = 3). All remaining animals were tested twice per day for 2 days at each of the remaining test points. Reaching ability was determined by averaging the score of the

impaired limb over the 4 trials and calculating the percentage of pellets eaten compared to the average of the last 4 trials prior to stroke.

2.2.4.2 Forelimb asymmetry test

Animals were tested for limb preference and their ability to support weight on either forelimb by placing the animals in a clear Plexiglas cylinder, 20 cm in diameter and 35 cm high, for 3 min (Schallert et al., 1997). This task measures the number of forelimb contacts on the wall as the animal rears to explore the environment. The number of bilateral placements, ipsilateral to lesion placements, and contralateral to lesion placements were counted. Normal animals tend to use each limb more or less equally while ischemic animals favor their ipsilateral forelimb after injury (Schallert et al., 1997). The percent of ipsilateral limb use was calculated using the equation: ipsilateral contacts/ (ipsilateral + contralateral contacts) x 100. Limb contacts were videotaped from below using an angled mirror and later analyzed in a blinded fashion.

2.2.4.3 Ladder-rung walking test

This task is a sensitive measure of long-term forelimb and hind limb motor function as well as motor coordination and compensation after several types of brain injury (Metz and Whishaw, 2002). Animals were pre-trained over 4 days to traverse a horizontal ladder with evenly spaced rungs. On day 5 and all subsequent test days the animals were given one run across the training pattern and then filmed for 4 trials as they traversed an irregular rung pattern that varied for each test day. Number of foot slips and

errors in foot placement were video recorded for a 1.0 m segment of the ladder and the average slips and placements per step over the 4 trials were calculated.

2.2.5 Anatomical procedures

2.2.5.1 Histology

At the completion of the study animals were overdosed with Somnotol® and transcardially perfused with heparinized saline followed by 4 % paraformaldehyde. Brains were removed and post-fixed over night before being placed in a solution of 20 % sucrose in phosphate buffered saline (PBS), and allowed to sink (approximately 3 days). Brains were rapidly frozen using dry ice and sectioned using a cryostat (CM 3050 S, Leica, Germany) at 40 μm. Every 8^{th} slice was mounted and stained using Cresyl Violet.

2.2.5.2 Infarct measurement

Using a random start point, sections stained with Cresyl Violet from +3.0 mm to -2.5 mm relative to Bregma were assessed for injury using NIH image software. The same number of sections were analysed for each animal. Remaining non-injured tissue in the cortex and striatum of both hemispheres was measured and the area of the healthy tissue in the ipsilateral side was subtracted from the healthy tissue in the contralateral side to give an area of injured tissue in the cortex and striatum separately. Total volume of injury was calculated by averaging the area recorded from each slice, and multiplying that value by the total distance between the first and last slices that contained injured tissue.

2.2.6 Statistics

Behavioral data were analyzed using repeated measures ANOVA or two-way ANOVA where appropriate. To determine differences between groups Fisher's test or Student's t-test comparisons were used.

2.3 Results

2.3.1 Fluoxetine administration

All pumps were examined for defects after removal and remaining fluid was extracted and measured. One animal was discarded (I+F+R) due to pump malfunction. On average 0.4 ml of the initial 2.0 ml of drug solution remained. Adjusting for animal weight gain and amount of fluid pumped, the average dose of fluoxetine was calculated to be 9.0 mg/kg/day ± 0.82 for the first set of pumps and 8.95 mg/kg/day ± 0.96 for the second set of pumps. No difference was found between the amount of fluoxetine delivered to the ischemic and control animals (data not shown).

2.3.2 Infarct measurement

Brain tissue from all animals included in the behavioral results was assessed to determine if treatment had any effect on infarct size. Figure 2.2 depicts the areas typically injured by the ET-1 protocol used for this study. The injured area included the forelimb region of the sensorimotor cortex and a portion of the dorsolateral striatum. The

average volume for injured tissue in the cortex, striatum and combined values are given in Table 2.1. There was a trend for the I+R+F group to have a larger infarct than the other two ischemic groups, but there was greater variability in the I+R+F group and no effect of treatment was found for the amount of cortex ($F_{(2,13)} = 0.376$, $p = 0.6935$), striatum ($F_{(2,13)} = 1.065$, $p = 0.3728$) or total (cortex + striatum) tissue ($F_{(2,13)} = 0.511$, $p = 0.6116$) injured. Regression analysis revealed that the volume of injury did correlate with increased impairment on the staircase test ($r = 0.784$, $p < 0.0001$), both slips/step ($R = 0.575$, $p = 0.0051$) and placement errors/step ($r = 0.585$, $p = 0.0043$) of the ladder test, but not the forelimb asymmetry test ($r = 0.338$, $p = 0.1235$) at the first test point after ischemia.

2.3.3 Staircase reaching test

A repeated measures ANOVA revealed a significant effect of treatment ($F_{(3,19)} = 8.993$, $p = 0.0006$) and an effect of day ($F_{(3,19)} = 11.315$, $p < 0.0001$). As shown in Figure 2.3, Fisher's PLSD post hoc analysis revealed that ischemic injury resulted in a significant decrease in performance in all ischemic groups compared to shams that persisted throughout the experiment ($p < 0.01$). No differences were found between the different ischemic groups and none of these groups improved over time. Effect of day was due to Shams (as determined by paired t-test) at 2 and 4 weeks treatment having lower scores compared to pre-treatment ($p < 0.01$ for both). Despite this there was no

difference between time points for Shams by the end of the study compared to pre-treatment levels of performance.

2.3.4 Forelimb asymmetry test

An effect of group was found in the cylinder task ($F_{(3,19)} = 3.925$, $p = 0.0245$), as well as an effect of day ($F_{(3,19)} = 10.189$, $p < 0.001$). Fisher's test showed that all ischemic groups increased the number of ipsilateral contacts relative to sham animals immediately after surgery ($p < 0.01$) but returned to levels comparable to shams after 2 weeks (Figure 2.4). I+F+R and I + R animals showed a significant difference from sham animals after 4 weeks of treatment but they were not significantly different at 2 weeks of treatment or 2 weeks after treatment ended and were not different from other ischemic groups. Paired student's t-tests revealed that while sham animals did not significantly differ between time points all ischemic animals remained impaired compared to their pre-surgery score at the final test point (data not shown, $p < 0.05$).

2.3.5 Ladder-rung walking test

There was an effect of group for slips/step ($F_{(3,19)} = 3.744$, $p = 0.029$) but not for day ($F_{(3,19)} = 0.368$, $p = 0.7763$). The I+F+R group was significantly more impaired than the sham group after 2 weeks of treatment ($p < 0.05$) but at no other time point. The I + R group was significantly different from sham animals at pre-treatment as well as after 2 weeks of treatment ($p < 0.05$ and 0.01 respectively). In contrast the I group lacking any

treatment was not different from the sham group (Figure 2.5a). Paired student t-test showed that none of the groups improved over time compared to pre-treatment scores. For placement errors/step there was no effect of group ($F_{(3,19)} = 1.261$, $p = 0.3159$) but there was an effect of day ($F_{(3,19)} = 37.836$, $p < 0.0001$). All groups improved over time as determined with student t-test ($p < 0.05$). The I + R group improved significantly from pre-treatment to 2 weeks treatment ($p = 0.05$), while all other groups did not reach significance until 4 weeks of treatment (Figure 2.5b).

2.4 Discussion

In the current study the goal was to combine chronic fluoxetine treatment with rehabilitation to approximate treatment of stroke patients in a clinical setting. In our previous work we have observed benefit of the rehabilitation task used in the present study when it is combined with environmental enrichment (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). In this study rehabilitation (i.e. reach training) alone did not improve the functional outcome of ischemic animals with or without combined fluoxetine treatment. This differs from studies in monkeys where reach training alone facilitates recovery (Nudo et al., 1996b). It is possible that environmental enrichment is a necessary component in rodent models of stroke that must be given concurrently with this type of reach training in order for the dendritic plasticity and the behavioral benefits to occur (Schallert et al., 2000). Combining fluoxetine with rehabilitation was also done because

previous animal studies have shown that amphetamine and related agents only enhance recovery when administered at the same time as the behavioral task (Hovda and Fenney, 1984; Stroemer et al., 1998). It is also conceivable that the reason fluoxetine fails to improve functional recovery while environmental enrichment and amphetamine are beneficial is that different mechanisms are involved. It is thought that the main benefit of amphetamine is due to increased norepinephrine (NE) transmission (for a review see (Gladstone and Black, 2000)) and environmental enrichment has also been shown to increase brain levels of NE in the mouse (Naka et al., 2002). The main action of fluoxetine is to increase serotonin transmission with little effect on NE (Beyer et al., 2002).

Fluoxetine has been suggested to improve motor recovery in humans after stroke (Dam et al., 1996; Pariente et al., 2001). In spite of these clinical findings, no improvement on a range of tests of long-term sensorimotor function was found in the present study nor in a related study by Jolkkonen and colleagues (Jolkkonen et al., 2000a). The study by Jolkkonen *et al.,* (Jolkkonen et al., 2000a) used a smaller dose of fluoxetine (5 mg/kg/day) given over a shorter time period (10 days) and did not employ a rehabilitation task. The present study found that fluoxetine did not improve recovery of function of forelimb reaching, forelimb preference or motor coordination while the Jolkkonen study found that fluoxetine failed to improve performance on limb placement and a cognitive water maze task (Jolkkonen et al., 2000a). It is possible that acute versus chronic administration of fluoxetine has different molecular effects. Differences such as these may explain the results in the acute clinical imaging study, which found that a

single dose of 20 mg of fluoxetine alters brain activity and modulates motor performance in stroke patients (Pariente et al., 2001) whereas the animal studies have found no alteration in performance. For example, fluoxetine can either increase or decrease levels of cAMP, CREB, BDNF and its receptor, tyrosine receptor kinase (trk)B, mRNA depending on whether an acute, short-term, or prolonged dose is given (Nibuya et al., 1996; Miro et al., 2002; Coppell et al., 2003).

Fluoxetine in a dose of 10 mg/kg/day has previously been shown to increase serotonin levels in the rat brain (Felton et al., 2003) as well as increase BDNF gene expression within the hippocampus when given for 2 weeks (Coppell et al., 2003), therefore it is unlikely that our dosing regimen was insufficient to produce changes in BDNF. Increases in BDNF and its trkB receptor have been positively correlated with increases in synapsin I (a protein involved in transmitter release) as well as GAP-43 (Gomez-Pinilla et al., 2002). These and other studies (Jin et al., 2002; Vaynman et al., 2003) suggest a role for BDNF in neurite outgrowth and plasticity and that by acting through BDNF, fluoxetine may also affect neural plasticity. However, several studies report opposing results with 2-week fluoxetine dosing on BDNF levels in the hippocampus. For example, Nibuya *et al.* (Nibuya et al., 1996) and Coppell *et al.* (Coppell et al., 2003) note increased BDNF while Miró *et al.* (Miro et al., 2002) reported decreased BDNF. Differences in dosing regimens may account for these discrepancies, but it is evident that effects of fluoxetine on BDNF levels require further investigation. It is possible that the benefits of fluoxetine could be masked if the presence of the pumps interfered with the animals' performance (i.e. impaired limb use) on the behavioral tasks.

This is an unlikely explanation for our results since there was no increase in recovery of ischemic animals that received vehicle once their pumps were removed after 2 weeks of treatment. It is more likely that despite effects of fluoxetine on BDNF they are insufficient to augment neural plasticity. A study that compared exercise-induced increases in BDNF found that lesioning the noradrenergic but not the serotonergic system attenuated the increases in BDNF normally seen with 7 days of wheel running. This suggests that the serotonergic system plays a minimal role in BDNF regulation (Garcia et al., 2003). Wheel running for 7 days increases BDNF levels by 200 % in the dentate gyrus of the hippocampus (Garcia et al., 2003), whereas 2 weeks of fluoxetine treatment (10 mg/kg) only increases BDNF levels by about 125% in the same region (Coppell et al., 2003). Antidepressants with a greater impact on the noradrenergic system may enhance BDNF to a greater extent than fluoxetine.

Another interpretation of the results from clinical studies suggesting that fluoxetine enhances the recovery process after stroke (Dam et al., 1996; Pariente et al., 2001) is that the benefit is due to alleviation of depression. Since depression is a predictor of worsened outcome (Paolucci et al., 2001) it is possible that by improving depressive symptoms patients would achieve a higher level of recovery simply because they were more motivated to engage in rehabilitation. In this and other animal studies depressed mood is unlikely to be a confounding factor. Indeed motivation is often provided as a result of mild food deprivation (e.g. staircase test) and other incentives (e.g. escaping ladder to return to home cage). Therefore, in the absence of depressed mood,

fluoxetine does not appear to have the capacity to promote processes of neuronal plasticity and functional recovery.

Table 2.1. Infarct volume (mm^3)

	Cortex	Striatum	Total
I	20.4 ± 7.4	10.12 ± 1.99	30.54 ± 8.9
I+R	15.38 ± 4.0	12.53 ± 2.6	27.92 ± 4.5
I+R+F	28.22 ± 17.6	17.1 ± 4.9	45.32 ± 21.8

Infarct volume did not differ between any of the groups. Values are mean ± SEM.

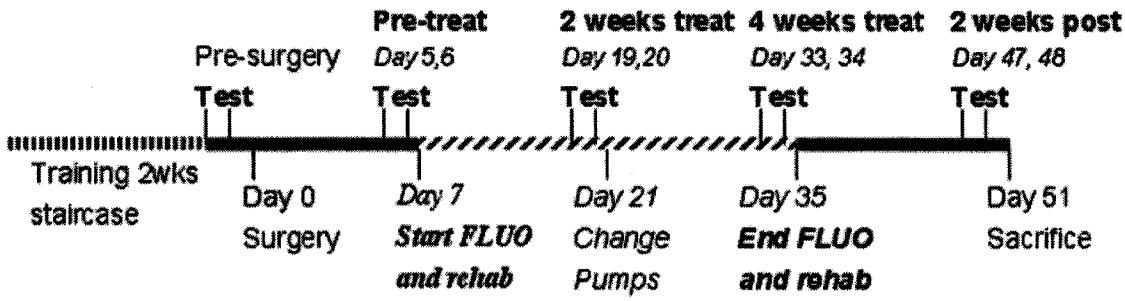


Figure 2.1. Time line of experiment. Days 5,6 correspond with pre-treatment testing and the remaining test points correspond with 2 weeks treatment, 4 weeks treatment and 2 weeks post-treatment respectively. At all test points ladder-rung walking was tested on the first day and forelimb asymmetry on the second day. Staircase was tested over two trials each of the test days and the average over the 4 trials was used as the animals score at each test point.

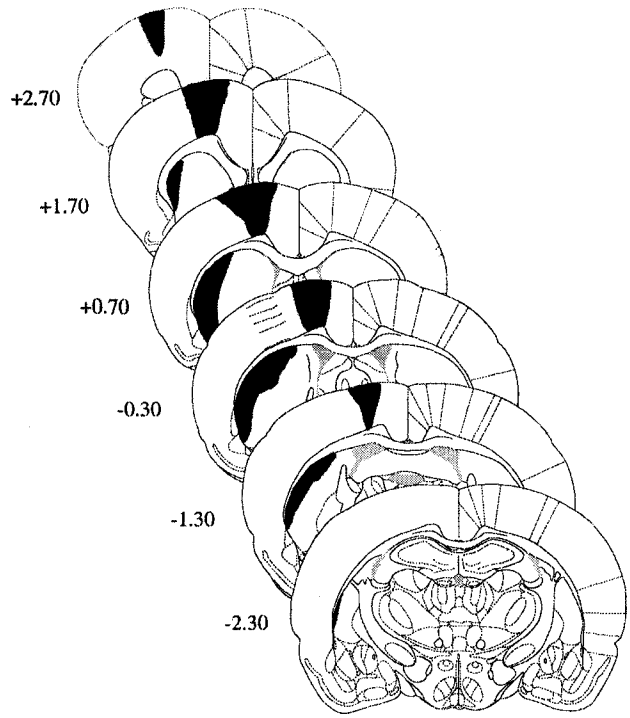


Figure 2.2. Representative diagram of regions of infarct after combined cortical and striatal injections of ET-1. Measurements given are in mm relative to Bregma.

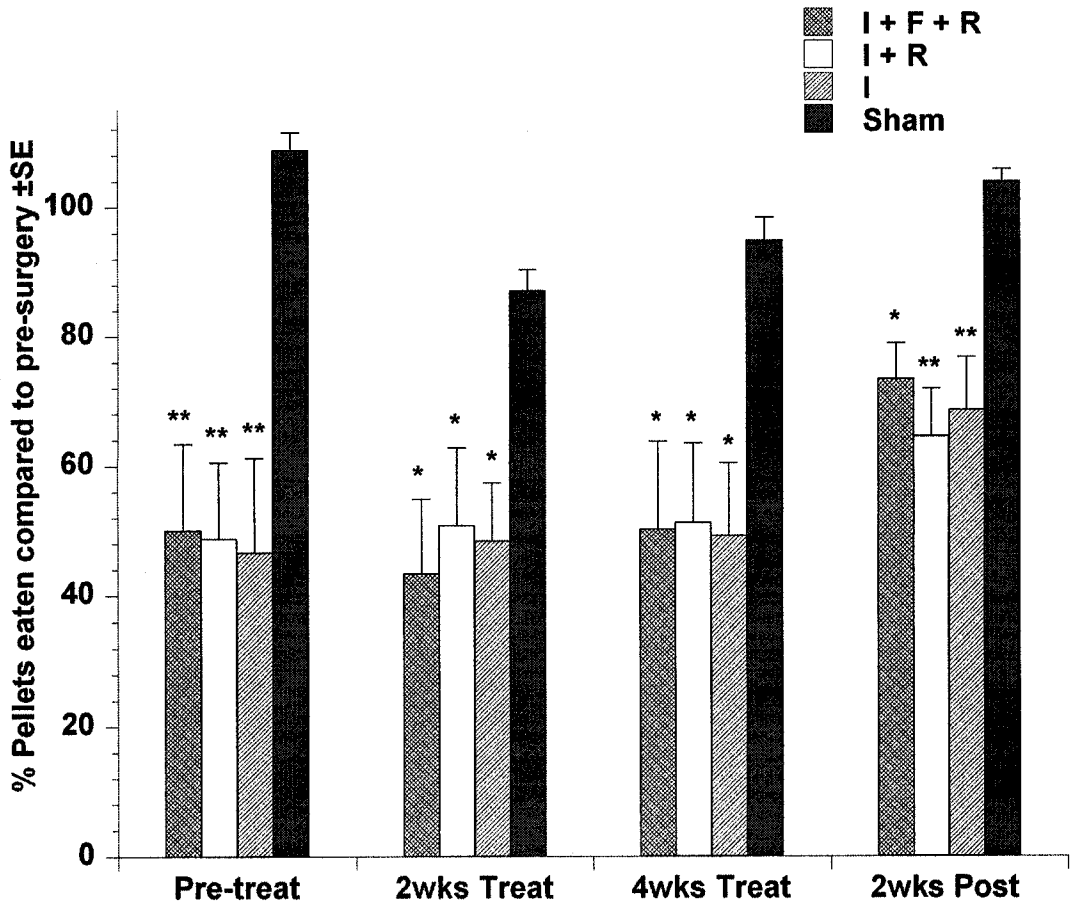


Figure 2.3. Staircase test of skilled forelimb reaching. All ischemic groups were significantly impaired compared to sham animals at all time points but no difference was found between the treatment groups. Values presented are mean ± SEM (* indicates a significant difference from sham group, $p < 0.01$, ** $p < 0.001$).

