MODULATION OF RECOVERY AND COMPENSATION AFTER STROKE

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Dedication

I would like to dedicate this thesis to Keri, Adrian, Linda, and Katrina for their help, support, and friendship during these last two years. I would hate to think what the last two years would have been like without them.

Thesis Abstract

Stress has been shown to exacerbate cell death and cognitive deficits after ischemic injury in rodents, however, little is known of the effects of stress on motor recovery. The objective of this present thesis is to examine the effects of chronic stress on skilled motor recovery after devascularization lesion in rats. It was found that pre-lesion stress induced the most behavioural impairments, while post-lesion stress exacerbated infarct volume. The effects of chronic multiple stress on skilled motor recovery after lesion was also examined. Chronic multiple stress did not modulate skilled motor recovery nor did it have any influence on infarct volume. Additionally, stress had effect on edema after devascularization lesion. The present thesis suggests that the time of exposure to chronic stress in respect to the ischemic lesion, in addition to the type of stress, will differentially affect recovery and compensation in rats.

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Abbreviations

ICP = Intracranial Pressure
Et-1 = Endothelian-1
MCAO = Middle cerebral artery occlusion
2-VO = 2 vessel-occlusion
4-VO = 4 vessel occlusion
HPA axis = Hypothalamic-Pituitary-Adrenal Axis
CRH = Corticotrophin Releasing Hormone
MR = Mineralcorticoid Receptor
GR = Glucocorticoid Receptor
ADX = Adrenalectomy
DPO = Days Post Operation
g = Gram
mg = Milligram
cm = Centimeter
s = Seconds
mm = Millimeter
SEM = Standard error mean
°C = Degree celcius
μl = Microliter
ml = Milliliter
TMT = 2,4,5-Trimethylthiazoline
mA = Milliamps
RPM = Rotations per Minute
AM = Before noon
PM = After noon
hr = Hour

Chapter 1

The Pathology of Focal Ischemic Stroke

Stroke is one of the leading causes of mortality and disability in North America. There are two types of stroke: hemorrhagic and ischemic. Hemorrhagic stroke is the result of a bleeding in the brain and accounts for approximately 15% of all stroke cases. Ischemic stroke is the most common form of stroke and the third leading cause of death in first world countries (Dirnagl, 2006). There are two forms of ischemic stroke: focal and global ischemia. Focal ischemia is the result of a lack of blood flow to an area of the brain due to a blood clot, known also as an embolism. Strokes may occur anywhere in the brain, including the cerebrum, brainstem, and the cerebellum. The most common area of infarct is blockage of the middle cerebral artery, which affects blood supply mainly to the striatum and forebrain. Global ischemia is the disruption of blood flow to the entire brain. Global ischemia can be the result of a heart attack, drowning, suffocation, or any sort of blockage that results in a lack of blood flow to the head.

An ischemic stroke can be permanent or temporary, in which case the blood clot is eventually broken down, and reperfusion takes place. Neurons need a constant supply of oxygen and glucose, which is carried by blood. Without oxygen and glucose, neurons cannot produce energy and will start to undergo the process of excitotoxicity. Cell death after ischemic stroke is thought to be due to both apoptotic and necrotic pathway activation within the cells (Unal-Cevik et al., 2004).

In the core of the infarct blood flow drops below 20% of normal flow and cell death occurs within minutes (Dirnagl, 1999). Damage within the core is irreversible. The penumbra is the area around the core in which blood flow has dropped to 20-40% of

normal flow rate (Ginsberg, 2003). Cells within the penumbra are metabolically vulnerable to further injury and cell death occurs at a slower rate than in the core. The penumbra has become the target of experimental investigation. It is believed that saving cells within the penumbra from cell death via therapeutic treatment is key to improving recovery after stroke. Cell death, however, is not the only concern after stroke. After the infarct, cerebral edema becomes a major concern for stroke patients.

Cerebral Edema

Cerebral edema after ischemic stroke is thought to be a predictor of neurological deterioration (Davalos et al., 1999). Edema is the leading cause of mortality within the first week after stroke (Heo et al., 2005). Edema represents an accumulation of fluid, mainly water, in the brain, causing the tissue to swell and resulting in an increase in intracranial pressure (ICP) (Hossmann, 1989). Most cases of mortality shortly after stroke are thought to be due to herniation as a result of edema (Ayata and Ropper, 2002). Herniation is the result of increased ICP that increases pressure in the brain resulting in dysfunctional and dead tissue. There is also a high risk of mortality if the herniation involves the hypothalamus or brainstem.

Edema is a major problem in cases of middle cerebral artery occlusions (MCAO) in which mortality rate of progressive edema can reach 80% (Ayata and Ropper, 2002). Generally larger infarcts relate to larger levels of edema (Ayata and Ropper, 2002; Heo et al., 2005). Small infarcts, however, such as within the cerebellum, can result in edema levels that can manifest into clinical symptoms (Ayata and Ropper, 2002). Edema, as a result of increased ICP, can distort surrounding brain tissue leading to dysfunctional

tissue and herniation. If ICP levels become too high, then cerebral blood flow is reduced and auto-dilatation of vessels can fail. This drop of cerebral blood flow can further aggravate ischemic injury (Ayata and Ropper, 2002).

There are two forms of edema that occur after ischemic stroke: cytotoxic and vasogenic edema. The earliest phase of edema is cytotoxic, which reflects cellular swelling. Cells undergoing metabolic crisis will start to depolarize via the opening of sodium and calcium channels. As these channels open, water enters the cell, and the cell will begin to swell and take up more space. Vasogenic edema occurs as a result of these cells bursting and releasing the water into the extracellular space. During vasogenic edema the blood-brain-barrier becomes more permeable, and allows more water and fluid to pass from the blood into the extracellular space.

In humans, clinical signs of edema are evident between 2-5 days after stroke (Ayata and Ropper, 2002). The clinical signs of edema include the loss of consciousness, headache, nausea, vomiting, and worsening of neurological deficits (Ayata and Ropper, 2002). In rats, edema peaks at 2-3 days after an ischemia lesion (Hatashita and Hoff, 1989).

Animal Models of Stroke

A large number of animal models are used to study motor recovery after stroke, each mimicking specific aspects of stroke pathology. These models use many species including rats, mice, gerbils, rabbits, dogs, cats and primates. For the purpose of this thesis, only the rodent models will be discussed. Common rodent models of focal ischemia include devascularization, endothelin-1 (Et-1) injection, and MCAO. These

models of stroke result in an ischemic injury that is localized in a specific area of the brain.

Devascularization or Et-1 models of stroke are useful for studying ischemic infarcts located anywhere in the neocortex such as the motor cortex, frontal cortex, or occipital cortex. Damage is generally limited to the neocortex but there may be some underlying damage to the corpus callosum and stretching of the striatum. The devascularization and Et-1 models are useful tools for examining recovery of a specific behaviour after ischemic injury (for example, studying motor recovery after ischemic injury to the motor cortex, or recovery of vision after ischemic injury to the occipital cortex). These models are adequate for examining recovery after a small lesion. The lesion size can vary by adjusting the coordinates used for inducing the infarct (Whishaw, 2000) or increasing the amount of Et-1 infused into the neocortex (Windle et al., 2006). Nevertheless, lesions as a result of devascularization or Et-1 are smaller than lesions created by MCAO.

In devascularization, a craniotomy is performed, the dura mater is removed and the blood vessels of the underlying neocortex are removed using a cotton swab. Et-1 is a potent vasoconstrictor, which induces ischemic injury wherever it is infused (Windle et al., 2006). An advantage both these models share that is the lesion is easy to perform and is fairly consistent. There is little to no mortality after the lesion. A disadvantage of devascularization is that the lesion is limited to the neocortex (although some underlying white matter and striatal damage can be seen) and the clinical relevance of this model is questionable. The advantage of the Et-1 animal model of focal ischemic stroke is that it can be injected anywhere in the brain, so the ischemic injury is not limited to the

neocortex. Thus, Et-1 is the animal model of choice when examining the effects of ischemic injury within the hippocampus or striatum.

Another model of focal ischemic injury is MCAO, which results in a relatively large lesion. Structures of the brain that are damaged after MCAO vary depending on the severity of occlusion, but usually include the striatum and forebrain. MCAO is a good model to use when investigating recovery after large ischemic lesions. In humans the most common site of ischemic stroke is the middle cerebral artery. The rodent MCAO model allows researchers to mimic the most common form of ischemic injury, making it the model of most clinical relevance.

MCAO can be induced via thermocoagulation, clipping, infusion of Et-1 onto the middle cerebral artery or temporary blockage via a suture. The suture model of MCAO is an animal model of temporary ischemia in which the blood clot is broken down, and reperfusion takes place. The disadvantage to MCAO is that the lesion is difficult to perform, the lesion can be inconsistent and there also is a high mortality rate. As a result, studies often need to use high sample sizes to make up for the lesion inconsistency and high mortality rate.

Rodent models of global ischemia allow the study of ischemic injury to the entire forebrain. Rat models of global ischemia are more difficult to perform because rats have a complete Circle of Willis. The Circle of Willis is a collection of arteries that allows for collateral blood flow throughout the brain, should arterial blood flow into the brain become compromised. Clipping the two carotid arteries in rats (also known as 2-VO) in combination with inducing hypotension will result in ischemic injury to the forebrain and hippocampus. The animals must experience additional hypotension; otherwise collateral

blood flow via the vertebral arteries will reduce the level of ischemic injury in the 2-VO model. Another rat model of global ischemia is the 4-VO model. The two vertebral arteries are permanently occluded, and the two carotid arteries are clipped. The 4-VO model of forebrain ischemia does not require additional hypotension because collateral blood flow is blocked via occlusion of the vertebral arteries (Ginsberg and Busto, 1989). The 4-VO produces ischemic damage to the striatum and hippocampus. A disadvantage to the 4-VO model is that the surgical procedures are quite difficult to perform and it has high mortality rates. The most commonly used model of global ischemia is the bilateral common carotid artery occlusion model in the gerbil. Gerbils do not have a complete Circle of Willis, so clipping the two carotid arteries alone is sufficient to induce forebrain ischemia in gerbils. The gerbil model of global ischemia is often used due to its simplicity and ability to produce replicable hippocampal cell death (Laidley et al., 2005).

Regardless of the animal model used, it is important that the rodent models not only replicate the type of lesion seen in patients, but also produce similar behavioural deficits as well. Interestingly, different rodent models of focal ischemia have similar patterns of forelimb recovery (Gonzalez and Kolb, 2003; Gharbawie et al., 2005) as described below.

Motor Deficits and Recovery and Compensation after Stroke

In patients the most common disability after stroke is hemiparesis (muscle weakness) of the limbs (Krakauer, 2005). Other deficits include dystonia (involuntary muscle contraction), and spasticity (abnormal muscle rigidity). Following ischemic stroke, impairments in upper limb function is a common and challenging problem facing

patients (Cirstea and Levin, 2000; Lannin, 2004). Similar to humans, rats show lasting motor impairments in reaching and grasping after MCAO, or ischemic injury to the motor cortex (Whishaw, 2000; Gilmour et al., 2004; Metz et al., 2005a; Gharbawie et al., 2006). In cases of focal ischemia, most motor deficits occur on the contralateral side, however, motor impairments on the ipsilateral side have been noted in animal models of focal ischemia (Gonzalez et al., 2004) and in patients (Jones et al., 1989; Desrosiers et al., 1996; Sunderland et al., 1999; Sunderland, 2000).

Many stroke patients and rats with ischemic lesion exhibit some spontaneous recovery within weeks to months after the stroke. The term recovery suggests that there is a restoration of original function in the impaired limb. For example, after ischemic stroke, rats will show initial impairments in reaching and grasping for food. Over time, however, rats improve in their ability to grasp food pellets with their paw (Whishaw, 2000; Gilmour et al., 2004; Gharbawie et al., 2005). In patients and in rats, the extent of recovery may vary. Spontaneous recovery may occur partially due to the resolution of edema and cell death. Neuroplasticity within surrounding cortex or within the contralateral hemisphere may also play a role in the partial return of original function (Kolb, 1995).

A closer examination of reaching movements will show that patients and rats reach differently from before to after the ischemic infarct. While patients and rats may be showing improvements in the ability to use the upper limb functionally, it does not seem to be due to recovery of original function in the limb, but rather the development of new compensatory motor movements. Compensation is the development of new additional movements to assist the patient or rat complete a motor task after the loss of original

motor function. Some examples of compensation include adjustments in body movements while reaching. Patients will use exaggerated shoulder trunk rotation while reaching (Cirstea and Levin 2000; Roby-Brami et al 2003; Michaelsen et al., 2004; Michaelsen and Levin, 2004). Rats also exhibit excessive body rotation after MCAO and devascularization (Gharbawie et al., 2005). Rats with ischemic injury exhibit qualitative differences in reaching ability while reaching for food pellets in the single pellet reaching task (Metz et al., 2005; Gharbawie et al., 2005; Gharbawie et al., 2006). In animal models of stroke, the single pellet reaching task allows for quantitative and qualitative analysis of reaching to assess the development in compensatory reaching strategies while rats reach for food pellets. In the single pellet reaching task, the rats have to reach through a slot to grasp a single food pellet sitting on a tray just outside the slot. Rats will typically grasp the food pellet, rotate the paw to a 90° degree angle, and then lift the paw as it is withdrawn. The paw again rotates as the rat sits back, and with the assistance of the other paw the rat will eat the pellet. After ischemic injury rats will exhibit qualitative differences in reaching. When the rat successfully grasps the pellet, they will drag it back as the paw is withdrawn. Rats often are unable to rotate the contralateral paw, so the pellet is eaten via dragging the pellet to the edge of the slot then bringing the snout to the slot to eat the pellet. Ischemic injury can induce the loss in the ability to successfully lift and rotate the paw so to eat the pellet, the rat compensates by using an additional strategy of eating the pellet so to avoid having to try rotating the paw. Compensatory movement strategies have also been found in adult squirrel monkeys while reaching for food pellets (Friel and Nudo, 1998).

Whether compensatory reaching strategies are beneficial or not is up to debate. Development of compensatory motor strategies can assist the patient to complete a task they may otherwise be unable to accomplish. By compensating, however, the patient is no longer attempting to use previous motor movements which may develop into learned nonuse. Recovery of previous motor movements may be limited due to reliance on new compensatory motor movements.

To better understand motor recovery after ischemic injury, it is necessary to understand what factors can impede or improve spontaneous recovery and the development of compensatory motor strategies.

Therapies for Stroke and Obstacles to Find a Cure

Pre-clinical research has had numerous success stories in regards to developing neuroprotective therapies. Various drugs and treatments like MK-801 (Herz et al., 1998), hypothermia (Colbourne and Corbett, 1995; Colbourne et al., 2000), and minocycline (Hewlett and Corbett, 2006) have been found to reduce infarct size in rats and have been promising treatments for ischemic stroke. Unfortunately, there has been little translation of these and other treatments from bench to bedside despite the success of animal studies. This can be contributed to clinically irrelevant timing of treatment, short survival times, and a focus on using infarct size as a sole indicator of outcome, rather than behavioural function (Corbett and Nurse, 1998). Currently no effective neuroprotective treatment is available for patients, despite billions of dollars spent on preclinical research each year (Dirnagl, 2006). With no effective therapies available in the near future, the best way of improving recovery after stroke is to understand what factors can influence motor

recovery after ischemic lesion in order to maximize spontaneous motor recovery in patients. Factors including environment, social interaction, and stress have been reported to modulate recovery after ischemia in animal models. Rats placed in an enriched environment, which provides rich opportunities for social interaction, a larger cage to promote motor activity, and numerous toys and objects for the rats to interact with, improves functional outcome after infarct in rats (Ohlsson and Johansson, 1995; Johannson, 1996; Farrell et al., 2001). Environmental enrichment in combination with forelimb rehabilitation further improves motor recovery in rats (Biernaskie and Corbett, 2001). Social interaction modulates stroke recovery, with increased social interaction leading to favorable outcome (Johansson and Ohlsson, 1996). Mice that were housed in pairs had reduced infarct size and improved behavioural outcome after MCAO compared to mice that were socially isolated (Craft et al., 2005). Another major factor that can affect recovery after stroke is the exposure to stress and stress hormones produced by adverse environmental conditions.

Physiological Consequences of an Adverse Environment

Stress is a physical or psychological challenge that moves bodily processes away from its allostatic point (Sapolsky, 1994). Physical challenges can include deprivation of food or water, physical assault, pain, or fatigue. Psychological challenges include changes in social status, strenuous cognitive activity (such as writing a thesis), and high levels of anxiety (Sapolsky, 1994). The stress response is adaptive, as it attempts to allow the organism to adjust to the stressor and its current situation. Three structures are key for the stress response and are referred to as the hypothalamic-pituitary-adrenal (HPA) axis.

Briefly, when the organism experiences stress, the hypothalamus releases corticotrophin-releasing hormone (CRH). CRH triggers the release of adrenocorticotropic hormone from the pituitary gland, which then stimulates the release of glucocorticoids from the adrenal glands. Glucorticoids, in concert with epinephrine and norepinephrine, are largely responsible for stress-related behaviour. Glucocorticoids are known as corticosterone in rodents, and cortisol in humans.

