Reduced Peri-Infarct Dysfunction With Pre-Stroke Exercise: Molecular and Physiological Correlates

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DEDICATION

I dedicate this thesis to all of my friends and family. Without their love and support, I could not have completed this thesis.

ABSTRACT

The effects of pre-stroke exercise and levesl of brain-derived neurotrophic factor (BDNF) on behavioural and functional recovery were examined following focal cortical ischemic infarct. Intracortical microstimulation (ICMS) was used to derive topographical maps of forelimb representations within the motor cortex and ischemia was induced via bipolar coagulation of surface vasculature. One month of exercise prior to ischemia significantly increased the amount of peri-infarct movement representations and initiates vascular changes within motor cortex. Further, this exercise-induced preservation of peri-infarct movement representations is associated with behavioural recovery and is dependent on BDNF levels in the motor cortex. These results provide further support for the idea that endurance exercise prior to stroke may enhance functional and behavioural recovery.

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CHAPTER ONE

GENERAL INTRODUCTION

Stroke is Canada's fourth leading cause of death, killing 16 000 people every year. There are between 40 000 and 50 000 Canadians diagnosed each year and 300 000 people living with the effects of stroke. As a result, stroke presents an enormous cost to the Canadian economy, with the acute care alone amounting to approximately $27 500 per patient. Thus, finding ways to both treat and prevent stroke are important goals for health research in Canada.

Given that over 80% of stroke victims exhibit motor impairments, much of the current stroke treatment is focused on enhancing recovery through motor rehabilitation. However, the benefits of rehabilitative strategies aimed at improving motor function have been demonstrated only on a general level (Woldag and Hummelsheim, 2002). This is because the effectiveness of motor rehabilitation is highly variable and depends on a number of factors, including severity of the injury (Duncan et al., 2000), timing of the therapy (Risedal et al., 1999) and the nature of the training experience (Langhammer and Stanghelle, 2000). Understanding the neural mechanisms mediating motor recovery may help to guide more effective rehabilitation programs. Although the specific neural mechanisms that support rehabilitation-dependent recovery are unknown, it is hypothesized that rehabilitation works via functional compensation ("plasticity") within residual neural tissue. Indeed, several experiments have shown plasticity to occur in association with behavioural recovery during post-stroke rehabilitation (Nudo, 1999).

More recently, it has become evident that experience *prior* to stroke may also influence recovery. That is, the state of the brain prior to the insult may be an important determinant of both the severity of impairment and degree of functional recovery (Rejdak et al., 2001). For example, exposure to ischemia (Kitagawa et al., 1990), hypothermia

(Nishio et al., 2000), and exercise (Wang et al., 2001) prior to stroke can reduce damage. Despite the wealth of evidence for structural sparing with pre-conditioning, very little research has been done to examine functional and behavioural sparing.

This thesis will test the hypothesis that pre-stroke experience in the form of exercise can influence recovery from stroke. Exercise has been shown to have a number of effects on the brain including angiogenesis, neurogenesis and changes in growth factor levels (Cotman and Engesser-Cesar, 2002). These changes are theorized to play a role in enhancing recovery after brain damage (Cotman and Engesser-Cesar, 2002). Specifically, the experiments will examine the potential neural mechanisms underlying the beneficial effects of pre-stroke exercise on physiological and behavioural recovery after stroke. These experiments utilize a rodent model of ischemia involving rat forelimb motor cortex and voluntary running behaviour. Intracortical microstimulation will provide the method of evaluating physiological status of the motor cortex following stroke and a battery of behavioral tests will be used to assess functional status. Finally, antisense oligonucleotides will be used to determine how a specific neurotrophic factor, brain-derived neurotrophic factor, is involved.

I. Stroke

Stroke is a heterogenous disorder that can be loosely defined as an event characterized by the interruption of blood flow to the brain. This loss of blood flow results in tissue damage in a particular area of the brain, which leads to functional impairment.

A. Types of Stroke

There are two types of stroke: ischemic and hemorrhagic. An ischemic stroke results from the blockage of an artery by a blood clot and can fall into one of two categories. The first is global, meaning that the stroke arises from the cessation of blood flow to the entire brain, as often occurs in the case of cardiac arrest. The second category is focal, meaning that the stroke arises from the occlusion of a particular vessel in the brain. The clot that causes an ischemic stroke can be classified as thrombotic or embolic. A thrombotic stroke is the result of a clot that causes vascular blockage at the site of clot formation, whereas a clot that forms elsewhere in the body and is carried to the point of lodgement by the bloodstream is characterized as embolic. In contrast to embolic strokes, thrombotic strokes tend to be preceded by transient ischemic attacks or TIAs. A TIA is the result of a temporary interruption of the blood flow to the brain. This causes focal neurological deficits from which patients often recover within 24 hours. The onset of a TIA is usually sudden and the duration brief, generally lasting between 2 and 30 minutes.

A hemorrhagic stroke is the result of uncontrolled bleeding in the brain. In addition to interrupting the normal flow of blood, this uncontrolled bleeding also floods and kills brain cells. Hemorrhagic strokes can be caused by structural problems within the cerebral vasculature, including aneurysms and arteriovenous malformations.

B. Types of Ischemic Cell Death

There are two types of processes that cause cell death in ischemic tissue : necrosis and apoptosis. Neurons in the infarct core tend to undergo necrotic cell death, which begins to occur immediately after the ischemic insult (Sweeney et al., 1995). In contrast,

neurons in the peri-infarct area generally undergo apoptotic death, which occurs in a delayed fashion hours to days after the insult (Sweeney et al., 1995). It is important to note, however, that neurons in the peri-infarct zone may also undergo necrosis; there is not necessarily a distinct boundary where necrotic cell death stops and apoptotic cell death begins.

Necrosis is considered to be pathological cell death resulting from an extrinsic insult to the cells. This insult is often in the form of an abrupt environmental perturbation that results in a departure from physiological conditions (Martin et al., 1998). The acute oxidative stress and excitotoxic conditions that are found in the infarct core are attributed to be the triggers for necrotic cell death in ischemic tissue (Martin et al., 1998; Banasiak et al., 2000).

Apoptosis is considered to be physiological cell death in which cells die in a controlled manner in response to activation of a specific genetic program (Sweeney et al., 1995). In contrast to necrosis, apoptosis requires active transcription and translation for initiation (Zipfel et al., 2000) and occurs with minimal release of genetic material and pro-inflammatory intracellular components (Johnson and Deckwerth, 1993).

C. Mechanisms Underlying Damage from Stroke

i. Energy Failure

The abrupt decrease of blood flow to a brain area that occurs during cerebral ischemia results in restricted oxygen and glucose delivery to the tissues. When oxygen delivery to the tissue is lost, aerobic metabolism can no longer continue, so the cells must resort to anaerobic metabolism. The process of anaerobic metabolism promotes the

excess production of lactic acid, which causes intracellular pH levels to drop (Sapolsky, 1992b). This acidosis causes cellular dysfunction, including disruption to mitochondrial and glycolytic enzyme activity (Rehncrona et al., 1981).

Energy failure also causes the membrane potential to be lost. As a result, the cells depolarize, which triggers the influx of Na^+, and Cl^- and the efflux of K^+ (Siesjo, 1992a). The influx of Na^+ and Cl^- is paired with inward water movement, resulting in cell swelling and tissue edema. Efflux of K^+ triggers the depolarization of adjacent cells, a phenomenon known as spreading depressions [Dirnagl, 1999 #2]. In the core region of the infarct, these depolarized cells never repolarize [Dirnagl, 1999 #2]. However, in the peri-infarct region, the cells are able to repolarize but only at the expense of further energy consumption (Dirnagl et al., 1999). These cells can then depolarize again due to the continually high levels of glutamate and K^+, which results in the occurrence of repetitive depolarizations (Hossmann, 1996).

ii. Excitotoxicity

The loss of membrane potentials and resulting neuron depolarization causes voltage-sensitive presynaptic Ca^{2+} channels to open (Dirnagl et al., 1999). Ca^{2+} influx into the cell via these channels then triggers the release of glutamate into the extracellular space. Increasing amounts of glutamate in the synapses cause the cells to be hyperexcitable, thus promoting continual depolarization of the cell membrane and excessive activation of the both α-amino-5-methyl-isoxazole-proprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors on the post-synaptic membrane (Sapolsky, 1992a).

iii. Calcium Signalling

The excessive activation of NMDA receptors allows for inordinate levels of Ca^{2+} influx into the post-synaptic neurons, as well as mobilization of free cytosolic Ca^{2+} and the release of intracellular Ca^{2+} stores (Siesjo, 1992b). In healthy cells, one of the functions of Ca^{2+} is to initiate various second messenger pathways and activate enzymes (Levitan and Kaczmarek, 1997). However, excessively high Ca^{2+} levels cause over-activation of both of these processes. The results is the initiation of a series of damaging consequences, including proteolysis of cellular proteins and changes in protein phosphorylation and dephosphorylation (Siesjo, 1993).

iv. Free Radical Damage

Under ischemic conditions, high intracellular Ca^{2+} levels activate the production of free radicals (Sapolsky, 1992a). In addition, the tissue concentration of free-radical scavengers declines (Liu et al., 1994). Significant intracellular damage ensues, including damage to cell membranes, (Sapolsky, 1992a), to organelles such as mitochondria (Richter and Kass, 1991), and directly to the cell's DNA (Halliwell, 1992). All of these harmful effects will eventually lead to necrosis in the affected neurons (Kuroda and Siesjo, 1997).

v. Inflammatory Response

The activation of intracellular second messenger systems by excessive intracellular Ca^{2+} levels and the increase in free radicals trigger the initiation of the inflammatory response (Ruscher et al., 1998). Although the various cellular components of the inflammatory response may be important in regulating tissue remodeling and scar

formation, they may also contribute to the degeneration of injured neurons by secreting neurotoxins including free radicals and excitotoxic neurotransmitters (Back, 1998).

II. Diaschisis

Not only does stroke affect the area immediately surrounding the infarct, it also affects the structurally intact brain network that is connected to the lesion. In the 1870's, Brown-Sequard described the loss of function in remote brain areas connected to the lesioned area (Feeney and Baron, 1986). Nearly 50 years later, the Russian neurologist von Monakow coined the term "diaschisis" to describe this phenomenon (von Monakow, 1914). He emphasized four important aspects of its definition. First, he proposed that damage to one brain area may produce dysfunction in regions adjacent but connected to the primary site of damage. Second, he suggested that diaschisis is a clinical diagnosis in which the presumptive mechanism is a loss of excitation to intact regions rather than neural inhibition. Third, he suggested that diaschisis follows neuroanatomical pathways spreading from the site of injury. Finally, he proposed that diaschisis undergoes a gradual regression in well-defined phases, and that resolution of diaschisis will be accompanied by a return of function.

von Monakow described three different types of diaschisis based on his observations of human patients (von Monakow, 1914). The first type is *diaschisis corticospinalis*, which refers to the spreading of functional depression from a motor cortex injury to the spinal cord along pyramidal tract fibers. The second type is *diaschisis commisuralis*, which refers to functional cortical depression from a motor cortex injury in one hemisphere that is spread to the contralateral side via the axons of the corpus callosum. The third type *is diaschisis associativa*, which describes the spreading

of dysfunction to intact cortical areas neighboring the infarct via association fibers. Diaschisis is now often characterized more generally as dysfunction that is either remote (corticospinal, transcommisural) from or proximal (associative) to the ischemic infarct.

i. Measuring Diaschisis

Diaschisis can be measured via several different methods, which are generally categorized as measures of electrical, metabolic or physiological brain activity. Measures of electrical activity may include recordings from individual neurons or electroencephalography. In terms of electrical measures, diaschisis is characterized as reduced electrical activity. Methods used to measure changes in metabolic activity include positron emission tomography (PET) and functional magnetic resonance imaging (fMRI). PET measures glucose and oxygen metabolism and cerebral blood flow, whereas fMRI allows for the simultaneous measurement of brain structure and oxygen usage. In terms of these measurements, diaschisis is usually present in the form of reduced metabolic activity, which is indicative of a decrease in neuronal activity (Gold and Lauritzen, 2002). Measures of physiological brain activity include intracortical microstimulation (ICMS) and transcranial magnetic stimulation (TMS). ICMS measures motor responses following electrical stimulation of pyramidal cells in the motor cortex. TMS measures motor evoked potentials (MEPS) in target muscles resulting from the stimulation of the motor cortex with magnetic field impulses. In terms of these functional methods, diaschisis is evident as the inability to evoke movement or MEPS with stimulation.

The use of these methods has shown that diaschisis can occur in a number of different brain areas. Cerebral infarction can produce diaschisis in the cortex, cerebellum

(Lenzi et al., 1982; Martin and Raichle, 1983; Kushner et al., 1984), thalamus (Baron, 1989; Baron et al., 1992), basal ganglia (Nguyen and Botez, 1998), red nucleus, inferior olive (Dauth et al., 1985) and brain stem (Meyer et al., 1979). In addition, a lesion in any one of these brain areas may cause diaschisis in the overlying cortex.

ii. Etiology

The occurrence of diaschisis in the acute phase (0-24 hours) following ischemic infarct is a matter of controversy. A PET study by Iglesias et al (2000) found no evidence of diaschisis in the ipsilateral hemisphere. Conversely, Rubin et al (2000) found decreased CBF in both the ipsilateral cerebellum and contralateral hemsiphere within 8 hours of ischemia. Further, Vandenberg et al (2002) demonstrated an ipsilateral loss of movement representations in the rat primary motor cortex that extends beyond the infarcted area and appears within 24 hours following focal ischemic infarct. Electrophysiological data has been equally contradictory, with studies showing increases (Meyer et al., 1985; Sakatani et al., 1990), decreases (Kempinsky, 1958; Molnar et al., 1988) or no change in contralateral electrical activity (Tulleken et al., 1982; Matsumiya et al., 1990). Ipsilateral electrical activity, however, is significantly reduced in areas outside the infarct during the acute stage (Domann et al., 1993). As suggested by Andrews (1991), the size of the ischemic injury as well as the nature of the insult may be important determinants of the occurrence of diaschisis during the acute phase.

Studies examining the later stages (1 day to > 2 months) following infarct are much more consistent. By this time point, diaschisis is present in the form of metabolic depression, which is exhibited by the presence of bilaterally reduced cerebral blood flow (CBF) and metabolism (Kiyosawa et al., 1990; Pantano et al., 1996; Cramer and

Bastings, 2000). These decreases are greater on the ipsilateral side (Meyer et al., 1971; Cramer and Bastings, 2000). Decreases in contralateral CBF that reach a minimum level at 7 to 10 days post-lesion (Meyer et al., 1971; Slater et al., 1977; Lenzi et al., 1982; Vorstrup et al., 1986) and seem to occur in areas that mirror the region of infarction (Andrews, 1991). Research suggests this reduction in contralateral CBF may be due to a redistribution of CBF such that blood is "stolen" from the contralateral hemisphere to provide collateral circulation to ischemic regions (Meyer et al., 1979).

It has been shown that the decrease in contralateral CBF recovers to normal or near-normal levels after varying time periods, ranging from three weeks (Meyer et al., 1979) to several months following infarction (Andrews, 1991). In contrast, CBF in the ipsilateral hemisphere has been shown to recover more slowly and to a lesser extent than that of the contralateral hemisphere, such that it remains slightly decreased even months after the infarction has occurred (Lenzi et al., 1982; Vorstrup et al., 1986).

Despite the fact that an overall reduction in excitability has been shown to occurs in remote areas following the acute stage, hyperexcitability is has also been detected in these areas. For example, after measuring the excitability of the perilesional region in vivo, Witte and Freund (1999) found the mean discharge frequency of neurons to be increased. In addition, several researchers have found bilateral decreases paired pulse inhibition (Domann et al., 1993; Buchkremer-Ratzmann et al., 1996; Witte and Freund, 1999). Finally, Witte and Freund (1999) found the resting membrane potential of remote neurons to be less negative, as well as a reduction in early and later GABAergic inhibitory post synaptic potentials.

Thus, it is apparent that there is somewhat of a discrepancy with regards to the remote changes in excitability that occur post-lesion. As suggested by Neumann-Haefelin and Witte (2000), the methods by which excitability was measured in these different studies may underlie the contrasting results. These authors suggest that excitability was frequently assessed at the level of single neurons, such that individual neurons showed signs of hyperexcitability. However, when overall excitability of the perileisonal region is assessed by measuring field potentials, the result is a reduction in hyperexcitability (Neumann-Haefelin and Witte, 2000). Neumann-Haefelin and Witte (2000) hypothesize that if the reduction in neuron number (caused by selective neuronal death) outweighs the increase in excitability of single surviving neurons, then an overall reduction of excitability would result. This debate, however, remains unresolved.

iii. Mechanisms

The mechanisms underlying the phenomenon of diaschisis also remain to be fully understood. More than one hundred years ago, Brown-Sequard proposed that diaschisis was caused by the excitatory and inhibitory effects produced by brain lesions (Finger and Stein, 1982). Almost 50 years later, Von Monakow expanded on this idea with his postulation that diaschisis resulted from the severance of CNS connections (von Monakow, 1914). In the late 1950's, Kempinsky proposed that the functional activity of remote neuronal networks is facilitated by a constant afferent synaptic activation from other neuronal groups (Kempinsky, 1958). Furthermore, he suggested that when a focal lesion causes the inactivation of the facilitator group of neurons, the remote neurons they facilitate become functionally depressed.

Currently, many researchers still hold the theory that reduced neuronal activity in the diaschitic area is related to a decrease in excitatory output from the injured brain region. For example, crossed cerebellar diaschsis has been shown to be due to damaged cortical projections to the cerebellum via the cortico-ponto-cerebellar tract (Nguyen and Botez, 1998). The result of this damage is the loss of excitatory influences that originate in the contralateral cerebral cortex and relay in the pontine nuclei before reaching the cerebellar granule cells (Nguyen and Botez, 1998). A recent study by Gold and Lauritzen (2002) confirmed this view by showing that acute cerebellar diaschisis is due to the deactivation of Purkinje cells caused by reduced excitatory input from the cerebal cortex. In addition, these researchers showed that this deactivation does not involve synaptic inhibition (Gold and Lauritzen, 2002).

With regard to remote changes in excitability, a possible mechanism may be modulations in receptor expression and function. It has been shown that γ-aminobutyric acid (GABA) receptor binding is decreased in association with hyperexcitability (Schiene et al., 1996). A decrease of the $GABA_A$ receptor density has also been shown to occur (Zilles et al., 1995; Witte and Stoll, 1997; Witte, 1998). In addition, there are alterations in the density of other neurotransmitter receptors in areas remote from the lesion, including an increase in NMDA receptor density and a decrease in AMPA receptor density (Witte, 1998; Witte and Freund, 1999).

iv. Functional Deficits Associated with Diaschisis

PET studies have shown that functional deficits in both language (Cappa et al., 1997) and motor abilities (Baron et al., 1986; Pantano et al., 1996) are associated with the presence of remote diaschisis in both the ipsilateral and contralateral hemispheres.

Further, proximal diaschisis has also been associated with motor deficits. Several cortical mapping studies have revealed the existence of a loss of movement representations that extends beyond the infarcted area (Nudo and Milliken, 1996; Goertzen, 2001; VandenBerg, 2002).

Many researchers have proposed that the resolution of diaschisis may be a necessary factor underlying spontaneous recovery from stroke, and there is some existing evidence that supports this idea. It has been shown that recovery of blood flow and metabolism in the contralateral hemisphere is correlated with clinical recovery in human patients (Meyer et al., 1993). This correlation is especially strong for younger patients, as the restoration of CBF toward normal levels appears to occur more rapidly in younger patients than in older patients (Meyer et al., 1971). In addition, recovery of both blood flow and cerebral metabolism in the contralateral hemisphere has been specifically associated with recovery of language function (Heiss et al., 1993; Cappa et al., 1997; Mimura et al., 1998). Rehabilitative training following stroke may enhance the resolution of diaschisis and functional recovery. Goerzten et al (2001) showed that animals trained on a rehabilitative skilled reaching task following stroke exhibited a sparing of distal and proximal forelimb representations in the lesioned motor cortex. Similarly, Nudo et al (1996) found that retraining of skilled hand use following focal ischemic infarct resulted in prevention of the loss of hand territory adjacent to the infarct. Further, VandenBerg et al (2002) showed that even delayed rehabilitation could alleviate diaschisis to some extent. In addition, manipulations that enhance recovery also decrease diaschisis (Kleim, 2003).