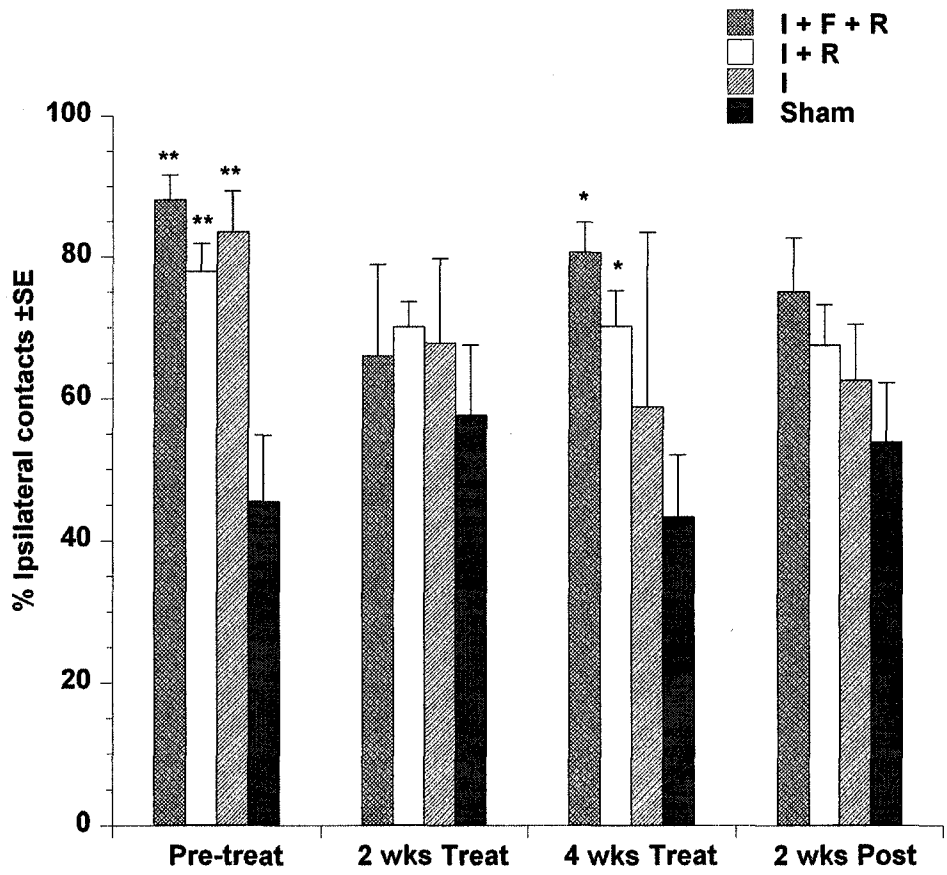
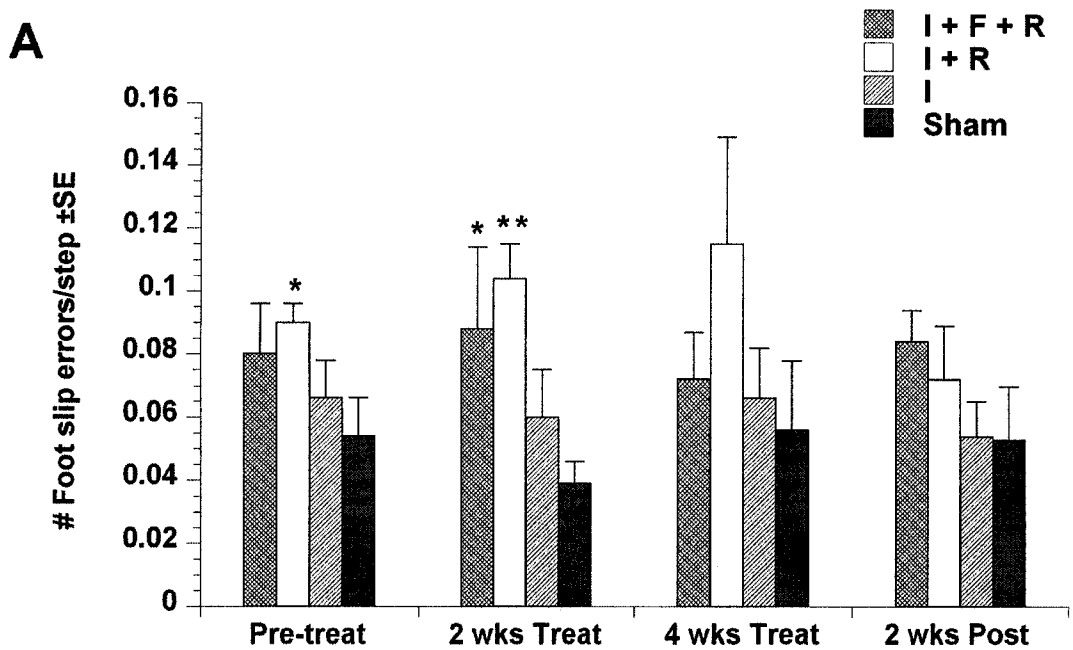
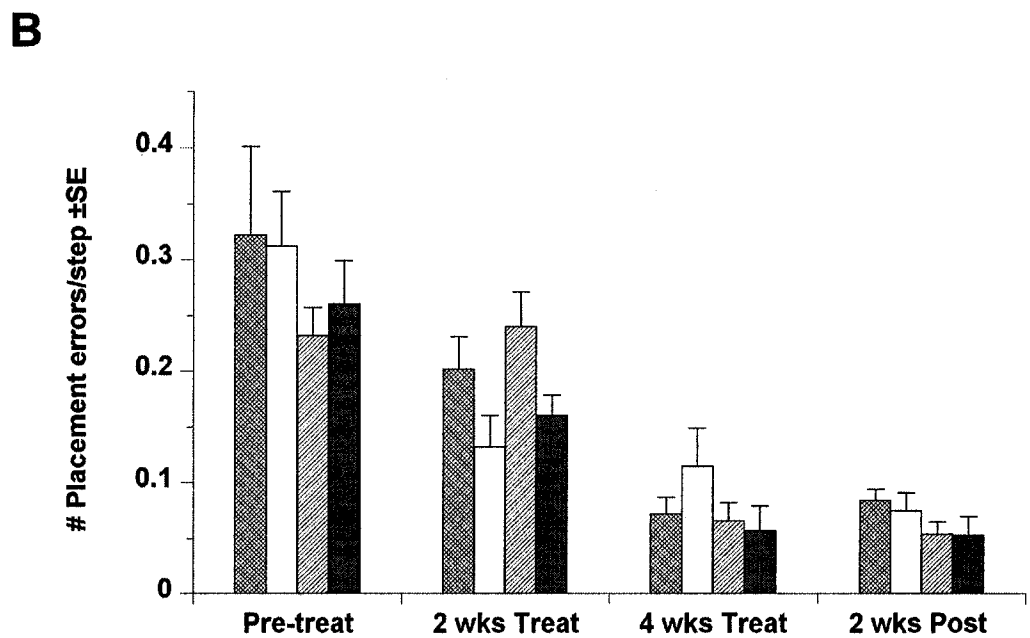


Figure 2.4. Forelimb asymmetry task. This task measured the number of ipsilateral forelimb contacts compared to contralateral contacts while the animal reared in a cylinder. All ischemic animals showed an increase in ipsilateral forelimb use after the ischemia (i.e. pre-treatment), but no difference was found between the treatment groups. Values presented are mean ± SEM (* indicates a significant difference from sham group, $p < 0.05$, ** $p < 0.01$).

Figure 2.5. Ladder-rung walking test. *A*. Foot slip errors/step decreased similarly for each treatment group. Although ischemic animals differed from shams at early time points they did not change significantly from themselves over time. (* indicates a significant difference from sham group, $p < 0.05$, ** $p < 0.01$) *B*. Placement errors/step revealed that animals improved at this task regardless of treatment group. Values presented are mean ± SEM.


A
I + F + R
I + R
I
Sham
Foot slip errors/step ±SE
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
* * **
Pre-treat
2 wks Treat
4 wks Treat
2 wks Post


B
Placement errors/step ±SE
0.4
0.3
0.2
0.1
0
Pre-treat
2 wks Treat
4 wks Treat
2 wks Post

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Chapter 3: Norepinephrine Depletion Facilitates Recovery of Function After Focal Ischemia in the Rat

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3.1 Introduction

Stroke is one of the leading causes of permanent disability in the world and as such research and therapy aimed at improving functional recovery after stroke is of the utmost importance. It is becoming apparent that traditional physiotherapy practices can be improved upon and recent experimental evidence suggests that following brain injury environmental factors (e.g. environmental enrichment) as well as pharmacological and rehabilitative treatment (e.g. amphetamine in combination with training) can markedly alter neuronal plasticity and behavioural recovery even when delayed by several weeks after an ischemic insult in rats (Gladstone and Black, 2000; Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002; Biernaskie et al., 2004).

Amphetamine has shown promise as an agent to improve motor recovery after brain injury as early as 1946 (Feeney, 1997; Gladstone and Black, 2000; Feeney et al., 2004). A single dose of amphetamine, and more effectively several doses paired with motor training, promotes recovery of function on a beam walking task in cats as well as rats after brain injury (Feeney et al., 1982; Hovda and Fenney, 1984; Goldstein and Davis, 1990). The beneficial effects of amphetamine can be blocked by haloperidol, a dopamine (DA) D_2 receptor antagonist, suggesting that the action may be mediated by DA (Feeney et al., 1982). However, amphetamine also increases serotonin (5-HT) and norepinephrine (NE) levels in the brain in addition to DA and other studies suggest that

the noradrenergic system is most likely responsible for the positive actions of amphetamine on recovery. For example, intraventricular infusion of DA or NE both improve beam walking after cortical injury, but if the DA is administered with a dopamine beta hydroxylase (DβH) inhibitor to block conversion to NE the effect is lost, demonstrating the importance of NE (Boyeson and Feeney, 1990). In addition, atipamezole (an alpha-2 adrenergic antagonist that increases NE) facilitates sensorimotor recovery after middle cerebral artery occlusion (MCAo) in the rat (Jolkkonen et al., 2000; Butovas et al., 2001; Puurunen et al., 2001). Finally, a number of drugs that increase NE facilitate motor recovery after brain injury, and depleting or blocking NE can attenuate recovery or reinstate deficits in recovered animals (Gladstone and Black, 2000).

We have previously found that environmental enrichment improves forelimb motor function after MCAo (Biernaskie and Corbett, 2001) and others have shown that mice reared in an enriched environment for 40 days had significantly higher brain levels of NE than control animals while 5-HT and DA levels were unchanged (Naka et al., 2002). Environmental enrichment facilitates neuroplasticity, neurogenesis and increases several neurotrophins, such as brain derived neurotrophic factor (BDNF) in the hippocampus and cortex, which may explain why it facilitates recovery of motor function after brain injury (Pham et al., 1999; Ickes et al., 2000; Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002; Mohammed et al., 2002; Gobbo and O'Mara, 2004). Similarly, amphetamine and other drugs that increase NE have also been shown to facilitate plasticity, neurogenesis and neurotrophin increases (Stroemer et al., 1998; Malberg et al., 2000; Russo-Neustadt et al., 2001; Butefisch et al., 2002). Prazosin (an

alpha-1 adrenergic blocker) decreases training dependant brain plasticity in humans (Sawaki et al., 2003) and depleting NE with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) has also been shown to block BDNF increases associated with exercise (Garcia et al., 2003).

The locus coeruleus (LC), the largest nucleus of noradrenergic neurons in the central nervous system, is the origin of the majority of noradrenergic afferents in the CNS, which project widely throughout the brain (Nakamura and Sakaguchi, 1990). These noradrenergic projections have the capacity to regenerate and have been shown to be plastic in response to stress and brain injury (Nakamura and Sakaguchi, 1990). In addition, infusion of antidepressants (several have been suggested to increase functional recovery after brain injury) increases NE axonal regeneration after a 6-OHDA lesion of NE axons in the frontal cortex (Nakamura, 1991). Lesioning of the LC prior to motor cortex injury impairs motor recovery on the beam walking task in rats (Goldstein and Bullman, 1997) and selective degeneration of the NE projections from the LC using DSP-4 also impairs motor recovery (Goldstein et al., 1991; Boyeson et al., 1992).

Despite data supporting the importance of NE in recovery of function after brain injury it is important to note that most of these studies have been performed using drugs (i.e. amphetamine, antidepressants and prazosin) that are known to have effects on other neurotransmitters in addition to NE. In those studies that lesioned the NE system it is also important to note that the NE lesions were performed prior to the motor cortex injury, and there are conflicting data about whether depleting NE increases or decreases subsequent ischemic injury (Blomqvist et al., 1985; Nishino et al., 1991; Nellgard et al.,

1999a). Increasing NE may facilitate motor recovery but it has yet to be determined whether NE is actually required to promote or increase recovery after ischemia. In order to more directly assess the involvement of NE in functional recovery, the present study used DSP-4 to selectively deplete NE projections from the LC one week *after* MCAo in rats. Environmental enrichment combined with a rehabilitation task was used to promote recovery and three different sensorimotor tests were used to determine if DSP-4 impeded recovery. In addition, BDNF was measured in a subset of animals to determine if depleting NE had any effect on levels of this neurotrophin.

3.2 Materials and methods

3.2.1 Subjects

A total of 139 male Sprague Dawley rats (Charles River, Montreal, QC, Canada) weighing approximately 300 g at time of surgery were used in this study. Initially, all animals were socially housed in pairs in standard Plexiglas cages on a reverse day-night cycle (see housing and treatment groups below). Behavioural testing was performed during the dark phase. Food and water were provided *ad libitum* except during behavioural testing periods when food was restricted to 12-15 g/day. All procedures were in accordance with guidelines set by the Canadian Council on Animal Care and approved by the Memorial University Animal Care Committee. Every effort was made to reduce animal numbers and alleviate suffering.

3.2.2 Surgical procedures

Animals were anesthetized with 3 % isoflurane in 30 % oxygen and 70 % nitrous oxide and were maintained with 1.5 % isoflurane. Temperature was maintained between 36.5^0 C and 37.5^0 C throughout the surgery using a self-regulating heating blanket (Harvard Apparatus, Holliston, MA). Animals underwent Endothelin-1 (ET-1) MCAo as outlined in previous studies (Biernaskie and Corbett, 2001) except an increased concentration of ET-1 (human and porcine from Calbiochem, Cedarlane, Hornby, ON, Canada) was employed. Briefly a single injection of ET-1 (600 pmol in 3 μl sterile H_20) was placed adjacent to the MCA: anterioposterior (AP) +0.9 mm, mediolateral (ML) –5.2 mm and dorsoventral (DV) -8.7 mm. All stereotaxic measurements are relative to Bregma (Paxinos and Watson, 1986). The control group included sham animals that underwent the same surgery up to and including the drilling of the burr hole but did not receive ET-1. Due to a relatively low success rate (~ 60 %) with this ischemic model (Biernaskie et al., 2004) animals with unsuccessful ischemia (as determined by behavioural and later histological assessment) were included in the control group in order to reduce animal numbers.

3.2.3 Norepinephrine depletion

In order to deplete the noradrenergic projections from the LC, rats were given a single I.P. injection of 50 mg/kg of DSP-4 dissolved in sterile saline (50 mg/ml) 6 days after ischemia. Dose of DSP-4 was based on previous studies that have shown long-term depletion of NE (Ross, 1976; Fritschy and Grzanna, 1991). Animals were closely

monitored and given mash and lactated ringers (5 ml) if needed. Most animals appeared healthy and were able to commence their housing treatment (see below) the next day, however a small number of animals were kept separate until they began to gain weight (usually 2-3 days). Weight loss occurred equally in all the treatment groups and the slight delay in starting housing treatment did not affect outcome (data not shown). Animals that did not receive DSP-4 received an equivalent volume (1 ml/kg) of sterile saline.

3.2.4 Housing and treatment groups

A timeline of the experimental protocol is given in Figure 3.1. Five days after ischemia rats were tested on 3 behavioural tests of motor coordination and forelimb function (see below), grouped according to the severity of impairment and randomly assigned to one of eight treatment groups: ischemic + enriched rehabilitation (I + ER), ischemic + standard housing (I + St), Control + ER, Control + St, I + ER + DSP-4, I + St + DSP-4, Control + ER + DSP-4, Control + St + DSP-4. Post-treatment testing was carried out 2, 6 and 9 weeks after the injection of DSP-4 and start of housing treatment. In addition to the animals listed in the above groups 30 animals in the 4 ischemic groups (n = 7-8 per group) were used without behavioural testing for the BDNF portion of the study and 7 animals in the I + ER + DSP-4 and 6 animals in the I + St + DSP-4 group were tested behaviourally but sacrificed one week post DSP-4.

3.2.4.1 Enriched rehabilitation

A similar protocol was followed as used previously (Biernaskie and Corbett, 2001). Animals were housed in groups of 6-8 rats in large metal cages equipped with ropes, beams, platforms and various toys. The groups switched cages twice a week at which point the cages were cleaned and the types of toys and the orientation of objects were changed. In addition, animals were given a 6 h rehabilitation session 5 days a week (with the exception of testing days) for a total of 9 weeks. The animals were placed individually in a standard cage containing a Plexiglas reaching apparatus containing Noyes precision pellets (45mg, Research Diets Inc, New Brunswick, NJ). The design of the apparatus was modified from the staircase apparatus with a central platform and two wells on either side that could be filled with pellets. Only the well on the side of the impaired limb was filled and the design prevented retrieval of pellets with the non-impaired limb and thus encouraged the use of the impaired forelimb. Animals that failed to consistently obtain over 5 g of pellets per day were excluded from the study.