The various actions of glucocorticoids include limiting cell metabolism to enhance glucose supply to muscles, anti-inflammatory actions, increased blood pressure and cardio-vascular output, suppressed appetite, and suppressed reproductive behaviour (Sapolsky et al., 2000). These functions are mediated primarily through the sympathetic nervous system. Through a negative feedback loop, glucocorticoids inhibit hypothalamic release of CRH in attempts to block further release of glucocorticoids thereby preventing the stress response from over-shooting.

There are structures in the brain that act to modulate the stress response. The prefrontal cortex, hippocampus, and amygdala have the highest concentrations of glucocorticoid receptors in the brain (Ahima and Harlan, 1990; Ahima et al., 1991). The prefrontal cortex (Spencer et al., 2004; Boyle et al., 2006) and hippocampus (Herman et al., 1989; Herman and Cullinan, 1997) act to inhibit HPA activation, while the amygdala acts to stimulate HPA activation (Herman and Cullinan, 1997; Vyas et al., 2004). There are two types of receptor for glucocorticoids: mineralcorticoid receptors (MR) are stimulated during basal levels of glucocorticoids (periods of nonstress), and glucocorticoid receptors (GR), which are only stimulated when there is an increase in circulating glucocorticoid levels (during stress). MR is thought to be involved in the

maintenance of stress activity, while GR mediates recovery from stress (De Kloet et al., 1998).

There are numerous ways of inducing stress in laboratory rats. One of the most commonly used stressors is restraint stress. During restraint stress, the movements of the rats are restricted via placing the rats into narrow Plexiglass tubes or plastic bags to restrict movement. The advantage of this type of stressor is that the corticosterone response in rats is well characterized across different strains and sex (Faraday et al., 2002; Dal-Zotto et al., 2003; Marquez et al., 2004). Other common stress procedures in laboratory rats include electric foot shock, overcrowding, social isolation, exposure to predatory odor, and forced swimming (Morrow et al., 2000; Vyas et al., 2002; Djordorvic et al., 2003; Westenbroek et al., 2005).

The duration of stress is classified as either acute or chronic, although what is considered the ideal length of time for a stressor to be considered acute or chronic is unknown. Generally, acute stress is a brief exposure to a stressor, such as exposure to stress for two days. The organism is only undergoing stress for a short time period, after which it is not undergoing stress. Acute stress is generally an adaptive response as that allows the organism to cope with the current emergency situation. In contrast, chronic stress can last for an extended time period. Generally, stress lasting for more than seven days or being exposed to periodic stressors for an extended period of time, is considered chronic. Frequent exposure to a stressor results in frequent increases in blood pressure and cardiovascular activity can increase the chance of a cardiovascular incident (Sapolsky, 2000). Chronic stress can also lead to fatigue due to the lack of energy storage, and frequently inhibiting the immune response.

Stress has a critical influence on behaviour. Acute and chronic swim stress (Metz et al., 2001) and chronic restraint stress (Metz et al., 2005b) impair skilled movements in rats. Stress can influence emotionality, as stress and high levels of stress hormones can result in increased depression and anxiety like behavoiur in humans and rats (Sapolsky, 2000). Chronic restraint stress increases anxiety in the elevated plus-maze in rats (Vyas et al., 2002, 2004).

Stress is capable of altering neuroplasticity within the limbic system of the brain. Chronic restraint stress can alter neuroplasticity in the brain leading to atrophy of the CA3 layer of the hippocampus (Magarinos and McEwen, 1995; Vyas et al., 2002) and prefrontal cortex (Wellman, 2001), while inducing hyperplasticity within the amygdala (Vyas et al., 2002, 2004). Since stress can have such a larger influence on the brain and behaviour, it has prompted researchers to investigate how stress can influence recovery after stroke.

Stress as a Determinant of Stroke Outcome

Stress and stress hormones may play an important role in recovery after stroke. Stroke alone is capable of increasing cortisol concentrations in humans (Olsson, 1990; Murros et al., 1993; Christensen et al., 2004) and corticosterone in rats (Wexler, 1980; Craft et al., 2005). High levels of corticosterone have been suggested to exacerbate infarct growth after ischemic lesion in rats. Corticosterone injections increase cell death after global ischemia (Sapolsky and Pulsinelli, 1985), 2-VO (Rami et al., 1998) in rats, and MCAO in mice (Sugo et al., 2002). Dexamethasone, a synthetic glucocorticoid, aggravates injury after forebrain ischemia in rats (Koide et al., 1986). In turn, drugs that

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reduce corticosterone levels are neuroprotective in rodent models of ischemic injury. Metyrapone, which blocks the synthesis of corticosteroids, ameliorates infarct size in rats when given prior to unilateral carotid artery occlusion (Krugers et al., 2000), MCAO (Smith-Swintosky et al., 1996), and 4-VO (Smith-Swintosky et al., 1996). Risedal et al., (1999) found that metyrapone treatment prior to MCAO in rats did not influence infarct size, however, metyrapone treatment shortly after MCAO exacerbated lesion size. The glucocorticoid receptor antagonist mifepristone (also known as RU-38486) was shown to be neuroprotective after MCAO in mice (Sugo et al., 2002) and traumatic brain injury in rats (McCullers et al., 2002). The effects of corticosterone blockade on recovery after ischemic stroke is not straightforward. Adrenalectomy (ADX) results in the complete halt of corticosterone production. ADX performed 24 hours after global ischemia saves cells within the hippocampus in gerbils (Morse and Davis, 1990). These authors also found that if cell death is measured after a longer survival period, the neuroprotection was lost, and there was no difference between ischemic, and ADX-ischemic rats. Additionally, ADX increased mortality and edema formation after forebrain ischemia in female rats (Abraham et al., 1992). This would suggest that while high levels of corticosterone may exacerbate infarct size, very low levels of corticosterone may also be detrimental to stroke outcome.

The role stress hormones play after ischemic stroke is not clear at the present time. Within the stress literature, there is known to be a U-shaped dose dependent curve for glucocorticoid action within the brain (De Kloet et al., 1998). For example, it has been shown that basal levels of corticosterone are necessary for cell survival and function within the hippocampus of rats. Shutting down the production of corticosterone via ADX,

will result in cell loss and altered neuronal morphology within the granule cell layer of the hippocampus of rats (Sloviter et al., 1989; Wossink et al., 2001). Additionally, chronic exposure to high levels of corticosterone will increase neurodegeneration within the hippocampus (Sapolsky, 1985; De Kloet et al., 1998). It would appear that low or high levels of corticosterone have detrimental effects within the brain. Potentially, an attenuated or exacerbated stress response may negatively influence motor recovery after ischemic injury in rats. This would also suggest that intermediate levels of corticosterone and HPA activation is potentially beneficial for recovery after an ischemic infarct in rats. This has not yet been studied.

Stress can also increase infarct size in rodents. Rats exposed to restraint stress for seven days prior to MCAO had enlarged infarct size (Madrigal et al., 2003). Ritchie et al., (2004) found that a chronic mild unpredictable multiple stress paradigm lasting for six weeks after 2-VO in rats increased cell loss of the CA1 cell layer. Interestingly, Madrigal et al., (2003) found that chronic exposure to restraint stress prior to MCAO (21 days for six hours) attenuated infarct volume in rats. It would seem that different exposure times to stress can differentially modulate infarct size after ischemic stroke in rats. Stress is capable of increasing functional deficits after ischemic infarct in rodents. Mice exposed to seven days of social stress prior to MCAO exhibited increased infarct size, and exaggerated cognitive deficits (DeVries et al., 2001; Sugo et al., 2002,).

The mechanism by which stress and stress hormones promote cell death are unknown. Some possibilities include excitotoxicity (Moghaddam et al., 1994; Rami et al., 1998; Madrigal et al., 2003; Mingli et al., 2003), reduced inflammatory response (Hwang et al., 2004), hyperglycemia (Payne et al., 2003), inability to use glucose leading to

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further metabolic insult (Sapolsky, 1985; Roy and Sapolsky, 2003) and suppressing Bcl-2 (an anti-apoptotic protein involved in cell survival) expression (Almeida et al., 2000; DeVries et al., 2001). McCullers et al., (2002) found that mifepristone-induced decrease in Bcl-2 did not predict cell survival suggesting Bcl-2 suppression may not be a mechanism of corticosterone-induced cell death. Stress-induced fever is another mechanism that can result in increased cell death, which yet has not been explored in animal models of stroke (Kitatkin and Wise, 2001; Clarke et al., 2003).

Stress and glucocorticoids could also modulate recovery after ischemic stroke via edema. Dexamethasone reduces edema formation in animal studies of cerebral ischemia (Betz and Coester, 1990; Dax et al., 1990; Shimauchi and Yamamoto, 1992) and hemorrhage (Vachon and Moreau, 2003), as well as reduce hematoma size when given after hemorrhage in rats (Lema et al., 2005). Some animal studies and clinical trials have failed to find any benefit of dexamethasone therapy on reducing edema after stroke (Ito et al., 1980; Ogun and Odusote, 2001). The mechanism by which dexamethasone reduces edema levels is currently unknown. Dexamethasone activates GR alongside stress and high amounts of stress hormones. If GR activation plays a role in reducing edema then it is possible that stress may also act to reduce edema after ischemia. It is currently not known whether stress has any influence on edema after ischemic lesion.

Previous literature investigating stress and stress hormones after lesion used infarct measurements as the sole indicator of recovery. Little is currently known on the effects of chronic stress on motor recovery after stroke. Improving functional recovery after stroke is the most important aim of pre-clinical research. As such it is necessary to assess motor recovery after infarct in animal models of stroke. Based on

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previous findings, it seems possible that stress may potentially modulate motor recovery after ischemic lesion in rats.

Objective of the Present Thesis

The purpose of this thesis was to examine the effects of chronic stress on skilled motor recovery and compensation after focal ischemia. Previous studies have not assessed the effects of stress or stress hormones on motor recovery after ischemic infarct, rather focusing on infarct size in rodents (Smith-Swintosky et al., 1996; DeVries et al., 2001; Madrigal et al., 2003). Infarct volume after ischemic lesion does not necessarily correspond with behavioural recovery (Nurse and Corbett, 1998). Thus, it is necessary to measure motor function to assess functional recovery after ischemic lesion rather then just infarct size. A previous study by Kirkland et al., (submitted) suggests that chronic restraint stress impairs motor recovery after devascularization lesion in rats. In the present thesis, the importance of the timing of the chronic stress on motor recovery after lesion was investigated. The effect of chronic stress prior to or after the lesion on motor recovery and infarct size was compared in rats. In addition the effects of the type of stressor was investigated, to see if different stressors modulate motor recovery after ischemic infarct in rats. The effect of the timing and type of stressor on edema after infarct was also studied.

In the present thesis, ischemic lesion in the motor cortex of rats was induced using the devascularization model of stroke. The area of infarct included the caudal and rostral forelimb areas of the motor cortex. The devascularization model of focal infarction in rats has been widely used in studies examining behavioral and structural processes of

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recovery. A number of studies investigated lesions to the sensorimotor cortex in the rat revealing permanent and well-defined motor deficits in skilled reaching and skilled walking (Whishaw, 2000; Metz and Whishaw, 2002; Gonzalez and Kolb, 2003). Motor tests used to detect deficits induced by ischemic injury and chronic stress included the single pellet reaching task to assess skilled reaching, and the ladder rung walking task to assess skilled walking. In addition, the effects of stress on edema formation after devascularization lesion was assessed using the wet/dry brain weight as a measure of brain water content and it is hypothesized that stress will reduce edema.

Chapter 2 is comprised of two studies. Experiment 1 examines the effects of chronic restraint stress on skilled movements after devascularization lesion. The effects of pre-lesion chronic restraint stress on skilled motor recovery and infarct size was compared to post-lesion chronic restraint stress. It is hypothesized that chronic restraint stress will induce impairments in skilled movements and will exacerbate after devascularization lesion. It is believed that chronic stress after the lesion will induce the most behavioural deficits. The results from Experiment 1 indicate chronic restraint stress occurring prior to or after lesion led to impairments in skilled reaching. Chronic restraint stress prior to lesion generally had a larger negative effect on the rate of functional improvement in the single pellet reaching task. Only chronic restraint stress after the lesion exacerbated infarct size. Experiment 2 studied the effects of chronic restraint stress on edema formation prior to or after lesion. Restraint stress did not influence edema levels after devascularization lesion. The data from *Chapter 2* suggests that the timing of chronic restraint stress can have differential effects on motor recovery and infarct growth depending on when it occurs in respect to ischemic injury.

Chapter 3 expands on whether stress can induce motor impairments after devascularization lesion. *Chapter 3* is made up of two experiments. Experiment 3 investigated whether multiple stressors are capable of inducing motor impairments after ischemic lesion as seen in Experiment 1 with chronic restraint stress. In Experiment 3 rats were exposed to a chronic multiple stress paradigm involving eight different stressors including restraint, forced swim, overcrowding, social isolation, saline injection, fox odor, shaking, and foot shock. It was hypothesized that chronic multiple stress will induce impairments in skilled movements and will increase infarct volume after lesion, Chronic multiple stress, however, failed to induce behavioral deficits in the skilled reaching and did not increase infarct volume, despite increasing adrenal weights. Experiment 4 examined whether acute exposure to various stressors, such as corticosterone treatment, fox odor, foot shock, restraint stress have any affect on edema. Experiment 4 replicated the results from Experiment 2 and found no effect of stress or corticosterone treatment on edema formation.

The present thesis suggests that chronic stress has the ability to influence motor recovery after ischemic injury in rats. The detrimental effect of chronic stress on forelimb recovery after ischemic infarct is limited by the timing but most importantly by the type of stressor.

Chapter 2

Chronic Restraint Stress Exaggerates Impairments in Skilled Reaching and Increases Infarct Size after Devascularization Lesion in the Rat

Introduction

Recent studies have pointed out an association between the degree of functional recovery after ischemia to levels of stress and stress hormones. In clinical studies, high levels of the stress hormone cortisol are predictive of poor functional outcome and high morbidity (Murros et al., 1993; Christensen et al., 2004). Additionally, May et al. (2002) found that psychological distress is a predictor of fatal strokes in humans. Similar observations have been made in animal models of stroke. Seven days of social stress increased infarct size and cognitive deficits after MCAO in mice (DeVries et al., 2001; Sugo et al., 2002). Madrigal et al. (2003) compared acute vs. chronic restraint stress prior to MCAO in rats. Acute restraint stress increased infarct size, while chronic restraint stress reduced infarct size. Another study showed that chronic multiple stress after 2-VO increases cell death within the hippocampus of rats (Ritchie et al., 2004).

In turn, blockade of stress hormones can also influence infarct volume in rats. Pre-treatment with metyrapone, a potent corticosteroid synthesis inhibitor, is neuroprotective when given after MCAO in rats (Smith-Swintosky et al., 1996; Krugers et al., 1998; Krugers et al., 2000). Risedal et al. (1999) found that pre-lesion treatment with metyrapone did not influence infarct size, while post-lesion administration exacerbated infarct volume in rats. Additionally, rats exhibited deficits on the rotating pole task when treated with metyrapone after MCAO rather then prior to the lesion

suggesting a differential effect of stress hormones on infarct volume prior to or after the infarct.

So far, few studies in the current literature have examined the effects of stress on skilled motor function after ischemic lesion. A previous study suggests that chronic restraint stress prior to and after devascularization lesion can have long-term effects on motor recovery in rats (Kirkland et al., submitted). It is unknown how chronic stress modulates skilled forelimb function after lesion in rats. It is also currently unknown whether chronic stress prior to or after the lesion can differentially affect deficits after ischemic infarct. There are several reasons to expect that chronic stress modulates skilled motor recovery after ischemic infarct in rats. In healthy rats, chronic swim stress and chronic restraint stress can induce impairments in motor function and skilled reaching (Metz et al., 2001, 2005b). It seems possible then that stress may exacerbate functional deficits in skilled movements after ischemic injury.

Chapter 2 is composed of two experiments. The purpose of Experiment 1 is to examine the role of chronic restraint stress on skilled motor recovery after ischemic injury in rats. The experimental design allowed for comparison of the differential effects of chronic restraint stress prior to or after the lesion. Devascularization was used to create an ischemic injury of the motor cortex. The single pellet reaching task and ladder rung walking task were used to assess recovery of skilled motor function following the lesion. Additionally, the effects of pre-lesion versus post-lesion chronic restraint stress on infarct growth were assessed. To measure infarct growth, a subset of brains were studied at two and fifteen days post-operation (DPO). Since most studies in the current literature have

used short survival periods when assessing the effects of stress on infarct size after MCAO (Sugo et al., 2002; Madrigal et al., 2003), the aim was to examine the effects of stress on infarct size at an acute and chronic time point. It was hypothesized that both pre-lesion and post-lesion chronic restraint stress will exaggerate functional deficits and infarct volume in comparison to non-stress rats. It was also hypothesized that rats undergoing post-lesion chronic restraint stress will have exacerbated infarct size and behavioural deficits in comparison to pre-treated stress rats. Exposure of the ischemic tissue to stress may place it under increased metabolic strain, thus increasing infarct volume and potentially increasing motor deficits.