III. Influence of Pre-stroke Factors on Recovery from Stroke

The idea that the state of the brain prior to the occurrence of injury may affect its functional outcome is not a new concept. In fact, researchers have uncovered a number of factors that may change the status of the brain prior to damage, which may in turn affect its ability to recover. For example, structural compensation after cortical injury is dependent upon history of exposure to gonadal steroid hormones (Forgie and Kolb, 2003). Although this research has been in progression for at least a decade, scientists are just beginning to understand how these pre-stroke factors may influence outcome after stroke.

i. Preconditioning

Preconditioning refers to a phenomenon in which tissue is rendered resistant to the deleterious effects of a prolonged insult by previous exposure to brief periods of the same insult in a milder form. The benefits of preconditioning were first demonstrated in the myocardium (Murry et al., 1986), and have since been found in the skeletal muscle (Mounsey et al., 1992), small intestine (Sola et al., 2000), kidney (Bonventre, 2002) and brain (Kitagawa et al., 1990). Several types of preconditioning have been encountered specifically in the brain, including application of the following stimuli: ischemia, spreading depressions, hyperthermia, hypothermia, chemical agents and immune system components such as cytokines.

a) Ischemic Preconditioning

Kitagawa and colleagues (1990) were the first to describe the effects of ischemic preconditioning. These authors found that the administration of two brief repetitive

periods of global ischemia prior to a longer period were sufficient to protect 60% to 90 % of the cells in the hippocampus from necrotic damage (Kitagawa et al., 1990). Kitagawa and colleagues (1991) later showed that this kind of preconditioning is also protective in other areas of the brain, including the cerebral cortex, the striatum and the thalamus (Kitagawa et al., 1991).

Further studies have since shown that induction of brief periods of focal ischemia establishes brain tolerance against subsequent global ischemia (Glazier et al., 1994) and vice versa (Simon et al., 1993). In addition, ischemic preconditioning is effective in aged animals (Dowden and Corbett, 1999). Finally, brain tolerance to these various ischemic preconditioning paradigms is generally evident 24 hours following the preconditioning stimulus and lasts for up to ten days (Corbett and Crooks, 1997; Rejdak et al., 2001). Ischemic preconditioning has also been found to have other beneficial effects that may further contribute to neuroprotection. Masada and colleagues (2002) showed that 15 minutes of MCAO followed by permanent MCAO three days later significantly reduced the formation of brain edema and blood-brain barrier disruption.

The concept of ischemic preconditioning has been receiving increasing attention because of its potential value within the clinical setting. For example, vascular surgeries requiring transient vascular clamping or direct suppression of cardiac output have the potential to produce ischemia. Thus, ischemic preconditioning is already commonly used in cardiovascular surgery in order to reduce possible ischemic damage to the nervous system, (Nishio et al., 2000). Research has shown that patients that had an ipsilateral TIA prior to suffering from a more severe stroke recovered better on average than those patients who did not have a TIA (Moncayo et al., 2000). Thus, it has been suggested that

chemical preconditioning might be a reasonable strategy to use in patients in whom a TIA has occurred in order to potentiate the endogenous protection against the next more severe insult (Rejdak et al., 2001).

b) Preconditioning via Spreading Depression

The induction of cortical spreading depression (CSD) via the application of potassium chloride has been shown to protect against both temporary global (Yanamoto et al., 1998) and focal (Matsushima et al., 1996) cerebral ischemia. It has also been shown that the greater the interval between the induction of continuous CSD and focal ischemia, the greater the resulting reduction in infarct size (Yanamoto et al., 1998), although some research has shown a three day interval to be effective (Matsushima et al., 1996).

c) Thermal Preconditioning

Hypothermia has been described as the "gold standard" of neuroprotection due to the robust protection it provides against cell death when delivered during and/or after ischemia (Barone et al., 1997). More recent research, however, is finding that changes in brain and body temperature, including both hypo- and hyperthermia, can also function as preconditioning stimuli. For example, Chopp and colleagues (1989) found that heating rats to 41.5°C for 15 minutes 24 hours prior to ischemia resulted in a significant reduction in cell damage. In addition, a study by Xu and colleagues (2002) showed that hyperthermic pretreatment administered at least 18 hours prior to MCAO was sufficient to significantly decrease the size of the lesion.

Brief periods of hypothermia administered prior to ischemia have also been found to be neuroprotective. Nishio and colleagues (1999) showed that a 20-minute period of hypothermic preconditioning at 31.5°C induced substantial resistance against transient focal ischemia. Further study by Nishio et al (2000) found that the neuroprotective effect of hypothermic preconditioning was present when the hypothermia was administered anywhere between 6 and 48 hours prior to ischemia.

The larger margin of safety associated with temperature-related preconditioning makes its use advantageous over ischemic preconditioning (Nishio et al., 2000). In the case of ischemic preconditioning, one minute of ischemia is insufficient to induce tolerance, while two minutes induces only partial tolerance (Kitagawa et al., 1990). However, three to five minutes of ischemic preconditioning is sufficient to kill vulnerable neurons; thus, the safety margin for this type of preconditioning is very narrow. In contrast, a moderate level of hypothermic preconditioning (eg 20 minutes at 31.5 degree Celcius) does not represent a direct risk to neurons (Nishio et al., 2000). In fact, it has been shown that a period of hypothermia lasting as long as three hours is still not sufficient to damage neurons (Chopp et al., 1992). Thus, hypothermic preconditioning may have more clinical potential than other forms of preconditioning, especially ischemic, because it is less injurious and invasive (Nishio et al., 2000).

d) Chemical Preconditioning

There are several chemical agents that may produce protective cellular changes comparable to those produced by ischemic preconditioning. For example mitochondrial ATP-dependent potassium (K_{ATP}) channel openers have been found to be neuroprotective when administered prior to stroke. Although the benefits of these mitochondral K_{ATP}

channel openers have been studied most extensively in the heart, their effects have also been studied recently in the brain. Diazoxide is one K_{ATP} opener that has been found to be very effective when used as a chemical preconditioning stimulus. In rat studies, it has been shown to reduce infarct volume following MCAO (Shimizu et al., 2002) and prevent morphological damage produced by hypoxia (Garcia de Arriba et al., 1999). In addition, diazoxide has been shown to preserve NMDA-induced cerebral arteriolar vasodilation, which is an indicator of intact neuronal function, following global cerebral ischemia (Domoki et al., 1999).

Another example of a successful chemical preconditioning paradigm is the use of 3-nitroproprioic acid (3-NPA). 3-NPA is a selective inhibitor of the mitochondrial neurotoxin succinic dehydrogenase (Kuroiwa et al., 2000). Several studies have found that subtoxic doses of 3-NPA administered prior to the infarct were effective in preserving neuronal density (Nakase et al., 2000) and reducing infarct volume (Kuroiwa et al., 2000).

e) Immunological Preconditioning

It is well known that the immune response triggered by ischemia plays a role in furthering cerebral injury. Unfortunately, the control of post-stroke inflammation by the administration of immunosuppressive agents is limited by the systemic side effects that many of these agents induce. However, recent research has shown that stimulation of the immune system prior to stroke may be neuroprotective. For example, the CNS antigen myelin basic protein has been shown to decrease infarct size when administered orally prior to stroke (Becker et al., 1997). In addition, tumor necrosis factor-alpha (TNFα) preconditioning protected rat cortical neurons from hypoxia (Liu et al., 2000) and

significantly reduced infarct size in a mouse MCAO model (Nawashiro et al., 1997). Finally, the cytokine erythropoietin, which is produced in the brain by astrocytes in response to hypoxia (Marti et al., 2000), has been shown to reduce infarct size by 50-75 percent in rat and mouse models (Bernaudin et al., 1999; Brines et al., 2000). Based on these findings and others, recombinant human erythropoietin is currently being used in clinical stroke trials to investigate its neuroprotective efficacy in human patients (Siren and Ehrenreich, 2001).

f) Dietary Restriction

Past research has shown that reduced caloric intake (with maintenance of micronutrient intake) can slow age-related cerebral changes in gene expression for proteins involved in immune responses, oxidative stress and energy metabolism (Lee et al., 2000). In addition, dietary restriction can help to counteract age-related deficits in learning and memory (Ingram et al., 1987) and motor abilities (Stewart et al., 1989). Thus, a reasonable assumption would be that the effects of dietary restriction might also be beneficial in helping the brain recover from injury. Yu and Mattson (1999) found that rats maintained on dietary restriction for several months exhibited reduced brain damage and improved behavioural outcome. Further investigation has shown that dietary restriction alters synaptic homeostasis such that synapses are better able to withstand oxidative and metabolic stress.

g) Environmental Preconditioning

Since Hebb's first experiments over 50 years ago, a number of different studies have shown environmental enrichment to promote numerous structural changes in the

brain. Some of these changes include increases in: dendritic arborization (Black et al., 1989), synaptic density (Turner and Greenough, 1985), blood vessel density, astrocyte number and mitochondrial volume (Sirevaag and Greenough, 1987) and neurogenesis (van Praag et al., 1999a). The conclusion that has evolved from the results of these studies is that exposure to an enriched environment may increase the brain's capacity for adaptation.

If environmental enrichment increases brain plasticity, then it may also promote recovery after injury. Gentile and colleagues (1987) first investigated this idea over 15 years ago and found that rats exposed to an enriched environment were less impaired on a motor task following cortical lesions. In addition, these animals were able to recover more quickly than control animals (Held et al., 1985).

h) Exercise

Several recent epidemiological studies have suggested that regular physical exercise may reduce risk for ischemic stroke in humans (Wannamethee and Shaper, 1992; Lee et al., 1999). In addition, two animal studies have shown that exercise may help protect the brain from the damage caused by stroke. Stummer and colleagues (1994; 1995) showed that pre-ischemic access to running wheels resulted in reduced neuronal death in selectively vulnerable areas of the brain and more rapid normalization of cell conductance. Furthermore, Wang and colleagues (2001) showed that treadmill training for at least two weeks reduced infarct volume and cerebral edema in a rat MCAO model. Despite these findings, however, there has been very little investigation into the effects of exercise on recovery from stroke.

IV. Effects of Exercise on the Brain

i. Metabolic Changes

Several lines of research support the idea that brain areas that are more active during exercise consequently have greater metabolic needs. For example, regional cerebral blood flow and oxygen uptake are increased during high intensity exercise (Herholz et al., 1987; Hellstrom et al., 1996; Ide and Secher, 2000). In addition, Vissing and colleagues (Vissing et al., 1996) found that local cerebral glucose utilization increased by 39% in the motor cortex during exercise, which is interpreted by these authors as an index of increased neuronal activity. Finally, increases in cytochrome oxidase have been shown to occur in response to chronic exercise (McCloskey et al., 2001). Cytochrome oxidase is coupled to the production of ATP, thus increases reflect changes in metabolic capacity (McCloskey et al., 2001).

The metabolic demands of aerobic activity may serve as a stimulant to angiogenesis. Several studies have reported the occurrence of cerebral angiogenesis in rats exercised in running wheels (Isaacs et al., 1992; Kleim et al., 2002a). Thus, angiogenesis may enhance the peak capacity of the brain to respond to the increased metabolic demands imposed by activity in the running wheel. This idea is further supported by studies reporting angiogenesis in rats exposed to hypoxia (LaManna et al., 1992; Boero et al., 1999). The formation of new capillaries under hypoxic conditions is an important mechanism by which oxygen availability to the brain can be increased.

ii. Structural Changes

In addition to the development of new cerebral vasculature, exercise causes several other structural changes in the brain. Pysh and Weiss (1979) showed that the cerebellar Purkinje cells in mice given exposure to exercise during development exhibited larger dendritic trees and greater spine density. In addition, a study by Anderson and colleagues (2002) showed that animals given access to running wheels exhibited increased cortical thickness in the medial and anterior regions of the motor cortex.

Another recent finding is the idea that exercise may affect cell proliferation, survival and differentiation. A study by van Praag and colleagues (1999a) showed that voluntary exercise in a running wheel increased cell proliferation, cell survival and net neurogenesis in the adult mouse hippocampus. These authors found that running doubled the number of surviving newborn cells in the dentate gyrus (van Praag et al., 1999a). Thus, these results imply that exercise-induced increases in neurogenesis may have important applications to the prevention of age-related cell loss.

iii. Improvements in Learning and Cognitive Functioning

There has been a large volume of research that has investigated the effects of exercise on cognitive functioning, mood and mental status. Many of the initial studies examined mood and cognitive functioning following acute exercise. The general results were that physical activity is associated with incremental changes in mood and mental functioning (Lichtman and Poser, 1983). Recent studies have examined specific alterations in mood and cognition following long-term exercise. For example, Stevenson and Topp (1990) showed that subjects aged 60+ who engaged in a long-term exercise

program exhibited significant improvements in attention, concentration, short-term memory and sleep quality. Furthermore, Williams and Lord (1997) demonstrated improvements in reaction time, memory span and measures of well-being following long-term exercise in older women. In addition, these authors also showed that long-term exercise normalized mood states in subjects who had high anxiety, depression and stress levels (Williams and Lord, 1997). Finally, it has been suggested that long-term exercise may contribute to the reduction of age-related cognitive decline (Schuit et al., 2001).

Increases in cognitive functioning and mood following exercise may have several underlying mechanisms. For example, cerebral increases in circulating beta-endorphin levels following a 10km run have been correlated with increased feelings of pleasantness in male runners (Wildmann et al., 1986). In addition, it have been shown that blocking opiate receptors with naltrexone prevented exercise-generated positive mood shifts (Daniel et al., 1992). Thus, mood improvements following exercise may be related to endorphinergic mechanisms.

There is also evidence suggesting that exercise is related to enhanced learning. For example, voluntary wheel running has been shown to improve spatial learning and enhance long term potentiation in the mouse dentate gyrus (van Praag et al., 1999b). In addition, Fabre and colleagues (2002) found significant improvements in logical memory, paired associates learning scores and memory quotient in elderly subjects who engaged in an aerobic training program. Possible mechanisms that may underlie enhanced learning in response to exercise include activity-induced hippocampal neurogenesis and BDNF upregulation (van Praag et al., 1999b; Cotman and Berchtold, 2002).

iv. Chemical and Molecular Changes

It has been known for some time that moderate and high-intensity endurance exercise stimulate the release of the opioid peptide beta- endorphin (Farrell et al., 1982; Wildmann et al., 1986; Debruille et al., 1999). These endorphin levels remain elevated for 15-60 minutes following exercise (Sforzo, 1989).

Neurotransmitter levels in the brain have also been shown to change in response to exercise. Microdialysis studies have shown that levels of dopamine and several of its metabolites increase in the striatum both during and following exercise (Hattori et al., 1994). In addition, dopamine release is increased 40 to 60 minutes following treadmill exercise (Meeusen and De Meirleir, 1995). Other studies have shown extracellular increases in noradrenaline and serotonin levels in various brain areas, including the hippocampus, frontal cortex and striatum (Pagliari and Peyrin, 1995; Wilson and Marsden, 1996; Meeusen et al., 1997).

Another exercise-induced molecular change that occurs in the brain is increases in the levels of growth factors, which are molecules that nourish and protect brain cells. Neurotrophic factors are a specific class of growth factors that target neurons. Because neurotrophins can enhance the survival and differentiation of neurons, it is believed that enhanced levels in the brain may serve a neuroprotective function. Voluntary running can upregulate the mRNA expression of brain-derived neurotrophic factor (BDNF) in the hippocampus, cerebral cortex, cerebellum, and lumbar spinal cord (Neeper et al., 1995; Neeper et al., 1996; Oliff et al., 1998; Gomez-Pinilla et al., 2001). Hippocampal levels of nerve growth factor (NGF) and fibroblast growth factor 2 (FGF-2) have also been shown to increase following exercise; their upregulation however, is less robust than that of

BDNF (Neeper et al., 1996; Gomez-Pinilla et al., 2001). Thus, BDNF is believed to play the most important role in mediating the beneficial effects of exercise on brain functioning (Cotman and Engesser-Cesar, 2002).

IV. Brain-Derived Neurotrophic Factor

i. Structure and Function of BDNF

BDNF is a basic protein with 119 amino acid residues in its structure (Maness et al., 1994). Although BDNF is primarily found in the central nervous system (CNS), it is also produced in lesser quantities in Schwann cells of the peripheral nervous system (Acheson et al., 1991) and in organs such as the heart and lungs (Rosenthal et al., 1991). In the CNS, BDNF is widely distibuted in a number of brain regions, including the hippocampus, striatum, cerebellum and substantia nigra (Maness et al., 1994). In the cerebral cortex, studies have shown BDNF to be primarily localized to layers III and V (Murer et al., 1999).

BDNF primarily supports the function of glutamatergic neurons and preferentially binds to the TrkB tyrosine kinase receptor on these neurons (Cotman and Engesser-Cesar, 2002). Following ligand binding, the TrkB receptor autophosphorylates, which leads to activation of the tyrosine kinase and thus the activation of the receptor itself (Huang and Reichardt, 2001). Once activated, the receptor is capable of triggering a number of intracellular signaling cascades, including the phosphatidylinositol-3-kinase/protein kinase B (PI-3-K/PKB), the phosholipase C-γ1 pathway and the mitogen-activated protein kinase (MAP)/extracellular signal-regulated kinase (ERK) systems (Kaplan and Miller, 2000). The ultimate results of these pathways are both short term (enhancement

of glutamate release) and long term (activation of transcription factors in the nucleus that alter gene expression) (Kaplan and Miller, 2000).

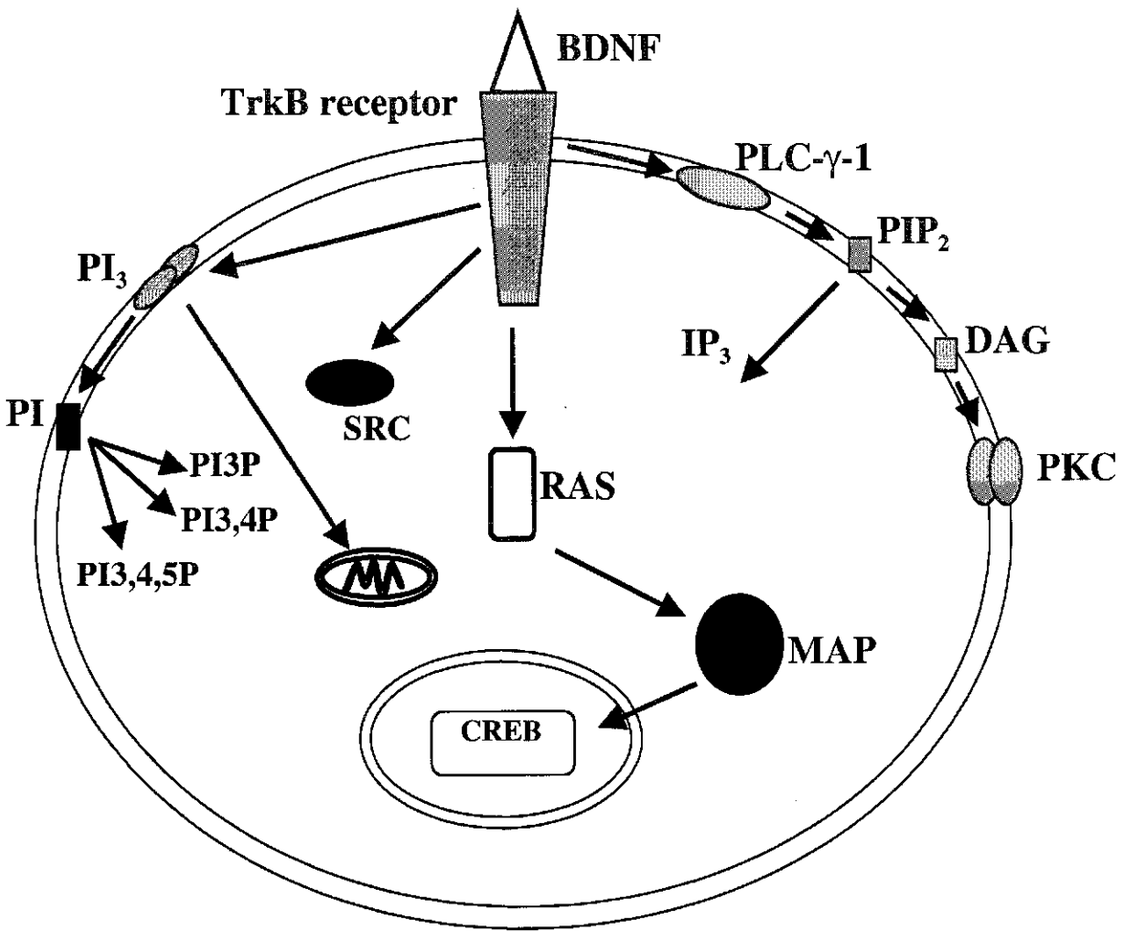

Figure 1 - Diagram of BDNF signal transduction pathways mediated by TrkB receptor. (Adapted from Maness et al, 1994)

BDNF has several important functions in the CNS. First, it promotes the differentiation and survival of a variety of neuronal populations during development and adulthood (Maness et al., 1994; Huang and Reichardt, 2001; Lu, 2003). Second, BDNF promotes neurite extension of sensory processes to their targets within the CNS (Maness

et al., 1994). Finally, BDNF plays a crucial role in mediating synaptic plasticity and transmission (Lu, 2003). These BDNF-induced changes in synaptic plasticity can occur in an acute manner. For example, application of BDNF to the neuromuscular junction elicits a rapid enhancement of neurotransmitter release (Stoop and Poo, 1995). BDNF can also play a long-term regulatory role in synapse development and function by triggering changes in the production of synaptic proteins (Cotman and Berchtold, 2002) and by modulating growth/arborization of cortical dendrites (Horch and Katz, 2002).

ii. Mechanism and Regulation of BDNF Action

The ability of BDNF to mediate synaptic modulation is dependent upon synaptic activity (Lu, 2003). Thus, increased electrical activity in the cells is proposed to upregulate BDNF, which in turn facilitates synaptic plasticity (Cotman and Berchtold, 2002). Increased neuronal activity can be initiated by several different types of stimuli, including experimentally induced seizures (Ernfors et al., 1991), tetanic stimulation (Morimoto et al., 1998) and physical activity (Neeper et al., 1996), and each has been shown to upregulate BDNF mRNA levels in the brain. Further, increased neuronal activity increases the number of TrkB receptors expressed on the presynaptic neuron (Meyer-Franke et al., 1998).