3.2.4.2 Standard housing

Animals were housed in pairs in standard Plexiglas cages. In addition to their regular chow they were fed the average amount of Noyes pellets eaten by the ER rats each day (approximately 12-14 g).

3.2.5 Behavioural assessment

3.2.5.1 Staircase reaching test (Montoya et al., 1991)

This test consists of a chamber with a central platform for the rat to climb onto and a set of seven steps on each side. Each step holds three 45 mg Noyes precision pellets. The rats remained in the staircase for 15 min and the total number of pellets eaten on each side was recorded. This test provides a sensitive measure of skilled reaching ability of the forepaw, and also of sensory neglect. The animals were pre-trained twice per day over a 14-day period ending 2 days prior to ischemic surgery. Animals that failed to consistently retrieve over 55 % of the total available pellets were excluded from the study. Animals were retested for two trials per day on days 5 and 6 after ischemia (pre-treatment), as well as over two days 2, 6 and 9 weeks after DSP-4 (post-treatment). Reaching ability was determined by averaging the score of the impaired limb over the 4 trials and calculating the percentage of pellets eaten compared to the average of the last 4 trials prior to ischemia.

3.2.5.2 Forelimb asymmetry test

Animals were tested for limb preference and their ability to support weight on either forelimb by placing the animals in a 20 cm diameter x 35 cm high clear Plexiglas cylinder for 5 min (Schallert et al., 1997). As the animal rears to explore the environment the number of bilateral paw placements, placements of the paw ipsilateral to the lesion, and placements of the paw contralateral to lesion are counted. Animals were required to have a minimum of 20 contacts. If 20 contacts were not achieved in the 5 min time frame

the animal was observed until 20 contacts had been made. Normal animals tend to use each limb more or less equally while ischemic animals favour their ipsilateral forelimb after injury (Schallert et al., 1997). The percent of ipsilateral limb use was calculated using the equation: (ipsilateral contacts + ½ bilateral contacts/ total contacts) x 100. Paw contacts were videotaped from below using an angled mirror and later analyzed in a blinded fashion. Animals were tested once prior to ischemia and once at each subsequent time point.

3.2.5.3 Ladder-rung walking test

Animals were tested for forelimb and hindlimb function as well as motor coordination by crossing a ladder with an irregular rung pattern (Metz and Whishaw, 2002). Animals were trained to cross the ladder over 4 trials in one day. At each test point, including prior to ischemia, the animals were filmed while crossing the ladder on 4 trials. Slips and misplacements of paws were scored for a 1 m segment of the ladder. To give an indication of impairment the number of slips were added to the placement errors and divided by the number of steps taken for a total number of errors/step. The rung pattern was changed for each test point.

3.2.6 Histological procedures and assay

3.2.6.1 Histology

Animals were sacrificed either at 1 or 9 weeks post-DSP-4 treatment. At the conclusion of each study, animals were injected with an overdose of Somnotol® and

perfused transcardially with heparinized saline followed by 4 % paraformaldehyde for 5 min. Brains were removed and placed in 4 % paraformaldehyde for 90 min then transferred to a 20 % sucrose solution in phosphate buffered saline (PBS) and allowed to sink (approximately 3 days). Brains were frozen on dry ice and 40 μm sections were cut using a cryostat (CM 3050 S, Leica, Germany). Every 8th section was mounted and stained with Cresyl Violet for infarct volume assessment. All other sections were stored in cryoprotectant at – 20 °C until processed for immunohistology.

3.2.6.2 Infarct measurement

Slides were scanned and every third slice was analyzed microscopically and the healthy tissue in the cortex, striatum and total hemisphere for each side of the brain were traced using Image J software (NIH). Volume of injury was calculated by subtracting the area measured in the ischemic hemisphere from the contralateral hemisphere and multiplying that value by the distance between the measured slices.

3.2.6.3 Immunohistology

Every 8th section was stained for DβH. Sections were washed in PBS followed by 3 min in 3 % H_2O_2. Slices were then washed in PBS (3 x 10 min), incubated in 5 % normal goat serum in PBS with 0.25 % triton-X for 1 h and then incubated overnight at 4 °C in 1:1000 dilution of mouse anti- DβH (Chemicon, Temecula, CA, USA) in PBS with 0.25 % triton-X. The next day slices were washed in PBS, incubated in 1:500 dilution of biotinylated goat anti-mouse (Jackson Immuno Research Labratories Inc., West Grove, PA, USA) in PBS with 0.25 % triton-X for 1 h, washed in PBS, incubated in 10 μg/ml

Extravadin (Sigma-Aldrich, St. Louis, MO, USA) in PBS with 0.25 % triton-X washed in PBS and reacted for 5 min in 3,3'-Diaminobenzidine (DAB) tablet set (Sigma-Aldrich, St. Louis, MO, USA). Negative controls were run with each batch of staining.

3.2.6.4 Measurement of DβH staining

Neuronal projections stained with DβH were traced and analyzed with Neurolucida software (MicroBrightfield Inc., Williston, VT, USA) at 40X magnification (Leica DMRXE light microscope, Leica Microsystems Canada, Richmond Hill, ON, Canada). The total length of axonal projections in a given area was calculated using Neuroexplorer (MicroBrightfield Inc., Williston, VT, USA). Projections were analyzed in 4 different regions of interest (ROI) in the contralateral hemisphere from the MCAo (in order to avoid infarcted tissue): the frontal cortex (ROI 1), the forelimb region of the motor cortex (ROI 2), the parietal cortex (ROI 3) and CA1 region of the hippocampus (ROI 4). Three counts where taken in each ROI (150 X 150 μm^2) and averaged. Figure 3.6 shows the location of each ROI.

3.2.6.5 BDNF immunoassay

Animals in this portion of the study were sacrificed by decapitation at 9 weeks under light isoflurane anesthesia. Both hippocampi and a portion of motor cortex from the intact, contralateral hemisphere (to avoid infarcted tissue) were quickly removed, weighed and flash frozen in liquid nitrogen. Samples were stored at -80 °C until further processing. Tissue was homogenized in a 7 times volume of ice-cold homogenization buffer (100mM Tris/HCl, pH 7, containing 2% bovine serum albumin, 1M NaCl, 4mM

EDTA, 2% Triton X-100, 0.1% sodium azide and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 15,000 x g for 30 min at 4 °C. Supernatants were collected and further diluted with buffer for a final dilution of 70 times. Samples were frozen and stored at -20 °C until processed further. BDNF levels were measured using ELISA (Chemicon, Temecula, CA, USA) according to manufacturer's protocol. Using the optical densities of known BDNF concentrations to create a standard curve the mean optical densities of samples (in duplicate) were used to calculate BDNF concentrations. Each plate contained samples from all four groups tested in this portion of the study.

3.2.7 Statistics

Behavioural and histological data were analyzed using repeated measures ANOVA or two-way ANOVA where appropriate. A Fisher's PLSD test was used to determine differences between groups and Student's-t test to determine differences within groups over time, where $p < 0.05$ was considered significant. Regression analysis was used to determine the relationship between the amount of DβH staining and staircase performance. All values given are mean ± SEM.

3.3 Results

3.3.1 Infarct volume

Values for infarct volume are provided in Table 3.1. There was no effect of DSP-4 or housing on infarct volume although there was a trend for animals in the ER groups to have smaller cortical, and thus total, infarcts. The area of injury was typical for MCAo and Figure 3.2 shows the range of injury in damaged animals.

3.3.2 Behavioural results

No differences were found between control animals with regard to housing condition or drug treatment therefore they were pooled into a single control group. Six animals that had shown minimal deficits in staircase testing early post-stroke were later eliminated from the control group because of ischemic injury. Another 9 animals were eliminated because they failed to reach the criteria for reaching success in rehabilitation or they continued to drop weight 4 days after DSP-4 treatment. The final group numbers were I + ER (n = 13), I + St (n = 14), I + ER + DSP-4 (n = 12), I + St + DSP-4 (n = 14) and control (n = 16).

3.3.2.1 Staircase reaching test

There was a significant effect of group ($F_{(4,64)} = 28.064$, $p < 0.0001$), time ($F_{(3,64)} = 7.831$, $p < 0.0001$) as well as a significant time x group interaction ($F_{(12,64)} = 2.447$, $p = 0.005$). All ischemic groups were severely impaired in the staircase test compared to control animals at all time points ($p \leq 0.0008$). While the I + St group did not improve over the 9 weeks the I + ER group improved slightly and the two DSP-4 treated groups

improved significantly ($p < 0.05$) from pre-treatment (Figure 3.3). The I +ER + DSP-4 group improved the most and showed significantly better recovery than the I + St group from 2 weeks post-DSP-4 treatment on ($p \leq 0.01$). At 9 weeks post-treatment both ER groups as well as the I + St + DSP-4 group exhibited significantly better recovery than the I + St group ($p \leq 0.01$).

3.3.2.2 Forelimb asymmetry test

There was no overall effect of group for this test although all ischemic groups were significantly impaired compared to control animals 6 days after ischemia (pre-treatment) ($p < 0.001$). There was an effect of time ($F_{(3,64)} = 6.999$, $p = 0.0002$) with all of the ischemic groups improving so that at 9 weeks no groups were different from the control group. Although all groups showed recovery on this task the I + St group was the slowest to recover, returning to control levels at 6 weeks rather than at 2 weeks like the other ischemic groups (Figure 3.4).

3.3.2.3 Ladder-rung walking test

There was no effect of group for this task but there was an effect of time ($F_{(3,64)} = 48.319$, $p < 0.0001$). All groups improved over the course of the study reducing the number of errors/step (Figure 3.5A). Comparison of errors/step at 9 weeks to pre-treatment performance showed that all groups made significant improvements but the two ER groups made the greatest gains ($p < 0.05$ for the St groups and controls, $p < 0.0001$ for the ER groups) (Figure 3.5B).

3.3.3 DβH staining

Figure 3.6 illustrates DβH terminal staining in a DSP-4 and a saline treated animal. There was a significant effect of group ($F_{(5,63)} = 15.009$, $p < 0.0001$), the region of interest measured ($F_{(3,63)} = 105.776$, $p < 0.0001$) as well as group x region interaction ($F_{(15,63)} = 5.612$, $p < 0.0001$) for DβH staining. All groups that received DSP-4 showed significantly decreased staining for DβH compared to the groups that received saline ($p < 0.01$) (Figure 3.7A). There was no effect of ischemia or housing on the amount of DβH staining although there was a trend for the I + ER + DSP-4 group to have more staining than the I + St + DSP-4 group (especially in ROI 1, frontal cortex). In addition, it was found that DβH staining in DSP-4 and saline treated groups was significantly decreased in ROI 4 (hippocampus) compared to the other regions ($p < 0.05$).

To investigate the possibility that NE projections were regenerating during the course of the study and whether housing had an effect on this, a subgroup of animals were sacrificed 1 week after DSP-4 injection. Comparison of DβH staining 1 and 9 weeks post-treatment revealed that there was no significant effect of time but there was a trend in both groups for more staining at 9 weeks (Figure 3.7B).

Regression analysis revealed that among ischemic animals in the ER groups there was a non-significant trend for decreased DβH staining to correlate with a higher staircase score at 9 weeks (data not shown). Among ischemic standard housed animals the correlation of decreased DβH staining and increased performance was significant for each ROI except ROI 2 ($R = 0.50$, 0.43 and 0.44 and $p = 0.007$, 0.02 and 0.02 for ROI 1, 3 and 4 respectively).

3.3.4 BDNF

No significant differences in the amount of BDNF protein were found between the 4 ischemic groups in either hippocampi or the cortex contralateral to the lesion. There was however an effect of region ($F_{(4,26)} = 384.786$, $p < 0.0001$) due to the cortex containing significantly less BNDF than both hippocampi ($p < 0.0001$). There was a trend for the animals exposed to environmental enrichment to have higher levels of BDNF, and when DSP-4 and saline treated animals were pooled for housing conditions there was a significant effect of group ($F_{(1,28)} = 4.952$, $p = 0.0343$). ER resulted in significantly higher levels of BDNF in the contralateral hippocampus (CH), the contralateral hippocampus + the contralateral cortex (CH + CC) as well as for total BNDF in all three regions measured ($p < 0.05$ for all) (Figure 3.8). Pooling animals for drug treatment showed that DSP-4 had no effect on BDNF levels in the regions measured (data not shown).

3.5 Discussion

The goal of the present study was to determine if NE is required for recovery of function after focal ischemia in rats. Accordingly, we depleted NE by injecting DSP-4 one week after MCAo and we found no difference in infarct volumes between the groups that received DSP-4 and those that received saline. DSP-4 was given after MCAo since DSP-4 given prior to ischemia can affect injury outcome (Blomqvist et al., 1985; Nishino et al., 1991; Nellgard et al., 1999a).

We have shown here that ER facilitates recovery of function as previously found (Biernaskie and Corbett, 2001; Biernaskie et al., 2004) but in addition we have shown that depleting NE also facilitates recovery of function 9 weeks post-treatment regardless of housing. These results are at odds with those previously reported in short term (12 and 19 days respectively) survival studies (Goldstein, 1991; Boyeson et al., 1992). In these studies the DSP-4 animals were more impaired than the respective controls at the start of behavioural testing, perhaps as a consequence of giving DSP-4 prior to the cortical injury (Goldstein, 1991; Boyeson et al., 1992). Further, infarct volumes were not measured in the Boyeson study (Boyeson et al., 1992) and no volumetric measures were provided in the Goldstein study (Goldstein, 1991). In the present study all groups were balanced for equal behavioural impairments prior to the initiation of treatments. Despite claims that depleting NE impeded recovery, the DSP-4 animals in the Boyeson study did fully recover if given an extra 5 days of training (Boyeson et al., 1992). Given that these DSP-4 animals began the study with a more severe impairment, the rate of recovery appears to be approximately the same as their control animals. In the Goldstein study the DSP-4 animals did not recover as much as the saline controls, but by the 12^{th} day appeared similar to sham animals given DSP-4 (Goldstein, 1991). This suggests that recovery was not impeded by DSP-4 as previously suggested, but instead DSP-4 affected the baseline performance of the animals. In addition, these studies used only a beam walking test to evaluate functional outcome (Goldstein, 1991; Boyeson et al., 1992), and spontaneous recovery was seen after the somatosensory cortex lesion.

Clinical stroke can result in a large variety of motor deficits but commonly involves persistent upper extremity deficits (Parker et al., 1986; Nakayama et al., 1994). The present study used several tasks that have previously been shown to reveal different impairments in animal models of stroke, including forelimb reaching deficits, and show little spontaneous recovery (Montoya et al., 1991; Schallert et al., 1997; Biernaskie and Corbett, 2001; Metz and Whishaw, 2002). The most pronounced effects of NE depletion were seen in the staircase reaching task. Animals housed in standard cages did not show recovery on the staircase test and it was only through intervention such as ER or NE depletion that some recovery occurred.

The use of DSP-4 has been reported to be a reliable tool for depleting LC-NE terminals for as long as 8 months without permanent effects on DA, 5-HT or peripheral NE systems (Ross, 1976; Jonsson et al., 1981; Fritschy and Grzanna, 1991; Harro et al., 2003). In the present study staining for DβH increased in both ischemic groups given DSP-4 over the course of the study but the increase was not significant and NE remained significantly depleted 9 weeks after DSP-4 administration. The present data show that LC-NE depletion facilitates forelimb function after ischemia since ischemic animals that received DSP-4 performed better in the staircase test than ischemic animals treated with saline in both housing conditions. There was a negative correlation between DβH terminal staining and staircase performance at the end of the study but this reached significance only in ischemic animals housed in standard conditions. This may be because animals housed in ER already improve to a greater extent than those in standard housing and although depletion of NE augments the recovery it is not apparent in ER

animals due to a ceiling effect. The negative correlation supports the hypothesis that DβH fiber loss is the significant variable in improved recovery.

The mechanisms by which depleting NE would facilitate recovery are unknown. Effects on other systems such as enhanced dopamine D_2 receptor density and sensitivity, which might facilitate motor function (Harro et al., 2000; Harro et al., 2003) is one possibility. Alternatively, DSP-4 may enhance NE function in a way that would be beneficial to motor recovery. NE infusion into the cerebellum of animals with a unilateral lesion of the LC resulted in a heightened behavioural response (Boyeson et al., 1993). This could have occurred because of selective increases in NE receptors, supersensitization of remaining receptors, sprouting of remaining NE terminals, or a combination of all three. Several studies have shown that DSP-4 treatment can up-regulate and increase sensitivity of α_1- and β-adrenergic receptors in the cortex and hippocampus which could facilitate the action of NE released from remaining terminals (Dooley et al., 1983; Dunwiddie et al., 1983; Mogilnicka, 1986; Zahniser et al., 1986; Theron et al., 1993; Wolfman et al., 1994). It is unknown how long the receptor changes persist, but even transient changes may play a key role in shaping recovery.

Important to the hypothesis that DSP-4 facilitates NE function is that the drug treatment did not totally eliminate DβH fiber staining. Regenerative sprouting of residual LC axons has been noted previously and even hyperinnervation of the frontal cortex has been observed 6 months after DSP-4 treatment (Fritschy and Grzanna, 1992). NE terminals were not completely removed in the present study and indeed there was a slight increase in DβH staining from 1 to 9 weeks post DSP-4 (see Figure 7B), which might be

important in the functional benefits seen. Since there are fibers remaining, an increase in NE turnover could compensate for decreased innervation. Microdialysis studies have shown that extracellular NE levels are unchanged (Kask et al., 1997; Nellgard et al., 1999b) or even increased in areas of decreased tissue NE after DSP-4 treatment (Logue et al., 1985; Hughes and Stanford, 1998). Thus, physiologically effective NE release acting on supersensitive β-adrenergic receptors might provide an enhancement of NE function that would facilitate motor recovery as seen in the present study. Enhanced NE release is also consistent with reported down-regulation or decreased sensitivity of α_2-adrenoreceptors following DSP-4 (Heal et al., 1993; Kask et al., 1997; Prieto and Giralt, 2001). This hypothesis is speculative since the microdialysis studies were conducted within a week of DSP-4 treatment and it is unknown how long such levels are sustained. On the other hand, as mentioned earlier, even early events may shape later recovery.

The greatest functional gains seen in the present study were in the ER + DSP-4 group, which may be related to further facilitation of NE function by ER. Environmental enrichment has previously been shown to increase NE in mice (Naka et al., 2002). In addition, repeated mild stress has also been shown to increase terminal sprouting in NE axons from the LC (Nakamura et al., 1989). Enriched rehabilitation could be viewed as a mild stress and in the present study ER animals did tend to have higher levels of DβH staining than standard housed animals, although the difference was not statistically significant.