Experiment 2 examined the effects of chronic restraint stress on edema formation before versus after devascularization. The effects of stress and stress hormones on edema after stroke is unknown. ADX which halts corticosterone production increased edema formation after forebrain ischemia in female rats (Abraham et al., 1992) suggesting that stress hormones may play an important role in decreasing edema after stroke. There may be a potential dichotomy with stress increasing infarct size but also decreasing edema levels after lesion in rats. Dexamethasone is a synthetic glucocorticoid that activates GR which is also activated during stress. Dexamethasone decreases edema after ischemic injury in rats (Betz and Coester, 1990; Dux et al., 1990; Shimauchi and Yamamoto, 1992; Vachon and Moreau, 2003). The mechanism of how dexamethasone attenuates edema is unknown.

In Experiment 2 it is hypothesized that stress will decrease edema after devascularization.

Methods

Experiment 1

Animals

Forty-two male Long-Evans rats (about 90 days old and weighing 400-550 g at the beginning of the experiment) raised at the University of Lethbridge vivarium were used in this study. The rats were housed in pairs under a 12 hr light/day cycle with lights on at 7:30 AM. All procedures were performed in accordance with the guidelines set by the Canadian Council for Animal Care.

Experimental Design

The timeline of Experiment 1 is illustrated in Figure 1. Prior to training in the single pellet reaching task, rats were food-deprived to 90-95% of their baseline weight. The rats were trained in the single pellet reaching task until their success rates reached an asymptote (45%). The rats were then divided into three groups based on their success rate in the pellet reaching task. The pre-lesion stress group (PRE-STRESS, n = 8) underwent restraint stress for 15 days prior to focal ischemia. The post-lesion stress group (POST-STRESS, n=8) was given restraint stress for 15 days after focal ischemia. A control group (CONTROL, n=8) did not undergo restraint stress. After training, rats were filmed for baseline performance in the single pellet reaching task, and the ladder rung walking task. Baseline blood samples were taken once behavioural testing was completed for a measure of baseline corticosterone levels. After baseline period the PRE-STRESS group underwent restraint stress for 15 days. During this time, all three groups continued to be tested daily in the single pellet reaching task. On day 14 of stress, all groups were filmed

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in the single pellet reaching task and the ladder rung walking task. Blood samples were taken on day 15 of stress. All rats underwent focal ischemic lesion of the motor cortex via devascularization the day following pre-lesion blood samples. The rats were given a one-day break from behavioural testing. Starting the day after lesion, the POST-STRESS group began its 15-day stress regimen. After the lesion the PRE-STRESS group was no longer subject to chronic restraint stress for the remainder of the study. On fourteen DPO, rats were filmed for post-surgical performance in motor tasks. On fifteen DPO, blood samples were taken to determine a post-lesion corticosterone levels. Following blood samples the rats were perfused and lesion size was analyzed.

In addition to the eight animals per group tested until fifteen DPO, six animals from each group were perfused on two DPO for infarct size measurements. Rats in the POST-STRESS group were perfused immediately after stress. The rats perfused on two DPO were not included in behavioural testing and analysis.
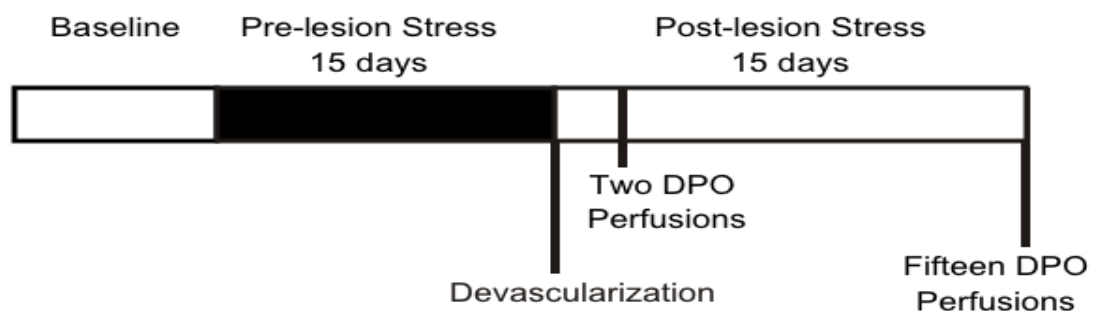
Figure 1. Timeline for Experiment 1. PRE-STRESS rats underwent pre-lesion stress for 15 days prior to devascularization lesion and did not undergo stress after the lesion. POST-STRESS animals underwent post-lesion stress for 15 days and did not undergo stress prior to lesion. Control rats did not undergo any chronic stress. All rats underwent devascularization. Six rats in each group were perfused on two DPO for an acute measurement of infarct volume. Blood samples took place on the last day of baseline testing, the day before devascularization, and fifteen DPO.

Blood Samples

Rats were anesthetized with isoflurane (4% induction and 4% maintenance, 1.5% oxygen). Blood samples were taken from the tail vein and an average of 0.5 ml of blood was collected. The remainder was centrifuged at 5000 rotations per minute (rpm) for eight minutes. The serum was collected and stored at -20°C. Blood samples were taken at baseline, on day 15 of stress prior to ischemia and on fifteen DPO. Blood samples were taken 30 minutes after initiation of restraint stress (Magarinos and McEwen, 1995; Metz et al., 2005). Rats were not tested on any behavioural tasks on blood sample days. Unfortunately, due to technical issues, analysis of corticosterone was not completed in time for the current thesis.

Focal Cerebral Ischemia

Rats were anesthetized with isoflurane (4% induction and 2% maintenance, 1.5% oxygen). Focal cerebral ischemia in the motor cortex was induced via devascularization as described by Whishaw (2000). Briefly, the skin over the skull was incised and the skull was exposed. Using a fine dental drill, a craniotomy was made at the following coordinates: -1.0 to 4.0 mm anterior, -1.5 to 4.5 mm lateral. The dura was removed, and the blood vessels were carefully wiped off using a cotton tip. The lesion was made to the motor cortex contralateral to the rat's preferred reaching paw in the single pellet reaching task. The skin was then sutured and the rat was given 0.05 ml of the analgesic, Temgesic (Schering-Plough Inc, Brussels, Belgium). Rats were allowed to recover in individual cages on top of a heating pad until fully awake and were then returned to their home cages.

Stress Regimen

Chronic stress was induced via daily restraint stress. Individual rats were carefully inserted into transparent Plexiglas tubes (six cm inner diameter and 18 cm long) for 20 minutes. The rats were then returned to their transport tubs and waited ten minutes before being tested in the single pellet reaching task. The rats were stressed by an experimenter not involved in behavioural testing.

Single Pellet Reaching Task

The single pellet reaching task assesses skilled limb-use based on reaching success and movement performance (Whishaw et al., 1993). The reaching boxes were made of clear Plexiglas (40 cm*45 cm*13 cm). The front wall of the box had a 1.3 cm wide vertical slot, allowing the rats to reach for the pellet located on a shelf secured to the outside of the box. The shelf was located 4 cm above the floor on the front of the box. On the top of the shelf were two indentations (5 mm in diameter, and 1.5 mm deep), each aligned with one side of the slot. These indentations stabilized the pellet and were located 1.5 cm away from the front wall (Metz and Whishaw, 2000). Rats were food deprived to 90-95% of their baseline body weight. In a training session, rats were placed in the reaching box and a food pellet (45 mg each, Bio Serv, Frenchtown, New Jersey, USA) was placed contralaterally to the preferred reaching paw. Rats were trained to walk to the rear end of the box and then return to the front to reach for a new pellet. The rats were given 20 pellets per training and testing session. A successful reach was defined as obtaining the pellet on the first attempt, withdrawing the paw through the slot and

released the pellet into the mouth and eaten Success rate was calculated using the following formula:

Success rate = $\dfrac{\text{number of successful reaches}}{20}$ * 100

The percentage of total pellets eaten was measured by counting the number of pellets eaten, regardless of whether the pellet was grasped and eaten on the first attempt. If the rat dropped the pellet, it was not included as a pellet eaten. The percentage of total pellets eaten was measured using the following formula:

Percentage of total pellets eaten = $\dfrac{\text{number of pellets eaten}}{20}$ * 100

To assess limb coordination, the number of reaches was measured by counting the number of attempts to grasp 20 pellets for each session.

Qualitative Analysis of Skilled Reaching

For qualitative analysis of single pellet reaching, a reaching movement was broken down into ten components and 28 subcomponents modified from (Metz and Whishaw, 2000). The components and subcomponents are shown in Table 1. Briefly, the ten components contained the following characteristics. (1) *Limb lift:* The limb is lifted from the floor and the digits move to the midline of the rat's body. (2) *Digits close*: The forelimb continues to lift, the palm supinates and the digits are semiflexed. (3) *Aim*: The forelimb continues to rise and the palm is in the midline of the rat's body and underneath the snout. As the forelimb is lifted the elbow moves in. (4) *Advance*: The forelimb moves forward through the slot and moves toward the pellet. As the forelimb advances the elbow continues to move in. (5) *Digits open*: The digits open and partially pronate above

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the pellet. (6) *Pronation*: The elbow moves out, the palm is fully pronated over the pellet and the palm moves down in an arpeggio movement. (7) *Grasp*: The digits close around the pellet. (8) *Supination 1*: The elbow moves in as the palm is withdrawn. As the palm is being withdrawn the palm turns 90º. (9) *Supination 2*: The palm is withdrawn from the slot and the palm is again supinated so that the palm faces the rat's mouth. (10) *Release*: The rat sits back and places the food in its mouth. The rat also raises its other paw to assist the reaching paw with eating.

Each of the subcomponents was scored on a 3-point scale. A score of 1 was given if the movement was present. A score of 0.5 was given if the movement was present but abnormal, and a score of 0 was given if the movement was absent. The average score from each component was averaged over three reaches. An average total score was calculated from the ten components.

Table 1

Components of skilled reaching and rating scale used to score each component.

Components	Behaviour scored	Rating
1. Limb Lift	limb moves forward	0, 0.5, 1
	digits in midline	0, 0.5, 1
2. Digits Close	palm subinated, semi-in	0, 0.5, 1
	digits semiflexed	0, 0.5, 1
3. Aim	elbow comes in	0, 0.5, 1
	palm in midline	0, 0.5, 1
4. Advance	elbow comes in	0, 0.5, 1
	limb forward	0, 0.5, 1
	limb directed to target	0, 0.5, 1
	head and upper body raised	0, 0.5, 1
5. Digits Open	digits open	0, 0.5, 1
	discrete limb movement	0, 0.5, 1
	not fully pronated	0, 0.5, 1
6. Pronation	elbow out	0, 0.5, 1
	palm down in arpeggio	0, 0.5, 1
7. Grasp	digits close	0, 0.5, 1
	hands lift	0, 0.5, 1
	arm still	0, 0.5, 1
8. Supination 1	elbow in	0, 0.5, 1
	palm medially before leaving slot	0, 0.5, 1
	palm turned 90°	0, 0.5, 1
9. Supination 2	palm straight up	0, 0.5, 1
	distal limb movement	0, 0.5, 1
	head points down	0, 0.5, 1
10. Release	open digits	0, 0.5, 1
	puts food in mouth	0, 0.5, 1
	raises other paw	0, 0.5, 1
	head and upper body lowered	0, 0.5, 1

Ladder Rung Walking Task

Training and Testing

Fore- and hind limb coordination and limb placement was assessed by using the ladder rung walking test (Metz and Whishaw, 2002). Rats were trained to cross a 1 m

long horizontal ladder with metal rungs arranged at random distances, ranging from 1 to 5 cm. In each test session, the rung pattern was reversed to prevent the rats from anticipating the location of the rungs. Rats were trained to cross the ladder in five trials. In each test session, rats were videotaped for three trials for further analysis.

Video Analysis of Ladder Rung Walking Task

A rating system according to Metz and Whishaw (2002) was used for qualitative analysis of skilled walking by frame-by-frame analysis. Briefly, the rating system was based on seven categories of limb placement on the rungs: (0) *Total Miss*: The limb misses the rung, causing the rat to fall. (1) *Deep Slip*: The limb is placed on the rung, but then slips off, causing the rat to fall. (2) *Slight Slip*: The limb is placed on the rung then slips off, but the rat does not fall and interrupt the walking pattern. (3) *Replacement*: The limb is placed on one rung, but quickly is lifted and placed on another rung. (4) *Correction*: The limb is aimed at one rung, but prior to placement, is placed onto a different rung; alternatively, the limb was placed on a rung and then readjusted on that rung. (5) *Partial placement*: The limb is placed on the rung, with the wrist/digits of the forelimb, or heel/toes of the hind limb. (6) *Correct placement*: The midportion of the limb is placed onto the rung.

The number of steps and foot faults (errors) for each fore- and hind limb was counted and the percentage of error (errors/step x 100) was averaged from three trials.

Video recording

All behavior was recorded by using a Canon ZR50 MD camcorder set at a shutter speed of 1/500 s. During filming, additional light was supplied by a cold light source (Lowel-light Mtg Inc, New York, USA). Frame-by-frame analysis was performed using a Sony GV-D1000 NTSC miniDV player.

Histology

After behavioural testing was completed, rats were sacrificed with an overdose of Euthansol (0.3ml i.p.) and perfused through the heart with 0.9% saline and 4% formalin. Brains were removed and post-fixed overnight before being placed in a 30% sucrose solution. The brains were frozen and 40 μm sections were cut on the cryostat. Every fifth section was mounted onto slides and stained with cresyl violet to calculate infarct volume.

Measurement of Infarct Volume

Photographs of cresyl violet stained sections were taken digitally at ten different standardized planes. The following planes (from bregma) were assessed: 3.70 mm, 3.20 mm, 2.70 mm, 2.20 mm, 1.70 mm, 1.20 mm, 0.70 mm, 0.20 mm, -0.30 mm, -0.80 mm, and -1.30 mm. The cross sectional volumes of both hemispheres were calculated using Zeiss Axiovision 4.3 (Zeiss, Germany). Infarct volume was measured according to Debow et al. (2003). The following formulas were used:

Volume of tissue lost = tissue remaining in normal hemisphere – tissue remaining in injured hemisphere

Volume of a hemisphere = average (average area of a complete coronal hemisphere – ventricles – area of damage) x interval between sections x number of sections

Statistical Analysis

Statistical analysis was performed using Statview software version 4.5 (SAS Institute, 1998). The data were analyzed using analysis of variance (ANOVA). The Scheffe's test was used for post-hoc analysis. Within-group analysis of quantitative data was done via paired t-tests. Kruskal-Wallis and Mann-Whitney U tests were used for analysis of qualitative reaching and ladder rung walking analysis. Wilcoxon Signed Rank Test was used for within group comparisons in the qualitative behavioural analysis. Correlation of infarct volume to motor recovery of fifteen DPO in single pellet reaching was assessed using Pearson's chi-squared test. In all analysis a p-value of ≤ 0.05 was chosen as the significance level. All data are presented as mean $\pm$ standard error mean (SEM).

Experiment 2

Animals

Thirty-six male Long-Evans rats (about 90 days old and weighing 450 g at the beginning of the study) raised at the University of Lethbridge vivarium were used. The rats were housed in pairs under a 12 hr light/day cycle with lights on at 7:30 am. All procedures were performed in accordance with the guidelines set by the Canadian Council for Animal Care.

Experimental Design

Rats were randomly divided into three groups, which were identical to Experiment 1. See Figure 2 for a timeline of Experiment 2. One group underwent 14 days

of chronic restraint stress prior to lesion (PRE-STRESS, n=12). After lesion the PRE-STRESS group did not undergo any restraint stress. One group received chronic restraint stress after lesion (POST-STRESS, n = 12) for a maximum of 14 days. Another group underwent only lesion and did not undergo restraint stress (CONTROL, n= 12). Similar to Experiment 1, rats in each group were divided into two survival groups. Edema measurements were taken on two DPO (n=6 from each group) and on fourteen DPO (n=6 from each group). The coordinates for devascularization of the motor cortex was the same as in Experiment 1. All the lesions were induced in the left hemisphere. The stress regimen was the same as in Experiment 1.
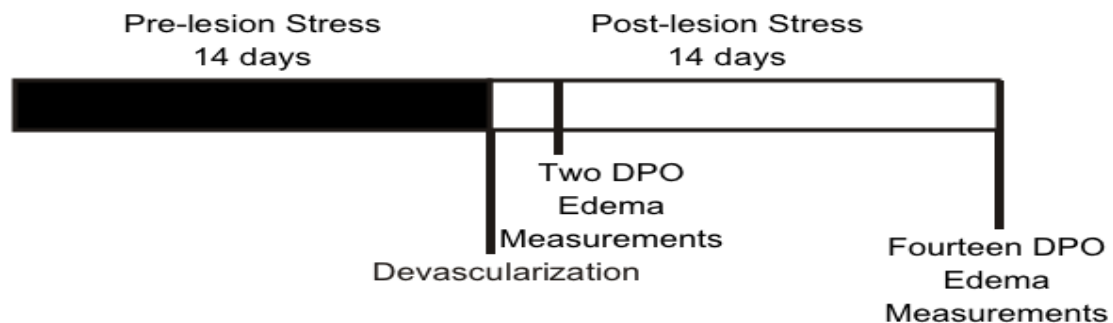
Figure 2. Timeline for Experiment 2. PRE-STRESS rats were stressed for only 14 days prior to lesion. POST-STRESS rats were stressed for a maximum of 14 days post-lesion. CONTROL rats were not stressed during the two-week span. On two DPO a subset of rats from each group were decapitated for edema measurements. The rest of the rats survived to fourteen DPO for edema measurements. Blood samples were taken on two and fourteen DPO during decapitation.