BDNF is considered to be a retrograde messenger. It is secreted from the pre-synaptic cell and has both pre-synaptic and post-synaptic targets (Manabe, 2002). Neuronal activity stimulates BDNF release from pre-synaptic sites (Yamada and Nabeshima, 2003). The secreted BDNF will bind to TrkB receptors on the pre-synaptic cell membrane and trigger an increase in pre-synaptic glutamate release. BDNF also binds to TrkB receptors on the post-synaptic membrane, which will become activated and

trigger intracellular signaling cascades as described above. Thus, increased neuronal activity enhances synaptic efficacy by increasing the transcription, secretion and binding of BDNF, which leads to enhanced glutamate release and activation of signal transduction pathways.

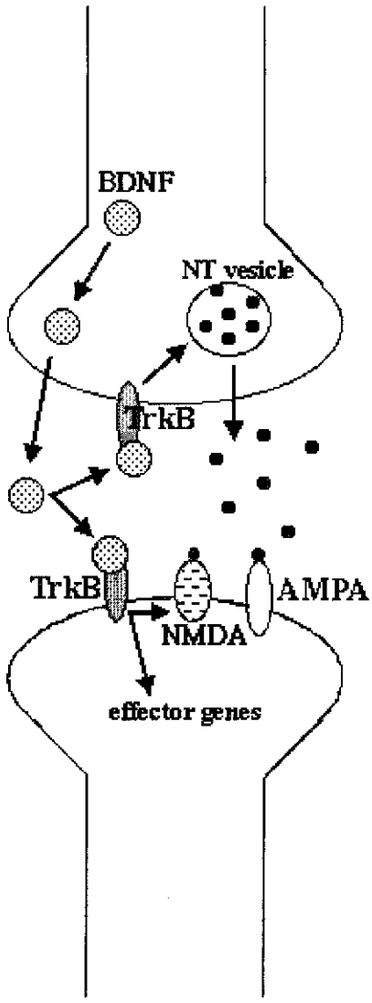

Figure 2 - Activity-dependent release of BDNF from neurons. Binding of BDNF to TrkB receptors on pre and postsynaptic sites leads to increased neurotransmitter (NT) release and activation of signal transduction pathways. (Adapted from Yamada, 2003).

iii. Role of BDNF in Learning and Memory

BDNF-induced changes in synaptic efficacy may contribute to the neural processes underlying learning and memory. Environmental manipulations such as running that increase neural activity have been found to increase both BDNF mRNA levels and performance on learning and memory tasks (van Praag et al., 1999b). BDNF gene expression is also enhanced during the performance of spatial memory tasks (Hall et al., 2000; Mizuno et al., 2000). Finally, inhibition of BDNF signaling by antisense infusion (Mizuno et al., 2000) or by gene knockout (Minichiello et al., 1999) impairs learning on spatial memory tasks.

It is well known that the hippocampus plays an important role in learning and memory, especially that of spatial memory. A recent study by Egan and colleagues (2003) provides ample evidence for the role of BDNF in hippocampal functioning and memory. These authors examined the effects of a valine to methionine substitution in the 5' pro-region of the human BDNF protein. This substitution was associated with impaired episodic memory and abnormal hippocampal activation in human patients. Further, hippocampal cells containing the met substitution exhibited a reduction in synaptic activity and an impairment in regulated secretion of BDNF protein, such that activity-dependent release of BDNF was severely reduced (Egan et al., 2003).

The idea that BDNF is required for learning and memory processes combined with the knowledge that neural activity is required to maintain BDNF levels, has important implications for studies of aging and neurodegenerative diseases. It is well known that normal aging is accompanied by changes in neuronal morphology and cortical circuitry (Page et al., 2002). More recently, it has been shown that BDNF levels

also decreases during aging process (Hayashi et al., 1997). Due to the role that BDNF plays in maintaining the functioning of healthy cells, it is probable that decreased BDNF levels may be related to these age-associated changes.

Decreased BDNF levels are also associated with the development of several neurodegenerative disorders. Reduced levels of both BDNF and its mRNA have been found in brains of Alzheimer's (Narisawa-Saito et al., 1996; Hock et al., 2000) and Parkinson's patients (Mogi et al., 1999). Alzheimer's disease is associated with reduced BDNF levels specifically in the hippocampus (Siegel and Chauhan, 2000). Due to the relationship between the hippocampus and learning, it is theorized that the reduction in hippocampal BDNF in brains of Alzheimer's patients may be related to cognitive deficits that are characteristic of the disease. In support of this idea, Ando et al (Ando et al., 2002) showed that an adenovirus-mediated gene transfer of BDNF could partially restore cognitive deficits in an animals model of Alzheimer's disease.

iv. Role of BDNF in Mood Regulation

Alterations BDNF levels may also underlie changes in mood, especially in relation to depression. Some antidepressant treatments that increase transmission at monoaminergic synapses, such as imipramine, also lead to an up-regulation of BDNF mRNA levels in the hippocampus (Russo-Neustadt et al., 1999). Because exercise induces a similar effect, the combination of physical activity and antidepressant treatment might potentiate hippocampal BDNF upregulation. This idea has recently been confirmed, with the results of several studies showing that the combination of physical activity and antidepressant treatment increased BDNF mRNA levels in a manner that is both additive and accelerated (Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2000;

Russo-Neustadt et al., 2001). In addition, BDNF levels may be related to mood changes induced by stress. It has been shown that stress-induced corticosteroid release downregulates hippocampal BDNF mRNA (Schaaf et al., 2000). Furthermore, this effect that can be counteracted by exercise prior to a stressful event (Russo-Neustadt et al., 2001).

v. BDNF and Brain Injury

Given the benefical effects of BDNF in the brain, it has been hypothesized that BDNF could be a viable treatment to ameliorate damage from various forms of brain injury. There is an abundance of research supporting this idea. Yanamoto et al (2000) found that pretreatment with BDNF prior to temporary focal ischemia significantly reduced infarct volume. Schabitz et al (2000) found that BDNF delivered intravenously following focal cerebral ischemia reduced infarct volume and neurological deficits. In addition, these authors also found that the expression of proapoptotic protein Bax was decreased, while expression of antiapoptotic protein Bcl-2 was increased, which may be one mechanim by which BDNF exerts its neuroprotective action. Finally, Wu and colleagues (Wu et al., 2003) found that low BDNF mRNA levels are related to poor recovery from fluid percussion injury. Further, Hicks et al (1998) showed that exercise-induced increases in hippocampal BDNF levels could prevent neuronal loss and impairments in motor performance and spatial memory following traumatic brain injury.

V. The Rat Model

Rats have a highly developed motor system that supports a vast repertoire of movements that have been widely studied and well defined. The rodent motor cortex in

particular is an ideal model because its structure and function is similar to that of the primate motor cortex. In addition, it is possible to functionally define the rat motor cortex and cause specific impairments that closely mimic the hemiparesis that is a common result of stroke. Thus, the rat provides an excellent model to examine the relationship between pre-stroke exercise and cortical function and recovery.

i. ICMS in the Rat Motor Cortex

Intracortical microstimulation (ICMS) is a technique that has been used to accurately and reliably define the functional organization of the rat motor cortex (Neafsey et al., 1986; Kleim et al., 1998). This technique allows for the construction of a "map" of the topography of forelimb movement representations within the rat motor cortex. Using standard ICMS techniques (Kleim et al., 1998), the rat is anesthetized and a craniotomy is performed over the motor cortex contralateral to the preferred paw. Following this preparation, a microelectrode controlled by a hydraulic microdrive is positioned at various locations on the cortex and then lowered in the layer V. A small amount of current is then passed through layer V, which stimulates pools of pyramidal cells. The resulting movement pattern can then be recorded. After numerous microelectrode penetrations are made, the final result is a "motor map" of the functional representations within the motor cortex.

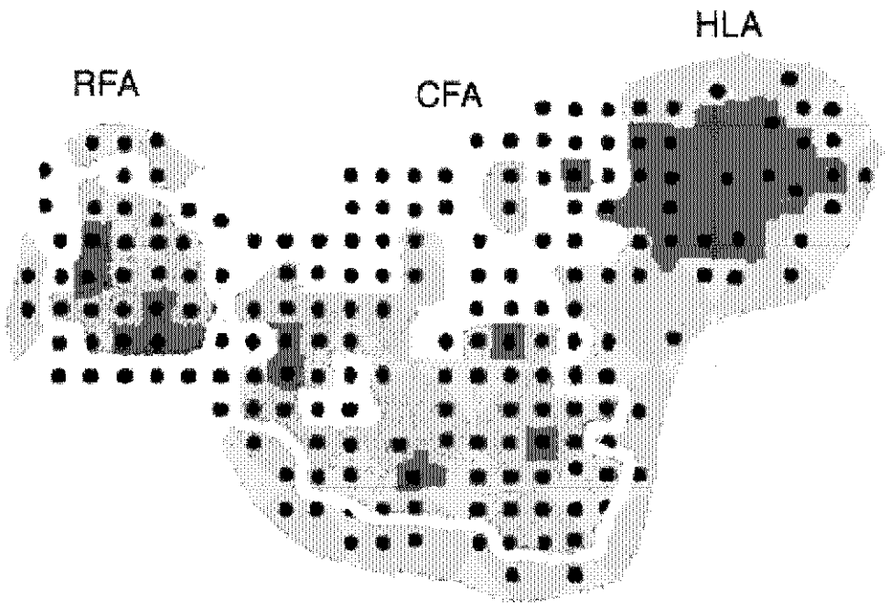

Figure 3 - Topographic representations within the rat motor cortex. Rostral forelimb (RFA), caudal forelimb area (CFA) and hindlimb area (HLA) consist of digit (red),wrist (green), elbow (light blue) and hindlimb (dark blue) movements. These areas are bordered by head/neck/vibrissae (yellow) representations and non-responsive sites (grey).

Studies of motor experience have shown that different types of experience exert differential effects on the organization of the motor cortex. Both rat and monkey experiments have shown that skilled reach training alters the map topography such that the distal representations expand at the expense of the proximal representations (Kleim et al., 1998; Plautz et al., 2000; Kleim et al., 2002b). Although strength training also causes similar changes in map topography, it has been shown that it is the skilled component of the strength task that causes the reorganization (Remple et al., 2001). In addition, wheel running does not alter movement representations in the motor cortex; this is because endurance activity involves primarily motor repetition as opposed to skill (Kleim et al.,

2002a). Thus, it is apparent that the nature of the motor experience determines how the motor system will respond.

ii. Focal Ischemia in the Rat Motor Cortex

Once the organization of the rat motor cortex has been functionally defined, focal ischemic infarct can be produced within approximately 30% of the distal representations movement representations in the caudal forelimb area via electrocoagulation of the surface vasculature. When ischemia is induced within these areas, behavioural deficits result (Goertzen, 2001; VandenBerg, 2002). Further, rats can recover from a focal infarct to the motor cortex and the improvements in motor behaviour are accompanied by an enlargement of movement representations (Goertzen, 2001; VandenBerg, 2002). Thus, the rat model of focal ischemia provides a unique opportunity to conduct a detailed examination of the relationship between changes in cortical function and improved motor performance.

A. B.

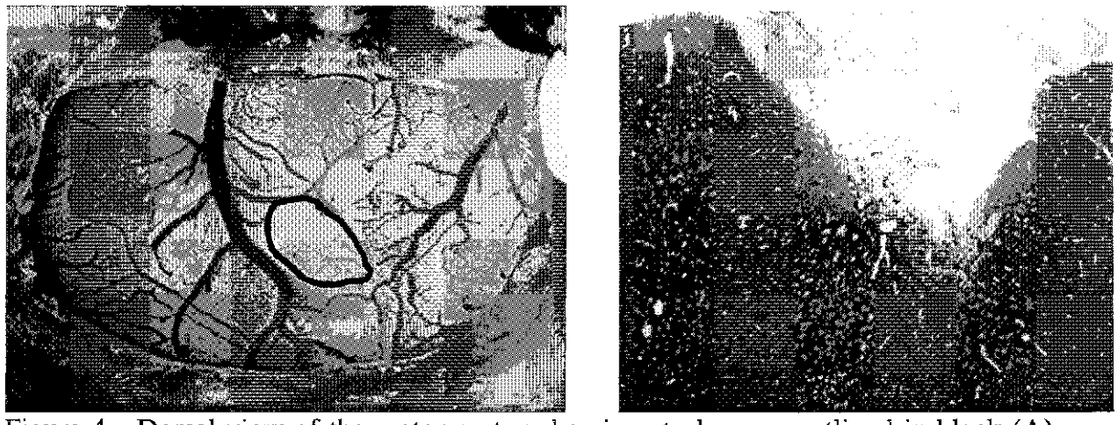

Figure 4 - Dorsal view of the motor cortex showing stroke area outlined in black (A). Coronal section of the motor cortex showing infarct (B).

iii. Voluntary Running Paradigm

Rats are a highly active species and will run spontaneously at their own pace when given access to a running wheel (Mondon et al., 1985; Ishihara et al., 1998). The use of running wheels is advantageous in that it allows the rats to run voluntarily during their normal active hours without the stress imposed by forced running on a treadmill (Ishihara et al., 1998). In addition, most rats will run one to three kilometers per night and will maintain a peak level of running intensity for several months (Mondon et al., 1985). Thus, the rat provides an excellent model in which to study the potential interaction between exercise and recovery from ischemic stroke.

VI. Thesis Outline.

The goal of this thesis is to determine how exercise prior to cerebral ischemia influences cortical function and recovery. It will also address the possible role of BDNF in the relationship between pre-stroke exercise and recovery from stroke. In order to accomplish this goal, a rodent model of ischemia involving rat forelimb motor cortex and voluntary running behaviour will be utilized. The method of evaluating functional status of the motor cortex following stroke will be provided by the use of intracortical microstimulation. A voluntary wheel running paradigm will provide a model of endurance exercise. A battery of sensorimotor tests will be used to evaluate motor deficits and recovery. Manipulations such as exercise that occur prior to ischemic lesion seem to alleviate diaschisis in surviving peri-infarct tissue and promote motor recovery.

Two primary questions will be addressed: First, does pre-lesion endurance exercise alleviate dysfunction in peri-infarct tissue and promote motor recovery? Secondly, is the exercise-induced alleviation of dysfunction in peri-infarct tissue

dependent upon synthesis of brain-derived neurotrophic factor?

CHAPTER 2 :

EXERCISE INCREASES RESISTANCE TO PERI-INFARCT DIASCHISIS FOLLOWING FOCAL CORTICAL INFARCT

INTRODUCTION

The idea that neural dysfunction associated with stroke occurs in brain areas outside the infarction is not new. Von Monakow introduced this concept over one hundred years ago when he coined the term "diaschisis" to explain behavioural symptoms that could not be correlated with lesion size. More recently, measures of electric activity (Neumann-Haefelin and Witte, 2000) and metabolic factors (Andrews, 1991) have confirmed the existence of dysfunction in both the ipsilateral and contralateral hemispheres.

It is hypothesized that extra-infarct dysfunction following stroke may contribute to motor impairment. TMS studies in humans have shown decreases in cortical hand representation area that correlate with deficits in motor performance (Di Piero et al., 1992; Traversa et al., 1997). In rat motor cortex, impairments in skilled forelimb use following ischemia have been associated with a loss of microstimulation-evoked forelimb movement representations that extend beyond the infarct (Goertzen, 2001). Similarly, in the monkey motor cortex, Nudo and Milliken (1996) demonstrated motor impairments associated with a reduction in digit representations adjacent to the lesion. It is also hypothesized that the alleviation of peri-infarct dysfunction may contribute to recovery of motor impairment following stroke. The administration of skilled rehabilitation training in both rats and monkeys has been shown to reverse the loss of movement representations and to reduce motor impairment (Friel et al., 2000; Goertzen, 2001). Further, VandenBerg (VandenBerg, 2002) showed that rehabilitation aids in motor recovery and the alleviation of peri-infarct diaschisis even when administration is delayed for up to 28 days.

Most research regarding recovery of motor impairments following stroke has focused on interventions administered after the insult. However, it is also possible that the state of the brain at the time of insult may be a critical factor in determining the degree of recovery. Age (Kolb et al., 2000), exercise (Wang et al., 2001), hormonal status (Forgie and Kolb, 2003), temperature (Chopp et al., 1992), and immune status (Becker et al., 1997) have all been shown to influence the level of impairment and/or recovery following damage. It is theorized that these factors may provide some means of enhancing and protecting brain function.

Exercise training in particular seems to be an excellent candidate for enhancing brain plasticity. This is due to the wide variety of beneficial changes it induces in the brain. Exercise has the ability to enhance the processes of neurogenesis (van Praag et al., 1999a) and angiogenesis (Kleim et al., 2002a), as well as induce other structural changes including dendritic arborization and synaptogenesis (Pysh and Weiss, 1979). Furthermore, exercise training stimulates chemical changes such as increases in levels of neurotransmitters (Hattori et al., 1994) and neurotrophic factors (Neeper et al., 1995; Neeper et al., 1996). It is hypothesized that such changes might alter the neural response to injury and reduce both functional and cognitive impairment. Indeed, there is evidence that treadmill training for at least two weeks prior to stroke can reduce infarct size (Wang et al., 2001). Furthermore, pre-ischemic exercise training has been shown to reduce neuronal death following stroke (Stummer et al., 1994). Finally, exercise training prior to brain injury has been shown to prevent impairments in spatial learning and memory performance (Carro et al., 2001).

The present experiment was designed to investigate whether pre-infarction exercise increases resistance to peri-infarct dysfunction within the motor cortex following a focal ischemic insult. Specifically, we examined the effects of pre-stroke endurance training on the preservation of forelimb representations within the motor cortex and recovery of motor impairments following focal cortical ischemia.

MATERIALS AND METHODS

Animals and Motor Training

Twenty one male Long-Evans hooded rats (Canadian Center for Behavioural Neuroscience) approximately 90 days of age (350-450g) were randomly assigned to either a Voluntary eXercise (VX) condition or an Inactive Control (IC) condition, with littermates equally distributed across condition. Animals in the VX group (n=11) were housed with free access to a running wheel (36cm in diameter) attached to their cages. The number of wheel rotations was recorded and used to calculate the distance traveled over 30 days. VX animals were weighed every three days to monitor any weight loss. IC animals (n=10) were housed in standard cages (11X40X40cm) with minimal opportunity for exercise. All animals were maintained under a 12:12 -h light/dark cycle with food and water available ad libitum. Prior the first intracortical microstimulation (ICMS) session, animals in each condition were randomly assigned to either the Control (n=11) or the Ischemic (n=10) conditions. Control animals did not receive an electrocoagulation lesion, while the Ischemic animals did immediately following the first ICMS session. All animals were allowed to recover for 21 days in their home cages. VX animals did not have access to the running wheels during the recovery period. Motor abilities were tested

every seven days using a battery of behavioural tests, including limb-use asymmetry test (Schallert et al., 2000), adhesive dot test (Schallert et al., 2000) and rung hanging test. Following the 21 day recovery period, all animals were remapped.

Table 1 : Experimental design for Voluntary eXercise, Inactive Control, Ischemic and Control conditions.