BDNF has been suggested to be crucial for experience-dependent plasticity as it can increase other mediators of plasticity such as synapsin I and growth associated

protein – 43 (Gomez-Pinilla et al., 2002). Rehabilitative therapies such as environmental enrichment (Falkenberg et al., 1992; Gobbo and O'Mara, 2004) and exercise (Russo-Neustadt et al., 2000; Gomez-Pinilla et al., 2002; Vaynman et al., 2003; Ploughman et al., 2005) increase BDNF levels. Similarily, NE has been suggested to be a modulator of BDNF given that several noradrenergic antidepressants increase BDNF in the brain (Nibuya et al., 1995; Russo-Neustadt et al., 2000) and that NE depletion attenuates exercise induced increases in BDNF (Garcia et al., 2003). In the present study we found that ER increased BDNF levels compared to standard housing, suggesting a possible role for BNDF in the improved functional recovery seen with ER. However, we found that depleting NE with DSP-4 did not alter BDNF levels. It is possible that NE function was maintained and therefore no change in BDNF would be expected, but it is also possible that changes in BDNF took place much earlier than when samples were taken (9 weeks post-DSP-4). Thus, it is possible that BDNF levels were transiently altered and then returned to normal, however this cannot be determined without further investigation.

In conclusion, we have shown that depleting tissue NE using DSP-4 facilitates recovery of function after ET-1 induced MCAo. Whether this effect is mediated by decreased NE, effects on other neurotransmitter systems, or due to increased NE efflux and receptor supersensitivity remains to be determined, but it does highlight the need for further research on the role of NE in recovery of function and neuroplasticity after focal ischemia.

Table 3.1. Infarct volume (mm^3)

Group	Striatum	Cortex	Hemisphere
I + St	25.10 ± 2.84	68.71 ± 10.01	99.64 ± 16.40
I + St + DSP-4	17.40 ± 3.37	56.83 ± 7.41	78.42 ± 12.03
I + ER	25.23 ± 2.59	43.94 ± 6.94	68.43 ± 9.95
I + ER + DSP-4	22.57 ± 1.83	40.81 ± 5.38	67.35 ± 6.82

Values given are mean ± SEM.

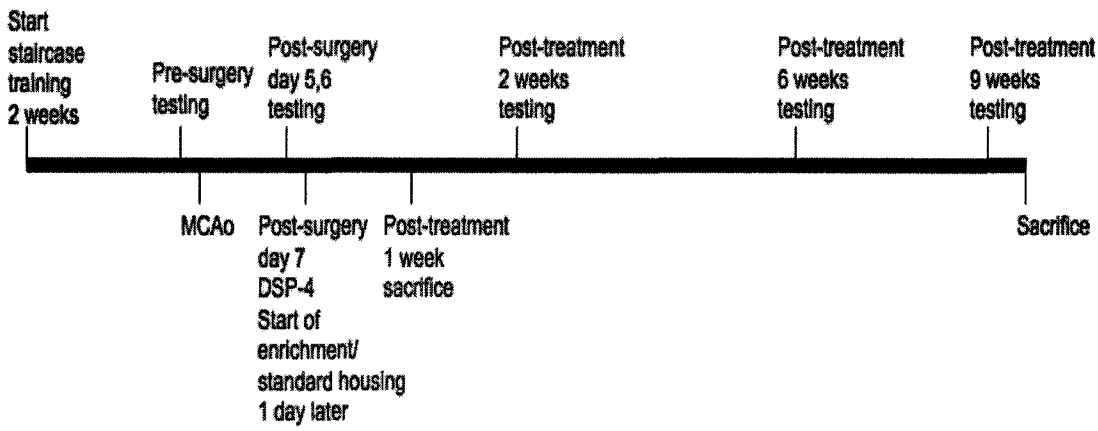


Figure 3.1. Timeline of behavioural testing, drug administration and housing treatment.

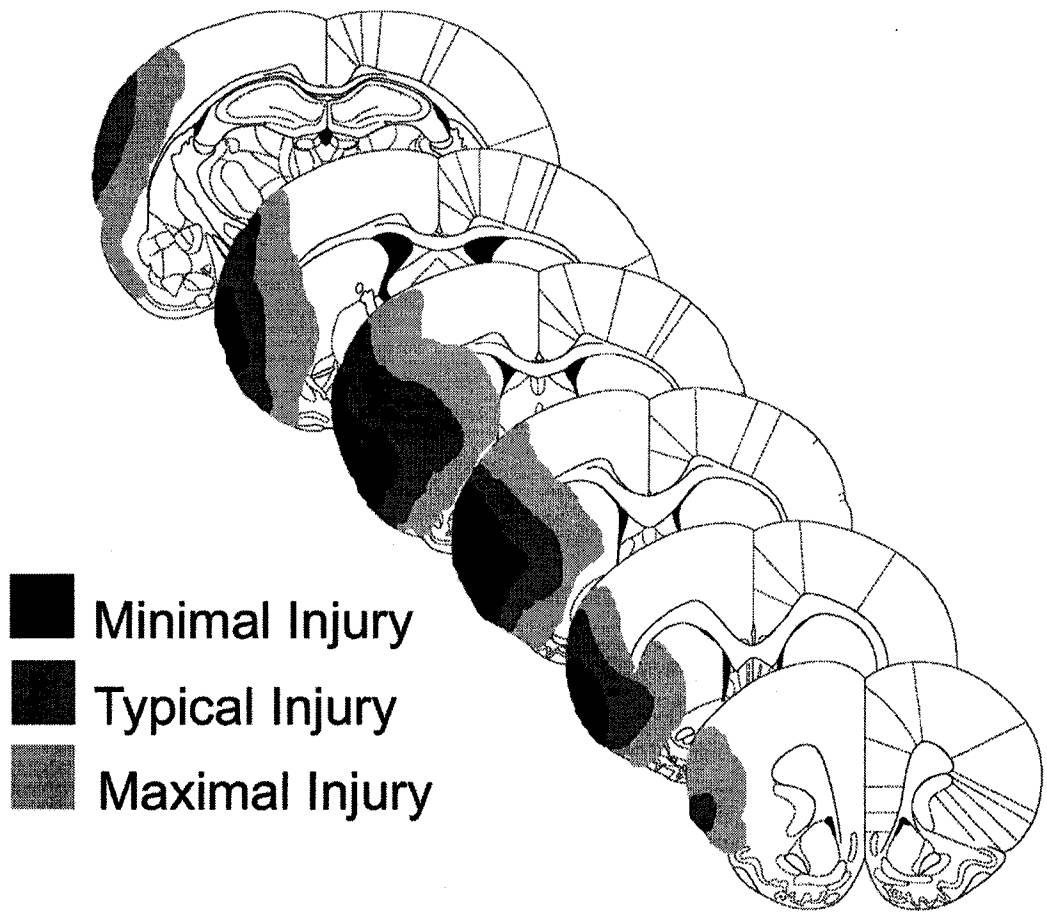


Figure 3.2. Schematic illustration of the minimal, average and maximal amount of ischemic damage after ET-1 induced MCAo.

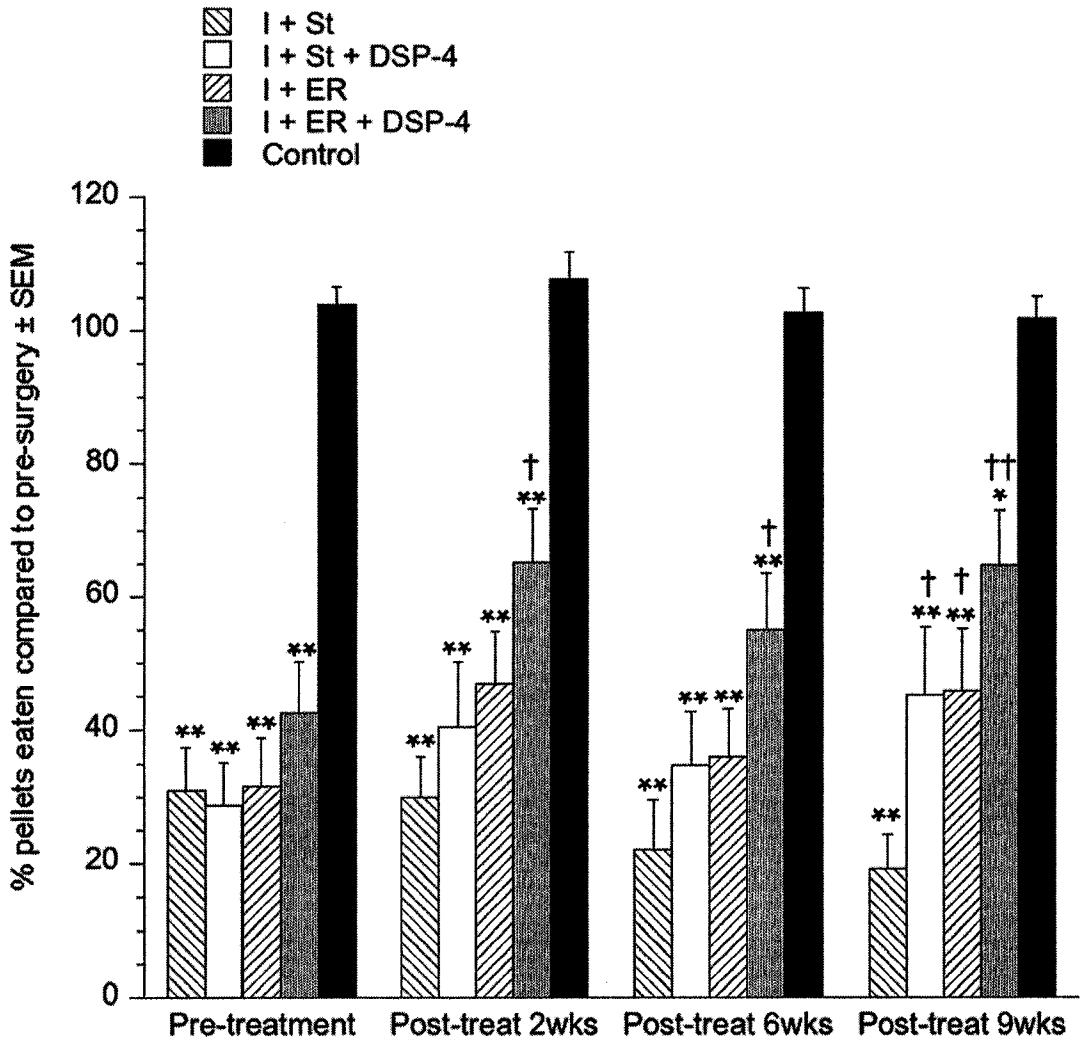


Figure 3.3. Performance in the staircase reaching task. All ischemic groups were impaired at pellet reaching with the contralateral limb compared to control animals. Enriched rehabilitation as well as depletion of NE using DSP-4 resulted in enhanced recovery compared to animals housed in standard cages. Values given are mean ± SEM (* $p < 0.001$, ** $p < 0.0001$ compared to control group; † $p < 0.01$, †† $p < 0.0001$ compared to I + St group).

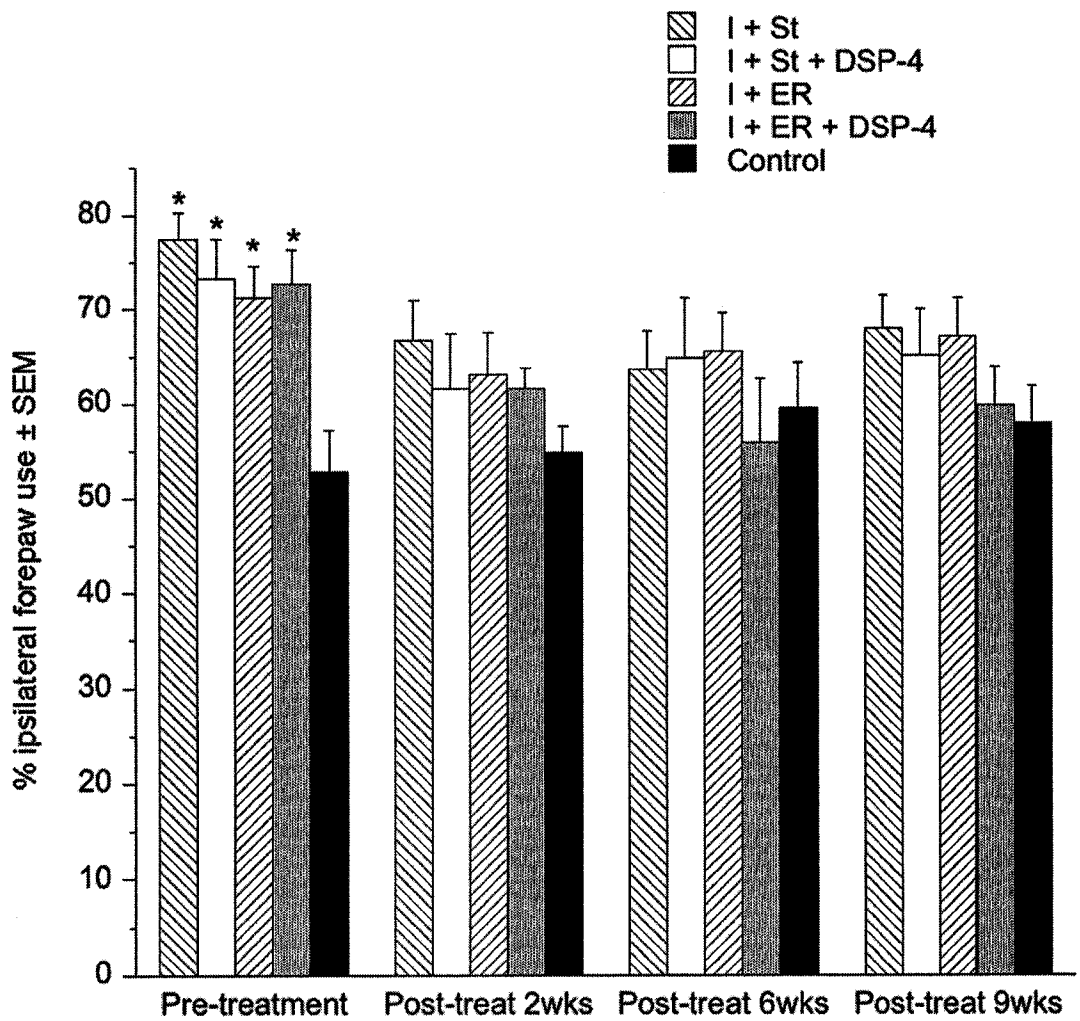
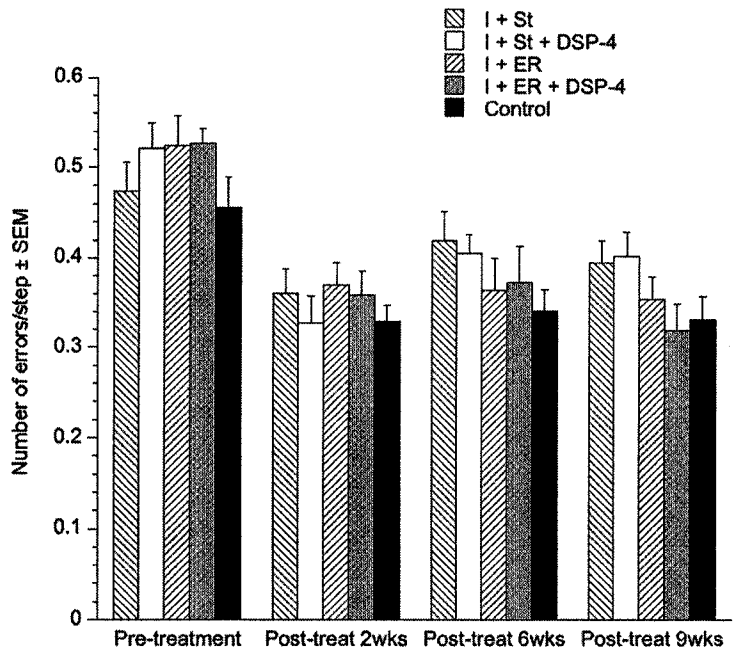


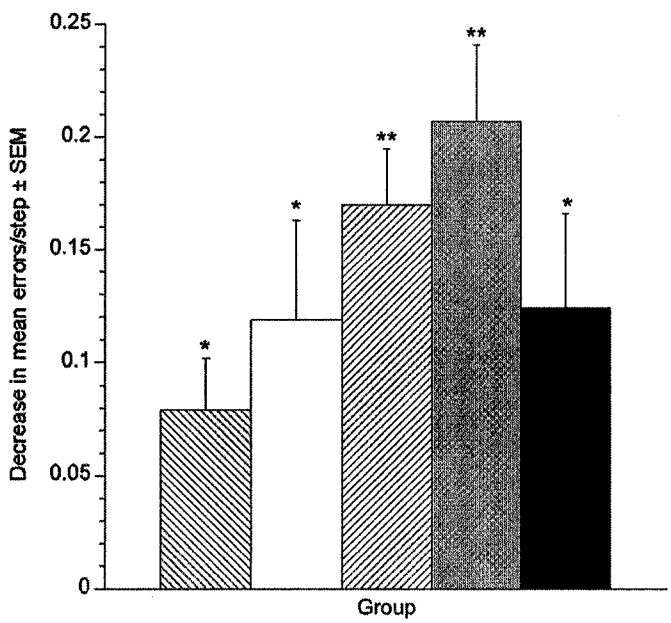
Figure 3.4. Ipsilateral forepaw use in forelimb asymmetry test. All ischemic groups displayed increased reliance on the ipsilateral forepaw after ischemia but returned to normal levels within the first 2 or 6 weeks of post-ischemic testing. Values given are mean ± SEM (* $p < 0.001$ compared to control group).

Figure 3.5. Mean number of errors/step in the ladder-rung walking task (A) as well as the decrease in errors/step from pre-treatment to 9 weeks post-treatment (B). All groups improved over time with the greatest improvement seen in the I+ ER and I + ER + DSP-4 groups. Values given are mean ± SEM (* $p < 0.05$, ** $p < 0.0001$ for difference in performance from pre-treatment to 9 weeks post-treatment).