Edema Measurement

Rats were anesthetized with isoflourane (5% induction, 1.5% oxygen) and decapitated. The brains were quickly removed, the hemispheres separated, and wet weight was measured. The brains were then dried in an oven (VWR, Scientific Products,

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USA) at 100°C for 24 hours. The dry weights of each hemisphere was then measured. Edema levels were determined using the wet/dry method, a standard method of edema measurement (Ito et al., 1979; Hatashita and Hoff, 1990; Gerriets et al., 2004; MacLellan et al., 2004). The formula is as follows:

$$\text{Brain Water Content (\%)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} * 100$$

In the case of the POST-STRESS group, rats were anesthetized and decapitated immediately after restraint stress exposure on two and fourteen DPO. Blood samples were collected after decapitation. Blood samples and corticosterone levels were treated the same way as in Experiment 1.

Statistical Analysis

Comparison of the edema percentages of each hemisphere between two and fourteen between DPO was performed using ANOVA between the groups. Comparisons of the lesion hemisphere to the non-lesion hemisphere was performed via paired t-tests. In all analyses, a p-value of ≤ 0.05 was chosen as the significance level. All data are presented as group means $\pm$ SEM.

Results for Chapter 2

Infarct Size

The infarct area included the primary and secondary motor cortex, and forelimb and hind limb areas of the somatosensory cortex. On a few occasions the corpus callosum on the lesion side was severed. In cases of the POST-STRESS group the area of infarct often included the dorsolateral striatum and cingulate cortex, which was not seen in

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CONTROL animals (see Figure 3). Three brains from two DPO, one from the CONTROL (n=5) and two from the PRE-STRESS (n=4) groups, were removed from analysis due to physical damage during extraction or sectioning. A significant difference in infarct volume was found ($F_{(5,33)}$ =4.623, $p \leq 0.001$) (see Figure 3). The POST-STRESS lesion on fifteen DPO was significantly larger than the lesion in the CONTROL group on two DPO ($p \leq 0.05$, Scheffe) and on fifteen DPO ($p \leq 0.05$, Scheffe) (see Figure 3).
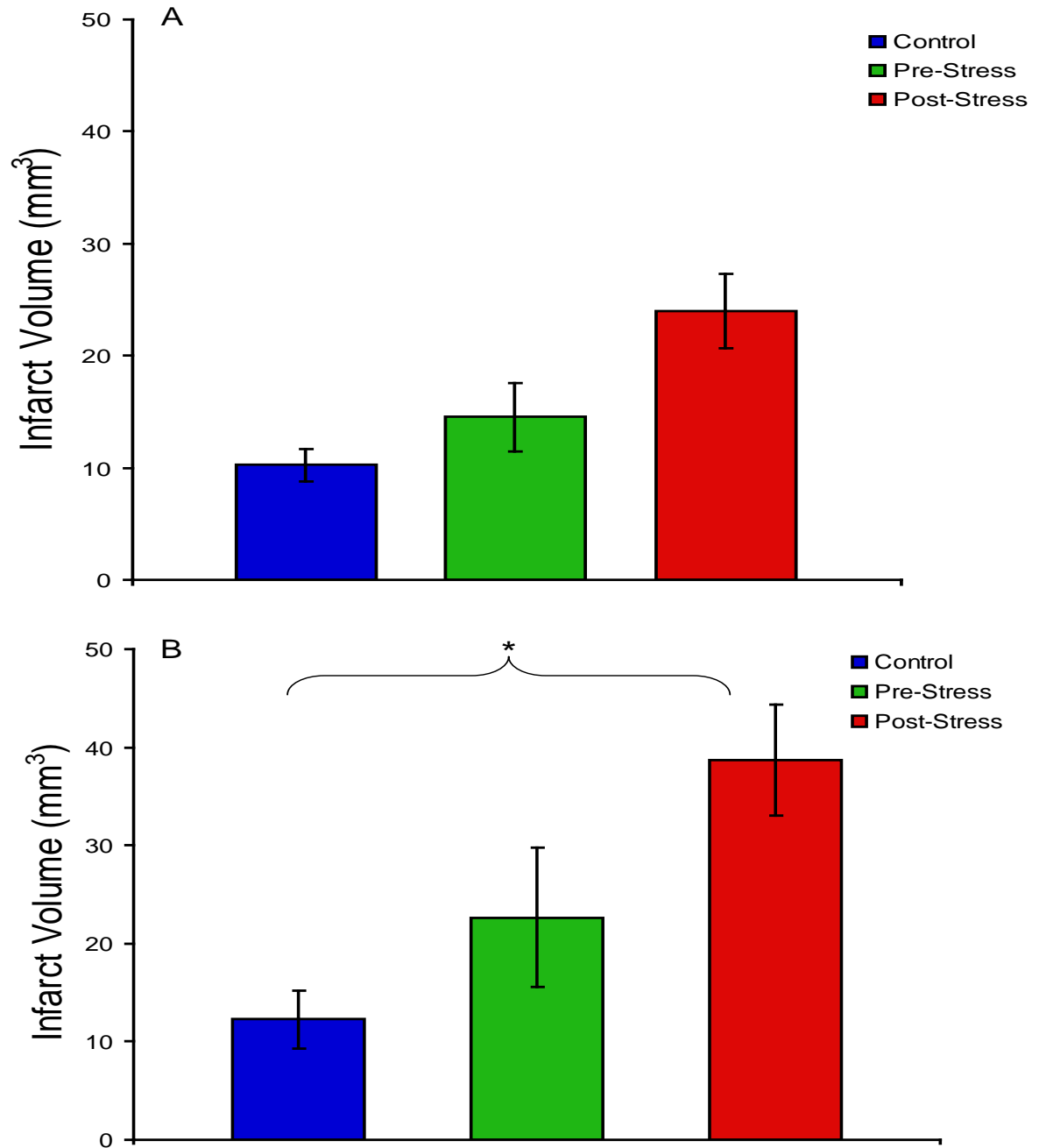
Figure 3. Volume of tissue lost after devascularization lesion of the motor cortex. A graph representing the volume of tissue lost on two DPO (A) and fifteen DPO (B). On fifteen DPO the POST-STROKE (red) group had a significantly larger infarct then the CONTROL (blue) group. There was no significant difference between the CONTROL and PRE-STRESS (green) lesion and the PRE-STRESS and POST-STRESS infarct. * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001. Comparisons between groups made with ANOVA and Scheffe's test. All data are presented as group means ± SEM.

Brain Water Content

On two DPO there was no significant difference in edema between the groups in the lesion hemisphere (see Figure 4). There were no differences in edema levels between the non-lesion hemispheres of the three groups, though there was a trend towards significance. On fourteen DPO there was no difference in edema between the lesion and non-lesion hemispheres. Using paired t-tests, there were significant differences in water content between the lesion and non-lesion hemisphere in the POST-STRESS group (t=3.107, p$\leq$0.05), PRE-STRESS group (t=10.019, p$\leq$0.001), and CONTROL group (t=10.453, p$\leq$0.001) on two DPO. No significant differences in edema percentages were found between lesion and non-lesion hemispheres on fourteen DPO.
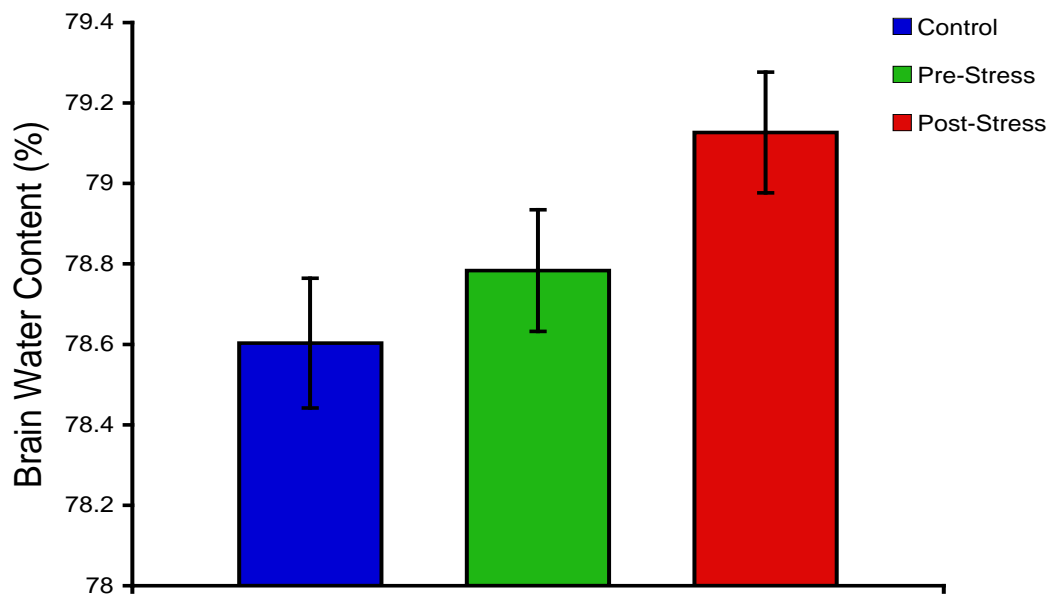
Figure 4. Percentage of brain water content (edema) after devascularization lesion on two DPO in the lesion hemisphere. There was no significant difference between the CONTROL (blue), PRE-STRESS (green), and POST-STRESS (red) groups in edema formation in the lesion hemisphere. Comparisons between groups was made using ANOVA. All data are presented as group means $\pm$ SEM.

Single Pellet Reaching Success

One rat from each group was eliminated from statistical analysis due to difficulty to meet the training criteria in the task. No significant difference between the groups in success rate was noted during baseline sessions. There was a significant difference between the groups during pre-lesion testing $(F(2,18) = 16.680, p \leq 0.001)$. The PRE-STRESS group had significantly lower reaching success compared to the CONTROL $(p \leq 0.001$, Scheffe) and the POST-STRESS groups $(p \leq 0.01$, Scheffe). There was no significant difference between the CONTROL and POST-STRESS groups. After the lesion, there was a drop in reaching success rates in all groups. There was a significant overall difference between the groups during post-lesion testing $(F(2,18) = 4.894, p \leq 0.05)$. Moreover, there was a significant difference between the PRE-STRESS and the CONTROL groups $(p \leq 0.05$, Scheffe). There was no significant difference between the CONTROL and POST-STRESS groups during post-lesion testing though there was a trend towards significance. No significant difference was noted between the PRE-STRESS and POST-STRESS groups during post-lesion testing (see Figure 5).

Within-group comparisons revealed that the CONTROL group had significantly higher success rates during pre-lesion testing in comparison to baseline $(t=-2.925, p \leq 0.05)$. During post-lesion testing the CONTROL group had significantly lower success rates than in pre-lesion testing $(t=9.470, p \leq 0.001)$. The PRE-STRESS group had significantly lower success rates during pre-lesion testing compared to baseline testing $(t=8.327, p \leq 0.001)$. Furthermore the PRE-STRESS group had significantly lower success rates after the lesion than during pre-lesion testing $(t=3.645, p \leq 0.05)$. There was no

significant difference in success rate during baseline to pre-lesion testing in the POST-STRESS group. As seen in the other groups, the POST-STRESS group showed a significant decline in reaching success compared to pre-lesion testing (t=6.502, p≤0.01). There was no correlation between infarct size and success rate on 14 DPO in the CONTROL (R=-0.679, p=0.093), PRE-STRESS (R=-0.372, p=0.411), and the POST-STRESS (R=0.519, p=0.232) groups. There was no correlation between infarct volume and the success rate on the final day of testing (R=-0.424, p=0.055) when the groups were combined.

Total Number of Pellets Eaten

There were no significant differences in the percentage of total pellets eaten in the single pellet reaching task during baseline testing. There was a significant effect of percentage of pellets eaten during pre-lesion testing (F(2,18) =14.07, p≤0.001) and a group-time effect (F(2,24) = 1.593, p≤0.05). Post-hoc analysis revealed that at pre-lesion testing the PRE-STRESS group ate significantly fewer pellets than the CONTROL (p≤0.001) and POST-STRESS (p≤0.01) groups. Further analysis revealed that during the first week of pre-lesion testing the PRE-STRESS rats were significantly impaired compared to the CONTROL (p≤ 0.01) and POST-STRESS (p≤0.01) groups. There was no significant difference between the CONTROL and POST-STRESS groups during the first week of pre-lesion testing. During the second week of pre-lesion stress, however, the PRE-STRESS rats were only significantly impaired compared to the CONTROL group (p≤0.001) and showed a close trend towards significant impairments compared to the POST-STRESS group. There was no significant difference between the CONTROL and

POST-STRESS rats during the second week of pre-lesion testing. There were significant

group differences during post-lesion testing ($F_{(2,18)}$ =6.210, $p \leq 0.01$).
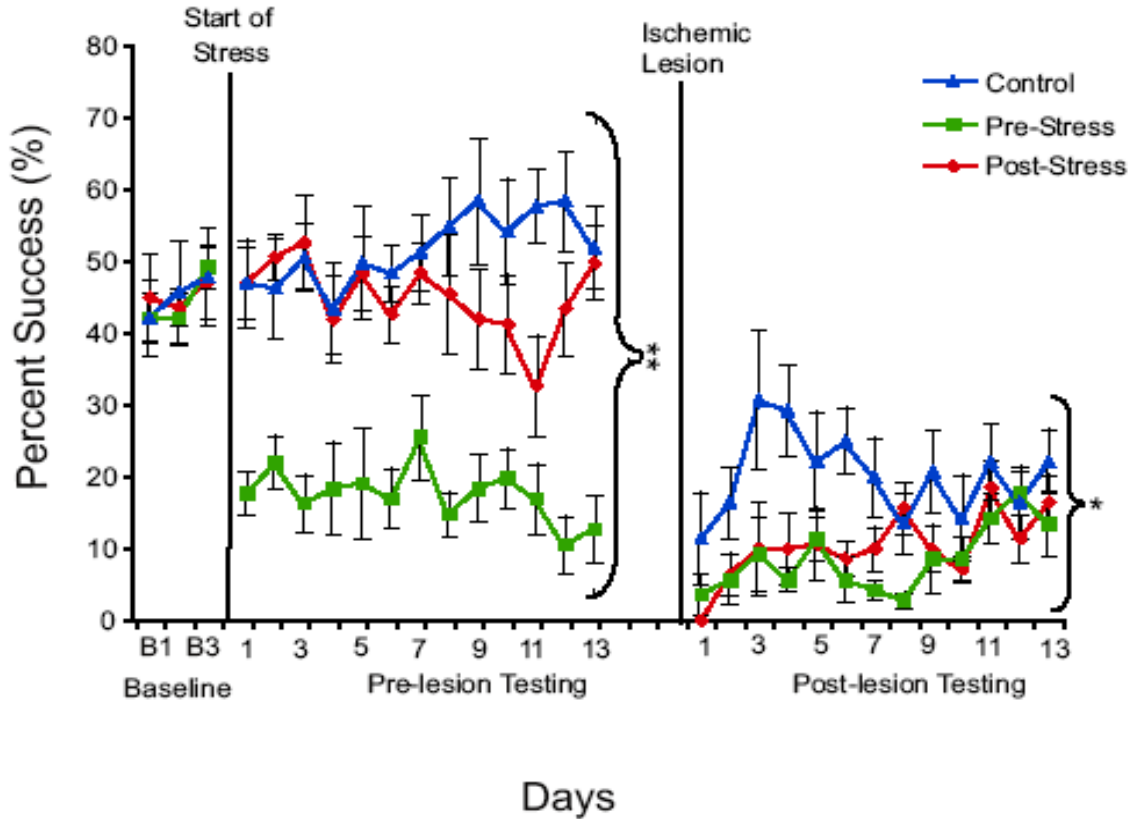
Figure 5. Percent success rate in the single pellet reaching task. There were no significant differences

between the groups during baseline testing. During pre-lesion testing, chronic restraint stress, the PRE-

STRESS group (green) had significantly lower scores then the CONTROL (blue) and POST-STRESS (red)

groups. There were no significant difference between the CONTROL and POST-STRESS groups during

pre-lesion testing. Following the lesion, the CONTROL group had significantly higher success rate then the

PRE-STRESS group. There were no significant difference between the CONTROL and POST-STRESS

group, and no differences between the PRE-STRESS and POST-STRESS groups after the lesion. * $p \leq$

0.05, ** $p \leq 0.01$, *** $p \leq 0.001$. Comparisons between groups made with ANOVA and Scheffe's test. All

data are presented as group means $\pm$ SEM.