	Voluntary eXercise	**Inactive Control**
Ischemic	5	5
Control	6	5

Electrophysiological Mapping

Standard intracortical microstimulation (ICMS) techniques were used to generate detailed maps of forelimb representations within the motor cortex (Kleim et al., 2002a). All animals were food deprived for 16 hours prior to each ICMS session. Prior to surgery, animals were anesthetized with ketamine hydrochloride (70mg/kg i.p.) and xylazine (5mg/kg i.p.), receiving ketamine (70 mg/kg i.p.) as needed. A craniotomy was performed randomly over either the left of right motor cortex. A small puncture was made in the cisterna magna to reduce edema prior to retraction of the dura. The exposed cortex was then covered with warm silicon oil (37°C). A glass microelectrode controlled by a hydraulic microdrive was used to make penetrations to a depth of ~1550 μm (corresponding to cortical layer V), with an interpenetration distance of 375 μm. Stimulation consisted of thirteen, 200 μs cathodal pulses delivered at 350 Hz from an electrically isolated stimulation circuit. Animals were maintained in a prone position with consistent limb support. At each site, stimulating current was gradually increased (up to 60 μA) until a movement could be detected (threshold current). If no movement could be detected at < 60 μA, the site was defined as non-responsive. Forelimb

movements were classified as either distal (wrist/digit) or proximal (elbow/shoulder). Representational maps were generated from the pattern of electrode penetrations. An image analysis program (CANVAS 3.5) was used to calculate the areal extent of the caudal forelimb area (CFA) (Remple et al., 2001).

Cortical Infarction

Immediately following the first mapping session, Ischemic animals received a small infarction corresponding to approximately 25% of the distal representations within the CFA. This infarct was created via electrocoagulation of all surface vasculature within the targeted area until vasculature was no longer visible [Nudo, 1996 #186]. The infarcted vessels included very thin capillaries as well as larger vessels. All bypassing vasculature was avoided in order to confirm the damage to the specific physiologically defined area. Potential reperfusion was examined for two minutes, and if reperfusion was observed, the tissue was again coagulated until the vasculature was no longer visible. Peri-infarct cortex was defined as the CFA representations outside the region in which surface vasculature was coagulated. Following the creation of the infarction, the craniotomy was cleared of silicon oil and closed with gel film and gel foam. SDI Wave flowable composite dental epoxy was applied to the opening and then cured with Dentsply QH175 UV light until hardened. The cisterna magna incision was sealed and the scalp closed with wound clips. Following surgery, animals were allowed to recover for 24 hours in transport tubs placed on heating pads. They were given water and fed mashed food pellets. In addition, they were injected with 4.0cc of Ringers solution to prevent dehydration.

Behavioural Testing

i. Limb- use Asymmetry

Rats were placed in a transparent cylinder (dimensions) and allowed to explore freely for 5 minutes. A mirror was placed below the cylinder at an angle to allow the experimenter to video tape all movements of the animals. Each training session was recorded using a Sony Digital Video Camera Recorder (Model number DCR-PC) onto Memorex SHQ T-120 VHS tapes. The cylinder was high enough that the animal could not reach the top edge by rearing and wide enough to allow approximately 2 cm between the tip of the snout and the base of the tail when the animal was not rearing. The behaviours scored to determine the extent of forelimb-use asymmetry displayed by the animal were the independent use of the left of right forelimb for contacting the wall during a full rear, to initiate a weight-shifting movement or to regain center of gravity while moving laterally in a vertical position (Schallert et al., 2000). All scoring was done using a VCR with slow motion and frame by frame capabilities. Limb asymmetry was measured by dividing the number placements by the impaired forelimb by the total number of limb placements made during wall movements.

ii. Adhesive Dot Removal

An adhesive stimuli (160 mm^2 piece of black electrical tape) was attached to the distal-radial aspect of both forelimbs of the animal. The labels were attached first to either the left or right forelimb in a random fashion. The animal was then placed in an empty plastic cage and allowed to contact and removed the adhesive stimuli one at a time using its teeth. The time required to remove each adhesive stimulus was recorded for each of three trials.

iii. Rung-hanging

Animals were required to hang by their forepaws from a wooden rung suspended above a foam pad. Animals were tested on three trials and the diameter of the rung was increased on each successive trial. Each trial was recorded using a Sony Digital Video Camera Recorder (Model number DCR-PC() onto Memorex SHQ T-120 VHS tapes. The amount of time that the animal was able to hang from the rung before dropping onto the foam pad was recorded.

Tissue Preparation

Immediately following the second mapping session, a small volume of cresyl violet dye was injectd via a Hamilton syringe into a cortical area corresponding to the lateral extent of the rostro-caudal midpoint of the CFA. Rats were then overdosed with pentobarbital (120mg/kg) and perfused transcardially with 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The mapped hemisphere was sectioned coronally (300μm) on a vibratome. Sections of tissue were taken from the primary motor cortex as identified by the presence of the dye injection. From these sections, blocks of motor cortex extending from the pia to the white matter were removed under a dissection microscope. The tissue blocks were then washed in 0.1 M cacodylate, postfixed in 2% osmium tetroxide/1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 2 hours and *en bloc* stained with 2% uranyl acetate for 45 minutes. Samples were then dehydrated through a series of alcohols before being transferred into propylene oxide and gradually embedded in Eponate resin. All tissue samples were coded with respect to treatment condition prior to stereological analysis.

Estimation of Blood Vessel Density

One block of tissue from each animal was randomly chosen and 32 serial 1-μm sections were taken using a diamond knife and an ultramicrotome. These sections were stained with toluidine blue and cortical layer V was identified by the presence of large pyramidal cells and the absence of layer IV granule cells. A computer-assisted microscope and a stereology software package (Adobe Photoshop 5.5) were used to obtain a measure of blood vessel density. Briefly, an unbiased counting frame (3.78 X $10^4 \mu m^2$) was placed on an image of layer V tissue and all the blood vessels within the frame were counted (mag 40X). Six sections were counted per animal, with six counts being done per section (corresponding to the left, right and middle areas of the section). The number of blood vessels per unit area was calculated by dividing the total number of blood vessels counted by the area of the counting frame.

RESULTS

Exercise Training

A within subject analysis of variance revealed a significant effect of TIME on distance traveled per day in the VX animals ($F(10,29)=5.54$; $p<0.0001$). VX animals traveled a mean total distance of 46.3 (± 21.2) km across the 30 day training period (Figure 2). Comparable results have been reported in similar experiments (Kleim et al., 2002a).

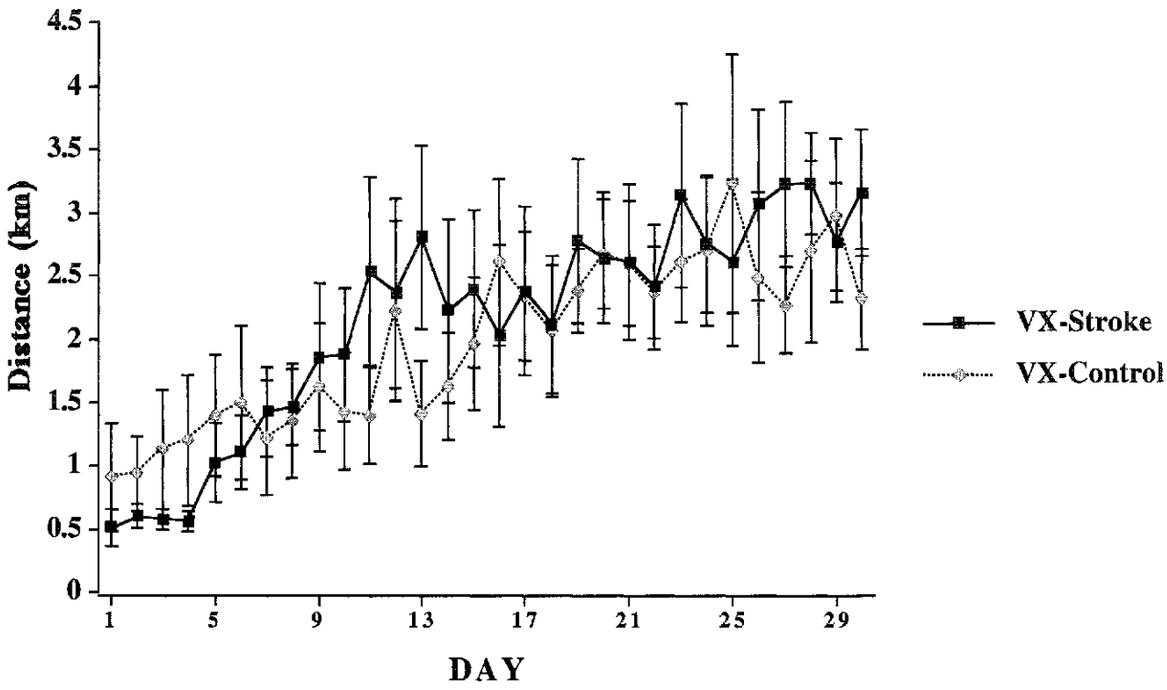

Figure 1 - Average distance traveled per day (±S.E.M.) by the VX-Control and VX-Stroke animals. A significant increase in distance traveled per day was observed across the 30-day training period.

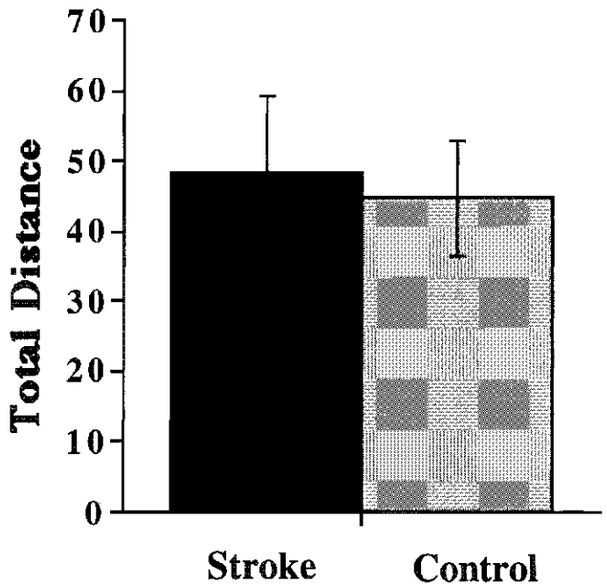

Figure 2 - Average total distance traveled across 30 days (±S.E.M.) by the VX-Stroke and VX-Control animals.

Peri-Infarct Movement Representations

A two way analysis of variance (ANOVA) with CONDITION (VX vs. IC) and TREATMENT (Stroke vs. Control) revealed a significant CONDITION X TREATMENT interaction on percentage of total functional peri-infarct map area [$F(1,18)= 8.54$; $p<0.01$] (Figure 3). Subsequent multiple comparisons (Fisher's PLSD; $p<0.05$) showed a significant reduction in percentage of total functional map area in the IC-Stroke animals only.

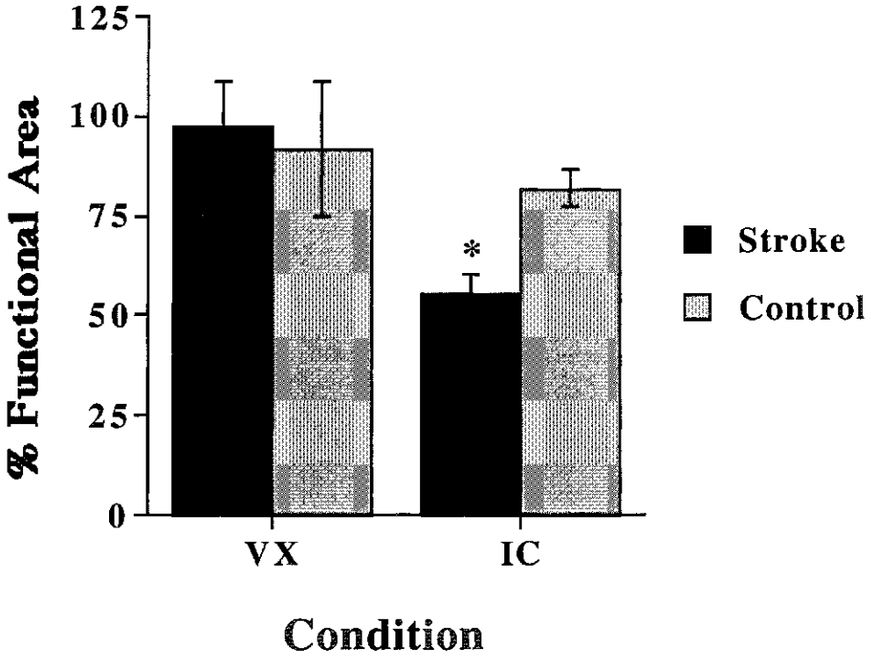

Figure 3 – Mean percentage (±SEM) of total functional area of CFA in VX and IC animals. IC Stroke animals showed a significant loss of functional map area compared to VX animals (*$p<0.05$).

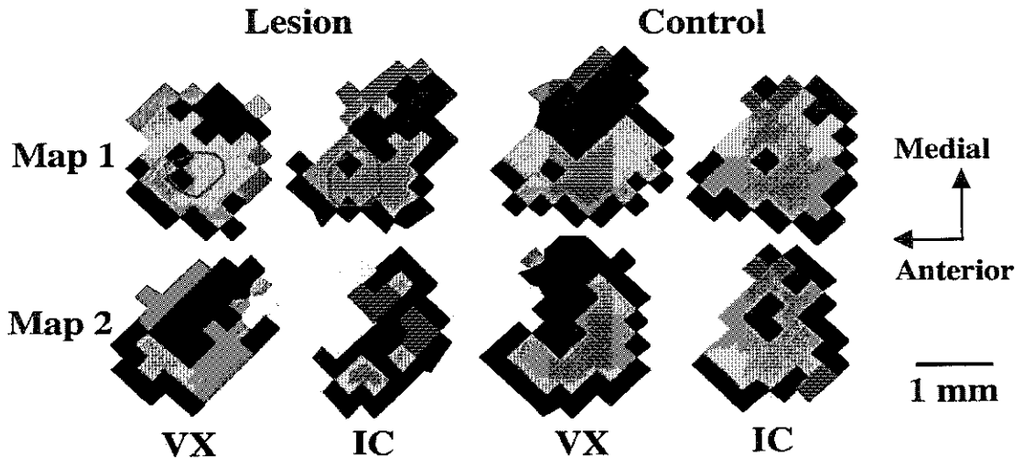

Figure 4 – Representative motor maps from Voluntary exercise (VX) Stroke (A), Inactive Control (IC) Stroke (B), VX Control (C), and IC Control (D) conditions. Distal movement representations (wrist and digit) are shown in green, proximal movement representations (elbow and shoulder) are shown in blue, head/neck representations are shown in yellow, vibrissae representations are shown in pink and hindlimb representations are shown in light blue. The red circle indicates the area of insult.

Blood Vessel Density

A two way ANOVA with TREATMENT and CONDITION as between subject factors revealed a significant TREATMENT X CONDITION interaction on blood vessel density [$F(1,17)= 6.23$; $p<0.05$]. Subsequent multiple comparisons (Fishers's PLSD; $p<0.05$) revealed that IC-Control animals had a significantly lower mean blood vessel density than all other conditions (Figure 6).

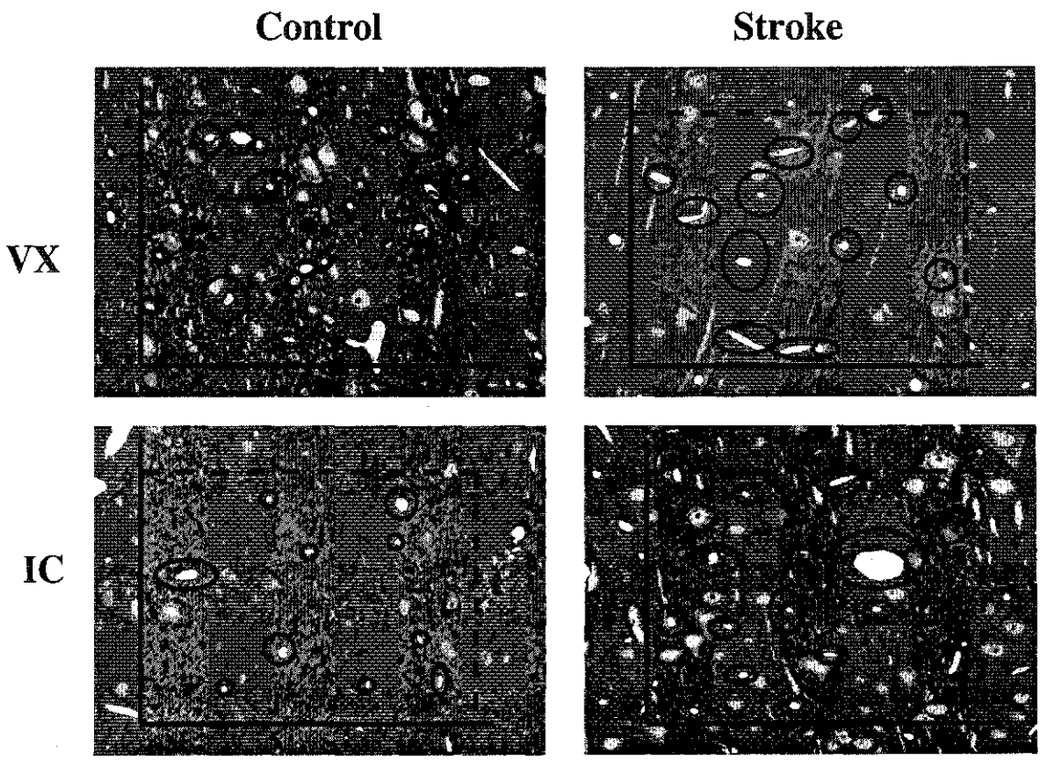

Figure 5. Representative 1 micron sections from eXercise (VX) and inactive control (IC) animals. The number of capillary profiles (circled in black) within an unbiased counting frame of known area were counted.

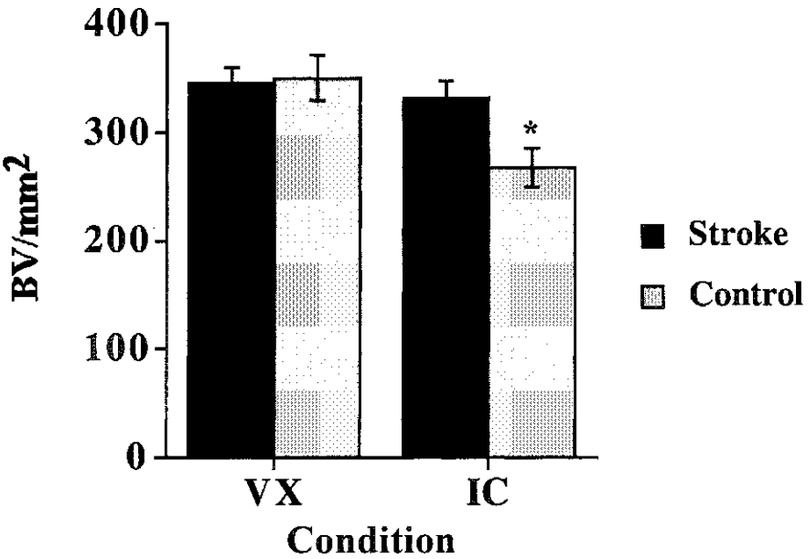

Figure 6 - Density of blood vessels within Layer V of the motor cortex for Inactive Control (IC) Control, Inactive Control (IC) Stroke, Voluntary eXercise (VX) Control and Voluntary eXercise (VX) Stroke conditions. IC Control animals had a significantly lower mean number of blood vessels/mm^2 than all other conditions (*$p<0.05$).

Behavioural Tests

i. Paw Placement Task

A repeated measures ANOVA with TIME as a within subject factor and CONDITION and TREATMENT as between subject factors was performed on lesioned paw use. Results showed no significant effect of TREATMENT (F (1,17)= 0.09, p=0.773) on paw placement from the pre-surgery time point to one, two and three weeks post-surgery (Figure 7). There was also no significant effect of CONDITION (F (1,17) = 0.63E-6, p = 0.998) or TIME (F(3,51)=2.46; p=0.073). Finally, there was no significant effect of TIME, TREATMENT and CONDITION (F (3,51) = 0.56, p = 0.642).