A


I + St
I + St + DSP-4
I + ER
I + ER + DSP-4
Control
Number of errors/step ± SEM
0.6
0.5
0.4
0.3
0.2
0.1
0
Pre-treatment
Post-treat 2wks
Post-treat 6wks
Post-treat 9wks

B


Decrease in mean errors/step ± SEM
0.25
0.2
0.15
0.1
0.05
0
*
*
**
**
*
Group

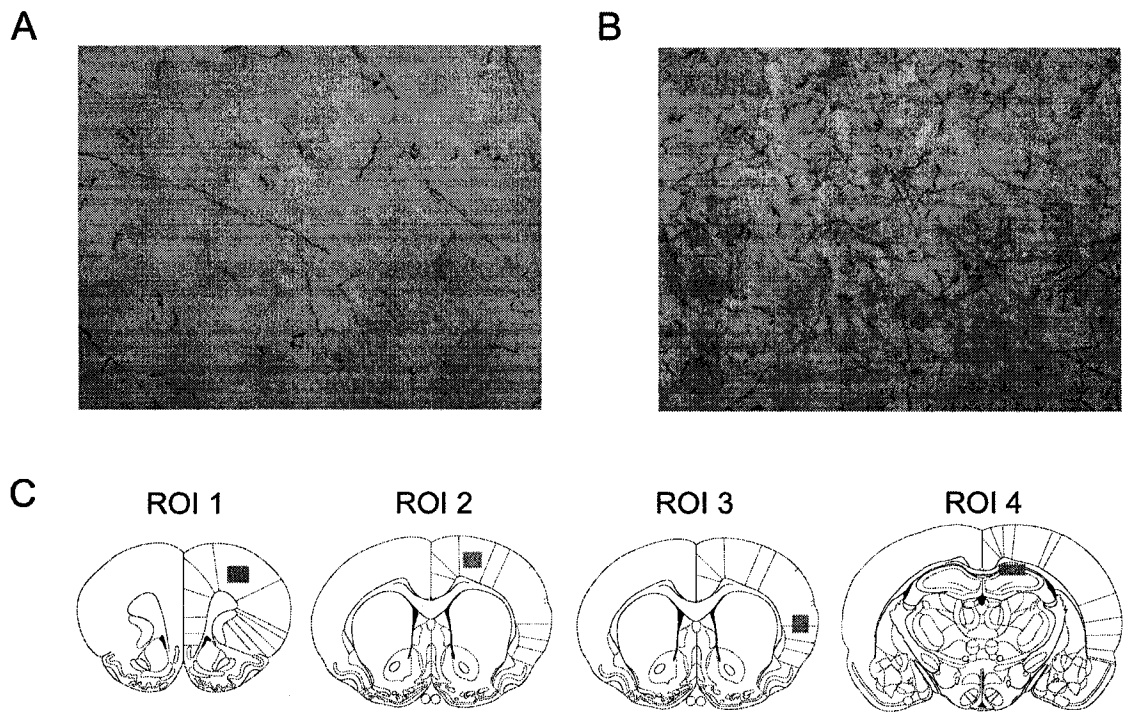
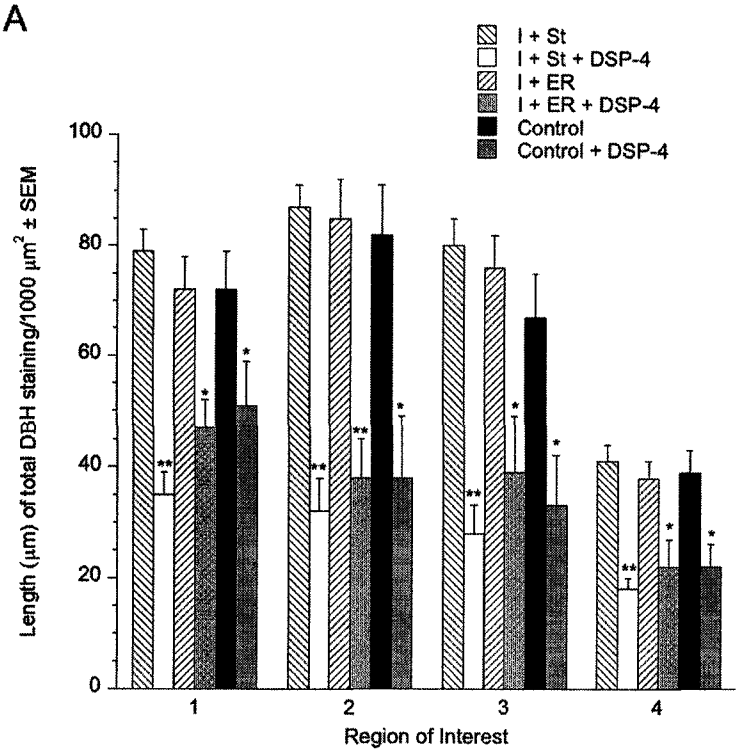


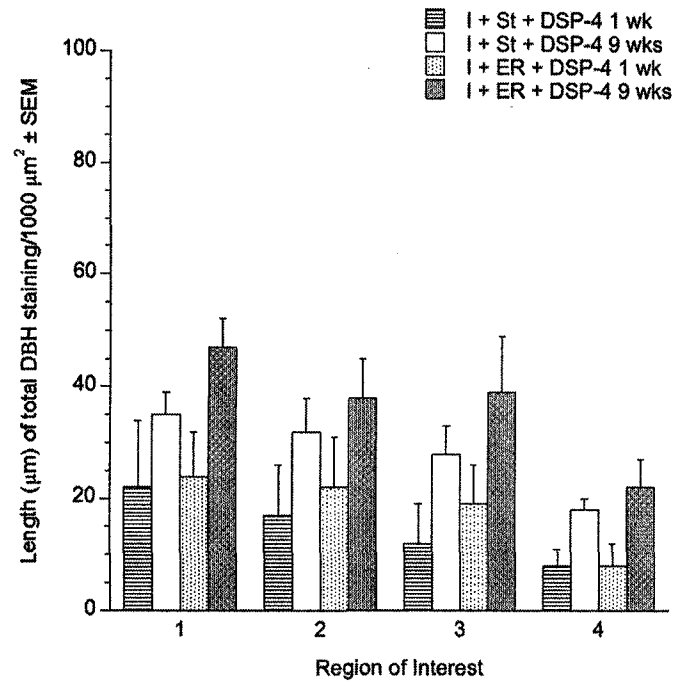
Figure 3.6. DβH staining of motor cortex contralateral to the infarct in DSP-4 (A) and saline (B) treated animals (20 x magnification). Regions of interest (ROI) in which DβH staining was measured (C). Note that the shaded boxes represent the approximate area in which 3 measurements were taken.

Figure 3.7. Mean length (μm) of DβH staining/10000 μm^2 9 weeks after DSP-4 treatment (A) as well as a comparison of staining at 1 and 9 weeks (B). Four different regions of interest were examined for DβH staining, and in all regions the administration of DSP-4 resulted in a significant decrease in staining. In addition it was found that among saline treated animals there was significantly less staining in ROI 4 compared to the other regions examined. Values given are mean ± SEM (* $p < 0.01$, ** $p < 0.0001$ compared to respective saline treated group).

A


I + St
I + St + DSP-4
I + ER
I + ER + DSP-4
Control
Control + DSP-4
Length (µm) of total DBH staining/1000 µm² ± SEM
100
80
60
40
20
0
1
2
3
4
Region of Interest

B


I + St + DSP-4 1 wk
I + St + DSP-4 9 wks
I + ER + DSP-4 1 wk
I + ER + DSP-4 9 wks
Length (µm) of total DBH staining/1000 µm² ± SEM
100
80
60
40
20
0
1
2
3
4
Region of Interest

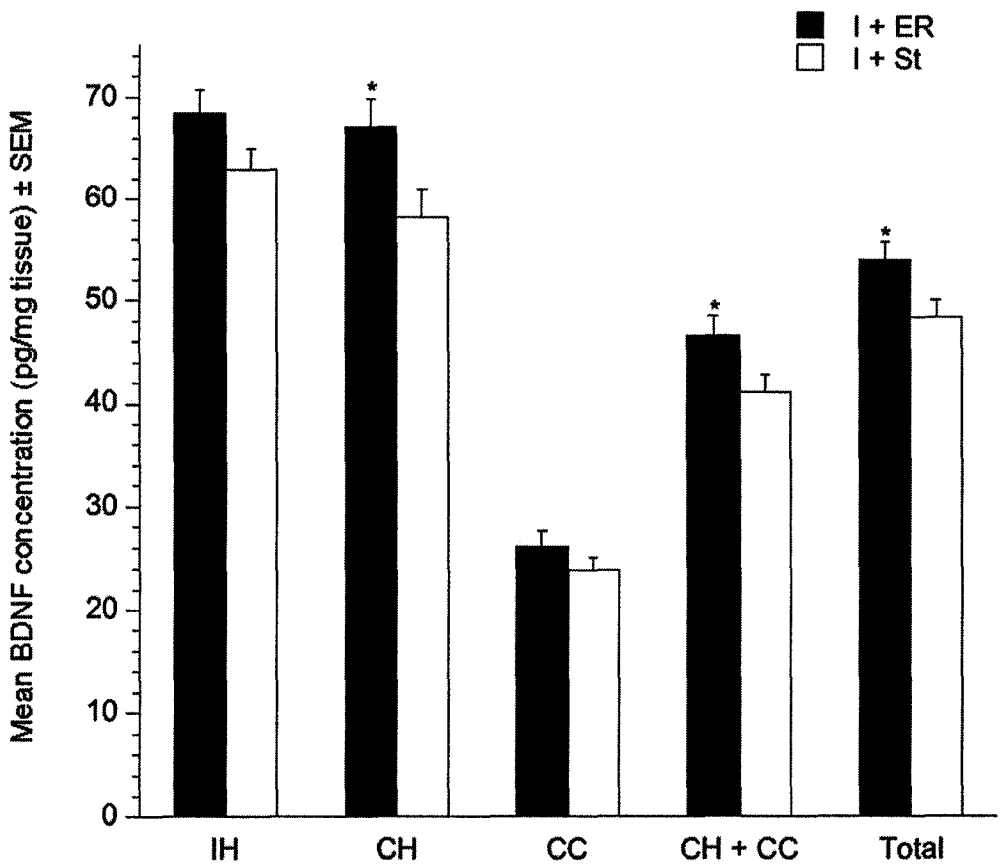


Figure 3.8. Effects of housing on BDNF protein levels in the ipsilateral hippocampus (IH), contralateral hippocampus (CH), contralateral cortex (CC) and the areas combined. Animals housed in ER showed increased BDNF levels in the contralateral hippocampus as well as the combined contralateral hippocampus and cortex (CH + CC) and all three regions combined (Total). Values given are mean ± SEM (* $p < 0.05$).

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Chapter 4: An Analysis of Four Different Methods of Producing Focal Cerebral Ischemia With Endothelin-1 in the Rat

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4.1 Introduction

There are a number of rodent focal ischemia models (Carmichael, 2005) but the most commonly used one involves occlusion of the middle cerebral artery (MCAo) (Kanemitsu et al., 2002) by insertion of an intraluminal suture (Ginsberg and Busto, 1989; Longa et al., 1989). Focal ischemia can also be produced by coagulation of the MCA, by introducing an embolism or by photothrombosis (i.e. injection of rose-bengal). Middle cerebral artery occlusion produces a well-defined region of injury that includes the neocortex as well as the lateral striatum, resulting in sustained behavioural deficits (Ginsberg and Busto, 1989) that are of paramount clinical importance. Despite the widespread use of the above MCAo models, they have several drawbacks when used to study sensorimotor deficits: 1) the amount of injury is variable, particularly in transient focal models, 2) they often do not affect the motor cortex, 3) by blocking much of the length of the MCA by an intraluminal suture other arteries may also be occluded (Dittmar et al., 2003; McColl et al., 2004) leading to injury in brain regions not typically affected in human MCA stroke, and 4) surgeries are invasive and often result in feeding difficulties and deterioration of long-term health (Sharkey and Butcher, 1995; Dittmar et al., 2003).

Endothelin-1 (ET-1), a potent vasoconstrictor (Yanagisawa et al., 1988), reduces local blood flow to levels that produce ischemic injury when injected directly into brain tissue (Fuxe et al., 1997). Stereotaxic injection of ET-1 adjacent to the MCA is a less invasive procedure compared to methods that expose the artery or introduce a suture into the lumen, yet it produces a pattern of ischemic damage similar to the more traditional MCAo models (Sharkey et al., 1993). Stereotaxic injection of ET-1 can be delivered to conscious animals (Sharkey et al., 1993; Moyanova et al., 2003) and yields similar results to other focal ischemia models when investigating neuroprotective drugs (Sharkey et al., 1994). It also produces long-term reaching deficits in the staircase task validating the model for studying recovery of function (Marston et al., 1995). In the ET-1 MCAo model blood flow reduction is rapid but not immediate (Macrae et al., 1993) and reperfusion occurs over several hours (Macrae et al., 1993; Biernaskie et al., 2001). This profile may be more representative of human stroke (not treated with t-PA) than the immediate reduction and reperfusion seen with the intraluminal suture or clip models of MCAo. Finally, it is possible to alter the amount of injury and improve consistency by increasing the concentration of ET-1 (Macrae et al., 1993).

Nonetheless, due to variability in vessel location and size, accurate placement of ET-1 adjacent to the MCA is difficult, affecting the ischemic success rate and resulting sensorimotor deficits. Application of ET-1 to the surface of the cortex causes a dose-related reduction of local blood flow for at least an hour, resulting in a well defined lesion (Fuxe et al., 1997). A variation of this model used by Adkins-Muir and Jones produced an injury that varied in size, yet was very consistent in location (Adkins-Muir and Jones,

2003). Intracortical injections of ET-1 into the forelimb region of the motor cortex produce lasting deficits in forepaw reaching ability with a recovery profile that is similar to that in other injury models (aspiration and quinolinic acid lesions) (Gilmour et al., 2004). However, the optimal placement of ET-1 to produce sensorimotor deficits has not been explored.

The purpose of this study was to evaluate different methods of delivering ET-1 to produce focal cerebral ischemia in order to create reliable sensorimotor deficits. Topical and intracortical applications were compared with injection adjacent to the MCA as well as to a combined approach of injecting ET-1 into both the cortex and striatum. Effects of the different models as well as effects of concentration of ET-1 were examined using three different sensorimotor tests and infarct measurements. In addition, magnetic resonance imaging (MRI) was performed on the cortical + striatal injection model in order to compare outcome to previous data for MCAo induced by injection of ET-1 adjacent to the MCA (Biernaskie et al., 2001).

4.2 Materials and methods

4.2.1 Subjects

A total of 115 male Long-Evans hooded rats (Charles River, Montreal, QC, Canada) weighing approximately 300 g at time of surgery were used in this study. All animals were socially housed in pairs in standard Plexiglas cages on a reverse day-night cycle (12 h) with the exception of those animals used in the MRI portion of the study.

Behavioural testing was performed during the dark phase. Food and water were provided *ad libitum* except during behavioural testing periods when food was restricted to 12-15 g/day. All procedures were in accordance with guidelines set by the Canadian Council on Animal Care and approved by the Memorial University and University of Manitoba Animal Care Committees.

4.2.2 Surgical procedures

Animals were anesthetized initially with 2 % halothane in 30 % oxygen and 70 % nitrous oxide and were maintained with 1 % halothane. Temperature was maintained between 36.5^0 C and 37.5^0 C throughout the surgery using a self-regulating heating blanket (Harvard Apparatus, Holliston, MA). Animals underwent one of the following surgeries that used ET-1 (human and porcine from Calbiochem, Cedarlane, Hornby, ON, Canada) in various concentrations dissolved in sterile saline. All stereotaxic measurements are relative to Bregma (Paxinos and Watson, 1986) and with depth determined from skull surface.

4.2.2.1 Topical application

Animals were placed in a stereotaxic apparatus, a midline incision was made and the bone and dura overlying the M1 region of the cortex in the right hemisphere were removed (-1.75 mm to 3.25 mm anterior to Bregma, and 0.5 mm to 5.5 mm lateral) either by drilling several small burr holes or by using a 5 mm trepan. ET-1 was applied to the cortex using a Hamilton syringe in varying concentrations, to achieve a total of 1200

pmol (n = 9), 2000 pmol (n = 19), or 4000 pmol (n = 4) dissolved in 30, 20 and 10 μl of saline, respectively. Groups were labelled topical 1200, topical 2000 and topical 4000, respectively. For the first two groups, application was followed immediately by placing sterile Gelfoam (Upjohn, Kalamazoo, MI), a gelatin sponge intended for slow release of the drug, over the exposed cortex and saturating it with additional drug (included in total pmol count). For the third group (topical 4000) Gelfoam was not used. The hole was covered by Parafilm (secured with glue to prevent infection) and the incision sutured.

4.2.2.2 Intracerebral injections

Animals were placed in a stereotaxic apparatus, a midline incision was made and small burr holes were drilled at the coordinates given below. Using a 10 μl Hamilton Syringe, ET-1 was injected into the motor cortex (2 μl) and in some animals into the dorsolateral striatum (2 μl) as well, in either 400 pmol or 800 pmol amounts. The syringe was lowered and after 1 min ET-1 was injected at a rate of 1.0 μl/2 min with a 1 min pause between each μl and a 3 min delay before syringe withdrawal. Five different protocols were used:

1. cortical 800 - 2 cortical injections of 400 pmol each (n = 4). Stereotaxic coordinates were anterioposterior (AP) 0.0 mm, +2.3 mm, mediolateral (ML) –2.5 mm, dorsoventral (DV) –2.3 mm (for both injections).
2. cortical 1600 - 2 cortical injection of 800 pmol each (n = 10). Same coordinates as above

3. cortical 2400 - 3 cortical injections of 800 pmol each (n = 11). Stereotaxic coordinates were AP –1.0 mm, +1.0 mm, +3.0 mm, ML –2.5 mm, DV –2.3 mm.
4. cortical + striatal (c/s) 1200 - 2 cortical injections of 400pmol each + 1 striatal injection of 400 pmol (n = 4). Stereotaxic coordinates were same as surgery #1 for the cortical injections, plus AP +0.7 mm, ML –3.8 mm, DV –7.0 mm for the striatal injection.
5. c/s 2400 - 2 cortical of 800 pmol each + 1 striatal injection of 800pmol (n = 13). Same coordinates as c/s 1200.

4.2.2.3 MCA occlusion (MCAo)

The protocol used for this model was as outlined in previous studies (Biernaskie and Corbett, 2001) except for an increased concentration of ET-1 and a minor adjustment to the DV coordinates. The accuracy of the coordinates was checked by injecting India ink and immediately removing the brain to confirm the proximity of the injection to the MCA (data not shown). Briefly a single injection of 600 pmol in 3 μl was placed adjacent to the MCA at AP +0.9 mm, ML –5.2 mm, DV -8.2 mm (n = 8).