The CONTROL group ate significantly more pellets than the POST-STRESS group ($p \leq 0.05$) and PRE-STRESS group ($p \leq 0.05$) during post-lesion testing (see Figure 6). There was also a group-and-time effect ($F(2,24) = 3.430$, $p \leq 0.001$) after the lesion. During the first 7 days of testing after the lesion there was a significant difference between the groups ($F(2,20) = 8.208$, $p \leq 0.01$). Post-hoc analysis revealed that the CONTROL group ate significantly more pellets then the PRE-STRESS ($p \leq 0.01$, Scheffe) and POST-STRESS ($p \leq 0.05$, Scheffe) groups during the first week after the lesion. There was no significant differences between the groups in the number of pellets eaten during the second week of post-lesion testing.

Within-group analysis of the CONTROL group revealed no significant difference in the number of pellets eaten from baseline to pre-lesion testing. There was a significant decrease in the number of pellets the CONTROL group ate after the lesion in comparison to pre-lesion testing ($t = 7.487$, $p \leq 0.001$). In the PRE-STRESS group, there was a significant decline in the number of pellets eaten during the pre-lesion testing period in comparison to baseline testing ($t = 3.913$, $p \leq 0.01$). There was also a significant decline in number of pellets eaten during post-lesion testing compared to the pre-lesion testing in the PRE-STRESS group ($t = 3.524$, $p \leq 0.05$). In the POST-STRESS group there was no significant difference in number of pellets eaten from baseline to pre-lesion testing. There was a significant decline in the POST-STRESS group in the number of pellets eaten from pre-lesion testing to post-lesion testing ($t = 4.468$, $p \leq 0.01$). There was no correlation between infarct volume and number of pellets eaten on 14 DPO in the CONTROL ($R = -0.388$, $p = 0.390$), PRE-STRESS ($R = 0.587$, $p = 0.166$), and POST-STRESS ($R = -0.035$,

p=0.940) groups. There was no correlation between infarct volume and number of pellets eaten on the final day of training (R=0.192, p=0.403) when the groups were examined together.
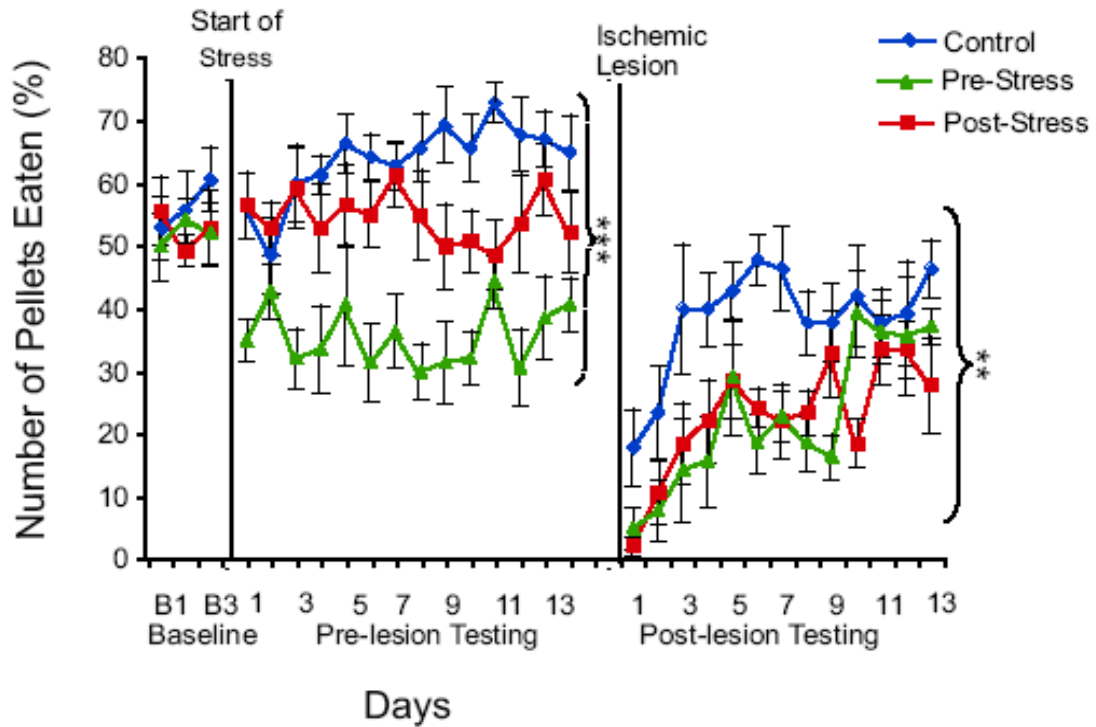
Figure 6. The percentage of total pellets eaten in the single pellet reaching task. There were no significant differences between the groups during baseline testing. During pre-lesion testing the PRE-STRESS group (green) ate significantly less pellets then the CONTROL (blue) and POST-STRESS (red) groups. There were no significant difference between the CONTROL and POST-STRESS groups during pre-lesion testing. Following lesion the CONTROL group reached for significantly more pellets after the lesion than the PRE-STRESS group and the POST-STRESS group. There were no significant differences between the PRE-STRESS and POST-STRESS groups after the lesion. * p≤ 0.05, ** p≤0.01, *** p≤ 0.001. Comparisons between groups made with ANOVA and Scheffe's test. All data are presented as group means ± SEM.

Number of Attempts

There were no significant differences in the number of reaches between the three groups at the end of baseline testing. There was a significant difference between the groups at pre-lesion ($F(2,18) = 1.8 \times 10^1$, $p \leq 0.001$) and post-lesion testing ($F(2,15) = 17.43$, $p \leq 0.001$). Post-hoc analysis revealed that the PRE-STRESS group reached more often than the CONTROL ($p \leq 0.001$) and the POST-STRESS ($p \leq 0.001$) groups. During post-lesion testing, PRE-STRESS rats reached more than the CONTROL ($p \leq 0.001$) and POST-STRESS ($p \leq 0.05$) groups. There was a trend for the POST-STRESS group to reach more than the CONTROL group (see Figure 7). There was also a group-and-time effect during post-lesion testing ($F(2,24) = 2.019$, $p \leq 0.01$). Further analysis revealed a significant difference between the groups during the first week of testing ($F(2,20) = 13.671$, $p \leq 0.001$). Post-hoc analysis found that the PRE-STRESS group reached significantly more often then the CONTROL group ($p \leq 0.001$, Scheffe) but not the POST-STRESS group during the first seven days of testing after the lesion. There was also a significant difference between the groups during the second week of testing ($F(2,20) = 5.666$, $p \leq 0.05$). Further analysis also revealed that during the last 6 days of post-lesion testing, the PRE-STRESS group reached significantly more often then the CONTROL group ($p \leq 0.05$, Scheffe) but not the POST-STRESS group.

Within-group comparisons revealed no significant difference in the number of reaching attempts from baseline to pre-lesion testing in the CONTROL group. The CONTROL group did show an increase in reaching attempts during post-lesion testing compared to pre-lesion testing ($t = -5.005$, $p \leq 0.01$). The PRE-STRESS group showed an significant increase in the number of reaching attempts during pre-lesion testing compared to baseline ($t = -7.530$, $p \leq 0.001$). The PRE-STRESS group also reached

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significantly more often during post-lesion testing then during pre-lesion testing ($t=-6.075$, $p \leq 0.01$). There was no significant difference in the number of reaches made in the POST-STRESS group from baseline to pre-lesion testing, however, there was an significant increase in reaches noted during post-lesion testing in comparison to pre-lesion testing ($t=-5.501$, $p \leq 0.01$).
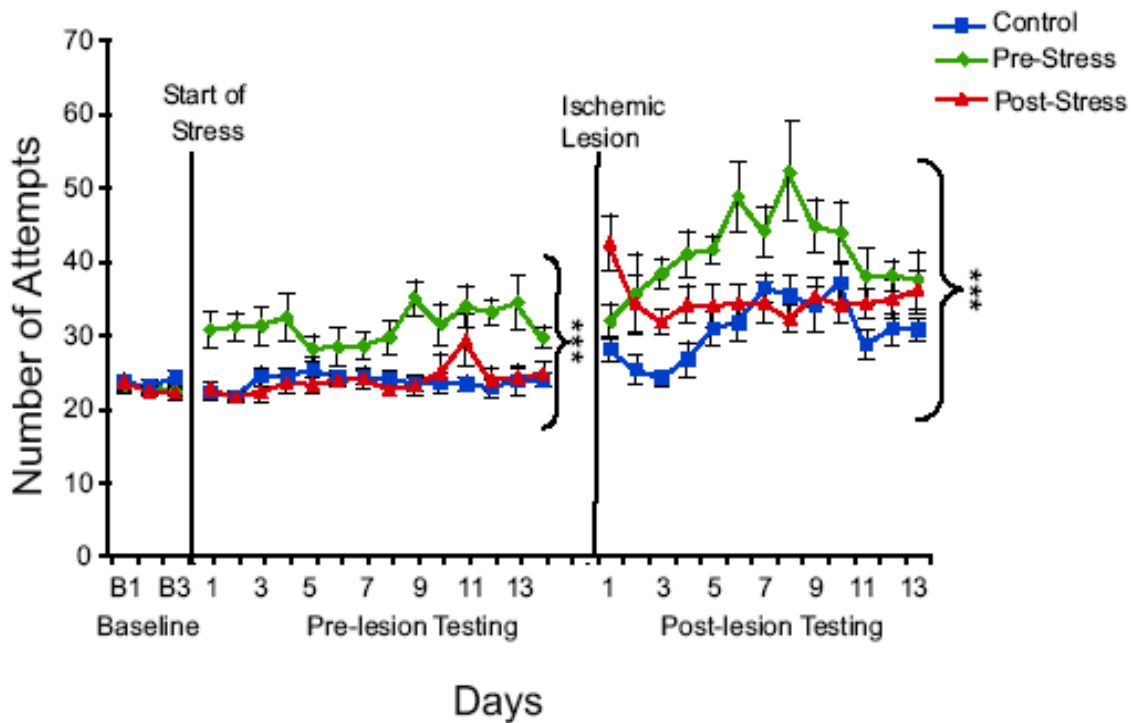
Figure 7. The number of attempts to grasp the pellet during the single pellet reaching task. No significant differences between the there groups were noted during baseline. At pre-lesion testing, the PRE-STRESS (green) group made significantly more attempts to grasp the pellet then the CONTROL (blue) and POST-STRESS groups. There was no significant difference between the CONTROL and POST-STRESS groups. After the lesion the PRE-STRESS group reached significantly more than the CONTROL and POST-STRESS groups. There were no significant difference between the CONTROL and POST-LESION groups. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Comparisons between groups were made with ANOVA and Scheffe's test. All data are presented as group means $\pm$ SEM.

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Qualitative Reaching Analysis

There was no difference in any of the movement components of reaching (limb lift, digits close, aim, advance, digits open, pronation, grasp, supination 1, supination 2, release) and in the total score between the groups at baseline or pre-lesion stress. During post-lesion stress, there was a significant difference between the groups in digits open (H=6.508, $p \leq 0.05$, Kruskal-Wallis Test), with the PRE-STRESS group being significantly worse than the POST-STRESS group (Z=-2.402, $p \leq 0.05$). Within-group comparisons of baseline to pre-lesion stress did not cause any differences in the reaching components between any of the groups. Comparisons of the reaching components in the CONTROL group between pre-lesion stress to fourteen DPO test session revealed deficits in limb lift (Z=-1.992, $p \leq 0.05$), advance (Z=-2.028, $p \leq 0.05$), digits open (Z=-2.201, $p \leq 0.05$), pronation (Z=-2.366, $p \leq 0.05$), grasp (Z=-2.201, $p \leq 0.05$), supination 1 (Z=-2.201, $p \leq 0.05$), supination 2 (Z=-2.201, $p \leq 0.05$), release (Z=-2.028, $p \leq 0.05$), and there was a significant drop in the total score (Z=-2.366, $p \leq 0.05$). In addition, digits close and aim were approaching significance. Comparisons of the reaching components in the PRE-STRESS group from pre-lesion stress to fourteen DPO found deficits in supination 1 (Z=-2.201, $p \leq 0.05$), and supination 2 (Z=-2.023, $p \leq 0.05$). Comparisons of the reaching components in the POST-STRESS group from pre-lesion stress to fourteen DPO found deficits in grasp (Z=-2.023, $p \leq 0.05$), supination 1 (Z=-2.023, $p \leq 0.05$), supination 2 (Z=-2.201, $p \leq 0.05$), and the total score (Z=-1.992, $p \leq 0.05$) (see Figure 8).

A

B
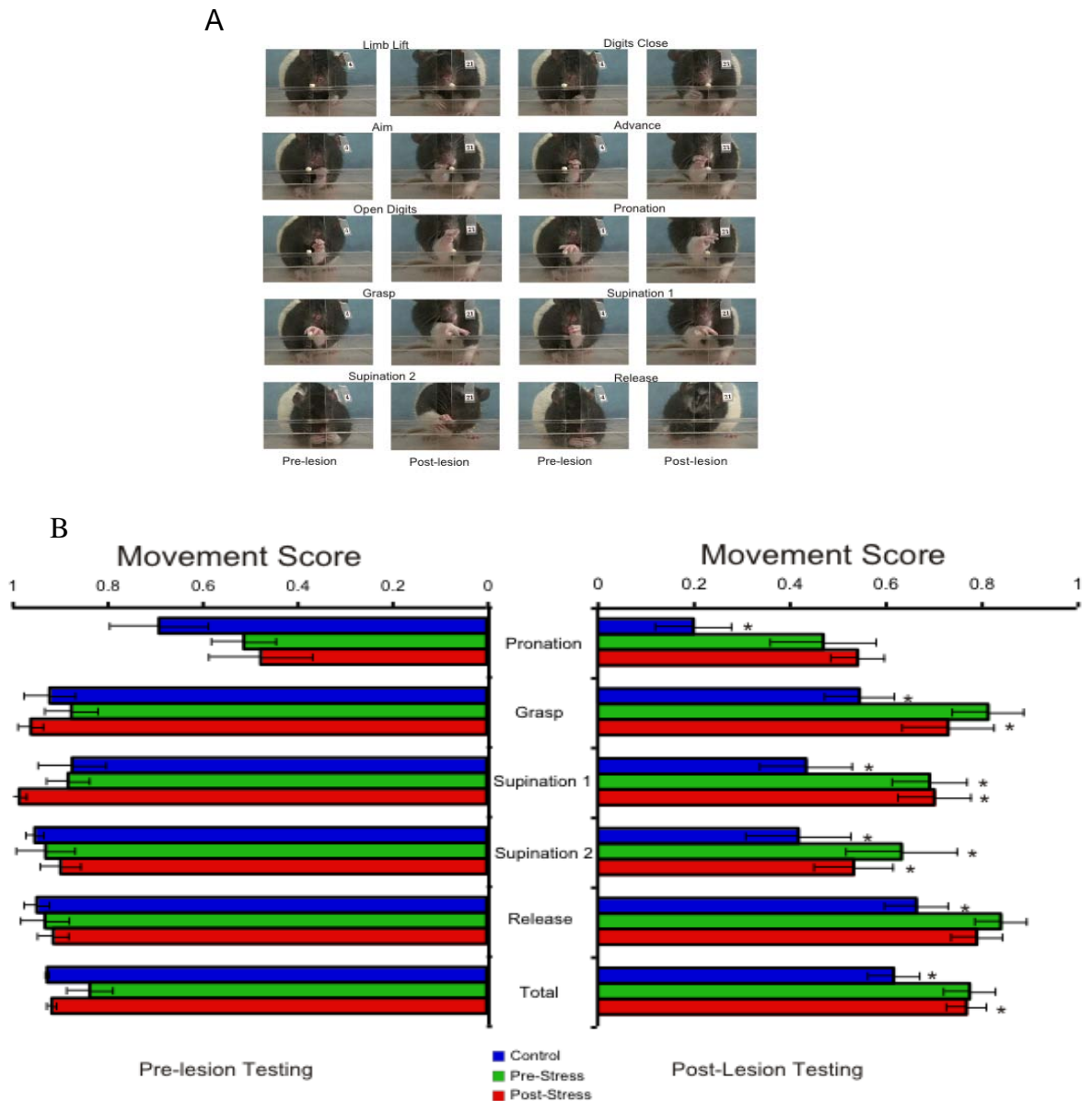
Figure 8. Qualitative analysis of reaching movements in the single pellet reaching task. (A) Within-group comparisons were made between pre-lesion and post-lesion testing. CONTROL rats (blue) showed impairments in limb lift, advance, digits open, pronation, grasp, supination 1, supination 2, release, and total score. The PRE-STRESS rats (green) had impairments in supination 1 and 2. POST-STRESS rats (red) had impairments in grasp, supination 1, supination 2, and total score (B). There were no significant differences in any of the reaching components between any of the groups prior to or after the lesion. * $p \leq$ 0.05, ** $p \leq$ 0.01, *** $p \leq$ 0.001. Group differences were analyzed via Kruskal-Wallace test. Within-group comparisons were performed by using the Wilcoxon Signed Rank Test. All data are presented as group means $\pm$ SEM.