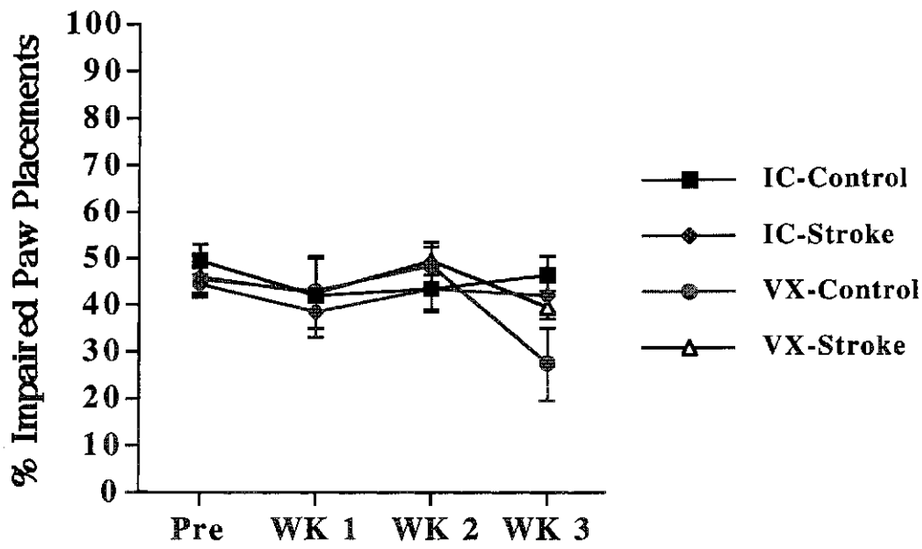

Figure 7 - Mean percentage of paw placements made by impaired paw for Inactive Control (IC) Control, Inactive Control (IC) Stroke, Voluntary eXercise (VX) Control and Voluntary eXercise (VX) Stroke conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There were no significant differences between IC and VX in either Control or Stroke conditions (p>0.05).

ii. Adhesive Dot Removal Task

A repeated measures ANOVA with TIME as a within subject factor and CONDITION and TREATMENT as between subject factors was performed on impaired paw preference. Results showed a significant effect of TIME (F (3,15)=9.36; p<0.0001) on time required to remove sticky dot from impaired paw from the pre-surgery time point to one, two and three weeks post-surgery (Figure 8). There was no significant effect of TREATMENT (F (1,15)= 0.01, p=0.9132). There was also no significant effect of CONDITION (F (1,15) = 0.12; p = 0.735) or TREATMENT X CONDITION (F(1,15)=3.62; p=0.077). Finally, there was no significant effect of TIME, TREATMENT and CONDITION (F(1,45)=0.15; p=0.926).

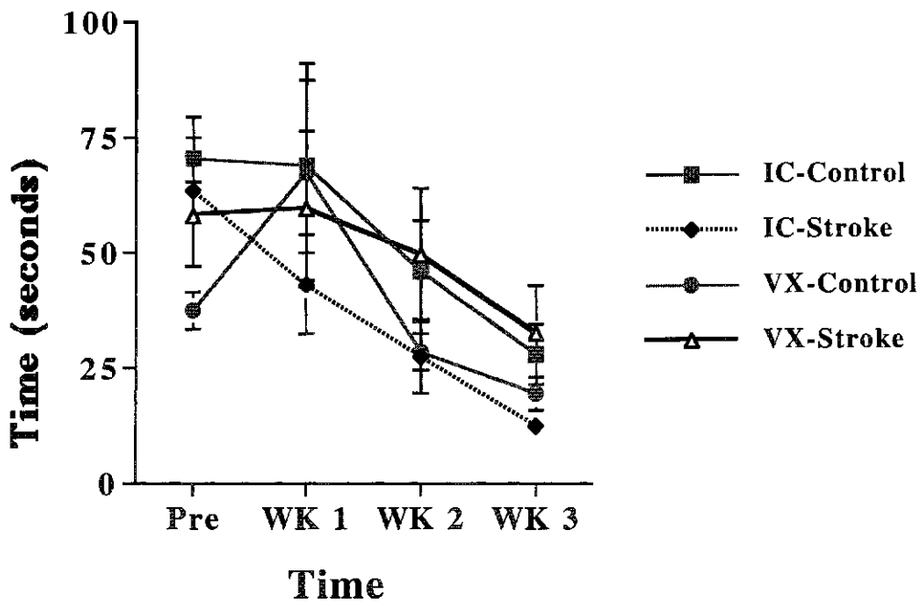

Figure 8 -Mean time required for adhesive dot removal from impaired paw for Inactive Control (IC) Control, Inactive Control (IC) Stroke, Voluntary eXercise (VX) Control and Voluntary eXercise (VX) Stroke conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There was a significant effect of TIME (p<0.0001).

A repeated measures ANOVA with TIME as a within subject factor and CONDITION and TREATMENT as between subject factors was also performed on intact paw preference. Results showed a significant effect of TIME ($F(3,15)=12.44$; $p<0.0001$) on time required to remove sticky dot from intact paw from the pre-surgery time point to one, two and three weeks post-surgery (Figure 9). There was no significant effect of TREATMENT (F (1,15)= 0.94, p=0.348). There was also no significant effect of CONDITION (F (1,15) = 0.01; p = 0.911) or TREATMENT X CONDITION ($F(1,15)=0.12$; p=0.735). Finally, there was no significant effect of TIME, TREATMENT and CONDITION ($F(1,45)=2.24$; p=0.096).

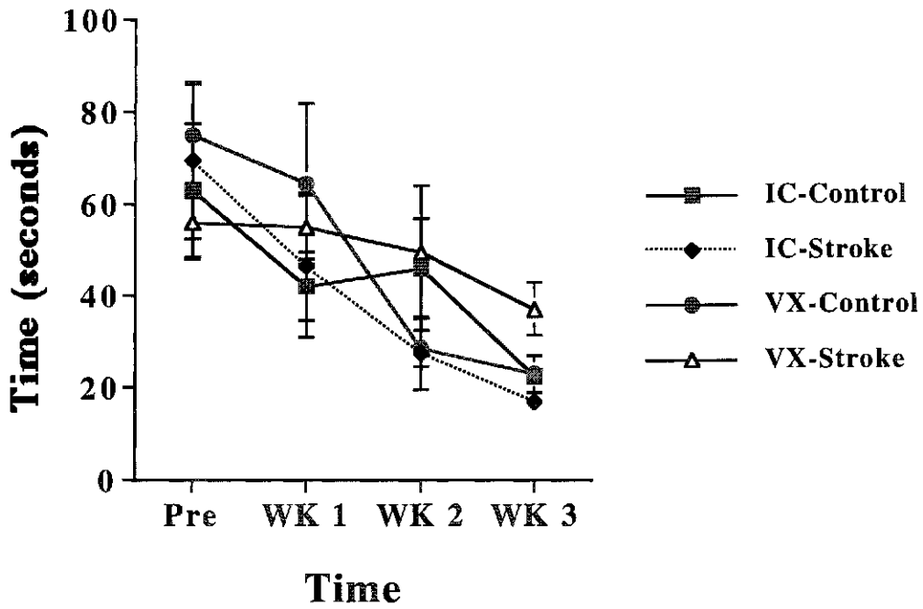

Figure 9 -Mean time required for adhesive dot removal from intact paw for Inactive Control (IC) Control, Inactive Control (IC) Stroke, Voluntary eXercise (VX) Control and Voluntary eXercise (VX) Stroke conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There was a significant effect of TIME ($p<0.0001$).

iii. Rung-hanging Task

A repeated measures ANOVA with TIME as a within subject factor and CONDITION and TREATMENT as between subject factors was performed on rung hanging time. Results showed no significant effect of TREATMENT (F (1,16)= 0.137, p=0.7158) on rung hanging time from the pre-surgery time point to one, two and three weeks post-surgery (Figure 10). There was also no significant effect of CONDITION (F(1,16)=0.01; p=0.933) or TIME (F(1,16)=3.32; p=0.027). Finally, there was no significant effect TIME, TREATMENT and CONDITION (F(1,48)=1.15; p=0.340).

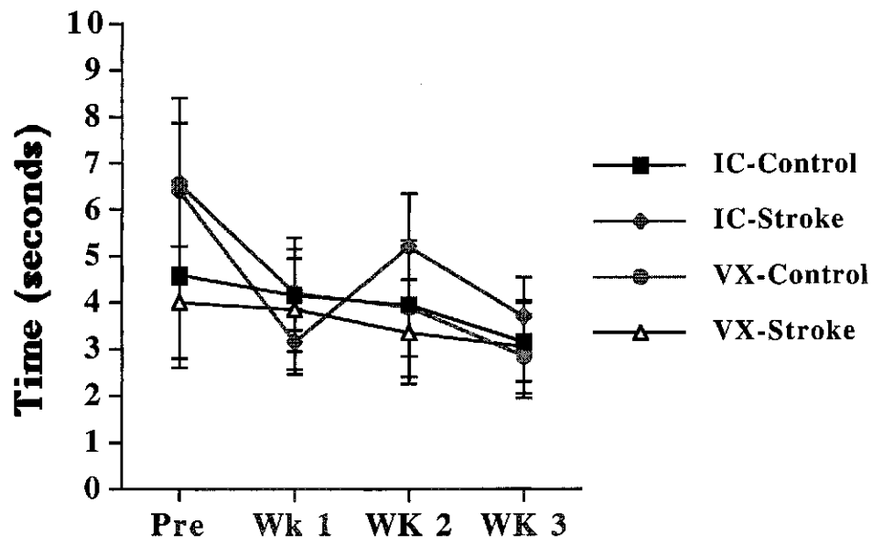

Figure 10 - Mean rung hanging time for Inactive Control (IC) Control, Inactive Control (IC) Stroke, Voluntary eXercise (VX) Control and Voluntary eXercise (VX) Stroke conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There were no significant differences between IC and VX in either Control or Stroke conditions (p>0.05).

DISCUSSION

Exercise can induce widespread changes across the central nervous system. The present results show that motor experience prior to ischemic insult reduces the amount of cortical dysfunction and initiates vascular changes in the motor cortex. Specifically, one month of exercise prior to ischemia significantly increases the amount of peri-infarct movement representations within motor cortex. Conversely, the IC-Stroke animals exhibit a significant loss of functional map area following the second mapping session. Finally, IC Control animals display a significantly lower mean number of blood vessels/mm^2 than animals in all other conditions.

The finding that pre-ischemic exercise reduces the magnitude of loss of movement representations in the peri-infarct area provides support for the idea that exercise attenuates the impact of a brain insult. This result is consistent with other current research findings. Carro et al (2001) showed that exercise prior to injury blocked neuronal impairment or loss and elicited functional recovery in several brain insult models of different etiology and anatomy. Further, Wang et al (2001) showed that treadmill training for two weeks prior to ischemic insult can reduce infarction size in a rat MCAO model (Wang et al., 2001). Thus, it is evident that exercise serves a neuroprotective function in that it has the capacity to reduce vulnerability to brain damage.

Exercise-induced alterations in neuronal metabolism and angiogenesis are possible mechanisms that may contribute to the reduction of diaschisis. Because restoration of perfusion in peri-ischemic regions is achieved partly via collateral capillaries (Krupinski et al., 1994), it is possible that an increased density of these

capillaries would promote enhanced reperfusion and better tissue survival. Indeed, it has been shown that higher blood vessel densities are correlated with a better prognosis in human stroke patients (Krupinski et al., 1994). The present results support this idea, as the VX-Stroke animals displayed an increased mean number of blood vessels/mm^2. It is interesting to note that the IC-Stroke animals also displayed an increased blood vessel density, which can likely be attributed primarily to ischemia-induced angiogenesis. However, because IC-Stroke animals exhibited a significant loss of functional map area, it is probable that the increases in capillary density that occur following stroke are not sufficient to prevent dysfunction in peri-infarct cortex.

Although the cellular mechanisms underlying the enhanced resistance to dysfunction are unclear, they may also be related to exercise-induced increases in various growth factors such as brain derived neurotrophic factor (BDNF) or insulin-like growth factor (IGF-I). BDNF has been shown to increase resistance to brain injury and promote neurological recovery (Schabitz et al., 2000). Similarly, IGF-I has been shown to be reduce neuronal death caused by insults of various types (Carro et al., 2001). Thus, it is possible that exercise-induced increases in several different neurotrophic factors may serve to be neuroprotective.

Although focal ischemic insults in the motor cortex have been shown to induce behavioural deficits in rodents (Goertzen, 2001), the present study did not show such a result. Neither VX-Stroke nor IC-Stroke animals showed any significant motor impairments compared to control animals. However, it is possible that the behavioural tasks that were chosen to assess gross motor ability were not sensitive enough. Thus, animals in the present study may have had more subtle deficits that could not be detected

by the motor tests that were administered. It is also possible that the size of the stroke was not sufficient to induce impairments in gross motor abilities. In support of this idea, lesion size and type have been shown to be related to degree of motor deficit (Whishaw, 2000). Further, although Schallert et al (Schallert et al., 2000) found deficits on the paw placement and adhesive dot removal tasks following focal ischemia, the model of ischemia was different (MCAO) and the extent of tissue damage was greater.

The present experiment evaluated the effects of pre-ischemic endurance exercise on cortical dysfunction following focal ischemic infarct. The results indicated that exercise decreases the amount of cortical dysfunction and initiates vascular changes in the motor cortex. These results provide further support for the idea that motor experience prior to stroke may enhance functional recovery. As a result, determining the neurochemical basis for the increased resistance to peri-infarct dysfunction may help to guide the development of pharmacological therapies for high-risk stroke patients.

CHAPTER THREE :

INCREASED RESISTANCE TO PERI-INFARCT DIASCHISIS FOLLOWING FOCAL CORTICAL INFARCT IS DEPENDENT ON BDNF SYNTHESIS

INTRODUCTION

Von Monakow was one of the first to recognize the occurrence of extra-infarct neural dysfunction, or diaschisis, associated with stroke (von Monakow, 1914). Now, over one hundred years later, research has confirmed the existence of extra-infarct dysfunction following stroke in both the ipsilateral and contralateral hemispheres. Furthermore, it is hypothesized that this extra-infarct dysfunction may contribute to motor impairment. Impairments in skilled forelimb use following ischemia has been associated with a loss of microstimulation-evoked forelimb movement representations that extend beyond the infarct (Nudo and Milliken, 1996; Goertzen, 2001). From this, it follows that alleviation of peri-infarct dysfunction may contribute to recovery of motor impairment following stroke. The administration of skilled rehabilitation training in both rats and monkeys has been shown to reverse the loss of movement representations and to reduce motor impairment (Friel et al., 2000; Goertzen, 2001), even when administration is delayed for up to 28 days (VandenBerg, 2002).

It has been hypothesized that exercise may function to provide some means of enhancing and protecting brain function (Cotman and Engesser-Cesar, 2002). Exercise has the capacity to initiate a number of metabolic, structural and chemical changes that are theorized to enhance brain plasticity and cognitive functioning (Cotman and Engesser-Cesar, 2002). Further, exercise may promote resistance to injury. Pre-stroke exercise has been shown to reduce infarct size (Wang et al., 2001) and neuronal death following stroke (Stummer et al., 1994). In addition, exercise also promotes the alleviation of diaschisis. We have previously shown that pre-infarction exercise increases

resistance to peri-infarct dysfunction within the motor cortex following a focal ischemic insult.

The cellular mechanisms underlying the enhanced resistance to ischemia-induced dysfunction are unclear. One possible mechanism may be the exercise-induced upregulation of several neurotrophic factors, including NGF, IGF-I and BDNF. BDNF is an especially suitable candidate for enhancing resistance to dysfunction. BDNF promotes both neuronal growth and survival (Huang and Reichardt, 2001). It also plays a critical role in synaptic plasticity, as it has the ability to enhance synaptic efficacy (Lu, 2003). Further, several recent studies have shown that administration of BDNF both prior to and following ischemic stroke reduces infarct size and promote neurological recovery (Schabitz et al., 2000; Yanamoto et al., 2000; Andsberg et al., 2002).

The present experiment was designed to investigate whether exercise-induced resistance to peri-infarct dysfunction is related to BDNF synthesis in the motor cortex. Specifically, we examined the effects of pre-infarct exercise-induced upregulation of BDNF on forelimb movement representations in the motor cortex following focal cortical ischemia. We also investigated if reducing BDNF synthesis would affect recovery of motor impairments.

MATERIALS AND METHODS

Experimental Design

Twenty-one male Long-Evans hooded rats (Canadian Center for Behavioural Neuroscience) approximately 90 days of age (350-450g) were randomly assigned to either a Scrambled Oligonucleotide (SC-ODN) or BDNF Oligonucleotide (BD-ODN), with littermates equally distributed across condition. Following cannula implantation, all

animals were housed with free access to a running wheel (Wodent Wheels, 0.3 m diameter) attached to their cages. The distance traveled over 30 days was recorded by a Sigma bicycle computer that was calibrated to the size of the wheel. All animals were maintained under a 12:12 hour light/dark cycle with food and water available ad libitum. During this 30 day exercise period, animals were infused with either SC-ODN (n=10) or BD-ODN (n=11) every second day. Immediately following the first intracortical microstimulation (ICMS) session, all animals received an electrocoagulation lesion. Animals were allowed to recover for 21 days in their home cages without access to the running wheels. Motor abilities were tested prior to and every seven days following the first ICMS session using the grid walking and forepaw inhibition tasks. Following the 21-day recovery period, all animals were remapped.

Cannula Implantation

Cannulas were implanted randomly in the right or left hemisphere of each animal. Prior to surgery, animals were anesthetized using Somnitol (70mg/kg i.p.) and Atropine (0.03 mg i.p. for animals weighing > 400g; 0.02 i.p. for animals weighing < 400g). A 4 mm guide cannula (Plastics One Inc) was implanted 0.88 posterior and 1.35 lateral to Bregma. A 5.5 mm infusion cannula was then introduced to the lateral ventricle via the guide cannula. Skull screws and dental cement were used to anchor the cannula to the skull. Following cannula implantation, animals were administered 0.06cc Metacam (i.p.). Animals recovered for five days before given access to running wheels.

Antisense Oligonucleotides

Phosphorothioate-modified antisense oligonucleotides for BDNF (5'-

TCTTCCCCTTTTAATGGT-3') and Scrambled (5'-ACCATTAAAAGGGGAAGA-3') were obtained through GeneLink. The SC-ODN served as a control and contained the same base composition as the BD-ODN, but in a randomized order. Upon receipt, antisense oligonucleotides were reconstituted with sterile phosphate buffer solution to a concentration of 1.5 nmol/μL. Reconstituted antisense was either kept frozen (when not in use) or stored on ice (when in use) at all times.

Antisense Infusion

During the 30-day running period, animals were infused with either SC-ODN or BD-ODN every second day. Animals were infused with 2.0 μl of solution per day (corresponding to 3.0 nmol of oligonucleotides). The solutions were infused through an infusion cannula that extended 1.5 mm beyond the tip of the guide cannula and was connected via polyethylene tubing to a Hamilton syringe. A microprocessor controlled infusion pump was used to deliver the infusions at a rate of 0.33 nmol/minute. The animal was unrestrained and sat on the handler's lap during the infusion period. At the end of each daily infusion period, the infusion cannula was removed and replaced by a 4 mm dummy cannula to prevent fluid escape from the ventricle.

Electrophysiological Mapping

At the end of the 30 day exercise period, standard intracortical microstimulation (ICMS) techniques were used to generate detailed maps of forelimb representations within the motor cortex (Kleim et al., 2002a). All animals were food deprived for 16 hours prior to each ICMS session. Prior to surgery, animals were anesthetized with ketamine hydrochloride (70mg/kg i.p.) and xylazine (5mg/kg i.p.), receiving ketamine

(70 mg/kg i.p.) as needed. A craniotomy was performed randomly over either the left of right motor cortex. A small puncture was made in the cisterna magna to reduce edema prior to retraction of the dura. The exposed cortex was then covered with warm silicon oil (37°C). A glass microelectrode controlled by a hydraulic microdrive was used to make penetrations to a depth of ~1550 μm (corresponding to cortical layer V), with an interpenetration distance of 375 μm. Stimulation consisted of thirteen, 200 μs cathodal pulses delivered at 350 Hz from an electrically isolated stimulation circuit. Animals were maintained in a prone position with consistent limb support. At each site, stimulating current was gradually increased (up to 60 μA) until a movement could be detected (threshold current). If no movement could be detected at < 60 μA, the site was defined as non-responsive. Forelimb movements were classified as either distal (wrist/digit) or proximal (elbow/shoulder). Representational maps were generated from the pattern of electrode penetrations. An image analysis program (CANVAS 3.5) was used to calculate the areal extent of the caudal forelimb area (CFA) and the rostral forelimb area (RFA) (Remple et al., 2001). CFA movement representations were dissociated from RFA representations by a border of head, neck and vibrissae representations.

Cortical Infarction

Immediately following the first mapping session, animals received an infarction corresponding to approximately 50% of the distal representations within the CFA. This infarct was created via electrocoagulation of all surface vasculature within the targeted area until vasculature was no longer visible (Nudo and Milliken, 1996). The infarcted vessels included very thin capillaries as well as larger vessels. All bypassing vasculature was avoided in order to confirm the damage to the specific physiologically defined area.