4.2.2.4 Sham surgery

In all of the above protocols sham surgeries involved replacing ET-1 with sterile saline (8 topical and 11 injected shams).

4.2.3 Behavioral assessment

Three different behavioural tests were used to assess motor deficits out to one month post-ischemia. These tests encompass a range of difficulty and have demonstrated sensitivity in revealing long term functional deficits compared to simple neurological scores (Montoya et al., 1991; Schallert et al., 1997; Modo et al., 2000; Biernaskie and Corbett, 2001).

4.2.3.1 Staircase reaching test (Montoya et al., 1991)

This test consists of a chamber with a central platform for the rat to climb onto and a set of seven steps on each side. Each step holds three Noyes precision pellets (45 mg, Research Diets Inc, New Brunswick, NJ). The rats remained in the staircase for 15 min and the total number of pellets eaten on each side was recorded. This test provides a sensitive measure of skilled forelimb reaching ability, and also of sensory neglect. The animals were pre-trained twice per day over a 14-day period. Animals that failed to consistently retrieve over 55 % of the total available pellets were excluded from the study. Animals were retested for two trials per day on days 7 and 8 (1 week) after ischemia, as well as 30 and 31 days (1 month) post ischemia. Reaching ability was determined by averaging the score of the impaired limb over the 4 trials and calculating the percentage of pellets eaten compared to the average of the last 4 trials prior to stroke.

4.2.3.2 Forelimb asymmetry test

Animals were tested for limb preference and their ability to support weight on either forelimb by placing the animals in a 20 cm diameter x 35 cm high clear Plexiglas cylinder for 3 min (Schallert et al., 1997). As the animal rears to explore the environment

the number of bilateral paw placements, placements of the paw ipsilateral to the lesion (right), and placements of the paw contralateral to lesion (left) are counted. Normal animals use each limb more or less equally while ischemic animals favour their ipsilateral forelimb after injury (Schallert et al., 1997). The percent of ipsilateral limb use was calculated using the equation ipsilateral contacts/ (ipsilateral + contralateral contacts) x 100. Paw contacts were videotaped from below using an angled mirror and later analyzed in a blinded fashion.

4.2.3.3 Balance beam test

Animals were trained to cross a 2 cm wide X 1.6 m long beam elevated 1 m above the floor. A black tube placed at the far end of the beam served as a goal box. Foot slips and falls were counted as faults. Animals were pre-trained for ten days and then given 4 trials as a baseline. At 1 week and 1 month after ischemia the animals were given four trials and the number of foot faults per trial were averaged. Faults were recorded using a video camera and later analyzed in a blinded fashion.

4.2.4 Magnetic resonance imaging

Ten animals underwent focal ischemia using the c/s 2400 method of ET-1 application (see above). Six rats were examined using perfusion- and diffusion-weighted imaging at 1, 7 and 24 h (group A) and 4 animals were examined 3, 10 and 48 h (group B) after ET-1 administration. Group B rats were also examined with T_2-weighted imaging at 48 h. An additional 3 un-operated animals were imaged (2 were imaged twice) to obtain normal values for blood flow and diffusion parameters. MR imaging was performed using a Biospec/3 (Bruker, Karlsruhe, Germany) equipped with a 21 cm bore magnet (7 Tesla, 3.2 cm diameter and 4.5 cm long). The rats were anesthetized with 1.5 to 2 % halothane in a 70:30 mixture of N_2O:O_2 and positioned in a quadrant radiofrequency coil (National Research Council, Winnipeg, MB, Canada). Rectal temperature was monitored and controlled using a water blanket and cooling air and respiration was monitored using electrocardiograph electrodes placed across the chest and a respiratory monitor (Hewlett-Packard, Palo Alto, CA). The imaging slice was positioned 1.2 mm anterior to bregma using as landmark the anterior commissure visualized on a sagittal scout image obtained at the midline.

MR perfusion imaging was done with continuous arterial spin tagging (CAST) (Detre et al., 1992) as described previously (Lei and Peeling, 1999). Following spin labelling 5 mm posterior to the brain stem (2.5 s 15 mT block pulse with a 2 G/cm posterior–anterior magnetic field gradient) or control labeling symmetrically anterior to the imaging slice, perfusion-weighted snapshot fast low angle shot (FLASH) images (4 x

4 cm^2 FOV, 2.0 mm thick slice, 128 x 128 matrix size, TR = 3.7 ms, TE = 2.3 ms, 12 degree flip angle, 128 averages) were acquired using a four-step gradient-offset cycling protocol to eliminate the asymmetrical magnetization transfer effect. Cerebral blood flow (CBF) images were calculated (Detre et al., 1992) with the degree of spin tagging (α) and the blood–brain partition coefficient (λ) taken as 0.75 and 0.9 ml/g, respectively (Lei and Peeling, 1999).

Quantitative diffusion maps were obtained using magnetization prepared TurboFLASH (Thomas et al., 1998) (64 averages were accumulated in a 128 x 128 matrix) with diffusion gradients (b = 21 1029 seconds/mm^2) applied separately along the x, y and z axes. Apparent diffusion coefficient (ADC) values were calculated from the decay constant obtained by pixel-by-pixel intensity fitting to an exponential decay, and a directionally-invariant trace ADC map was calculated by averaging the ADC_x, ADC_y and ADC_z maps (Ulug et al., 1997).

4.2.5 Assessment of neuronal damage

4.2.5.1 Histology

Animals were sacrificed either at 48 h (MR study), 10 days or 1 month post-ischemia to determine infarct volume at each time point. At the conclusion of each study, animals were injected with an overdose of Somnotol® and perfused transcardially with heparinized saline followed by 10 % phosphate buffered formalin. Heads were removed and placed in 10 % formalin for 24 h before removing the brain. The brain was stored in

10 % formalin for 24 h and then transferred to a 20 % sucrose solution in 10 % formalin and allowed to sink (approximately 3 days). Brains were frozen on dry ice and 40 μm sections were cut using a cryostat (CM 3050 S, Leica, Germany). Every 8th section was mounted and stained with Cresyl Violet.

4.2.5.2 Infarct measurement

Sections stained with Cresyl Violet from +3.0 mm to –2.5 mm relative to Bregma were assessed for injury. Ischemic injury was determined by the absence of normal neuronal cell bodies and the border of the infarct was taken as the point at which marked gliosis ended. Every third slice was analyzed microscopically and the infarcted area in the cortex and striatum were separately traced using NIH image software. Volume of injury was calculated by summing the area recorded from each slice and multiplying that value by the distance between the measured slices (Buchan et al., 1992).

4.2.6 Statistics

Behavioural and MR data were analyzed using repeated measures ANOVA or two-way ANOVA where appropriate. A Fisher's PLSD test was used to determine differences between groups, where $p < 0.05$ was considered significant. All values given are mean ± SEM.

4.3 Results

4.3.1 Infarct volumes

The average infarct volumes for each surgical group for the cortex, striatum and the two measurements combined are given in Table 4.1. There was no difference between estimated infarct volumes at 10 days and 1 month and therefore the data were combined (data not shown).

4.3.1.1 Cortical injury

Intracortical injection of ET-1 in either the cortical or c/s models resulted in the largest cortical infarcts. Intracortical injection also resulted in larger variability of cortical injury than the topical and MCAo methods (see Table 4.1). There was no effect of concentration of ET-1 within each surgical method group although there was a trend for the higher concentration of cortical injections to result in a larger injury. Topical, cortical and c/s models resulted in similarly placed cortical lesions (see Figure 4.1), which were mainly confined to the forelimb motor region. Conversely the ET-1 MCAo model resulted in injury to the lateral cortex and most often spared the forelimb motor region (Figure 4.1). Three sham animals in the topical groups had slight injury to the cortex. These were animals that were operated on early in the study using a small drill bit for bone removal. Despite the small injury there were no detectable behavioural deficits in these animals. To avoid this non-selective injury in subsequent animals a trepan was used.

4.3.1.2 Striatal injury

The topical method occasionally resulted in slight striatal injury just below the corpus callosum in animals with the largest cortical infarct. Striatal injury occurred more often using the cortical injection than the topical method, due likely to the larger injury. As expected, the combination of c/s resulted in a significant striatal injury that was statistically larger than in all other groups ($p < 0.0001$). There was no difference in injury produced with the two concentrations of ET-1 used for this method. The c/s and MCAo ET-1 models resulted in similar location of injury in the dorsolateral striatum (Figure 4.1) and only differed in the amount of rostral-caudal spread of the injury. None of the sham animals from any group had ischemic striatal injury.

4.3.1.3 Total injury

The concentration of ET-1 did not significantly alter the amount of injury. The average infarct in all topical groups was significantly smaller than those of the cortical 1600 and 2400 groups as well as the c/s 1200 and 2400 groups ($p < 0.05$). In addition, the cortical 1600 and 2400 and the c/s 2400 groups had significantly larger infarcts than the MCAo group ($p < 0.05$). Among the animals subjected to the cortical and the c/s injection method there was no effect of surgery or concentration on the total amount of injury. Representative injury profiles for each ET-1 ischemic model are shown in Figure 4.1.

4.3.2 Model success rate

Table 4.2 summarizes the number of animals that survived the surgery and also the number of animals that exhibited significant reaching impairments, defined as any animal that had at least a 20 % reduction in performance in the staircase test at 1 week post-surgery. Animals with less than a 20 % deficit in the staircase task at 1 week were excluded from the behavioural portion of the study. While all four methods of applying ET-1 resulted in similar behavioural results (see below) the success rates varied. Applying ET-1 adjacent to the MCA resulted in a success rate of only 50 %, much lower than any of the other methods (with the exception of the c/s 1200). In most cases increasing the concentration of ET-1 increased the percent of survivors with deficit and overall success. All animals exposed to the highest doses of ET-1 (top 4000, cor 2400 and c/s 2400) that survived displayed behavioural deficits.

4.3.3 Behavioural results

Apart from success rate, the concentration of ET-1 did not appear to affect behavioural results (i.e. the severity of deficits did not differ between concentrations) therefore the animals from each concentration were pooled to represent each surgical method in the interest of reducing the number of animals in the study.

4.3.3.1 Staircase reaching test

There was an effect of group 1 week ($F_{(4,76)} = 27.07$, $p < 0.0001$) and 1 month ($F_{(4,45)} = 14.69$, $p = 0.0001$) post-surgery. All groups were significantly impaired in the staircase reaching test compared to sham animals ($p \leq 0.0001$) at both time points (see Figure 4.2).

4.3.3.2 Forelimb asymmetry test

There was an effect of group for the forelimb asymmetry task at 1 week ($F_{(4,62)} = 16.94$, $p < 0.0001$) with all surgical groups showing increased ipsilateral forelimb use compared to shams ($p < 0.01$, see Figure 4.3A). One month post-surgery ($F_{(4,38)} = 2.99$, $p = 0.03$) all groups with the exception of the MCAo group remained impaired ($p < 0.02$) (see Figure 4.3B).

4.3.3.3 Balance beam test

There was an effect of group for balance beam at 1 week and 1 month ($F_{(4,58)} = 7.42$, $p < 0.0001$, $F_{(5,58)} = 8.59$, $p = 0.0002$ respectively). While all groups had on average more foot faults than the sham group, only the cortical group was significantly different from the sham group at each time point ($p < 0.0001$ for both, data not shown).

4.3.4 Magnetic resonance imaging

ET-1 induced MCAo has previously been studied using MRI (Biernaskie et al., 2001). Since the c/s model most closely resembled the MCAo model (both cortical and

striatal injury) and also had a high success rate we used MRI to further examine this model. Figure 4.4 shows perfusion and ADC images from two representative rats (1, 7 and 24 h from one rat and 3, 10 and 48 h from another). CBF and ADC values were measured in 8 regions of interest (ROI) in both ipsilateral and contralateral hemispheres (see Figure 4.5 for diagrams showing location of ROI). ROI 1 encompasses the cingulate cortex and is just medial to the cortical injection of ET-1. ROI 2 is located approximately where ET-1 was injected into the motor cortex and is representative of the ischemic core. ROI 3, 4 and 5 represent increasingly lateral regions of the cortex. Since they had similar perfusion and diffusion values the data from these regions were pooled (ROI 345). ROI 6, 7 and 8 were located in the lateral striatum and data from these regions were also pooled (ROI 678).

4.3.4.1 Perfusion imaging

Figure 4.5 gives the absolute CBF values for the above 4 regions in both hemispheres. For all regions normal values were taken from unoperated control animals and were assigned as time 0 post ET-1. In the ipsilateral hemisphere CBF was dramatically reduced in all ROI at 1 h ($p \leq 0.0001$). There was slight reperfusion beginning at 7 h for all regions except ROI 2 (the ischemic core), which didn't show any reperfusion until 48 h (Figure 4.5B). CBF in ROI 345 and 678 (Figure 4.5B and C) returned to normal levels by 48 h and ROI 1 was close to normal at 48 h (Figure 4.5A). In the contralateral hemisphere CBF was reduced in all regions compared to normal levels beginning at 1 h ($p \leq 0.0001$) but was most severely reduced in ROI 1 (Figure 4.5A).

With the exception of ROI 1, CBF in the ipsilateral hemisphere was significantly lower than CBF in the contralateral hemisphere ($p < 0.05$).

4.3.4.2 Diffusion imaging

In the ipsilateral hemisphere all regions showed a significant decrease in ADC values by 3 h ($p < 0.05$) that remained depressed out to 48 h (Figure 4.6) while in the contralateral hemisphere only ROI 1 showed a similar pattern. ADC values in all other contralateral regions were not significantly affected or only affected at a single time point (Figure 4.6B, C and D).

4.3.4.3 T2 imaging

T2-weighted MR images were used to determine the extent of injury caused by the c/s 2400 model at 48 h post ET-1. Infarct volumes estimated from T2-weighted images correlated with, but were significantly larger than the infarct volumes obtained from analyzing the same tissue stained with Cresyl Violet ($r = 0.973$ and 0.914 for the cortex and striatum, respectively; $p < 0.05$ for both regions). Volume estimates from T2 images were 232.99 ± 55.36 mm^3 in the cortex, 79.47 ± 17.29 mm^3 in the striatum, and 312.46 ± 72.69 mm^3 combined. Infarct volumes measured from histological sections stained with Cresyl Violet were 134.37 ± 17.75 mm^3 in the cortex, 33.34 ± 3.2 mm^3 in the striatum, and 167.71 ± 20.31 mm^3 combined for the rats examined with MR imaging. The cortical infarcts for this group were also significantly larger than the cortical volumes for the c/s 2400 group examined in the behaviour portion of this study ($p \leq 0.0001$). While only a portion of the motor cortex was injured in the previous c/s 2400 animals

(Figure 4.1) the animals used in MRI analysis had damage in this region and also in the lateral cortex (Figure 4.7). Most animals also had injury to the contralateral cingulate cortex (ROI 1). In contrast, only one animal demonstrated contralateral injury on histological inspection of animals from the behavioural portion of the study.

4.4 Discussion

Occlusion of the MCA using the vasoconstrictor ET-1 is a common approach to produce focal ischemia in rodents (Sharkey and Butcher, 1995; Biernaskie et al., 2001; Callaway et al., 2003; Moyanova et al., 2003; Ploughman et al., 2005). The location of the MCAo injury in this study was similar to other results (Sharkey et al., 1993; Sharkey et al., 1994; Sharkey and Butcher, 1995) and size of the lesion was consistent with the lesion produced by Macrae and colleagues (Macrae et al., 1993). Despite a higher dose of ET-1, the average lesion size in this study was smaller than that obtained by Sharkey and colleagues (Sharkey et al., 1993; Sharkey et al., 1994; Sharkey and Butcher, 1995). This may be due, in part, to different rat strain and stereotaxic coordinates used in this study. Although proximity of the injection to the MCA was confirmed, a more distal portion of the MCA may have been occluded compared to other studies. Our findings also show that ET-1 MCAo produces long-term deficits in the staircase test at least 1 month post-surgery as reported elsewhere (Marston et al., 1995).

For the first time, we compared ET-1 induced MCAo directly with other ET-1 induced focal ischemia models in order to directly compare sensorimotor deficits from model to model. All of the models tested in this study injured the forelimb motor cortex

and/or the dorsolateral striatum, and likely explains why all the methods resulted in similar motor deficits. The ET-1 induced MCAo model yielded a lower incidence of sensorimotor deficits compared to the other models. Previously we found that the MCAo method produces a success rate of only 50-60 % (Biernaskie et al., 2001; Biernaskie et al., 2004), consistent with the current findings.

Topical application of ET-1 provides precision in size and injury location as shown by the small variability in lesion size found in the present study. While increasing the dose of ET-1 did not have a significant effect on lesion size or behavioural outcome, others have found a dose-related increase (Fuxe et al., 1997). This may be due to a ceiling effect. The lesions produced in the present study were considerably larger than those reported by Fuxe and colleagues using a topical method (Fuxe et al., 1997). The doses for the present study were selected so as to create consistent and long lasting sensorimotor deficits while in the Fuxe study doses were used only to study reduced blood flow. While the topical model produced similar deficits to the ET-1 MCAo model, it had a higher success rate (75 % versus 50 %) suggesting it is more reproducible. One concern with this model is possible injury to underlying brain tissue caused by the craniotomy. Despite care when removing the bone, 3 out of 8 shams showed evidence of injury to the cortex, and while there was no obvious effect on behaviour, it suggests that some of the injury in this model is due to mechanical injury. One way to minimize injury due to craniotomy is to perform small intracortical injections. This method produced lesions in approximately the same location as the topical application, but they were larger and more variable in size (see Table 4.1). A smaller dose of ET-1 (e.g. 400 pmol) may

reduce variability in this model as suggested from findings of Gilmour and colleagues (Gilmour et al., 2004). Encouragingly, the smaller dose of ET-1 used in the Gilmour study still produced a reaching deficit that was apparent 12 weeks post surgery (Gilmour et al., 2004), but our results suggest that a lower dose of ET-1 produces a lower success rate (75 % for cortical 800 versus 91 % for cortical 2400). Despite these findings, all doses of ET-1 used for intracortical injections tested in this study yielded a higher success rate than the MCAo method of applying ET-1.

The photothrombosis (i.e. rose-bengal) model of focal ischemia is similar to the cortical ET-1 model in that it does not require the removal of bone and can be precisely localized. However, it is a permanent ischemia model and its mechanisms of injury are complex (Carmichael, 2005).