Ladder Rung Walking Task

There were no significant group differences in average limb score at any time point (see Figure 9). Paired comparisons showed that the CONTROL group, were not significantly different in performance of either paw during baseline and pre-lesion time points. The contralateral (impaired) forelimb after ischemic lesion showed significant impairments compared to the ipsilateral (non-impaired) forelimb ($Z=-2.240$, $p \leq 0.05$). There was no significant difference between the hind limbs in the CONTROL group at any time point. In the PRE-STRESS group there were no significant differences between the forelimb and hind limbs at any time point. The contralateral score was significantly impaired after the lesion, when compared to pre-lesion testing ($Z=-2.521$, $p \leq 0.05$). In the POST-STRESS group, the contralateral forelimb displayed significant impairments compared to the ipsilateral forelimb ($Z=-2.366$, $p \leq 0.05$). In addition, the contralateral hind limb had exacerbated deficits in comparison to the ipsilateral hind limb ($Z=-2.521$, $p \leq 0.05$).
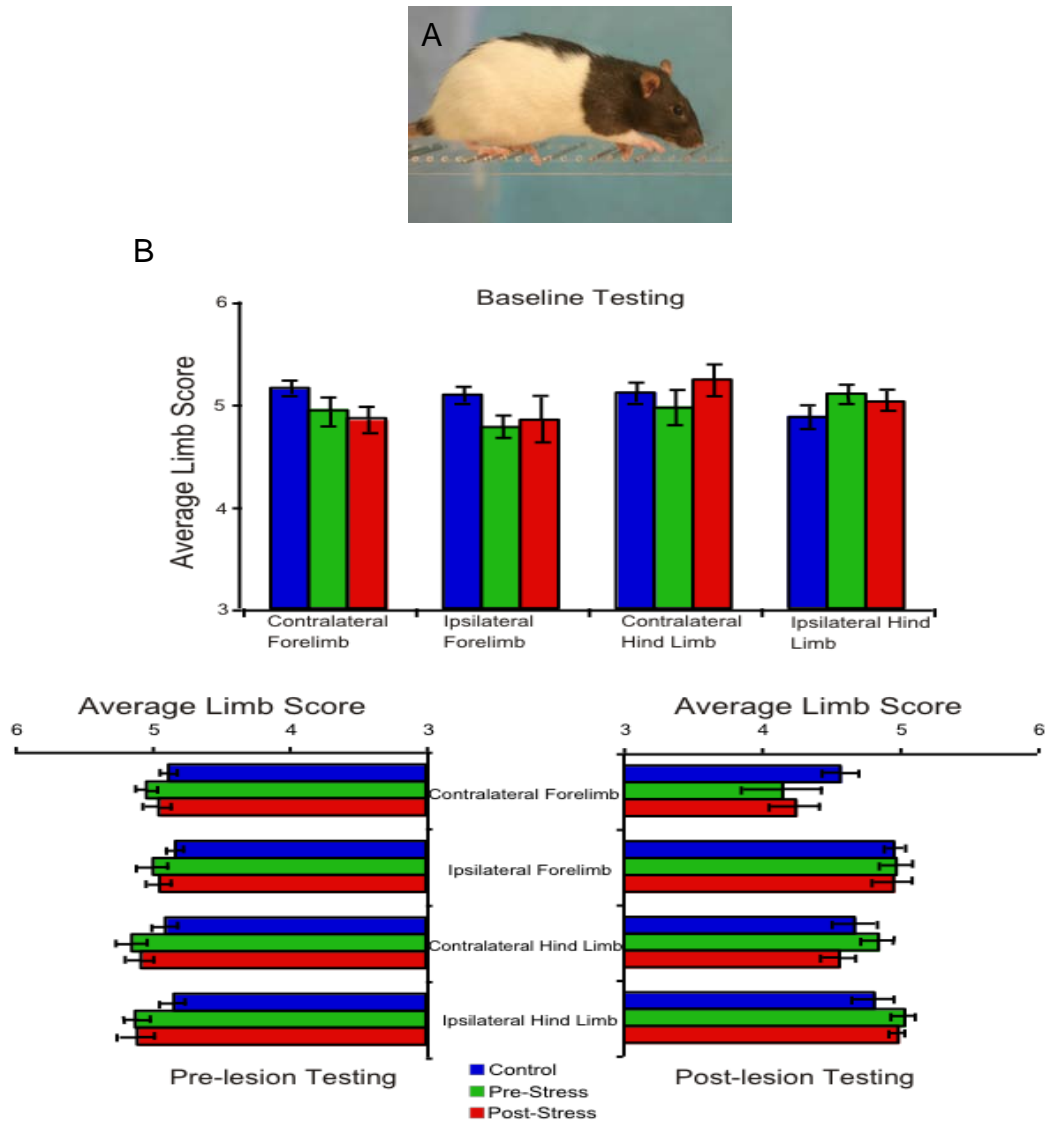
Figure 9. Average limb scores in the ladder rung walking task (A). There were no significant differences in average limb scores between the CONTROL (blue), PRE-STRESS (green) and POST-STRESS (red) groups at any time point (B). Between-group comparisons was performed with the Kruskal-Wallis test. All data are presented as group means $\pm$ SEM.

There was no significant difference in percentage of error made by each paw between the groups at pre-lesion testing and on fourteen DPO (see Figure 10). There was a significant difference between the groups in percentage of errors made by the

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contralateral forelimb at baseline (H=8.371, p≤0.05, Kruskal-Wallis Test) and the difference was found to be between the CONTROL and POST-STRESS groups (Z=-2.626, p≤0.01) and the PRE-STRESS and POST-STRESS groups (Z=-2.048, p≤0.05). There was no significant difference between the forelimbs and hind limbs of the CONTROL group during baseline and pre-lesion testing. After lesion, there was a significant increase in percent error between the contralateral and ipsilateral forelimb (Z=-2.366, p≤0.05). In the POST-STRESS group, there was a significant increase in percent error of the contralateral hind limb then compared to the ipsilateral hind limb (Z=-2.521, p≤0.05) on fourteen DPO. There were no significant differences between the limbs at baseline or pre-lesion testing. There were no significant differences in percent error between the forelimbs and hind limbs in the PRE-STRESS group at any time point. There was, however, a significant increase in percentage of error in the contralateral forelimb from pre-lesion testing to fourteen DPO in the PRE-STRESS group (Z=-2.366, p≤0.05).
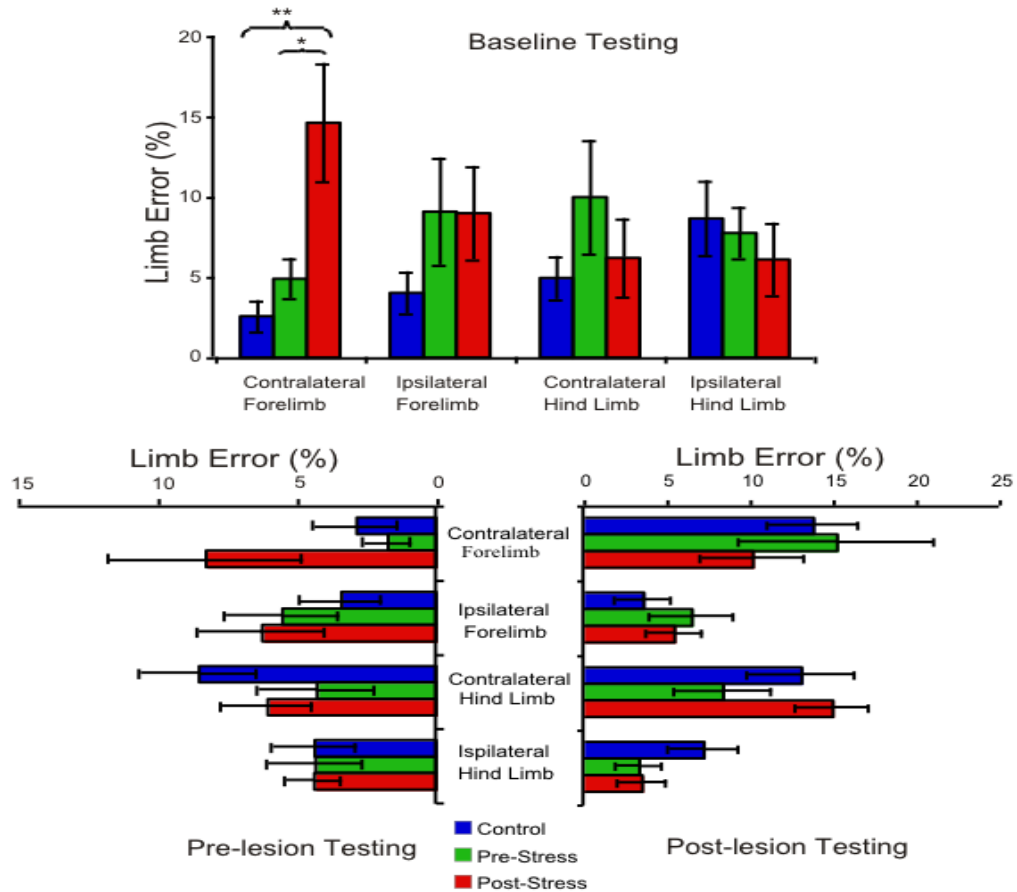
Figure 10. Percentage of limb errors while crossing the ladder-rung walking task. The POST-STRESS (red) group made significantly more errors with the contralateral forelimb than the PRE-STRESS (green) and CONTROL (blue) groups. There was no significant difference between the groups at pre-lesion or post-lesion testing. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Between-group comparisons done with the Kruskal-Wallis test. Follow-up between-group comparisons was done with the Mann-Whitney U test. All data are presented as group means $\pm$ SEM.

Discussion

Summary

Chapter 2 examined the effects of chronic restraint stress on motor recovery after devascularization. This is the first study to use thorough behavioural assessment to

investigate the effects of chronic restraint stress on skilled motor function after ischemic injury. Experiment 1 compared the effects of chronic restraint stress prior to the lesion to chronic restraint stress after the lesion. The data suggests that chronic restraint stress at either time point exaggerated motor deficits in skilled reaching after ischemic injury in rats. Chronic restraint stress prior to lesion decreased the success rate and the total number of pellets retrieved in the single pellet reaching task. Moreover, chronic restraint stress prior to lesion increased the number of attempts to grasp the pellet before and after the lesion. Post-lesion chronic restraint stress decreased the number of pellets eaten, and there was a trend towards impaired success rate and increased number of attempts in the single pellet reaching task. The data indicates pre-lesion chronic restraint stress has a larger influence on impairments in skilled reaching than post-lesion chronic restraint stress. In contrast, no differences were found in the ladder rung walking task. Pre-lesion chronic restraint stress had no influence on infarct size, however, infarct size was increased in rats that underwent post-lesion chronic restraint stress. Experiment 2 suggests that restraint stress does not influence edema after devascularization lesion.

Restraint Stress Induces Impairments in Skilled Reaching

Pre-lesion chronic restraint stress reduced success rate and the total number of pellets eaten prior to the lesion in healthy rats. A previous study by Metz et al. (2005b) found that chronic restraint stress also induced impairments in reaching success in healthy animals. Experiment 1 indicates that chronic restraint stress can impair skilled reaching performance after lesion. Pre-lesion chronic restraint stress reduced the number of successful reaches and pellets eaten after ischemic injury. In addition, post-lesion

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chronic restraint stress decreased the number of pellets eaten after devascularization lesion. This is the first study to show that chronic restraint stress can induce impairments in skilled reaching after ischemic infarct in rats. Post-lesion chronic restraint stress did not reduce the number of successful reaches after lesion, though there was a strong trend towards increased impairments after lesion. Regardless, chronic restraint stress after lesion attenuated the number of pellets eaten and the number of successful reaches, suggesting that chronic restraint stress induced impairments in limb coordination and grasping after the lesion in rats. Taken together, the stress rats were more likely than the control rats to knock the pellet away instead of grasping and eating the pellet. It is important to note that stress-induced impairments in reaching were not permanent. Rats that had undergone chronic restraint stress had recovered to control levels in success rate and the total number of pellets eaten suggesting chronic restraint stress slows down improvement of forelimb function, but not permanently impair function.

It is interesting that post-lesion chronic restraint stress did not further decrease success rates after lesion then pre-lesion chronic restraint stress. Indeed, there was no significant difference between the two chronic restraint stress groups in terms of success rate or number of pellets eaten after ischemic lesion. Any differences between pre-lesion stress and post-lesion stress on skilled reaching could possibly be masked by a floor effect. The lesion used in the current study induced severe impairments in reaching success suggesting chronic restraint stress may have only been able to induce reaching impairments to a point until the rats were unable to be any worse. Perhaps with a smaller lesion that induces smaller reaching deficits could differences in reaching success induced by chronic restraint stress prior to or after the lesion be noted.

Chronic Restraint Stress Increases Attempts to Grasp Food Pellets

Chronic restraint stress prior to lesion increased the number of attempts to grasp a pellet, particularly after the lesion. This may potentially be due to chronic restraint stress-induced anxiety and hyperactivity (Roth and Katz, 1979; Strekalova et al., 2005; Boyle et al., 2006). Chronic restraint stress increases anxiety related behaviours and increases dendritic arborization in the amygdala in rats (Vyas et al., 2002, 2004). A previous study by Metz et al. (2005b) found that chronic restraint stress increased the number of attempts to grasp the pellet. The same study found that success rates improved after giving the rats diazepam, which may have reduced hyperactivity and reduce the number of attempts to grasp the pellet, however, this was not assessed. Interestingly, pre-lesion stress greatly increased the number of attempts after the lesion, despite the rats no longer undergoing stress. Chronic restraint stress may have altered neural and behavioural processes that lasted well past the extinction of stress. It has been shown that chronic restraint stress can induce long-term changes in behaviour and neuroplasticity. Vyas et al. (2004) found that rats that were allowed to recover for 21 days after ten days of chronic restraint stress, still exhibited increased anxiety and neural hypertrophy within the amygdala. The present study demonstrates evidence of altered behavioural processes that lasted after the termination of chronic restraint stress. Chronic restraint stress prior to the lesion induced reaching deficits and increased the attempts to grasp a pellet, long after the stress had been terminated. Furthermore it would seem that ischemic lesion to the motor cortex altered these processes further, potentially disinhibiting them as seen in the increase in reaching attempts after the lesion in the pre-lesion stress rats. A lesion to the

motor cortex leads to dishinibition of contralateral forelimb movements during swimming in rats (Stoltz et al., 1999) and potentially could be responsible for the increase in attempts with the contralateral limb during reaching for food pellets.

It is noted that post-lesion stress did not increase the number of attempts to grasp the pellet compared to non-stress rats, although there was a trend. Pre-lesion chronic restraint stress may have induced rats to reach more often after devascularization lesion, because the stress-induced changes in neuronal and behavioural processes have already taken place prior to the lesion. Infarction to the motor cortex could have altered the inhibition of these networks. Alternatively, post-lesion chronic restraint stress may have been unable to alter neural processes. It is known that after ischemia, anxiety and depressive symptoms increase in rodents (Quinn et al., 2005; Winter et al., 2005) and humans (Paolucci et al., 1999; Williams, 2005; Fure et al., 2006). If stress-induced symptoms of anxiety are already high after ischemic infarct, then exposure to chronic restraint stress may be unable to further alter behaviour. Further investigation into the mechanism of chronic restraint stress-induced impairments in skilled reaching is needed.

It would appear the increase in attempts to grasp the pellet could be partially responsible for the decrease in success rate in the single pellet reaching task. An increase in the number of attempts to grasp the pellet would decrease the likely hood of a successful reach. The increase in attempts could explain why the pre-lesion rats show reaching deficits prior to and after lesion.

Restraint Stress Modulates Components of Reaching

An interesting finding was the observation that the non-stress rats showed more qualitative impairments after the lesion than rats undergoing pre-lesion or post-lesion chronic restraint stress. This was not expected as the non-stress rats had significantly higher reaching success and obtained more pellets than the two stress groups. This suggests that rats that underwent chronic restraint stress prior to devascularization lesion where able to more or less maintain a similar quality of reaching behaviour after the lesion as they did prior to the lesion. This is in contrast to the non-stress rats who showed qualitative changes in how they reached for the pellet after the lesion in such areas as limb lift, advance, pronation, supination, and release in comparison to reaching performance prior to the lesion. The implications of this finding are that chronic restraint stress may somehow limit the development of compensatory motor strategies after lesion.

After ischemic injury, humans (Cirstea and Levin, 2000) and rats (Whishaw, 2000; Metz et al., 2005b; Gharbawie et al., 2005) adjust for difficulties in reaching by developing new compensatory motor strategies to assist in completing a task. For example, rats will adjust their posture and will perform exaggerated shoulder and body rotation in order to lift, aim and advance the paw (Gharbawie et al., 2005). The result is that rats develop new compensatory motor strategies to successfully grasp the pellet, because it has become exceedingly difficult to use former reaching strategies. The current study suggests that there were different reaching strategies between the groups. All rats, however, had difficulty in rotating the paw while withdrawing and eating the pellet. The data suggests that chronic restraint stress, especially prior to lesion, can impede animals from developing new compensatory movements after lesion which appears to slow the rate of functional improvement. Animals that underwent chronic restraint stress prior to

devascularization lesion displayed limited deficits in movement score. They had, however, lower reaching success rate in comparison to non-stress animals. This suggests that the development of compensatory motor strategies might improve functional outcome. At the same time, not developing compensatory motor strategies after an ischemic lesion and relying on previously successful strategies may slow down the rate of functional improvement.