Potential reperfusion was examined for two minutes, and if reperfusion was observed, the tissue was again coagulated until the vasculature was no longer visible. Peri-infarct cortex was defined as the CFA representations outside the region in which surface vasculature was coagulated. Following the creation of the infarction, the craniotomy was cleared of silicon oil and closed with gel film and gel foam. SDI Wave flowable composite dental epoxy was applied to the opening and then cured with Dentsply QH175 UV light until hardened. The cisterna magna incision was sealed and the scalp was sutured. Following surgery, animals were allowed to recover for 24 hours in transport tubs placed on heating pads. They were given water and fed mashed food pellets. In addition, they were injected with 4.0 ml of Ringers solution and 0.06cc of Metacam for pain relief.

Behavioural Testing

i. Rung walking

The runway consisted of a straight section 1 m in length with plexiglass walls 19 cm high and a square box at one end in which food was located. The width of the runway was adjusted to allow 1 cm on either side of the animal to prevent it from turning around. The floor of the runway was made of a readily changeable arrangement of horizontal steel rods 3 mm in diameter. An irregular but unchanged rung pattern was maintained throughout all trials. Each animal received three trials during each testing session and a video camera was positioned at a slight ventral angle, so that the positions of all four limbs could be filmed simultaneously from a ventral view (Gharbawie and Whishaw, 2003). Each training session was recorded using a Sony Digital Video Camera Recorder (Model number DCR-PC) onto Memorex SHQ T-120 VHS tapes.

All scoring was done using a VCR with slow motion and frame by frame capabilities. Forelimb and hindlimb was rated on a five-point scale as described previously by Gharbawie et al (2003). Briefly, if the foot placement appeared normal where the midportion of the palm was placed on the rung, it was given a score of zero. If placement on the rung was done using the wrist or digits of the forelimb or the heel or toes of the hind limb, it was given a score of one. If a limb was placed on a rung and slipped off during weight shifting without disturbing balance, it was given a score of two. If a limb was placed on a rung and slipped off during weight shifting causing a fall, it was given a score of three. If a limb missed the targeted rung completely and fell through the gap compromising body posture and balance, it was given a score of four. An error score was calculated for each forelimb and hindlimb by averaging the scores across the three trials.

ii. Forepaw Inhibition Task

Video recordings were made of rats swimming in a large rectangular aquarium (120cmX43cmX50 cm). Each training session was recorded using a Sony Digital Video Camera Recorder (Model number DCR-PC) onto Memorex SHQ T-120 VHS tapes. Water temperature was maintained at approximately 21 °C. A visible wire mesh escape platform onto which the animals could climb was placed at one end of the aquarium. Animals were pre-trained on the task for three trials per day for four subsequent days prior to the first day of testing. During the training phase, most animals used all four limbs to stroke, rapidly changed direction, and sometimes swam aimlessly (Gharbawie and Whishaw, 2003). Once animals were more familiar with the task, they held their forelimbs immobile under their chins and only used their hind limbs to propel through the

water. Each animal performed three trials during each testing session. Disruption to the normal swim pattern was quantified by counting the number of strokes by each forelimb (Gharbawie and Whishaw, 2003).

Tissue Preparation

Immediately following the second mapping session, a small volume of cresyl violet dye was injectd via a Hamilton syringe into a cortical area corresponding to the lateral extent of the rostro-caudal midpoint of the CFA. Rats were then overdosed with pentobarbital (120mg/kg) and perfused transcardially with 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The mapped hemisphere was sectioned coronally (300μm) on a vibratome. Sections of tissue were taken from the primary motor cortex as identified by the presence of the dye injection. From these sections, blocks of motor cortex extending from the pia to the white matter were removed under a dissection microscope. The tissue blocks were then washed in 0.1 M cacodylate, postfixed in 2% osmium tetroxide/1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 2 hours and *en bloc* stained with 2% uranyl acetate for 45 minutes. Samples were then dehydrated through a series of alcohols before being transferred into propylene oxide and gradually embedded in Eponate resin. All tissue samples were coded with respect to treatment condition prior to stereological analysis.

Estimation of Blood Vessel Density

One block of tissue from each animal was randomly chosen and 32 serial 1-μm sections were taken using a diamond knife and an ultramicrotome. These sections were stained with toluidine blue and cortical layer V was identified by the presence of large

pyramidal cells and the absence of layer IV granule cells. A computer-assisted microscope and a stereology software package (Adobe Photoshop 5.5) were used to obtain a measure of blood vessel density. Briefly, an unbiased counting frame (3.78 X $10^4 \mu m^2$) was placed on an image of layer V tissue and all the blood vessels within the frame were counted (mag 40X). Six sections were counted per animal, with six counts being done per section (corresponding to the left, right and middle areas of the section). The number of blood vessels per unit area was calculated by dividing the total number of blood vessels counted by the area of the counting frame.

RESULTS

Exercise Training

SC-ODN animals traveled a mean total distance of 12.8 km (±12.3 km) across the 30 day training period, while BD-ODN animals traveled a mean total distance of 7.3 km (±8.65 km). A repeated measures ANOVA with TIME as a within subject factor and TREATMENT was performed on distance per two days. Results revealed a significant effect of TIME [$F(1,14)=1.780$; $p=0.042$) on distance traveled. However, there was no significant effect of TIME and TREATMENT [$F(1,14)=1.287$; $p=0.215$) on distance traveled.

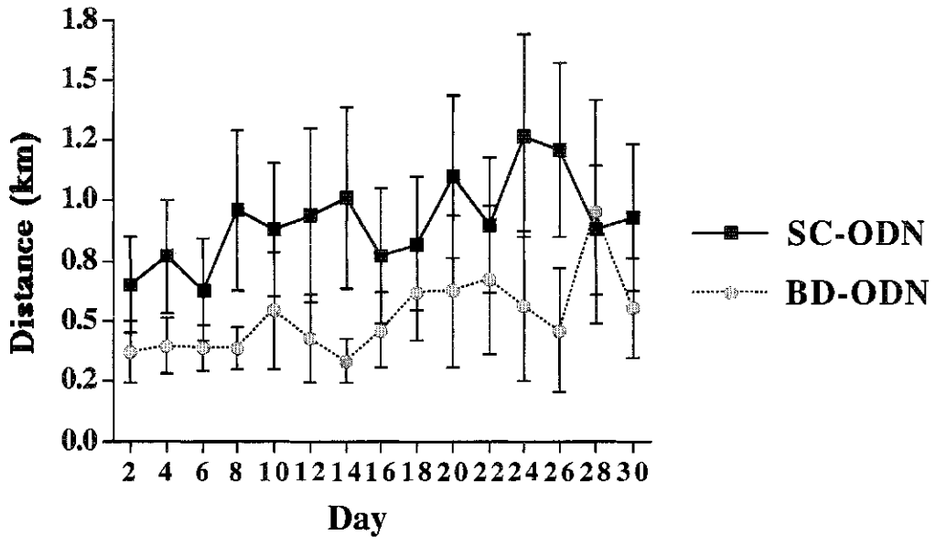

Figure 1 - Average distance traveled per day (±S.E.M.) by the voluntary exercise (VX) animals. A significant increase in distance traveled per day was observed across the 30-day training period ($p<0.05$).

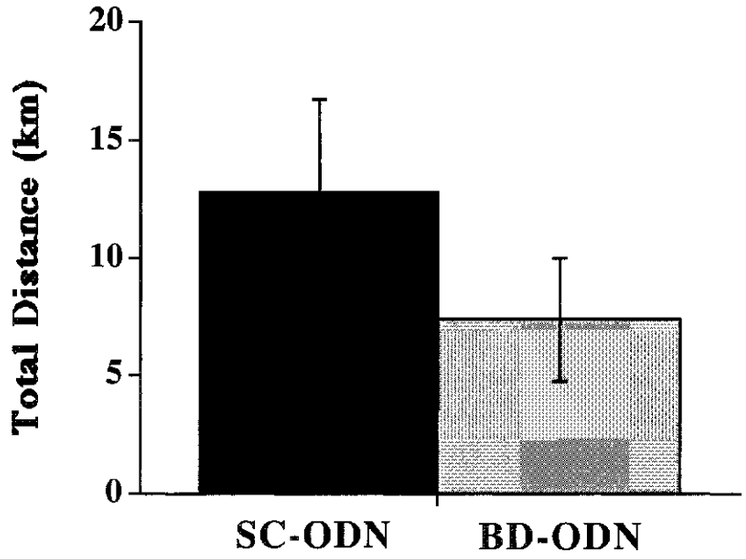

Figure 2 - Average total distance (±S.E.M.) traveled across the 30-day exercise period. There was no significant difference between SC-ODN and BD-ODN animals ($p>0.05$).

Peri-Infarct Movement Representations

i. Total Peri-infarct Movement Representations

A repeated measures ANOVA with MAP as a within subject factor and TREATMENT as a between subject factor was performed on total functional peri-infarct map area. Results showed a significant effect of TREATMENT ($F(1,18)=24.02$; $p=0.0001$) and mapping session (MAP) ($F(1,18)=133.48$; $p<0.0001$) on total functional peri-infarct map area from Map 1 to Map 2 (Figure 4). Finally, there was a significant effect of MAP X TREATMENT ($F(1,18)=5.27$; $p=0.034$).

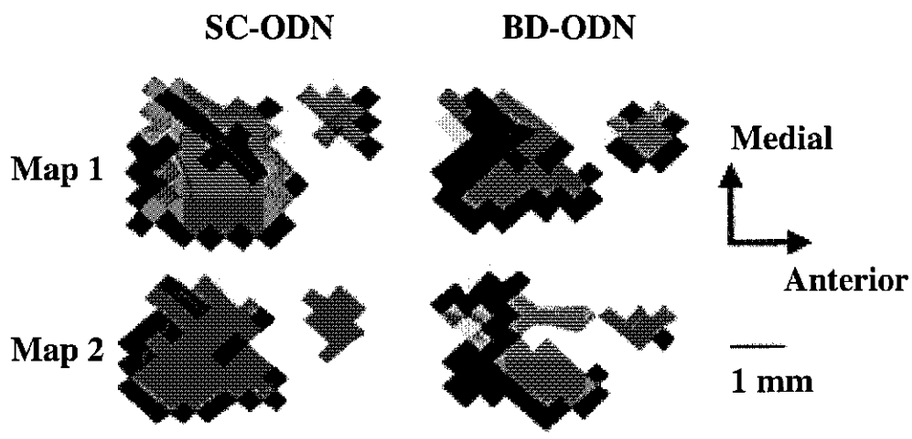

Figure 3 – Representative motor maps from SC-ODN and BD-ODN conditions. Distal movement representations (wrist and digit) are shown in green, proximal movement representations (elbow and shoulder) are shown in blue, head/neck representations are shown in yellow, vibrissae representations are shown in pink and hindlimb representations are shown in light blue. The red circle indicates the area of insult.

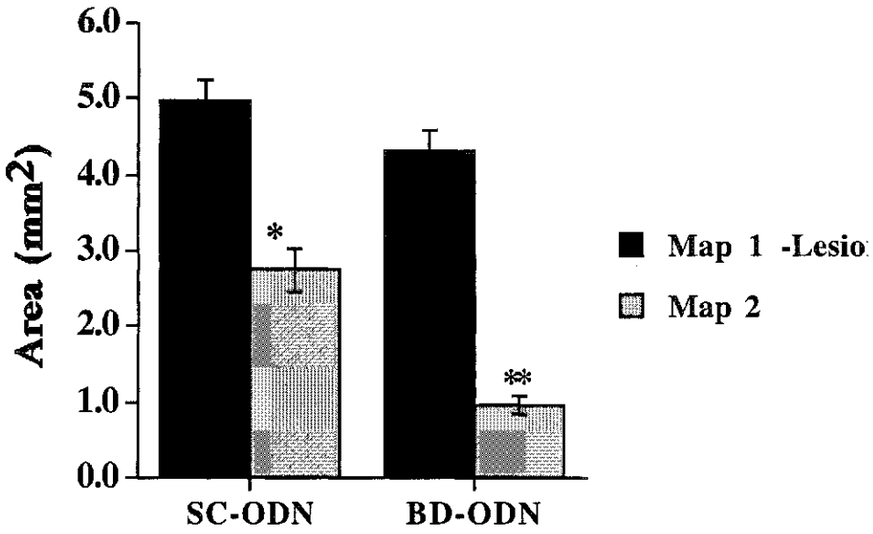

Figure 4 - Mean percentage (±SEM) of total functional area in motor cortex in SC-ODN and BD-ODN animals. BD-ODN animals showed a significant loss of total functional map area compared to SC-ODN animals (*p<0.05).

ii. CFA Peri-infarct Movement Representations

A repeated measures ANOVA with TIME as a within subject factor and TREATMENT as a between subject factor was performed on area of CFA peri-infarct movement representations. Results showed a significant effect of TREATMENT ($F(1,18)=18.48$; $p=0.0004$) and MAP ($F(1,18)=100.29$; $p<0.0001$) on peri-infarct map representations in the CFA from Map 1 to Map 2. However, there was no significant effect of MAP X TREATMENT ($F(1,18)=2.07$; $p=0.168$). Subsequent multiple comparisons (Fischer's PLSD, $p<0.05$) showed BD-ODN animals to have significant reduction in area of CFA peri-infarct movement representations at Map 2 (Figure 5).

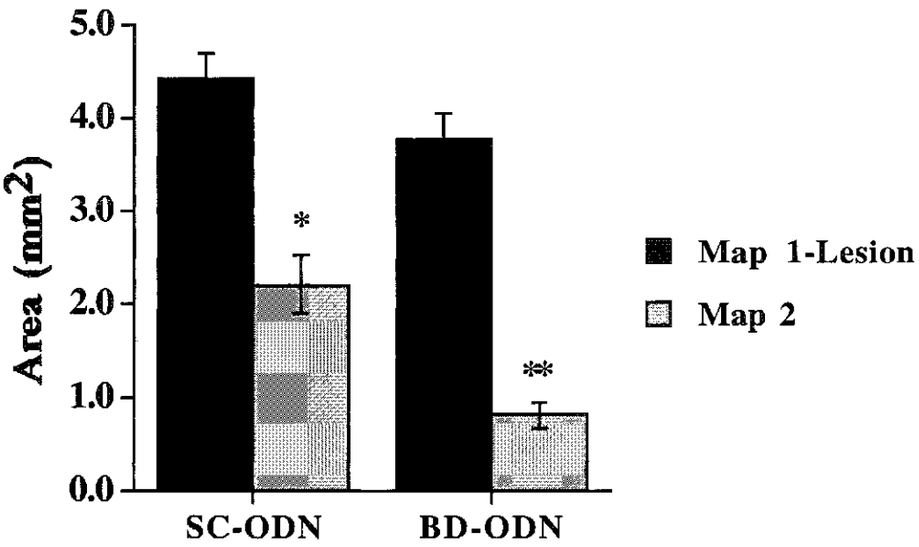

Figure 5 - Mean percentage (±SEM) of total functional area of CFA in SC-ODN and BD-ODN animals. BD-ODN animals showed a significant reduction in area of CFA peri-infarct representations at Map 2 ($p<0.05$).

iv. RFA Movement Representation

A repeated measures ANOVA with TIME as a within subject factor and TREATMENT as a between subject factor was performed on area of RFA movement representations (Figure 6). Results showed a significant effect of TREATMENT ($F(1,18)=8.74$; $p=0.009$), MAP ($F(1,18)=7.88$; $p=0.012$) and TREATMENT X MAP ($F(1,18)=6.47$; $p=0.020$).

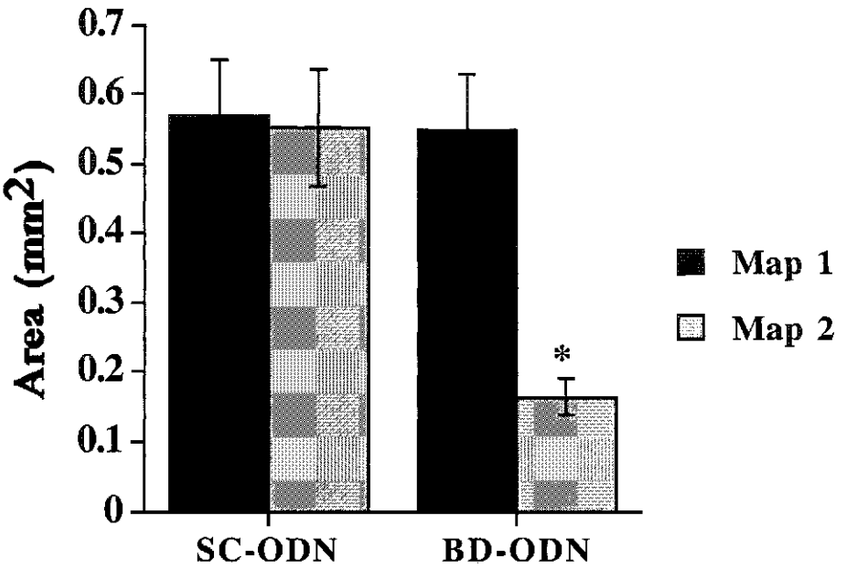

Figure 6 - Mean percentage (±SEM) of total functional RFA area in SC-ODN and BD-ODN. BD-ODN animals showed a significant loss of functional RFA map area compared to SC-ODN animals (*p<0.05).

Behavioural Tests

i. Rung walking

A repeated measures ANOVA with TIME as a within subject factor and TREATMENT as a between subject factor was performed on errors made with intact and impaired forelimb. Results showed no significant effect of TREATMENT on error score with intact paw (F (1,19)= 0.31; p=0.572) or impaired forelimb (F(1,19)=1.07; p=0.313). There was also no significant effect of TIME (F(3,19)=0.144; p=0.933) or TIME X TREATMENT (F (3,57) = 0.24, p = 0.871) on error score with intact forelimb (Figure 7). However, there was a significant effect of TIME X TREATMENT (F(3,57)=4.61; p=0.0059) on error score with impaired forelimb. Subsequent multiple comparisons (Fischer's PLSD, p<0.05) showed BD-ODN animals to have made significantly more errors than SC animals with impaired forepaw at week 2 post surgery (Figure 8).

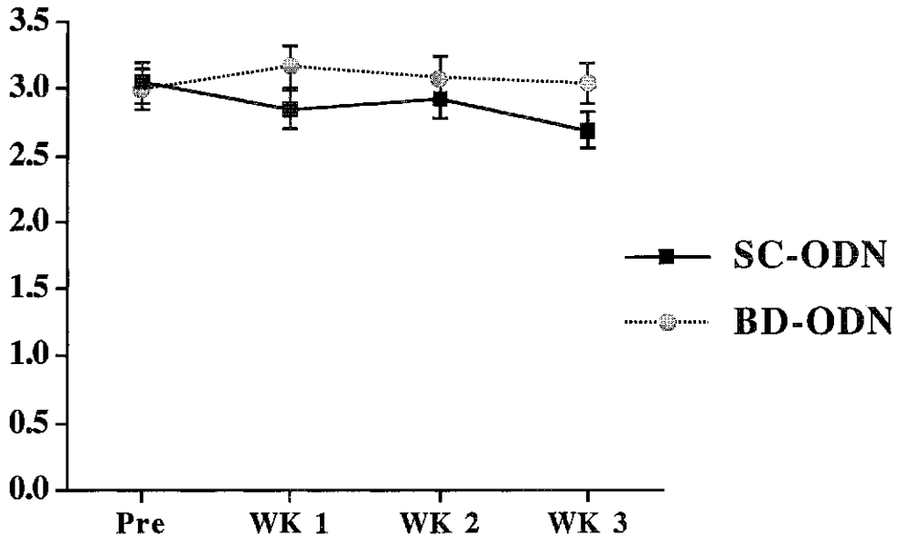

Figure 7 - Mean error score for intact forelimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There were no significant differences between BD-ODN and SC-ODN ($p>0.05$).