Clinical stroke often results in cortical and/or subcortical injury and consequently purely cortical injuries in the rodent may not be representative of the human stroke condition (Cramer, 2003). The combination of intracortical and striatal injections produced a lesion that targeted the forelimb motor region of the cortex and dorsolateral striatum. Our results show that this method produces a significant and enduring deficit in reaching and forelimb asymmetry. At the highest dose (c/s 2400) this model produced a 77 % success rate, which is superior to that achieved with the MCAo model. Furthermore, *all* of the surviving animals demonstrated long-lasting impairments. We have previously found that combining cortical and striatal injections of ET-1 produces a high success rate (85 %) without excessive morbidity (Windle and Corbett, 2005),

especially when isoflurane was used instead of halothane. Use of isoflurane does not increase the success rate of the ET-1 MCAo model (Biernaskie et al., 2004).

We have previously described the spatial and temporal parameters of CBF changes following low dose application of ET-1 adjacent to the MCA (Biernaskie et al., 2001). Here we characterized the combined cortical and striatal model (c/s 2400) of ET-1. We found that CBF drops quickly at 1 h, remains low for 48 h in the ischemic core (ROI 2) and gradually returns to normal in other regions. It is difficult to determine the flow rates and duration of CBF reduction required for permanent injury to occur. Indeed, the threshold of reduced CBF that marks metabolic failure increases with the duration of ischemia (Hossmann, 1994) which may vary between different models (Macrae et al., 1993; Tsuchidate et al., 1997; Biernaskie et al., 2001). Injury to the contralateral cingulate cortex (ROI 1), although unexpected, provides an opportunity to examine CBF thresholds required to produce neuronal injury in this model. This region was the only contralateral region measured in which the CBF dropped below 50 ml/100g/min. Although both injured (ipsilateral ROI 345) and uninjured (contralateral ROI 2) areas show similar reductions in CBF, they differ in the time to reperfusion. In spared regions (e.g. contralateral ROI 2), CBF increases above 100 ml/100g/min by 7 h, while in injured regions (e.g. contralateral ROI 1 and ipsilateral ROI 345) CBF remains depressed below 100 ml/100g/min for 24 h. In summary, it appears that CBF must drop significantly (~ 50 ml/100g/min) and remain below 100 ml/100g/min for at least 10 h in order for the tissue injury to occur in this model. Perfusion deficits below a certain threshold (which is variable depending on the duration of perfusion deficit) cause cells to undergo metabolic

energy failure, membrane depolarization and subsequent cell swelling that is observed as a reduction in ADC values (Back et al., 2004). In the current study, only those groups that meet the above criteria (CBF below 100 ml/100g/min for at least 10 h) have ADC values that consistently remain below normal values. Decreases in ADC values and CBF below a certain threshold correlate with one another and together can predict areas of irreversible injury for the c/s 2400 model of ET-1 induced focal ischemia.

We used T2-weighted MR imaging and histology to assess injury volume and found that they correlated, although lesions visualized by MR were larger. This was not unexpected as we noted the same effect previously using ET-1 induced MCAo (Biernaskie et al., 2001) and may be due to fixation changes. Importantly, the injury volume determined from the histological sections for the animals that underwent MRI was significantly larger than the estimated infarcts for the ischemic animals used in the behavioural portion of this study (not examined by MRI). In the rats that were imaged, damage extended into the contralateral cortex. The disparity between the infarct volumes measured for the MRI and behavioural portions of the study may be due, in part, to the shorter survival time (48 h versus 10 days or 1 month respectively). Although it is known that infarcted tissue may shrink over time resulting in an underestimate of injury volume (Back et al., 2004), it is unlikely to be the only reason for the large difference between the two groups in cortical infarct. The increased exposure to halothane (~ an additional 6 h) may also have played a role in expanding the ischemic lesion. Increased exposure to halothane is used to create hypotension during global ischemia to increase injury size and reliability (Bendel et al., 2005) and has also been used to increase injury

in the suture model of MCAo (Zhu and Auer, 1995). Prolonged and repeated exposure to halothane in the MRI portion of the study (~ 7 h versus ~ 1 h in the behavioural portion) likely lowered blood pressure and resulted in the larger infarcts and may also explain why CBF was decreased in both hemispheres. In support of this interpretation, increasing halothane exposure from 5 to 180 min after ET-1 induced MCAo, increases cortical infarct volume from 17 % to 82 % (Sharkey and Butcher, 1995). This emphasizes the importance of consistent surgery length and anaesthetic exposure when using ET-1 to induce ischemia.

As with all models of ischemia there are disadvantages with using ET-1. ET-1 is expressed by astrocytes in response to ischemia and previous studies have noted that ET-1 has direct effects on neurons and astrocytes affecting excitability, gap junctions and gliosis (Hama et al., 1997; Dreier et al., 2002; Sanchez-Alvarez et al., 2004; Carmichael, 2005). While ET-1 expression is normal after ischemia, using it to create ischemia could exacerbate the injury and should be considered when using ET-1 models, especially when studying neuroprotective agents.

In summary, each ET-1 method of inducing focal ischemia produced long-lasting impairments in forelimb reaching and postural support, although the MCAo group began to recover symmetry in postural support at 1 month. While all methods resulted in similar behavioural outcomes, they differed in success rate. ET-1 injected adjacent to the MCA, the most widely used of the methods tested, produced the lowest success rate. While occlusion of the MCA in the rat resembles human stroke in a number of respects it does not reliably produce upper limb deficits, a common clinical problem (Parker et al., 1986;

Nakayama et al., 1994). Cortical and striatal injections of ET-1 resulted in ischemic injury similar to MCAo but produced a more consistent injury and ensuing behavioural deficits resulting in a higher success rate. The increased reliability of the cortical + striatal injection method, as well as the greater similarity to clinical stroke (compared to purely cortical models), makes it attractive for recovery studies. It is also useful for neuroprotective studies where it may not be possible to use behavioural testing or imaging to identify incomplete ischemia prior to drug treatment.

Table 4.1. Infarct Volume (mm^3)

Model	Cortex	Striatum	Total
topical 1200	20.20 ± 1.5	0.67 ± 0.67	20.87 ± 1.6
topical 2000	17.88 ± 2.5	0.0	17.88 ± 2.5
topical 4000	19.80 ± 7.4	0.45 ± 0.3	20.22 ± 7.4
cortical 800	63.60 ± 26.5	0.0	63.6 ± 26.5
cortical 1600	86.07 ± 15.4*†	9.49 ± 5.0	95.56 ± 18.8*†
cortical 2400	80.29 ± 12.4*†	5.84 ± 4.4	86.13 ± 16.1*†
c/s 1200	36.20 ± 21.3	49.4 ± 7.7‡	85.6 ± 27.0*
c/s 2400	60.48 ± 15.4*	37.38 ± 4.8‡	97.86 ± 18.7*†
MCAo	30.42 ± 6.8	10.76 ± 2.6	41.18 ± 9.1

Values given are mean ± SEM, * significantly different from all topical groups ($p < 0.05$), † significantly different from MCAo ($p < 0.005$ for the cortex and $p < 0.05$ for total), ‡ significantly different from all other surgical methods ($p < 0.0001$).

c/s, cortical + striatal injection model; MCAo, middle cerebral artery occlusion model. Values in model name refer to the total pmol amount of endothelin-1 administered.

Table 4.2. Endothelin-1 Model Success Rate

Model	% survival	% survivors with deficit	% total success
topical 1200 (n = 9)	100	89	89
topical 2000 (n = 19)	100	63	63
topical 4000 (n = 4)	100	100	100
pooled groups	100	75	75
cortical 800 (n = 4)	100	75	75
cortical 1600 (n = 11)	91	70	64
cortical 2400 (n = 11)	91	100	91
pooled groups	92	83	77
c/s 1200 (n = 4)	75	67	50
c/s 2400 (n = 13)	77	100	77
pooled groups	76	92	71
MCAo (n = 8)	75	67	50

Percent survivors with deficit were animals that had at least a 20 % reduction in reaching performance in the staircase task at one week post- versus pre-surgery.

c/s, cortical + striatal injection model; MCAo, middle cerebral artery occlusion model.

Values in model name refer to the total pmol amount of endothelin-1 administered.

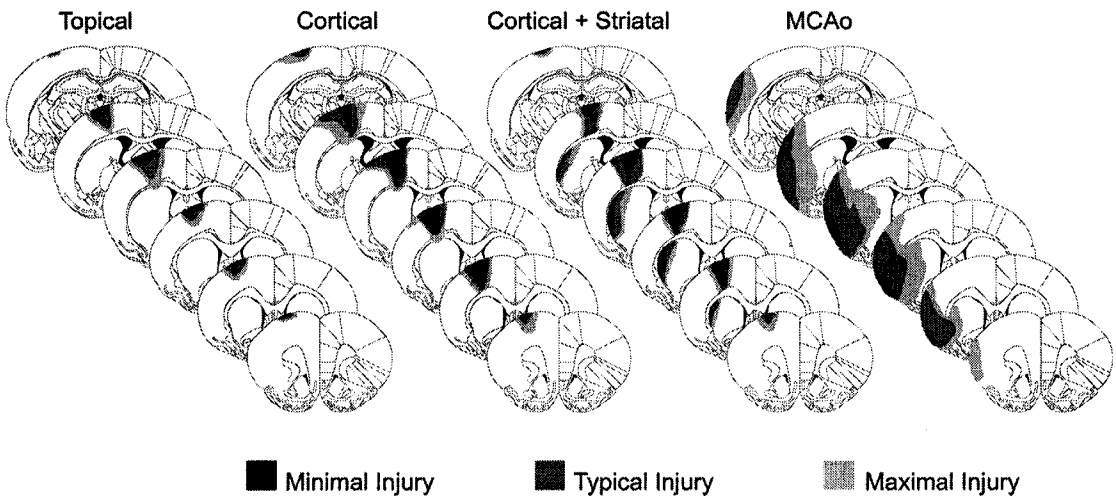

Figure 4.1. Representative illustrations of typical, minimal and maximal injury areas for each surgical method. The concentration of endothelin-1 (ET-1) did not significantly affect infarct volume. All animals were lesioned in the right hemisphere (left in each figure).

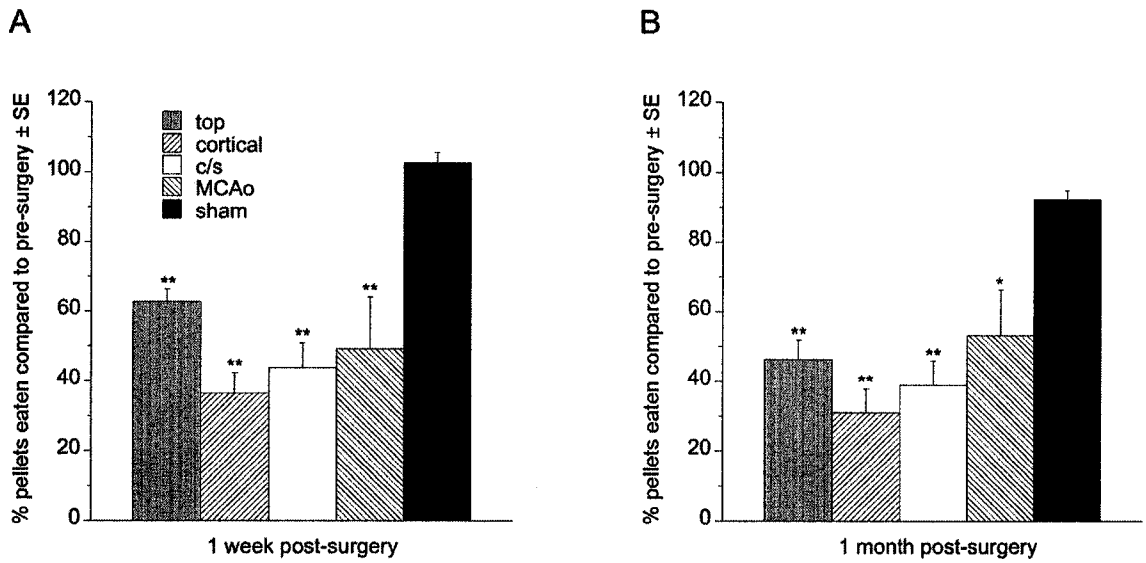

Figure 4.2. Staircase reaching test. All endothelin-1 models result in a significant deficit compared to sham animals at 1 week (A) and 1 month post-surgery (B). Values are mean ± SEM (*$p < 0.005$, **$p < 0.0001$ difference from sham group).

c/s, cortical + striatal model; MCAo, middle cerebral artery occlusion model.

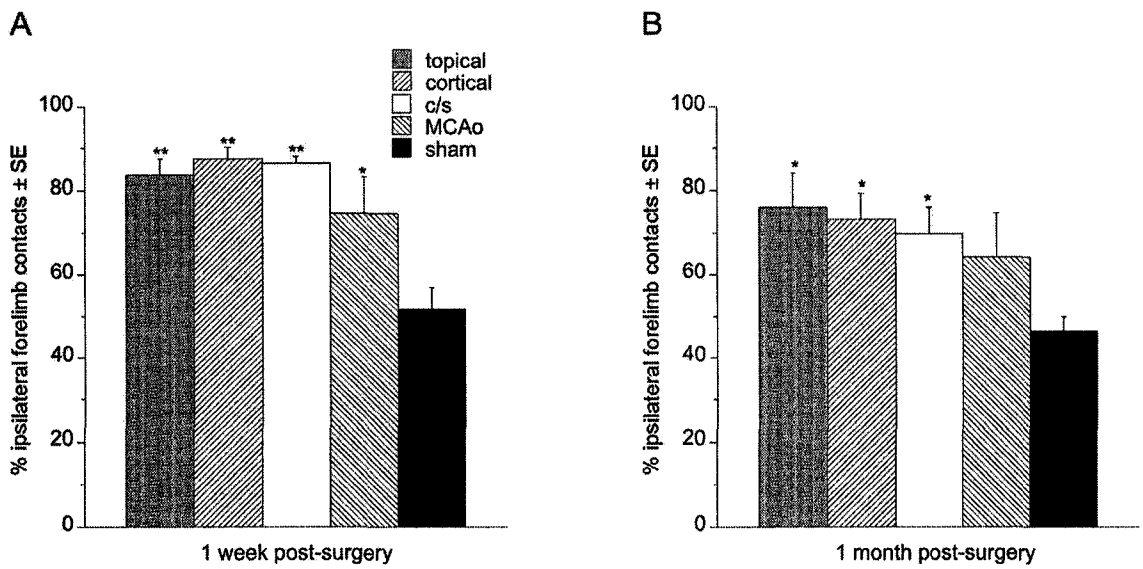

Figure 4.3. Forelimb asymmetry test. All endothelin-1 groups exhibit a significant increase in ipsilateral forelimb use 1 week post stroke (A) that remains for all groups except MCAo at 1 month survival (B). Values given are mean ± SEM (*$p < 0.02$, **$p < 0.0001$ difference from sham group).

c/s, cortical + striatal model; MCAo, middle cerebral artery occlusion model.

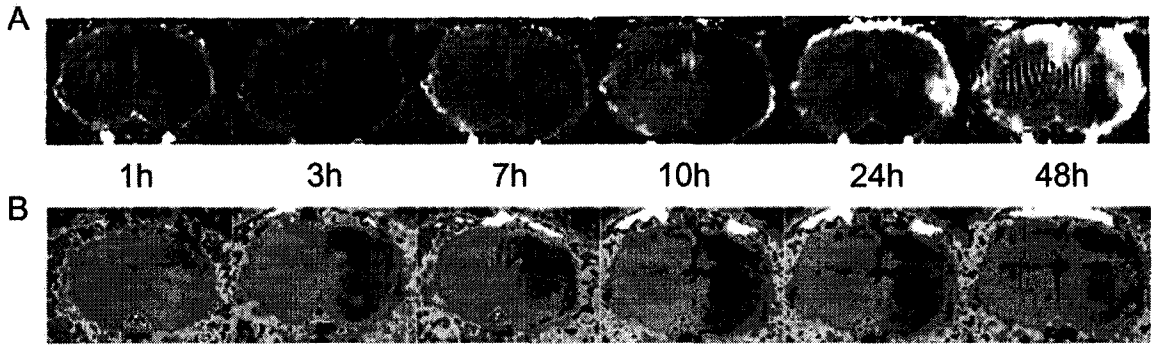

Figure 4.4. Representative perfusion (A) and diffusion (B) images from 2 animals (1,7 and 24 h post-endothelin-1 (ET-1) from one animal and 3, 10 and 48 h post-ET-1 from another). ET-1 was injected into the right hemisphere (appears on the right of each figure component).

Figure 4.5. Absolute cerebral blood flow (CBF) for select regions in the ipsilateral and contralateral cortex (A, B and C) and striatum (D). Blood flow in the ipsilateral hemisphere decreases quickly in all regions and gradually reperfuses over 48 h. Blood flow in the contralateral hemisphere decreased in all areas but was most severely reduced in region of interest (ROI) 1 (A), which was the only contralateral region to show histological injury. Values given are the mean ± SEM. Values for 0 h post-endothelin-1 (ET-1) are from animals that did not receive ET-1 and are considered normal (* $p < 0.05$, ** $p < 0.01$ and *** $p \leq 0.0001$ relative to the normal value for the same hemisphere; † $p < 0.05$, †† $p < 0.01$ and ††† $p \leq 0.0001$ relative to the same time point in the contralateral hemisphere).