The qualitative data suggests that when the stress rats reach, they are trying to use a similar reaching style as before the lesion, which has become exceedingly difficult. Chronically stressed rats may not be compensating, thus increasing their movement score but decreasing their success rate. The non-stress rats have adopted an alternative style of reaching which was slightly more effective than that used by stress animals. For example, this would involve advancing the limb at a higher angle then normal so to lower their paw on top of the pellet, to compensate for deficits in aim, pronation and grasping the pellet. Typically non-lesion rats will advance the paw forward and towards the pellet, pronate the paw in an arpeggio movement before grasping the pellet. New compensatory reaching strategies the rats develop after ischemic lesion differs from their previous reaching style, thus reducing their movement score. By developing these compensatory movement strategies they are able to reach and grasp for more pellets then the stress animals. By fourteen DPO, the stress groups do reach control levels, suggesting that the stress-induced limitation in compensation is only detrimental in the short term. It may be that chronic stress is able to preserve the neural networks necessary for reaching. As discussed earlier it seems that chronic restraint stress prior to lesion may modify long-term motor behavioural and neural processes which may include the kinematics of skilled

movements. This is purely speculation, as it is unknown how stress affects plasticity within the motor system.

It may also be possible that the increase in the number of attempts seen in the stress group may have potentially induced a training effect. Indeed, previous studies have shown that the use of the contralateral paw after Et-1 induced MCAO (Biernaskie et al., 2001) and intracerebral hemorrhage (Debow et al., 2003) improves recovery in this limb in rats. The increase in the use of the contralateral paw in the stress group after lesion may have altered the quality of the reach, which may have assisted the functional improvement of the limb.

Restraint Stress Failed to Influence Skilled Walking

No group differences were found in the ladder rung walking task after lesion. This could be due to functional improvement of the stress animals to control levels by fourteen DPO. As seen in the skilled reaching data for success rate and number of pellets eaten, stress induced impairments early after lesion, but the animals are able to slowly improve to control levels by the end of behavioural testing. If the rats had been tested in the ladder rung walking task a few days after devascularization lesion, the stress rats may have shown impairments. Alternatively, it may be easier for the rats to compensate while crossing the ladder, thus masking any deficits induced by the lesion. The post-lesion stress group showed increased percentage of placement errors during baseline testing. This is most likely due to poor ability of the group to learn and perform the task during the initial training, as further testing showed a decrease in percent of errors made in the

post-lesion stress group as they learned to properly cross the ladder and were no longer different than the other groups.

Post-Lesion Restraint Stress Exacerbates Infarct Size

Chronic restraint stress after the lesion increased infarct volume in the present study. The only other study to examine stress after ischemic injury (Ritchie et al., 2004) found that chronic multiple stress starting one week after 2-VO exacerbates cell death within the CA3 layer of the hippocampus in rats. Stress can influence infarct size by promoting the release of glutamate resulting in excitotoxicity (Madrigal et al., 2003), inducing hyperglycemia (Sapolsky, 1985; Payne et al., 2003) and inhibiting bcl-2 expression (DeVries et al., 2003). Another possible mechanism of stress-induced cell death may be hyperthermia. Hyperthermia exacerbates infarct size in rats (Kim et al., 1996; Baena et al., 1997) and worsen clinical outcome (Ginsberg and Busto, 1998). Stress is capable of inducing hyperthermia in rats. Repeated social defeat in male mice increases body temperature from 37C to 39°C (Keeney et al., 2001). A mild stress paradigm consisting of food and water deprivation, continuous light or dark cycles, and soiled cages in rats also increased body temperature (Ushijima et al., 2006). Measuring temperature using rectal probes induces stress-induced hyperthermia in gerbils (Clark et al., 2003). An interesting paper by Kitatkin and Wise, (2001), using temperature probes designed to measure brain temperature, found that stressors such as social stress, saline injection, and tail pinch increases temperature in the dorsal and ventral striatum. In addition, the stress from just handling and testing increases rats' body temperature (Colbourne, Personal Communication). Another possibly explanation for the increase in

infarct volume in the post-lesion group may be due to the effects of stress on the cardiovascular system. Stress increases blood pressure (Sapolsky et al., 2000), which could have increased bleeding in the infarct site. During the devascularization process, the blood vessels are severed using a cotton tip. Increased blood flow could cause these damaged vessels to hemorrhage and increase infarct size. Post-lesion stressed rats exhibited high levels of blood within the lesion compared to pre-lesion stress and non-stressed rats which could result in larger infarct volume.

No effect of chronic restraint stress prior to the lesion on infarct size was found. Madrigal et al., (2003) found that chronic restraint stress for 21 days prior to MCAO increased infarct volume while seven days of restraint stress increased infarct size. Two weeks of restraint stress prior to devascularization lesion appears to have no influence on infarct volume in the present chapter. Different lengths of exposure to stress prior to ischemic lesion appear to differentially influence infarct size.

Regardless, it would appear that infarct volume is not the main culprit for the differences in behavioural deficits seen in the current study. Chronic restraint stress prior to lesion had no influence on infarct size, but induced the most severe behavioural deficits after lesion. It seems most likely that the results are due to the effects of stress on behavioural and possibly neural processes rather then the size of the infarct. It has been suggested previously that there is no direct link between infarct size and behavioural outcome in rats (Corbett and Nurse, 1998), which was supported in the current study.

The Failure of Chronic Stress to Modulate Edema

Chronic restraint stress had no effect on edema formation regardless of whether it was induced prior to or after the lesion. There are several possibilities for the findings. Devascularization lesion may not be the ideal animal model of stroke for measuring edema. Part of the problem is that a devascularization lesion requires a craniotomy. A craniotomy reduces ICP and possibly allows excess edema fluids to escape through the opening. It is possible that by replacing the missing bone, no edema fluid would escape from the brain, which may have changed the results. The second possibility is that the lesion was not big enough. Edema is most severe after larger lesions, such as MCAO (Ayata and Rooper, 2002). It is possible that with higher levels of edema, there are more chances for stress hormones to influence edema. Lastly, it is possible that GR activation and stress do not influence edema formation after ischemic stroke. There have been several studies which have found that treatment with dexamethasone failed to influence edema formation after stroke in humans (Ito et al., 1980; Ogun and Odusote, 2001). Dexamethasone may reduce edema via a mechanism that is independent of its activation of GR. Restraint stress after the lesion showed a trend towards significantly increased edema severity on two DPO. Thus the effects of stress on edema will be examined again in Chapter 3. Nevertheless, edema is most likely not the mechanism for the early impairments seen in the stress animals after devascularization in the present study.

Potential Mechanism of Restraint Stress induced Impairments

The mechanism into how chronic stress modulates recovery and compensation in the current study is unknown. It is possible that stress alters neuroplasticity in surrounding tissue or in the contralateral hemisphere. Chronic restraint stress can

influence neural plasticity within the limbic system (Sapolsky, 2003; Sandi, 2004). So far, no studies have examined whether chronic restraint stress modulates neural plasticity within the motor system. High levels of GR receptors are located in the motor cortex, striatum, and the cerebellum (Ahima et al., 1990; Ahima et al., 1991) suggesting that stress hormones may play a role in neuronal function of the motor system. Chronic restraint stress could modulate neural plasticity within the motor system which could act to increase the number of attempts to grasp the pellet and also preserve motor movements after ischemic infarct in rats. Chronic restraint stress-induced changes in neural plasticity could explain why motor deficits persist after the extinction of the stressor.

Another possible explanation for this finding may be due to stress-induced anxiety, depression, and learned helplessness. Chronic restraint stress has been used as an animal model of anxiety (Vyas and Chattarji, 2004; Strekalova et al., 2005; Kim and Han, 2006), and depression (Strekalova et al., 2005; Kim and Han, 2006). Chronic exposure to stress can also induce learned helplessness in rats (Kademian et al., 2005). It is possible that the stress-induced anxiety, depression, and learned helplessness could have hindered the rats' ability to adapt and cope with the lesion induced deficits thus slowing down the rate of functional improvement. Indeed, clinical studies have shown that high levels of anxiety or depression can impede functional recovery and represent predictors of poor outcome (Sinyor et al., 1986; Shimoda and Robinson, 1998; Paolucci et al., 1999; Lewis et al., 2001). A study by Nannetti et al. (2005) found that depressed stroke patients started with lower scores on functional and motor abilities, however, they were still able to recover over time when receiving rehabilitation treatment. Additionally, once discharged from the hospital, depressed patients showed slower recovery compared to non-depressed

patients (Nannetti et al., 2005). The current study replicates this finding in rats as chronically stressed rats initially had lower scores early in skilled reaching, however, where still able to show moderate improvements. Metz et al. (2005b) found that rats given the anxiolytic drug diazepam increased in reaching success suggesting that stress-induced anxiety may play an important role in the present study.

Conclusion

In conclusion, the present study found that chronic restraint stress is capable of inducing skilled motor impairments after devascularization. There seem to be time dependent effects of chronic restraint stress. Chronic restraint stress prior to lesion induced the most behavioural impairments in skilled reaching, while only chronic stress after the lesion increases infarct volume. The behavioural deficits induced by chronic restraint stress seem to be due to stress-induced changes in behavioural and possibly neuroplastic processes, rather then changes in edema formation and infarct size. The most likely mechanism of chronic restraint stress-induced impairment is due to increased anxiety/depression-like behaviors. The next chapter will further examine the role of chronic stress on motor recovery after devascularization lesion.

Chapter 3

A Chronic Unpredictable Stress Paradigm does not Induce Motor Impairments after Devascularization Lesion in the Rat

Introduction

Chapter 2 and Kirkland et al. (submitted) have shown that chronic restraint stress induces motor impairments after devascularization lesion in rats. Restraint stress, however, is only one type of stressor so it is important to note whether effects of chronic restraint stress on motor recovery generalize to other types of stress. To better understand the effects of chronic stress on motor impairments after devascularization, *Chapter 3* examined whether the type of stressor determines the degree of motor impairments. A study by Vyas et al. (2002) compared the effects of neural plasticity and anxiety of chronic restraint stress to a chronic unpredictable multiple stress in rats. The authors found that chronic restraint stress significantly induced dentritic atrophy in the CA3 layer of the hippocampus, as well as hypertrophy in the amygdala. Chronic multiple stress, however, resulted only in mild atrophy in the CA3 layer and amygdala. Furthermore, rats exposed to chronic restraint stress showed elevated anxiety-like behaviour in the elevated plus maze, as opposed to rats treated with multiple stress (Vyas et al., 2002; Vyas and Chattarji, 2004). Richardson et al. (2006) stressed pregnant rats with either chronic restraint stress or an unpredictable stress paradigm and compared the behavioural alterations in the offspring. Chronic restraint stress resulted in offspring that exhibited increased anxiety-related behaviours while multiple stress did not. This suggests that stress-induced behavioural changes depend on the type of stressor. Potentially, these stressor specific behaviours could differentially affect motor recovery after lesion.

One would expect that unpredictable stress has more severe effects on behaviour then restraint stress. Exposure to a repeated stressor such as restraint results in habituation (Magarinos and McEwen, 1995; Girotti et al., 2006) while a chronic multiple stress paradigm does not induce habituation (Magarinos and McEwen, 1995) in rats due to the unpredictability of the stressors. A study by Madrigal and colleagues (2003) found that acute exposure to restraint stress increases infarct volume, while chronic exposure to restraint stress decreased infarct volume. In *Chapter 2*, chronic restraint stress prior to lesion had no influence on infarct size, while post-lesion stress exacerbated infarct size. Stress induced impairments in success rate and percentage of pellets eaten in the single pellet reaching task in Experiment 1 climbed to control values after two weeks of testing. Habituation might potentially attenuate the impact of chronic stress on motor recovery and infarct size after lesion, which could have affected the results from *Chapter 2*.

Chapter 3 is comprised of two studies. Experiment 3 employed a multiple stress paradigm to compare the influence of stressor type on skilled motor function after devascularization lesion. The stress paradigm consisted of seven different stressors including restraint, forced swim, foot shock, shaking, fox odor, overcrowding, saline injection, and social isolation. To maximize stress-induced changes in behaviour, stress was given prior to and after lesion. The previous chapter found that chronic restraint stress prior to the lesion induced the most motor impairments in skilled reaching, while post-lesion chronic restraint stress exacerbated infarct volume. Devascularization was used to create an ischemic injury of the motor cortex. The single pellet reaching task and the ladder rung walking task were used to assess recovery of skilled motor function

following the lesion. Animals were perfused two weeks after lesion and histological assessments were performed to calculate infarct volume. It was hypothesized that due to the unpredictive nature of the chronic multiple stress paradigm, the rats will be unable to habituate to the stress and the rats will exhibit severe chronic impairments in skilled motor function. Lastly, it was predicted that chronic multiple stress will exacerbate infarct volume.

Experiment 4 represents a continuation of Experiment 2. It was observed that restraint stress showed a trend towards significantly higher edema on two DPO. The design of Experiment 4 was to further examine the effects of stress on edema formation after devascularization lesion. The stressors used included fox odor, corticosterone treatment, restraint stress, and foot shock. Rats underwent devascularization of the motor cortex, then underwent the designated stressor for two days. On two DPO, edema formation was measured using the wet-dry assessment. It was hypothesized that stress and corticosterone treatment would diminish edema levels after lesion.

Methods

Experiment 3

Animals

Thirty-six male Long-Evans rats (90 days and weighing 400-600 g at the beginning of the experiment) raised at the University of Lethbridge vivarium were used in this study. The rats were housed in pairs under a 12 hr light/day cycle with lights on at 7:30 AM. All procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Experimental Design

Rats were food deprived to 90-95% of their baseline weight. Rats were trained in the single pellet reaching task for five weeks. Rats were then filmed in the single pellet reaching task and the ladder rung walking task as a measure of baseline motor performance. Blood samples were taken to obtain baseline measurements of corticosterone the day after filming. The rats were matched based on baseline reaching success and subdivided into a STRESS & LESION group (n=18) and a LESION group (n=18). Rats in the STRESS & LESION group were stressed for a total of 24 days. Prior to the lesion the STRESS & LESION group underwent eight days of stress. Both the STRESS & LESION and LESION groups were tested in the single pellet reaching task daily. On day seven, rats were filmed in the single pellet reaching task, and in the ladder-rung walking task. Blood samples were taken on day eight. On day nine, both groups received a focal ischemic injury of the motor cortex. Rats in the STRESS & LESION group were stressed five minutes prior to the lesion. The multiple stress paradigm continued for an additional 15 days. Single pellet training continued two DPO. Rats were filmed in behaviour on day 23 of stress (fourteen DPO). On day 24 (fifteen DPO) rats underwent blood sample collection for post-lesion analysis of corticosterone. Rats were perfused the following day and the brains were removed for analysis of infarct size. A timeline of Experiment is illustrated in Figure 11.
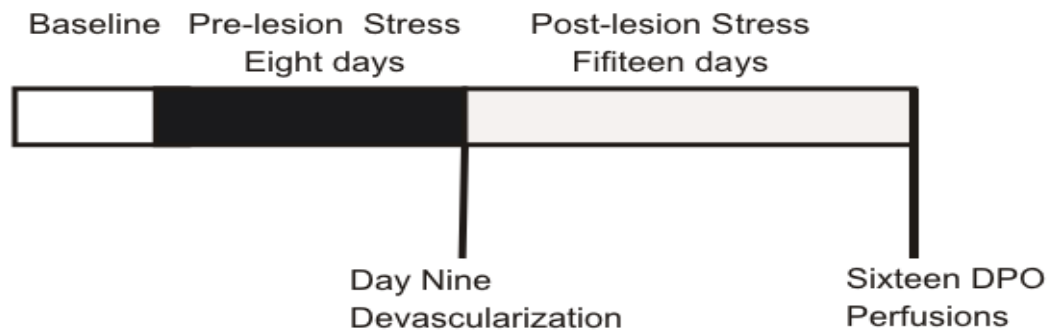
Figure 11. Timeline of Experiment 3. Rats in the STRESS & LESION group were stressed on 24 days, while the LESION group did not undergo chronic stress. After baseline testing, the STRESS & LESION group underwent stress treatment for eight days. On day nine of stress both groups underwent devascularization. The STRESS & LESION group continued to be stressed for 15 days after lesion. Blood samples were taken on the last day of baseline testing, day eight of stress, and on day 24 of stress (fifteen DPO). Devascularization was induced on day nine of stress. Animals were perfused on sixteen DPO.

Multiple Stress Paradigm

The multiple stress paradigm used in the present study was a combination of stressors that have been shown to induce stress and increase corticosterone levels in rats. The multiple stress paradigm consisted of two different stressors per day. The first stressor occurred from 9:30AM to 11:30AM and the second stressor occurred from 3:00PM to 6:00PM. The only exception was single housing, in which the animals were separated from their cage mate in the afternoon, and were socially isolated overnight before being returned to their home cage on the following morning. Rats were tested in the behavioural tasks ten minutes after the end of the morning stressor. On the last day of baseline, day eight of stress, and on fifteen DPO, blood samples were taken immediately after the morning stressor. Control animals were handled daily.