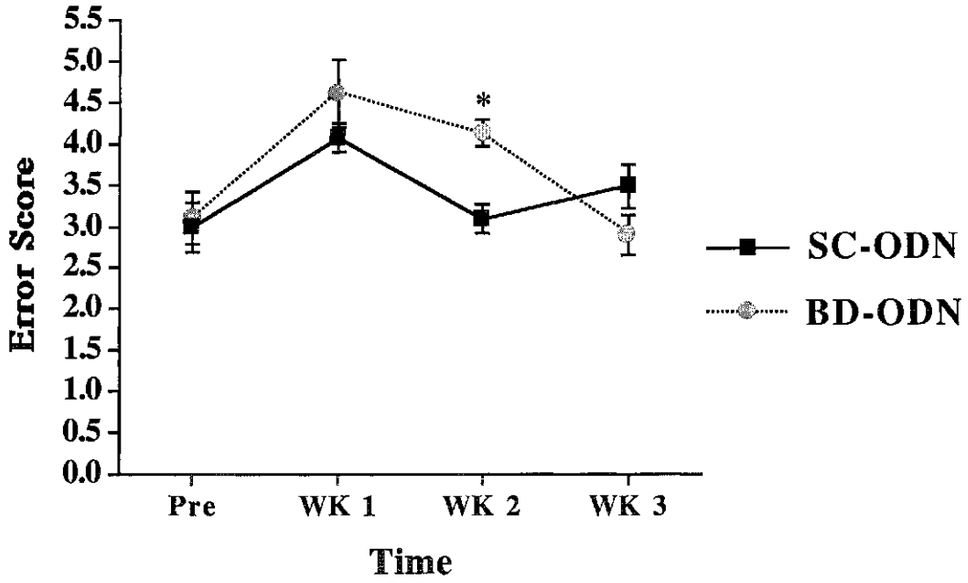

Figure 8 - Mean error score for impaired forelimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. BD-ODN animals had significantly greater error scores at Week 2 post stroke ($p<0.05$).

A repeated measures ANOVA with TIME as a within subject factor and TREATMENT as a between subject factor was performed on errors made with intact and impaired hindlimb. Results showed no significant effect of TREATMENT on error score with intact hindlimb (F (1,19)= 0.565; p=0.462) or impaired hindlimb (F(1,19)=1.596; p=0.222) from the pre-lesion time-point to one, two and three weeks post-surgery. There was also no significant effect of TIME (F(3,19)=1.577; p=0.205) or TIME X TREATMENT (F (3,57) = 0.515, p = 0.674) on error score with intact hindlimb (Figure 9). There was a significant effect of TIME (F(3,57)=7.332; p=<0.001) on error score with impaired hindlimb (Figure 10). However, there was no significant effect of TIME X TREATMENT (F(3,57)=2.467, p=0.0713) on error score with impaired hindlimb.

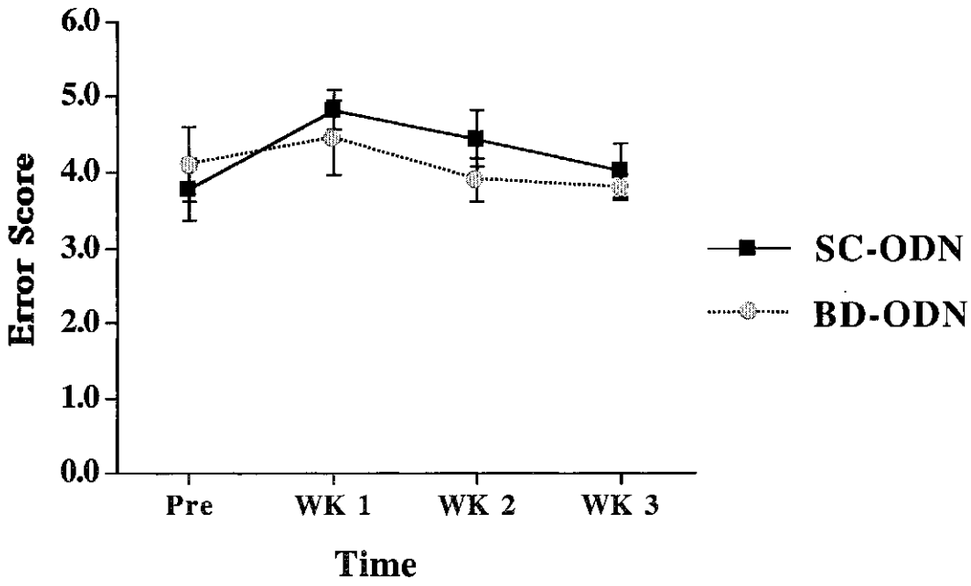

Figure 9 - Mean error score for intact hindlimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There were no significant differences between SC-ANT and BD-ANT animals (p>0.05).

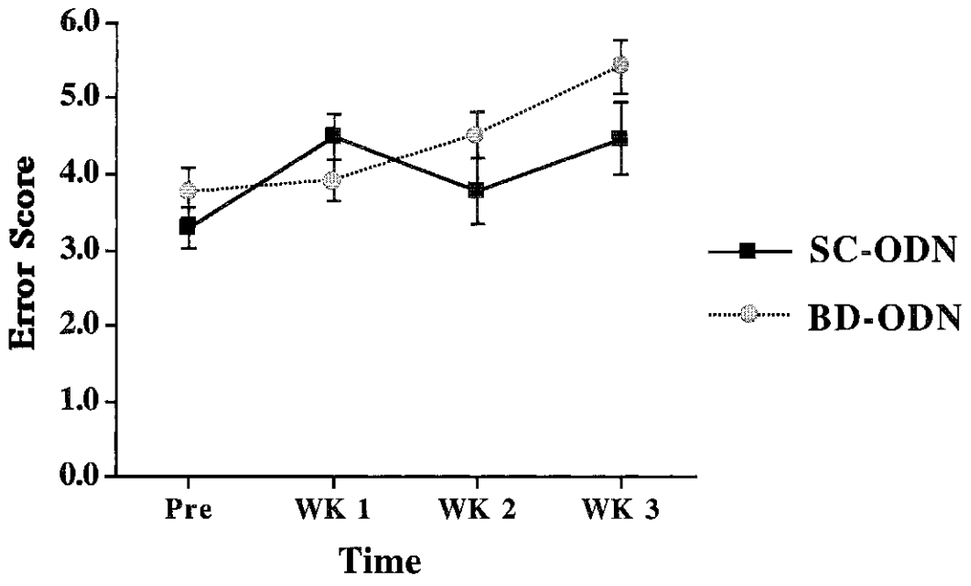

Figure 10 - Mean error score for impaired hindlimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There was a significant effect of TIME for both SC-ODN and BD-ODN animals ($p<0.05$).

ii. Forepaw Inhibition

A repeated measures ANOVA with TIME as a within subject factor and TREATMENT as a between subject factor was performed on strokes made with intact and impaired forelimb. Results showed no significant effect of TREATMENT on strokes made with intact forelimb ($F(1,19)=0.11$; $p=0.739$) or with impaired forelimb ($F(1,19)=3.73$; $p=0.069$). There was no significant effect of TIME ($F(3,57)=0.47$; $p=0.707$) or TIME X TREATMENT ($F(3,57)=0.48$; $p=0.696$) on intact forelimb strokes (Figure 11). In contrast, there was a significant effect of both TIME ($F(3,57)=4.41$; $p<0.05$) and TIME X TREATMENT ($F(3,57)=2.88$; $p<0.05$) on strokes made with impaired forelimb. Subsequent multiple comparisons (Fischer's PLSD, $p<0.05$) showed

BD-ODN animals to have made significantly more strokes than SC-ODN animals with impaired forepaw at week 2 post-surgery (Figure 12).

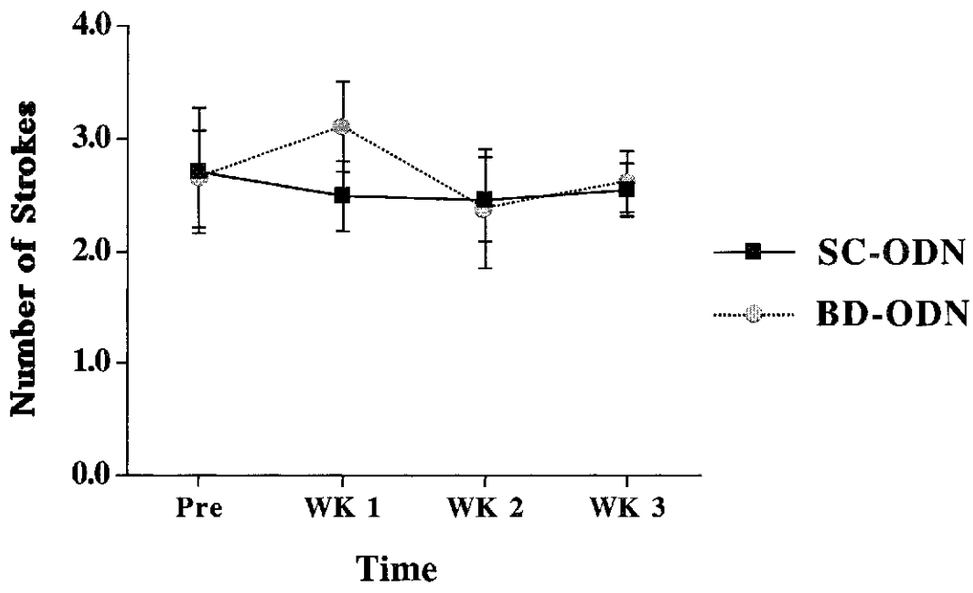

Figure 11 - Mean number of strokes (± SEM) for intact forelimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. There were no significant differences between SC-ODN and BD-ODN animals ($p>0.05$).

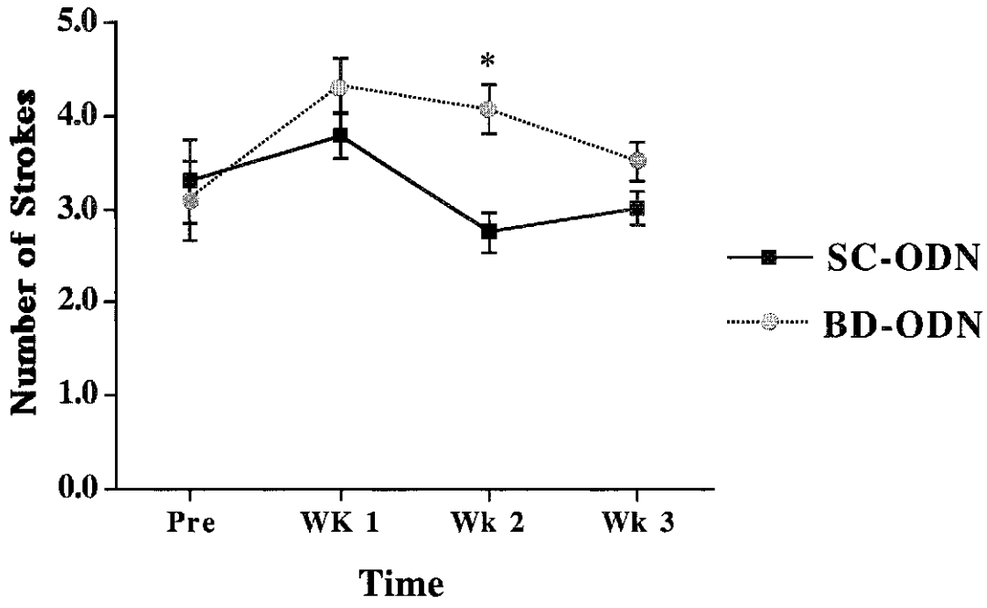

Figure 12 - Mean number of strokes (± SEM) for impaired forelimb for SC-ODN and BD-ODN conditions on pre-lesion, post-lesion Week 1, post-lesion Week 2 and post-lesion Week 3 time points. BD-ODN animals made a significantly greater number of strokes at Week 2 post-lesion ($p<0.05$).

Blood Vessel Density

A student's t test (unpaired, $p<0.05$) revealed no significant differences in number of blood vessels per mm^2 ($t(19)= -0.154$, $p=0.879$) between SC-ODN and BD-ODN animals (Figure 13).

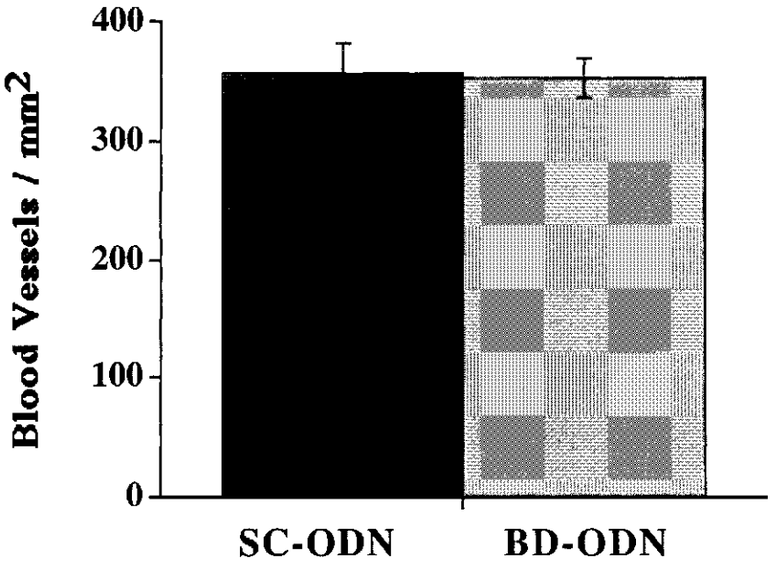

Figure 13- Density of blood vessels within Layer V of the motor cortex for SC-ODN and BD-ODN conditions. There was no significant difference between the two groups ($p>0.05$).

DISCUSSION

Exercise induces a robust pattern of neurochemical and anatomical changes in the brain. These changes are accompanied by enhanced cognition and resistance to brain damage (Cotman and Engesser-Cesar, 2002). Here we show that exercise-induced increases in BDNF synthesis are related to reduction of post-stroke cortical dysfunction. Specifically, one month of exercise prior to ischemia significantly reduces the amount of peri-infarct diaschisis within motor cortex of SC-ODN animals. Conversely, the BD-ODN animals exhibit a significant loss of total functional map area following the second mapping session. In comparison to SC-ODN animals, BD-ODN animals also exhibit significant behavioural deficits following stroke. Finally, there is no difference in mean number of blood vessels/mm^2. Thus, the reduction in peri-infarct dysfunction is likely related to BDNF synthesis and not to exercise-induced angiogenesis.

The present findings extend previous work showing that motor experience prior to ischemic insult reduces the amount of peri-infarct dysfunction within the motor cortex. BD-ODN animals show a significant loss of total functional map area from the first to the second mapping session. In addition, BD-ODN animals display a significantly greater loss of functional CFA area from Map 1 to Map 2 than SC-ODN animals. Thus, reducing BDNF synthesis prior to infarct adversely affected the level of functional recovery that could be achieved in the motor cortex of exercised animals.

A third interesting and unexpected finding was that the BD-ODN animals display a significant reduction in functional RFA area following the second mapping session. Since the SC-ODN animals did not exhibit such a reduction in functional RFA area, it is possible that this result could be attributed to remote effects caused by the ischemic infarct. It may be the case that a lesion of that size is sufficient to cause remote dysfunction not only in the CFA, but in the RFA as well. Thus, pre-stroke exercise may have been sufficient to reduce peri-infarct dysfunction in both the CFA and the RFA of the SC-ODN animals. In contrast, the reduced BDNF levels in the motor cortex of BD-ODN animals prior to stroke may underlie the dysfunction present in the CFA and RFA.

It is also possible that the significant reduction in functional RFA area in the BD-ODN animals could be related to the antisense infusion. Antisense oligonucleotides block protein synthesis by arresting translation (Szklarczyk, 1999). Thus, it is possible that the loss of functional RFA area in the BD-ODN animals could be related to reduced synthesis of BDNF in the motor cortex. This idea is supported by Kleim et al (2003), who recently showed that the injection of the BDNF antisense oligonucleotides into layer V of the motor cortex produced a loss of forelimb movement representations.

The present study indicates that the reduction in total functional map area in BD-ODN animals is most likely related to BDNF levels within the motor cortex. However, the mechanisms by which BDNF may be exerting its effects are not fully understood. BDNF is known to bind to the TrkB receptor, which can then activate a number of intracellular signal transduction cascades. Several of these signal transduction systems have been associated with BDNF-dependent neuroprotection, including the the ERK/MAP kinase pathway (Hetman et al., 1999) and the PI-3-K pathway (Klocker et al., 2000). Both of these pathways have been implicated as cell survival pathways. As suggested by Hetman et al (1999), it is probable that BDNF exerts its neuroprotective effects in cortical neurons via multiple survival pathways and that the specific pathway that is activated may depend on the type of cellular stress.

The finding that there was no significant difference in number of blood vessels/mm^2 between SC-ODN and BD-ODN animals provides further evidence for the idea that exercise-induced resistance to brain damage may be influenced primarily by BDNF. Several studies have recently shown that regional angiogenesis initiated either prior to (Bellomo et al., 2003) or following stroke (Sun et al., 2003) contributes to histological and functional improvement. However, the present study did not achieve this result. Despite the fact that the BD-ODN animals displayed an increase in the number of blood vessels/mm^2 compared to inactive control animals, these animals still exhibited a loss of total functional map area. Thus, it is possible that angiogenesis alone is not sufficient to reduce loss of peri-infarct movement representations. This idea is supported by the recent finding that gene expression of vascular endothelial growth factor (the primary trigger for angiogenesis) may be a trigger for the expression of BDNF in some

organ tissues (Weston et al., 2002). Thus, it is possible that the process of angiogenesis, regardless of whether it is initiated prior to or following stroke, loses some of its neuroprotective value in the absence of BDNF.

It is interesting to note that the BD-ODN animals displayed a slightly lower mean total distance traveled across the 30-day training period. Although the difference in total distance traveled was not significant between the SC-ODN and BD-ODN animals, it implies that the BD-ODN animals were less active on average. This may be an effect of the BDNF antisense infusion. Several animal models of depression have shown "depressed" animals to have reduced levels of locomotor activity (Nestler et al., 2002). Further, BDNF has been shown to be linked to depression (Siuciak et al., 1997; Shimizu et al., 2003). Thus, it is possible that the reduced synthesis of BDNF via infusion of BDNF antisense contributed to the reduced activity displayed by the BD animals.

The finding that all animals displayed post-stroke motor impairments on gross motor tasks is consistent with other research (Schallert et al., 2000). However, the BD-ODN animals do not display significantly greater motor impairment until post-stroke week two in comparison to SC-ODN animals. In addition, BD-ODN animals recovered motor ability to the level of the SC-ODN animals by post-stroke week three. Some degree of spontaneous motor recovery is known to take place following ischemia (Borlongan, 2000). Thus, it is possible that the SC-ODN animals are able to spontaneously recover to a greater extent than the BD-ODN animals. In the case of the BD-ODN animals, it is probable that reduced BDNF synthesis slows the progression of spontaneous motor recovery.

It is also interesting to note that the behavioural recovery displayed by the BD-ODN animals does not correlate with the reduction in total functional map area that is still present in these animals at three weeks post stroke. These findings indicate that impairments in gross motor ability may not be behaviourally representative of the reduction in functional map area. In support of this idea, Kleim et al (2003) recently found that animals injected with the protein synthesis inhibitor anisomycin show a profound loss in cortical movement representations and subsequent motor deficits on a skilled reaching task. However, these animals do not show gross impairments in motor behaviour, such as inability to perform reaching movements. They only show impairments when required to perform a skilled motor learning task. Thus, it is possible that motor maps may be representative of changes in cortical circuitry that specifically support skilled movement.

The present experiment evaluated the effects of pre-ischemic reduction of BDNF syntheis in the motor cortex of exercised animals on cortical dysfunction following focal ischemic infarct. The results indicated that exercise-induced decreases in the amount of cortical dysfunction are primarily related to BDNF levels in the motor cortex and not to angiogenesis. These results provide additional insight into the mechanisms underlying exercise-induced functional recovery following stroke.

CHAPTER FOUR

GENERAL DISCUSSION

In Canada, 50,000 people suffer from a stroke each year. Most treatments focus on the post-stroke phase. An alternative approach is to employ interventions that increase resistance to deficits arising from stroke, and there are several groups that are at high risk for stroke that would benefit from such interventions. The results of this thesis provide evidence that exercise is a viable method for increasing resistance to brain damage.

This thesis addressed the effects of pre-stroke endurance exercise on recovery from ischemic infarct and the role of brain derived neurotrophic factor in exercise-induced reduction of peri-infarct dysfunction. Two specific questions were addressed. First, does pre-infarction exercise increase resistance to peri-infarct dysfunction within the motor cortex following a focal ischemic insult? Second, does pre-infarction reduction of BDNF synthesis affect exercise-induced resistance to peri-infarct dysfunction within motor cortex following a focal ischemic insult? Results from the first experiment showed that pre-infarct exercise reduced the loss of peri-infarct movement representations in the motor cortex following focal cortical infarct. Results from the second experiment showed that this exercise-induced reduction in peri-infarct dysfunction is dependent on BDNF synthesis.

How Are Peri-infarct Movement Representations Lost After Stroke?

In order to be able to demonstrate dysfunction in a brain area, one must be able to reliably measure brain function. Here we used the well-established technique of intracortical microstimulation (ICMS) to define the functional organization of the motor cortex. Microelectrodes are lowered into layer V of the motor cortex and a small amount of current is passed to activate clusters of pyramidal cells. Because the corticospinal tract that passes directly from the cortex to the spinal cord is formed by the axons of some of

the layer V pyramidal cells (Porter and Lemon, 1995), stimulation of the pyramidal cells causes an impulse to be sent down the corticospinal tract. The final result is the production of an observable movement in the corresponding contralateral muscle.