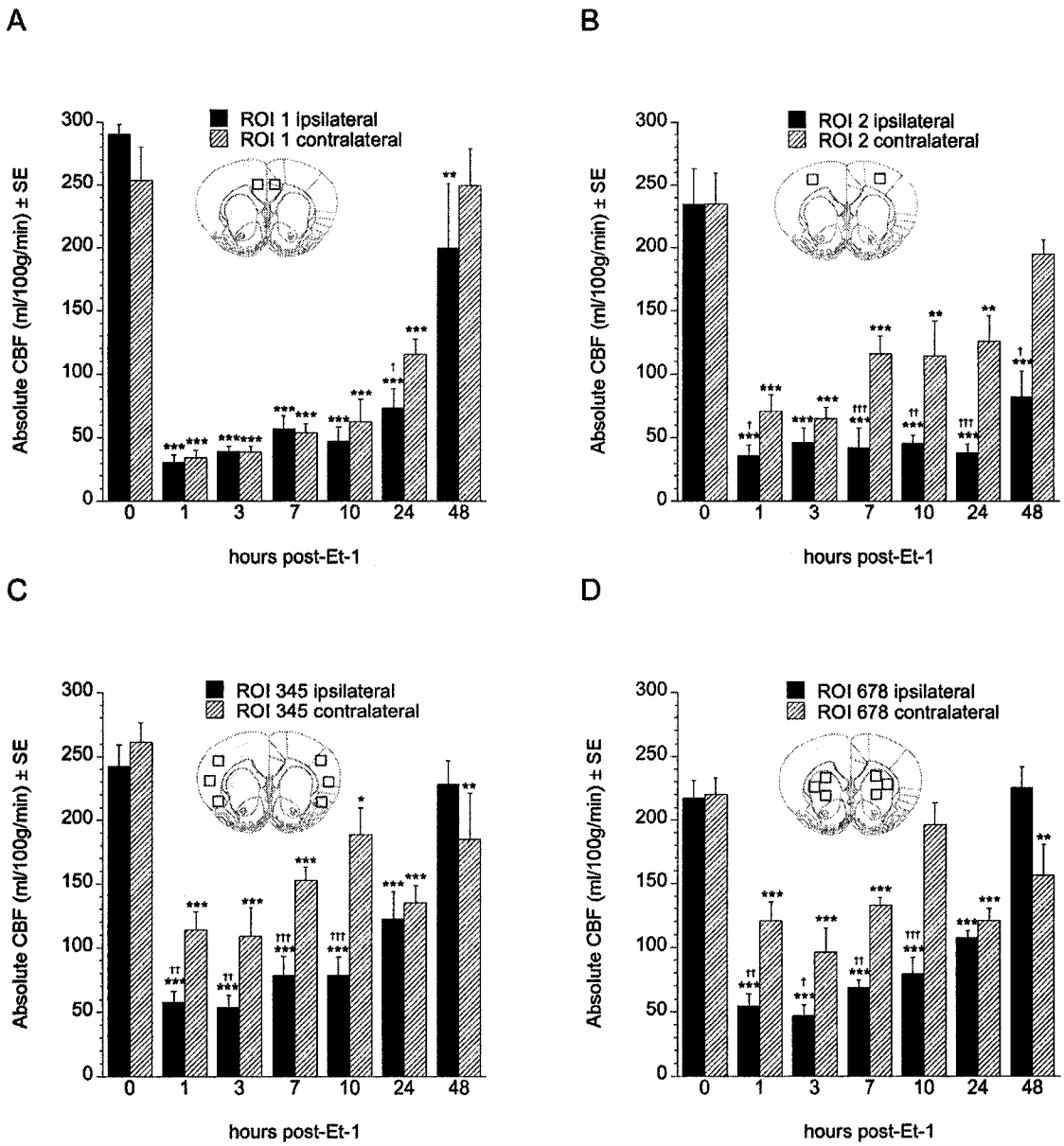
A
B
C
D
ROI 1 ipsilateral
ROI 1 contralateral
ROI 2 ipsilateral
ROI 2 contralateral
ROI 345 ipsilateral
ROI 345 contralateral
ROI 678 ipsilateral
ROI 678 contralateral
Absolute CBF (ml/100g/min) ± SE
hours post-Et-1
0
1
3
7
10
24
48
50
100
150
200
250
300

Figure 4.6. Apparent diffusion coefficient (ADC) values for select regions in the ipsilateral and contralateral cortex (A, B and C) and striatum (D). ADC values in all ipsilateral regions significantly differed from the normal values while only the value in contralateral region of interest (ROI) 1 differed from its normal value (A). Values given are the mean ± SEM. Values for 0 h post-endothelin-1 (ET-1) are from animals that did not receive ET-1 and are considered normal (* $p < 0.05$, ** $p < 0.01$ and *** $p \leq 0.0001$ relative to the normal value for the same hemisphere; † $p < 0.05$, †† $p < 0.01$ and ††† $p \leq 0.0001$ relative to the same time point in the contralateral hemisphere).

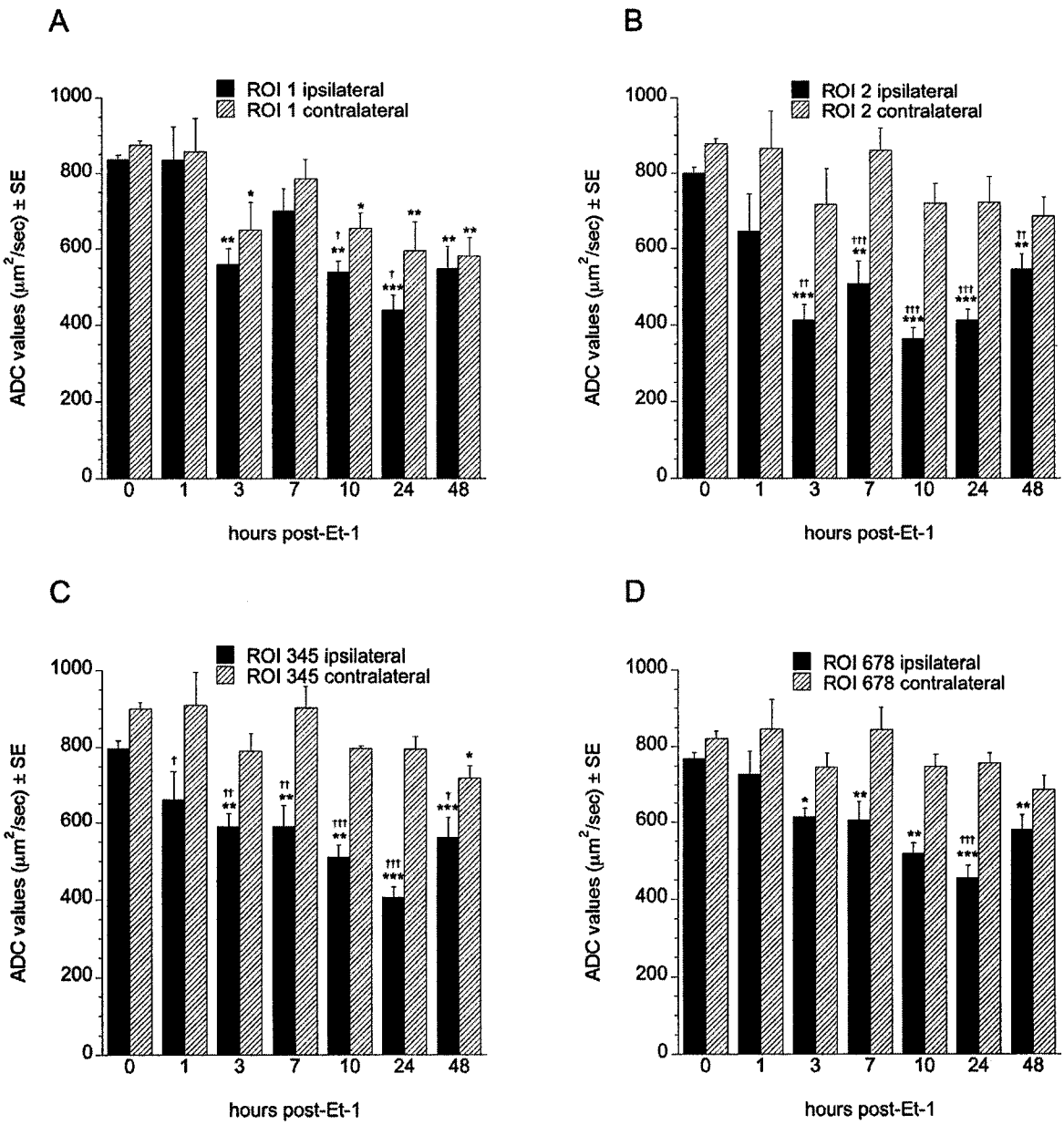
A
ROI 1 ipsilateral
ROI 1 contralateral
ADC values (μm²/sec) ± SE
hours post-Et-1
B
ROI 2 ipsilateral
ROI 2 contralateral
ADC values (μm²/sec) ± SE
hours post-Et-1
C
ROI 345 ipsilateral
ROI 345 contralateral
ADC values (μm²/sec) ± SE
hours post-Et-1
D
ROI 678 ipsilateral
ROI 678 contralateral
ADC values (μm²/sec) ± SE
hours post-Et-1

Figure 4.7. Representative T2-weighted images showing the rostral-caudal extent of the injury (top-left to bottom-right) as a result of endothelin-1 (ET-1) injected into the cortex and striatum. The injury extends into the lateral cortex as well as the contralateral cingulate cortex as shown by increased signal (bright areas). ET-1 was injected into the right motor cortex and striatum (appears on the right of each figure component).

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Chapter 5: Summary

5.1 Summary of main findings

5.1.1 Fluoxetine fails to improve functional recovery

Previous work failed to show any benefit of fluoxetine treatment after ischemia (Jolkkonen et al., 2000). However, in this thesis (chapter 2) a more clinically relevant chronic dosing schedule combined with reaching rehabilitation was employed after focal ischemia. To date, animal and human studies have yielded contradictory results as to the efficacy of fluoxetine to enhance motor recovery after stroke. Therefore, a thorough re-examination seemed warranted since positive clinical studies were not able to eliminate the effects of mood on rehabilitation and the previous negative rat study of fluoxetine (Jolkkonen et al., 2000) left doubt as to whether a more sensitive battery of tests, with prolonged and higher doses, or combining fluoxetine with therapy would be more effective. Despite the ability of fluoxetine to increase various forms of neural plasticity (e.g. increased brain derived neurotrophic factor (BDNF) and neurogenesis) (Nibuya et al., 1996; Coppell et al., 2003) no benefit on motor recovery was observed. It is possible that despite statistically significant changes in BDNF or other indices of plasticity as a result of fluoxetine administration, the changes are not sufficient to have an impact on motor recovery. The results of chapter 2 help reconcile previous discordant findings regarding fluoxetine's effects on motor recovery.

5.1.2 Norepinerine depletion facilitates motor recovery

The main finding of chapter 3 is that norepinephrine (NE) depletion facilitates recovery of function after ischemia. The previous findings of several earlier N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) studies (Goldstein, 1991; Boyeson et al., 1992) have reported decreased NE to be detrimental to motor recovery after brain injury. However, these studies were flawed because NE was depleted prior to injury and this could have affected the development of the injury (i.e. the extent of cell death) and did affect the magnitude of post-injury behavioural deficits. Further, NE and other monoamines are decreased after brain injury (Robinson et al., 1975; Dunn-Meynell et al., 1994; Prasad et al., 1994), therefore reducing NE after injury is more representative of the natural pathology process. The mechanism by which DSP-4 lesions facilitate motor recovery is unknown. It is possible that NE inhibits recovery as suggested by the negative correlation between DβH staining and staircase performance, but there are numerous data to support the hypothesis that increasing NE facilitates neural plasticity (Nibuya et al., 1995; Nibuya et al., 1996; Malberg et al., 2000; Duman et al., 2001), which would question this interpretation. The other possibility is that partially depleting NE projections establishes receptor supersensitization and increased NE turnover (Dooley et al., 1983; Dunwiddie et al., 1983; Logue et al., 1985; Mogilnicka, 1986; Zahniser et al., 1986; Theron et al., 1993; Wolfman et al., 1994; Kask et al., 1997; Hughes and Stanford, 1998) such that NE function is facilitated. Further investigation is

required to distinguish among these possibilities. Chapter 3 also found that depleting NE did not alter levels of BDNF. This could mean that the benefits of NE on motor recovery are not mediated by BDNF as previously thought (Nibuya et al., 1996; Duman et al., 2001), or that if BDNF levels were altered, it was a transient change that occurred well before the time (9 weeks post-treatment) when BDNF levels were measured.

5.1.3 ET-1 models of focal ischemia

As a result of the experiments described in chapters 2 and 3 it became evident that different models of endothelin-1 (ET-1) induced ischemia were not equal with respect to reproducibility. This led to the experiment outlined in chapter 4 which compared different models of ET-1 induced focal ischemia with regard to producing consistent sensorimotor deficits. Each of the models was capable of producing similar sensorimotor deficits. However, the main finding of the study was that success rate (i.e. survival plus significant functional impairment) differed appreciably between models. Interestingly, the method that is most commonly used and the method used in chapter 3 (ET-1 applied to the middle cerebral artery (MCA) had the poorest success rate (~ 50 %). This finding was consistent with previous work from our laboratory (Biernaskie et al., 2001; Biernaskie et al., 2004). The combined injections of ET-1 into the motor cortex and the dorsolateral striatum, as used in chapter 2, resulted in a higher success rate (71 % in chapter 4 and 85 % in chapter 2) therefore establishing the greater reliability of this model. To further characterize this model, magnetic resonance imaging (MRI) data were

collected. Injection of ET-1 results in a prolonged decrease in cerebral blood flow (CBF) that differs depending on distance from the injection site. Blood flow is permanently decreased in the immediate region of the injection representing a permanent occlusion, however all other regions regain blood flow gradually (e.g. 7 to 48 h) representing a transient occlusion. In addition, the lesion evolves slowly over time which is consistent with human studies of stroke (Pantano et al., 1999).

5.2 Implications for stroke patients

The findings presented in this thesis may help to clarify issues regarding antidepressant use after stroke. Previous clinical studies in normal subjects and depressed stroke patients have suggested that serotonin specific reuptake inhibitors (SSRIs) like fluoxetine might be beneficial to motor function and recovery after stroke (Dam et al., 1996; Loubinoux et al., 1999; Gainotti et al., 2001; Pariente et al., 2001). If this was the case then fluoxetine could be used in all stroke patients in order to facilitate recovery. No motor benefit was seen with fluoxetine in the present thesis and thus there is no basis for its use other than to treat depression. Also important is that fluoxetine did not impede recovery in these animals, suggesting that treatment in humans should not have a negative impact on their recovery. Indeed, fluoxetine may still facilitate motor recovery in humans indirectly by improving mood and as a consequence increasing the motivation of the patient's to participate in physiotherapy and daily activities.

Noradrenergic antidepressants (i.e. tricyclics or NE reuptake inhibitors) are not commonly given to stroke patients because of the possibility of aggravating cardiac

complications (Robinson, 2003). Based on previous ideas about the role of NE and recovery of function, NE antidepressants would seem advantageous for motor recovery and some investigators have recommended their use clinically (Gonzalez-Torrecillas et al., 1995). In addition, some studies have suggested that drugs that suppress NE, such as antihypertensives that are sometimes given after stroke, be avoided (Goldstein, 1991; Goldstein, 2000). The findings of the present thesis show that depleting NE projections from the LC is, in fact, beneficial although whether this is due to decreased NE or increased facilitation of the NE system remains to be determined. Obviously, the role of NE in stroke recovery is unclear and further research is warranted before recommendations can be made regarding the use or non-use of post-stroke drug treatments.

5.3 Implications for research

The findings of this thesis indicate that careful methodological procedures are of utmost importance when studying post-stroke recovery in animals. Previous studies have chosen a single behavioural test to show motor recovery after injury. Frequently such tests (e.g. beam walking) were ones in which animals show a high degree of spontaneous recovery, unlike most deficits observed clinically. Throughout this thesis multiple behavioural tests were chosen that were sensitive to both permanent and transient deficits as well as testing different aspects of sensorimotor function. For example, if only forelimb asymmetry had been used as an outcome measure in chapter 3 no effect of NE depletion would have been observed, highlighting the importance for multiple and

varying tests. This may partly explain the different results found in the current thesis and previous DSP-4 studies that relied on a single beam walking task. Longer survival times were also used in this thesis, which is more reflective of the clinical situation. The importance of longer survival times was also reflected in chapter 3 since the benefits of depleting NE on motor recovery were not seen until later test points.

As discussed in the introduction (chapter 1), the selection of an appropriate animal model is important for recovery of function and neuroprotection studies. There are several models of ET-1 induced focal ischemia, however the current literature does not provide useful information regarding the relative success rates of different models. A reliable animal model of stroke that can be used to investigate recovery processes is of obvious value. Accordingly, the findings of chapter 4 provide a better alternative model for researchers interested in using a transient ischemia model that avoids the many limitations of the currently widely used intraluminal suture model.

5.4 Future directions

The use of antidepressants to facilitate rehabilitation after stroke remains controversial. The regulation of monoamines is extremely complex, and altering one neurotransmitter through drug therapy often indirectly alters others, making it difficult to make definitive conclusions (Gobert et al., 1998). Many of the early studies using pharmacological approaches to facilitate recovery examined amphetamine, which increases NE, serotonin (5-HT), and dopamine (DA). Recently it has been discovered that extracellular DA can be taken up by 5-HT transporters (Kannari et al., 2006) and

fluoxetine treatment can increase levels of DA in the striatum (Shishkina et al., 2006). In addition, depletion of NE has been shown to affect the metabolism of DA and its D_2 receptor sensitivity (Harro et al., 2000; Harro et al., 2003) and increasing NE can also increase DA (Gobert et al., 1998). A small clinical study found that patients treated with levadopa prior to physiotherapy sessions improved more on motor tasks than those given a placebo (Scheidtmann et al., 2001). Given the well known role of DA in motor function, it's contribution to recovery of function after stroke should be examined.

While facilitating 5-HT may not be a sufficient approach, and more research is required to assess NE and recovery, there are other agents that may be beneficial by providing an environment conducive to neuronal growth and repair. Agents that increase plasticity, angiogenesis, tissue oxygenation and neurogenesis are of interest. One agent of particular interest is Erythropoietin (EPO), a hematopoietic growth factor that is endogenous to the human brain. EPO has shown some promise in both animals and stroke patients as a neuroprotectant, likely due to its ability to promote neuron survival, neurogenesis, ischemic tolerance in astrocytes, decrease inflammation, and protect blood brain barrier integrity (for review see Hasselblatt et al., 2006). It may also play a significant role in rehabilitation. EPO and EPO receptor null mice have severely impaired embryonic neurogenesis and receptor knock down mice have decreased neurogenesis and neuroblast migration after distal MCA occlusion (Tsai et al., 2006). The role EPO plays in regulating neurogenesis suggests that it could be possible to promote recovery through neurogenesis by increasing EPO. EPO has also been found to be an important mediator of angiogenesis (Kertesz et al., 2004), which can be beneficial

in promoting neural plasticity. An attractive element of EPO is that it is already approved for treatment in renal disease and has an excellent safety profile in humans (Goodnough et al., 1997), making it a good candidate to study as an adjunct to rehabilitation after stroke.

Physiotherapy is an effective treatment for improving motor function and independence of stroke survivors, but there is room for improvement. Beginning therapy earlier and giving more frequent and intense treatment has been shown to improve functional outcome in both animal models of brain injury as well as stroke patients (Teasell and Kalra, 2005). The reality of this is that there are limited resources available for a growing population of stroke patients and as such the optimal time-to-therapy and therapy intensity may not be met. For this reason pharmacological enhancement of physiotherapy remains an attractive treatment option and deserves continued research.

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