Current from the microelectrode also indirectly stimulates pyramidal tract neurons by activating local afferents (Jankowska et al., 1975). In fact, the majority of pyramidal tract neurons that drive movement responses are trans-synaptically activated (Cheney and Fetz, 1985; Lemon et al., 1987). Further, the output volley produced by ICMS increases with each successive pulse, which suggests that pyramidal tract neurons are added through synaptic facilitation (Jankowska et al., 1975). Thus, the spatial organization of an ICMS derived motor map is dependent upon the spread of synaptic recruitment across localized groups of pyramidal tract neurons (Kleim et al, 2003). Therefore, in the absence of cell loss, loss of motor maps must be due to an inability to drive corticospinal neurons. The loss may therefore be due to either synaptic or neuronal dysfunction.

In the same way that ICMS can be used to define the function of areas in the intact motor cortex, it can also be used to detect dysfunction in areas of the injured brain. In this thesis, diaschisis, or dysfunction, is defined as the absence of forelimb movement representations within structurally intact areas of motor cortex following stroke. This dysfunction may occur as the result of several different processes related to ischemia.

i) Neuronal Dysfunction

Decreases in cerebral blood flow and ATP availability are known to adversely affect neuronal activity (Dirnagl et al., 1999). If neural activity cannot be metabolically supported following stroke, then ICMS will not be able to produced overt movements (VandenBerg, 2002). Indeed, dysfunction in peri-infarct regions is associated with

decreased cellular excitability (Neumann-Haefelin and Witte, 2000). Thus, ICMS may not be able to initiate movement responses if the excitability of peri-infarct cells is sufficiently reduced.

ii) Synaptic Dysfunction

Loss of the motor map in the peri-infarct area may be the result of dysfunction of intracortical synapses. Indeed, a loss of movement representations has been associated with a loss of synapses in the peri-infarct area (Goertzen, 2001). Further, Bolay et al (1998) recorded SEPS in the motor cortex following stroke and found persistent defects in synaptic transmission despite the recovery of axonal conductance. Finally, protein synthesis is inhibited in response to cerebral ischemia (Kohno et al., 1994). Because protein synthesis is required for the formation and maintenance of synaptic connectivity (Klintsova and Greenough, 1999), the inhibition of protein synthesis may underlie the loss of synapses that occurs in peri-infarct areas. In support of this idea, Kleim et al (2003) recently showed that inhibition of protein synthesis has been associated with a loss of movement representations and a profound reduction in synapse number and size. When taken together, these studies indicate that synaptic dysfunction may impair both normal synaptic activity and the facilitation required to sufficiently recruit enough synapses to drive localized groups of pyramidal tract neurons to produce movement.

How Does Exercise Increase Resistance to Peri-infarct Dysfunction?

Exercise is known to initiate a number of metabolic, structual and chemical changes that are theorized to enhance brain plasticity and thus promote recovery from injury. The results of this thesis support this idea by showing that exercise reduces the

amount of peri-infarct diaschisis in the motor cortex following focal cortical ischemia. This thesis specifically shows that BDNF is an important mediator of exercise-induced resistance to peri-infarct dysfunction. However, the cellular mechanisms by which BDNF promotes resistance to dysfunction are unclear. In addition, the role of other molecular mechanisms cannot be ruled out.

i. Brain-Derived Neurotrophic Factor

Exercise is known to cause a robust upregulation of BDNF (Neeper et al., 1996; Gomez-Pinilla et al., 1997). Further, BDNF has been shown to be neuroprotective in several models of brain ischemia (Zhang and Pardridge, 2001; Andsberg et al., 2002; Pardridge, 2002). Due to the crucial roles that BDNF plays in neuronal differentiation and survival, as well as in synaptic plasticity and transmission, it is believed to be an mediators of the beneficial effects of exercise on brain functioning (Cotman and Berchtold, 2002). However, the exact cellular mechanisms by which BDNF exerts its neuroprotective effects are not fully understood.

BDNF preferentially binds to TrkB tyrosine kinase receptors (Cotman and Berchtold, 2002). Once activated, this receptor is capable of triggering a number of intracellular signaling cascades, including the phoshpatidylinositol-3-kinase/protein kinase B pathway (PI-3-K), the phosholipase C-γ1 pathway and the mitogen-activated protein kinase (MAPK) pathway (Kaplan and Miller, 2000). Each of these pathways has been implicated as a cell survival pathway and has been associated with BDNF-induced neuroprotection (Hetman et al., 1999; Klocker et al., 2000).

a) PI3-K/PKB Kinase Pathway

The binding of BDNF to TrkB activates either the GTP-binding protein Ras or the Shc-Grb-2-Gab-1group of adaptor proteins (Patapoutian and Reichardt, 2001). Activation of either protein leads to the activation of the phosphatidylinosito-3-kinase (PI3-K) (Yamada and Nabeshima, 2003). PI3-K generates various D3-phosphorylated phosphatidylinositides, which serve as second messengers (Klocker et al., 2000). These second messengers evenutally lead to the activation of protein kinase B (PKB, also known as Akt kinase). PKB has been shown to protect against apoptosis via the phosphorylation and subsequent inhibition of human caspase-9, which is an initiator of the neuronal cell death effector caspase-3 (Cardone et al., 1998). PKB also phosphorylates and thereby inactivates the pro-apoptotic proteins Bcl-2-associated death protein (BAD) (Klocker et al., 2000; Patapoutian and Reichardt, 2001) and glycogen synthase kinase 3-β (GSK 3-β) (Patapoutian and Reichardt, 2001). Further, PKB phosphorylates IκB, which allows for the activation of the transcription factor NF-κB. NF-κB has been shown to promote neuronal survival (Middleton et al., 2000). Finally, PKB phosphorylates the mammalian target of rapamycin (mTOR), which plays an important role in the regulation of mRNA translation and subsequent protein synthesis (Patapoutian and Reichardt, 2001). Thus, protein synthesis initiated by the activation of PKB may be one mechanism that contributes to the prevention of synaptic dysfunction in peri-infarct tissue of exercised animals following stroke.

b) PLC-γ1 Pathway

The activated TrkB receptor can also phosphorylate and activate PLC-γ1 in the cell membrane. PLC-γ1 then hydrolyzes phosphatidylinositol-4,5-biphosphate (PIP_2) into

2 products: diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) (Levitan and Kaczmarek, 1997). IP_3 diffuses into the cytoplasm and binds to the endoplasmic reticulum, where it induces the release of Ca^{2+} stores and thereby activates the many pathways controlled by Ca^{2+} (Levitan and Kaczmarek, 1997). DAG remains in the cell membrane, where it causes translocation of protein kinase C (PKC). PKC is required for activation of the Erk cascade and for neurite outgrowth (Patapoutian and Reichardt, 2001). Further, PKC may also have a neuroprotective function. Rapid loss of neuronal PKC activity is a characteristic feature of cerebral ischemia that is linked to NMDA-induced cell death (Tremblay et al., 1999). However, as shown by Tremblay et al (1999), the prevention of ischemia-induced loss of PKC by exogenous BDNF significantly reduced cell death caused by glutamate. Thus, exercise-induced upregulation of BDNF prior to stroke may help to prevent the loss of PKC activity in peri-infarct tissue, which may in turn contribute to resistance against diaschisis.

c) MAPK Pathway

The mitogen-activated protein kinase pathway is intiated by the coupling of the activated TrkB protein to Ras. In addition to having other functions, Ras initiates the phosphorylation of mitogen-activated protein kinase/extracellular signal related kinase kinase (MEK). MEK then phosphorylates and activates MAP kinase. After phosphorylation, MAPK is subsequently translocated to the nucleus, where it activates transcription factors either directly or indirectly through kinases of the Rsk family (Pizzorusso et al., 2000). Transcription factors are proteins that bind to transcription initiation sites in regions of DNA and stimulate the transcription of those regions. Thus, one final outcome of the MAPK pathway is protein synthesis. The MAPK pathway has

also recently been associated with phosphorylation and inactivation of the pro-apoptotic BAD protein, although the mechanism by which this process occurs is not known (Ballif and Blenis, 2001).

d) cAMP Response Element-Binding Protein (CREB)

One MAPK-activated transcription factor that may be especially important to BDNF-induced neuroprotection is that of cAMP response element-binding protein (CREB). CREB is also activated by components of other intracellular signaling pathways, including protein kinase C, Ca^{2+} calmodulin kinases and ribosomal S6 kinase 2 (Walton and Dragunow, 2000). CREB has been described as the genetic switch that regulates the expression of genes necessary for the establishment of long-term synaptic plasticity (Alberini, 1999). It is involved in other aspects of neuronal functioning, including neuronal excitation (Moore et al., 1996) and development (Imaki et al., 1994). In addition, CEBP has been linked to cell survival. Several recent studies have shown that activated CREB promotes cell survival while inhibition of CREB phosphorylation triggers apotosis (Bonni et al., 1999; Walton and Dragunow, 2000; Jaworski et al., 2003). CREB may also play a crucial role in neuronal survival following ischemia (Walton et al., 1996; Jin et al., 2001).

It is interesting to note that CREB is able to activate the BDNF gene directly, thus promoting BDNF protein synthesis (Shieh et al., 1998; Tao et al., 1998). As suggested by Walton et al (2000), the CREB-regulated transcription of the BDNF gene may represent a positive feedback loop that may operate in some cell populations to promote resistance to brain injury. Both CREB (Alberini, 1999) and BDNF (Lu, 2003) have been

linked to synaptic potentiation; thus they may act to promote the reduction of diaschisis in peri-infarct tissue by preventing dysfunctional synaptic transmission.

ii. Vascular Endothelial Growth Factor and Angiogenesis

Exercise is known to upregulate vascular endothelial growth factor (VEGF) expression (Gavin and Wagner, 2001) and angiogenesis (Isaacs et al., 1992; Kleim et al., 2002a) in the motor cortex. Because VEGF promotes the formation of new blood vessels in the cerebral cortex, it is theorized that this increase in VEGF expression might be one mechanism by which exercise can promote resistance against ischemia. Upregulation of VEGF is an endogenous neuroprotective response that occurs within 12 hours following ischemia (Zhang et al., 2002). VEGF upregulation triggers the formation of new vessels within the peri-infarct tissue within 3 days following ischemia (Hayashi et al., 2003). Recent research shows that exogenous application of VEGF following ischemia stimulates angiogenesis (Sun et al., 2003), reduces infarct volume (Hayashi et al., 1998; Sun et al., 2003) and improves neurological outcome (Zhang et al., 2000; Sun et al., 2003). Finally, it has been shown that higher blood vessel densities are correlated with a better prognosis in human stroke patients (Krupinski et al., 1994). Thus, it is possible that the upregulation of VEGF-stimulated angiogenesis may be one mechanism by which pre-stroke exercise could promote resistance against ischemia.

In addition to promoting angiogenesis, VEGF may have other neuroprotective functions. VEGF has been shown to stimulate axonal outgrowth and enhance cell survival in the peripheral nervous system (Sondell et al., 1999; Sondell et al., 2000). Further, VEGF has been shown to both stimulate proliferation of neuronal precursors in the non-ischemic brain (Jin et al., 2002) and promote survival of proliferating cells of

neuronal lineage in the ischemic brain (Sun et al., 2003). Thus, it is apparent that VEGF may have neurotrophic effects in addition to its well-characterized angiogenic effects and that these functions might both contribute to the promotion of resistance against injury.

The results of this thesis showed that under conditions of reduced BDNF synthesis, angiogenesis is not sufficient to significantly reduce the amount of peri-infarct diaschisis in the motor cortex following focal cortical ischemia. It is possible the neurotrophic and angiogenic effects of VEGF may not be sufficient to promote resistance to injury in the absence of BDNF. Further, it has recently been shown VEGF expression may be a trigger for the expression of BDNF in some organ tissue (Weston et al., 2002). Thus, one of the mechanisms by which VEGF functions to reduce damage from stroke may be by triggering further BDNF expression.

iii. Exercise Preconditioning

A common theme across all models of preconditioning is the idea that the preconditioning technique itself is stressful to the brain. However, the stressful preconditioning signal is transduced into a tolerant response, possibly via the induction of protein kinases, transcription factors, and immediate early genes. Thus, it is possible that exercise acts as a form of insult to the brain, and induces plasticity by taxing the system such that systems that promote recovery are activated, even though serious damage has not occurred. The activation of these recovery-promoting systems may in turn enhance the brain's resistance to injury.

Limitations

The existence of limitations and confounding variables is a reality in any study. In the case of this thesis, the lesion size and behavioural tasks present two limiting factors. Furthermore, the age of the animals as well as the oligonucleotide infusion are two factors that may be considered possible confounding variables.

i. Lesion Size

In the experiment one, we did not observe gross motor impairments in either IC-Stroke or VX-Stroke animals following focal cortical infarct. This was attributed to be a result of a small lesion size (approximately 30% of the distal movement representations in the CFA). Thus, even though reduction of peri-infarct dysfunction was observed in response to pre-stroke exercise, this result could not be correlated with recovery of motor function. Because so many patients exhibit motor deficits following stroke, recovery of motor function should be the primary focus of any experimental model of stroke. Thus, in experiment two, we chose to increase the size of the lesion to approximately 50% of the forelimb representations in the CFA in order to produce motor impairments. Although this manipulation proved to be successful in producing motor impairments, recovery of motor function could not be correlated with reduction of peri-infarct dysfunction in the CFA. It is possible that the lesion size was too large to allow for a significant reduction in peri-infarct dysfunction in the CFA. When taken together, the results of these two experiments indicate that lesion size can be an important determinant of both level of behavioural deficit and functional recovery.

ii. Behavioural Tasks

As described above, the focal ischemic infarct administered to stroke animals in experiment one was not sufficient to induce motor deficits. Furthermore, although the infarct administered to all animals in experiment two was sufficient to induce motor impairments, recovery of motor function could not be correlated with reduction of peri-infarct dysfunction in the CFA. These results may also be related to the behavioural tasks that were administered. Although we have been able to achieve reliable motor impairments following focal ischemic infarct on the skilled reaching task, the use of this task was undesirable in both experiments in this thesis. This is because we have shown that skilled reach training can be used as a form of rehabilitation that can promote recovery of motor impairment and increase peri-infarct movement representations following stroke. Thus, we chose to use tasks that measured primarily gross motor abilities in order to avoid inducing rehabilitative-like effects during the post-stroke behavioural testing. However, in the case of the first experiment, these tasks may not have been sensitive to the subtle impairments that may have been induced by the small ischemic lesion.

In the case of the second experiment, different behavioural tasks were chosen in hopes that they would be more sensitive measurements of lesion-induced motor deficits. However, the occurrence of motor recovery in the BD animals that could not be correlated with reduction of peri-infarct dysfunction using these tasks. As a result, it is theorized that impairments in gross motor ability measured by these tasks may not be behaviourally representative of the reduction in functional map area. In support of this idea, Kleim et al (2003) showed that animals who displayed a profound loss of movement

representations following inhibition of protein synthesis did not exhibit gross impairments in motor behaviour such as failure to support body weight on the impaired limb. Instead, these animals only showed impairments when they encountered a challenging motor task requiring the performance of skilled movement (Kleim et al, 2003). Furthermore, Borlongan (2000) showed that repeated behavioural testing promoted partial spontaneous recovery of motor deficits following stroke, even if the testing was conducted only once per week. When taken together, these studies indicate that gross motor impairment may partially resolve independently of changes in motor map organization. Further, loss of movement representations may not be manifested as impairments in gross motor behaviour, but only as deficits in skilled motor behaviour. Thus, it is probable that SC-ANT and BD-ANT animals would all display impairments on a skilled motor task at Week 3, despite the fact that their gross motor impairments had recovered.

iii. Age of the Animals

All animals used in this thesis were approximately 90 days of age at the onset of experimentation. This age, which is analogous to young adulthood in humans, may not be directly applicable to exercise-induced resistance to injury in humans. Firstly, although humans do experience the occurrence of stroke in childhood and young adulthood, the majority of strokes occur later in life (Wade et al., 1985). Further, age is an important factor in determining recovery of function (Wade et al., 1985). However, it is assumed that the mechanisms of ischemic damage are the same in young and old rats. Thus, the mechanisms mediating exercise-induced resistance to injury should also be similar across all ages. It is important to note, however, that voluntary running levels are

decreased in aged rats (Peng et al., 1980; Peng and Kang, 1984; Shinoda and Miura, 1994). Thus, it could be hypothesized from the results of this thesis that older rats and, perhaps older humans, may have a reduced capacity for exercise-induced resistance to injury in comparison to younger rats. However, by examining exercise-induced plasticity under optimal circumstances (ie in young rats) prior to stroke, we can achieve a better understanding of the maximal amount of recovery that can be achieved.

iv. Suitability of the Model

The model used in this thesis mimics the effects of stroke via biopolar coagulation of surface vasculature within a physiologically defined area of the motor cortex. This method allows for the administration of damage to a confined area of cortex and the subsequent measurement of functional changes in surrounding areas. Although this method of focal ischemic infarct may not reflect the extent of damage that many human patients may experience following a stroke, it is still a useful model because it allows for the direct examination of the consequences of stroke. The underlying hypothesis is that plasticity within residual tissue underlies resistance/recovery. Therefore, the use of a model in which animals are younger (more plastic) and given small strokes maximizes the ability to study the phenomenon. It is assumed that the same mechanisms underlying recovery in young animals with small strokes also underly recovery in older animals with large strokes.

v. Antisense Infusion

Antisense oligonucleotides have become a commonly used tool for blocking gene expression in mammalian central nervous systems. However, the exact parameters to be

followed in regards to antisense usage in the brain are not clearly defined. In terms of this thesis, there are several limiting factors related to antisense infusion that should be mentioned. First, although the BDNF antisense was able to effectively block BDNF gene expression in the motor cortex, there is the possibility that it was also inhibiting other unexpected genes that were not analyzed. However, as suggested by Nicot et al (1997), this possibility is diminished the more oligonucleotide sequence is diminished in length. These authors recommend keeping the restricting the antisense sequence to 18 nucleotides in length, a parameter which was followed in this thesis.

Secondly, there is the issue of antisense-induced side effects. For example, Chiasson et al (1994) reported the occurrence of neurotoxic damage following repeated daily antisense infusion into the amygdala. However, these authors also reported that this damage was greatly diminished when the time interval between antisense infusions was extended. In this thesis, antisense was infused every 48 hours, thus neurotoxic damage induced by antisense infusion is not probable. However, since antisense infusions were completed prior to administration of a focal ischemic infarct, potential neurotoxic effects of antisense cannot be completely ruled out. Despite these possible limitations, administration of BDNF antisense in this thesis appears to have been successful method of reducing BDNF expression in motor cortex with no observable side effects.

Implications

Animal models of stroke have been used primarily to examine the neural mechanisms underlying cell death during ischemia. However, much less attention has been focused on the neural mechanisms underlying recovery following stroke. The general approach is to examine changes within residual neural tissue and correlate these

changes with behavioural recovery. Although this approach provides insight into how the brain adapts after stroke, it does not provide any clinically useful information. In other words, simply demonstrating brain plasticity in association with behavioural recovery does not, on its own, provide any information that can be used to enhance resistance to damage in people that may be at risk for stroke. Understanding the cellular and molecular mechanisms underlying this plasticity and how it can be enhanced will provide more potentially useful information.

The findings from this thesis are important for two reasons. First, we have demonstrated that exercise prior to stroke produces functional sparing, and not just a reduction in infarct size or cell death, as has previously been shown. This is demonstrated by the first experiment, which showed that pre-stroke exercise causes a reduction in the loss of forelimb movement representations within CFA following focal ischemic infarct. Second, we have provided evidence that the molecular mechanisms underlying this phenomenon involve BDNF. This is demonstrated by the second experiment, which showed that reducing BDNF levels in the cortex prior to stroke limits the exercise-induced reduction of forelimb movement representations in the motor cortex. Further, reduced BDNF levels prior to stroke are associated with increased motor impairments.

The results of this thesis provide further evidence for the characterization of exercise as a physiologically relevant context compared to sedentarism in the promotion of resistance to brain injury. This research may also have important implications for the development of pharmacological therapies for high-risk stroke patients. However, further study of the neurochemical basis for the increased resistance to peri-infarct dysfunction is

required in order to more fully understand the molecular mechanisms underlying neuronal self-repair. It is also important to note that there may not be one specific factor that can provide complete pharmacological substitution to for the effects of exercise on the brain. Thus, further exploration of the interactions between the different molecular mechanisms involved in mediating the beneficial effects of exercise on the brain should be emphasized in future research.

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