The schedule of the multiple stress paradigm can be seen in Table 1. The stressors used in the paradigm included restraint stress (Vyas et al., 2002; Metz et al., 2005b;

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Richardson et al., 2006), swim stress (Metz et al., 2001; Vyas et al., 2002; Metz et al., 2005b), shaking (Magarinos and McEwen, 1995; Vyas et al., 2002; Alfarez et al., 2003), foot shock (Tentani et al., 2003; Westenbroek et al., 2005), saline injection (0.09%) (Kiyatkin and Wise, 2001; Richardson et al., 2006), social isolation (Vyas et al., 2002), overcrowding (Djordorvic et al., 2003), and fox odor (Morrow et al., 2000; Day et al., 2004; Thomas et al., 2006). A brief description of the stressors is given below.

Restraint stress: Rats were placed in a cloth pillow case, and duct tape was wrapped on the cloth around their body to restrict movement. The restraint stress lasted for 20 minutes. *Swim stress*: The rats were placed in a bin filled with 23ºC water. The rats were unable to touch the bottom with their feet. The temperature of the water was 23ºC. Rats were forced to swim for five minutes. *Saline Injection*: Rats received one i.p. injection of 0.2 ml of a 0.9% saline solution. *Fox Odor*: Rats were exposed to 2,4,5-trimethylthiazoline (TMT) also known as fox odor (Pherotech, Delta, British Columbia) for 20 minutes. Kim wipes with 20 μl of TMT were placed into a bottle. The bottle was placed in the corner of a transport tub (40 cm*23.5 cm*20 cm) with the rat. Only one rat was placed in the tub at a time. *Shaking*: Individual rats were placed in a transport tub (24.5 cm*17.5 cm*12.5 cm) and placed onto a unstable surface (a belly dancer) at 75 rpm for 20 minutes each. *Foot Shock*: Rats were individually placed in an operant conditioning box. Each rat received a foot shock from the grid floor after 20 seconds. The foot shock lasted for two seconds at 0.8 mA intensity. The rats stayed in the box for another 40 seconds. *Overcrowding*: Six rats were placed in a transport tub (40 cm*23.5 cm*20 cm) at one time. Different rats were combined in each session. Overcrowding lasted for one hour. *Single Housing*: The rats were housed in pairs. One rat was removed

from its home cage and placed into another cage. Both rats were isolated overnight (approximately from 4 PM to 8 AM). For each session of single housing, the home cage was alternated between the rats.

Table 2
Chronic Unpredictable Multiple Stress Paradigm

Day	Morning/Afternoon
1)	Restraint/Overcrowding
2)	Forced Swimming/Foot Shock
3)	Saline Injection/Shaking
4)	Fox Odor/Social Isolation
5)	Shaking/Overcrowding
6)	Foot Shock/Forced Swimming
7) Filming Day	Restraint/Social Isolation
8) Blood Samples	Fox Odor/Forced Swimming
9) Stroke	Foot Shock/Social Isolation
10)	Saline Injection/Foot Shock
11)	Restraint/Overcrowding
12)	Fox Odor/Shaking
13)	Forced Swimming/Overcrowding
14)	Shaking/Social Isolation
15)	Foot Shock/Saline Injection
16)	Shaking/Fox Odor
17)	Saline Injection/Overcrowding
18)	Forced Swimming/Shaking
19)	Foot Shock/Saline Injection
20)	Restraint/ Forced Swimming
21)	Saline Injection/Social Isolation
22)	Restraint/Overcrowding
23) Filming Day	Fox Odor/Social Isolation
24) Blood Samples	Restraint/Fox Odor
25)	Perfusions

The timeline and schedule of the stress paradigm used in the current study. The rats are exposed to two stressors a day, one in the morning (starting at 9:30 AM – 11 AM) and one in the afternoon (3 PM – 6 PM). Each respective stressor occurred six times throughout the 24 day stress period.

Blood Samples

Rats were anesthetized with isoflurane (4% induction and 4% maintenance, 1.5% oxygen). Blood samples were taken from the tail vein and an average of 1 ml of blood was collected. The remainder was centrifuged at 5000 rpm for eight minutes. The serum was collected and stored at -20°C. Blood samples were taken at baseline, on day eight of stress prior to ischemia (pre-lesion), and on fifteen DPO. Blood samples were after stress (Magarinos and McEwen, 1995; Metz et al., 2005b). No behavioural testing was conducted on days in which blood samples was taken. Unfortunately, due to technical issues, analysis of corticosterone was not completed in time for the current thesis.

Focal Cerebral Ischemia

Rats were anesthetized with isoflurane (4% induction and 2% maintenance, 1.5% oxygen). Focal cerebral ischemia in the motor cortex was induced via devascularization as described by Whishaw (2000). Briefly, the skin over the skull was incised and the skull was exposed. Using a fine dental drill, a craniotomy was made at the following coordinates: -1.0 to 4.0 mm anterior, -1.5 to 4.5 mm lateral. The dura was removed, and the blood vessels were carefully wiped off using a cotton tip. The lesion was made to the motor cortex contralateral to the rat's preferred reaching paw in the single pellet reaching. Then the skin was sutured and the rat was given 0.05 ml of the analgesic, Temgesic (Schering –Plough, Brussels, Belgium). Rats were allowed to recover in individual cages on top a heating pad until fully awake and were then returned to their home cages.

Single Pellet Reaching Task

The rats were trained in the single pellet reaching task which assesses independent skilled forelimb function (Whishaw et al., 1993). The reaching boxes were made of clear Plexiglas (40 cm*45 cm*13 cm). The front wall of the box had a 1.3 cm wide vertical slot, allowing the rats to reach for the food pellet located on a shelf attached to the outside of the box. The shelf was located 4 cm above the floor on the front of the box. On the top of the shelf were two indentations (5 mm in diameter, and 1.5 mm deep), each aligned with one side of the slot. These indentations stabilized the pellet and were located 1.5 cm away from the front wall (Metz and Whishaw, 2000). Prior to training, rats were food deprived to 90-95% of their free feeding body weight. In a training session, a rat was placed in the reaching box and a food pellet (45 mg each, Bio Serv, Frenchtown, New Jersey, USA) was placed contralaterally to the rat's preferred reaching paw. The rats were trained to walk to the rear end of the box before reaching for a new pellet. The rats were given 20 pellets per training and testing session. A successful reach was defined as obtaining the pellet on the first attempt, withdrawing the paw through the slot and releasing the pellet to the mouth and eaten. Success was calculated using the following formula:

Success rate = $\underline{\text{number of successful reaches}}$ * 100
                                     20

The percentage of total pellets eaten was measured by counting the number of pellets eaten, regardless of whether the pellet was grasped and eaten on the first attempt. If the rat dropped the pellet, it did not count as a pellet eaten. The percentage of total pellets eaten was measured using the following formula:

Percentage of total pellets eaten = $\dfrac{\text{number of pellets eaten}}{20} * 100$

To assess limb coordination, the number of reaches was measured by counting the number of attempts to grasp 20 pellets for each session.

Qualitative Analysis of Skilled Reaching

For qualitative analysis of single pellet reaching, a reaching movement was broken down into ten components and 28 subcomponents modified from Metz and Whishaw (2000). See Experiment 1, Table 1, for an outline of the components of skilled reaching and the rating scale used to score each component.

Ladder Rung Walking Task

Training and Testing

Fore- and hind limb coordination and limb placement was assessed by using the ladder-rung walking task (Metz and Whishaw, 2002). Rats were trained to cross a 1 m long horizontal ladder with metal rungs arranged at random distances, ranging from 1 to 5 cm. In each test session, the rung pattern was reversed to prevent the rats from anticipating the location of the rungs. Rats were trained to cross the ladder in five trials. In each test session rats were videotaped for three trials for further analysis.

Video Analysis for Ladder Rung Walking Task

A rating system according to Metz and Whishaw (2002) was used for qualitative analysis of skilled walking by frame-by-frame analysis. See Experiment 1 for an outline of the rating system of skilled walking.

Video recording

All behavior was recorded by using a Canon ZR50 MD camcorder set at a shutter speed of 1/1000 s. During filming, additional light was supplied by a cold light source (Lowel-light Mtg Inc, New York, USA). Frame-by-frame analysis was performed using a Sony GV-D1000 NTSC miniDV player.

Histology

On sixteen DPO, rats were sacrificed with an overdose of Euthansol (0.3mls) and perfused through the heart with 0.9% saline and 4% formalin. Brains were removed and post-fixed overnight before being placed in a 30% sucrose solution. In addition, adrenal glands were removed and weighed. The weights of the left and right adrenal glands were summed up and the adrenal weights corrected for body weight was calculated using the following formula.

$$\text{Adrenal Weight} = \frac{\text{Adrenal Weight}}{(\text{Body weight} - \text{Adrenal weight})}$$

The brains were frozen and 40 μm sections were cut on the cryostat. Every fifth section was mounted onto a slide and stained with creysl violet.

Measurement of Infarct Volume

Photographs of cresyl violet stained sections were taken digitally with a Zeiss microscope (Zeiss, Germany). Every third section was analyzed for lesion volume. The cross sectional volumes of both hemispheres were calculated using Zeiss Axiovision 4.3 (Zeiss Germany). Infarct volume according to Debow et al. (2003). The following formulas were used:

Volume of tissue lost = tissue remaining in normal hemisphere – tissue remaining in injured hemisphere

Volume of a hemisphere = average (average area of a complete coronal hemisphere – ventricles – area of damage) x interval between sections x number of sections

Statistical Analysis

Statistical analysis was performed by Statview software version 5.0 (SAS Institute, 1998). The data was analyzed using analysis of variance (ANOVA). The Scheffe's test was used for post hoc analysis. Within-group comparisons were made via paired t-tests. Correlation of infarct volume to motor recovery of fifteen DPO in single pellet reaching was assessed using Pearson's chi-squared test. The Mann-Whitney U test was used for qualitative reaching analysis and the ladder rung walking task. The Wilcoxon Signed Rank Test was used for within group comparisons of the qualitative reaching analysis and the ladder rung walking task. In all analysis a p value of ≤ 0.05 was chosen as the significance level. All data are presented as mean $\pm$ SEM.

Experiment 4

Animals

Forty-eight male Long-Evans rats (90-110 days and weighing 400-600 g at the beginning of the experiment) raised at the University of Lethbridge vivarium were used.

The rats were housed in pairs under a 12 hr light/day cycle with lights on at 7:30 AM. All procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Experimental Design

Rats were randomly assigned to one of six groups. A timeline for Experiment 4 is illustrated in Figure 12. The control group (CONTROL, n = 8) did not receive any ischemic lesion or any stressors. All the other groups underwent devascularization lesion of the motor cortex. One group underwent ischemic lesion (LESION, n = 8) and was not exposed to any stressors. The other groups were exposed to a particular stressor for two days after the lesion. One group of rats underwent restraint stress (RESTRAINT, n= 8) for 20 minutes a day in Plexiglass containers (7.5cm diameter, 20 cm long). Larger tubes were used in this study because of the size of the animals. The larger restraint containers still restricted the rats' movements. Another group of rats was fed 5 mg of corticosterone that was dissolved in peanut oil and placed on a teddy graham (CORT, n=8). One group of rats was exposed to fox odor. The fox odor procedure was nearly identical to that used in Experiment 1 except that the amount of time the rat was exposed to the fox odor was 20 minutes instead of ten minutes. One of the rats in the FOX group died shortly after the lesion was induced (FOX, n=7). The last group underwent foot shock (SHOCK, n=8) using the procedure described in Experiment 3.

The coordinates for devascularization of the motor cortex were identical to those used in Experiment 3. All lesions were induced in the left hemisphere. Starting one DPO, rats in the stress groups underwent their specific stressors, which lasted for two days. On

two DPO, rats were decapitated immediately after the stressor and brain edema levels were measured using the wet/dry method.
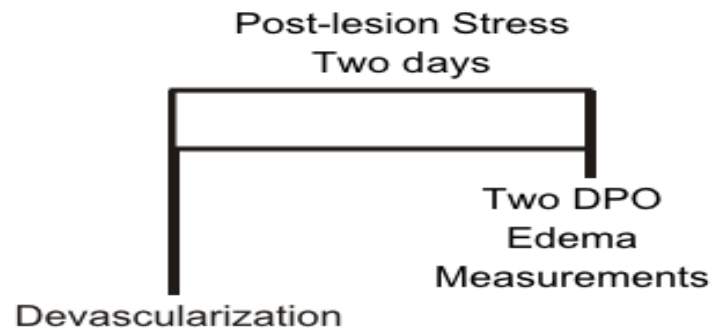
Figure 12. Timeline of Experiment 4. After devascularization, rats underwent treatment with restraint, fox odor, foot shock stress, or corticosterone treatment for two days. After the stressor on two DPO, rats were decapitated for measurements of edema. Blood samples were also taken on two DPO.

Edema Measurement

On two DPO, all rats were anesthetized with isoflurane (5% induction, 1.5% oxygen) and decapitated. The brains were removed, the hemispheres separated, and wet weight of the brains was measured. The brains were then dried in an oven (VWR, Scientific Products, USA) at 100°C for 24 hours. The dry weight of each hemisphere was taken. To measure edema, the wet/dry method, a standard method for edema measurement (Ito et al., 1979; Hatashita and Hoff, 1990; Gerriets et al., 2004; MacLellan et al., 2004) was used. The formula is as follows:

$$\text{Brain Water Content (\%)} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} * 100$$

In the case of the stress groups, all rats were anesthetized and decapitated immediately after exposure to the stressor. The exception was the CORT group, which was decapitated one hour after corticosterone treatment to allow the corticosterone to be metabolized (Metz et al., 2005b). Blood samples were taken after decapitation. Blood

samples and corticosterone levels were prepared using the techniques described in Experiment 3.

Statistical Analysis

Comparisons of the edema percentages of each hemisphere between two and fourteen DPO was performed using ANOVA between the groups. Post-hoc tests were performed using Scheffe's test. Comparison of the lesion hemisphere to the non-lesion hemisphere was compared via paired t-tests. In all analysis a p-value of ≤ 0.05 was chosen as the significance level. All data are presented as mean $\pm$ SEM.

Results of Chapter 3

Infarct Size

The lesion included the primary and secondary motor cortex as well as the forelimb and hind limb areas of the somatosenory cortex. On a few occasions there was tearing of the corpus callosum. Occasionally, blood was visible within the corpus callosum within the ispsilateral and contralateral hemisphere on several sections in both groups. No significant difference between infarct size were found between the two groups (see Figure 13).
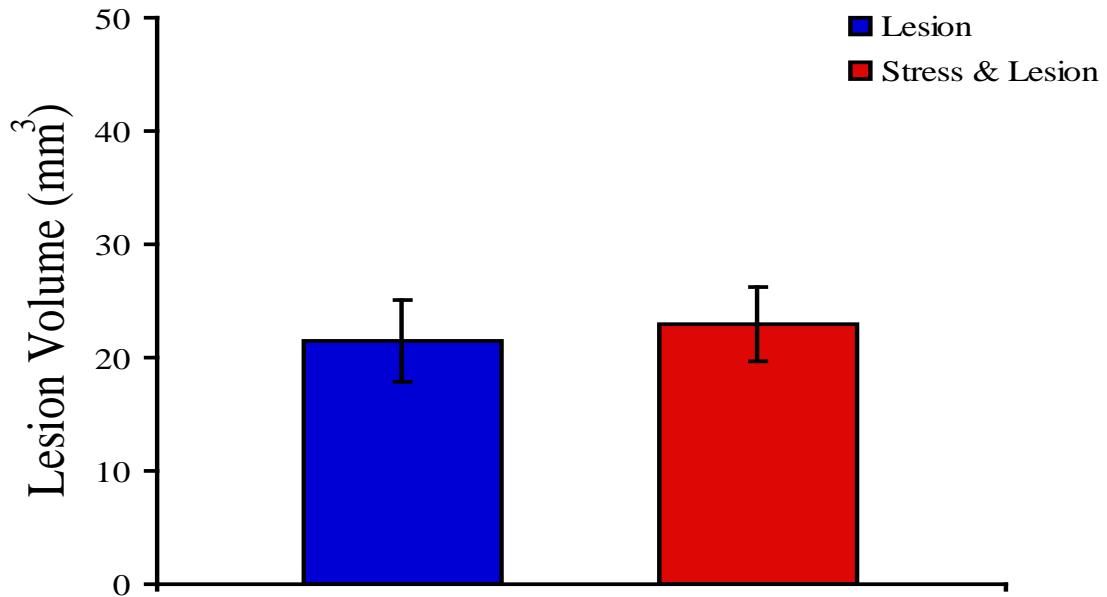
Figure 13. Volume of tissue lost 15 DPO after devascularization lesion of the motor cortex. No significant differences in infarct volume was found between the LESION (blue) and STRESS & LESION (red) groups. The average lesion volume in both groups was 21-23 mm^3. Comparisons between groups was done with ANOVA. All data are presented as group means $\pm$ SEM.

Brain Water Content

In the lesion hemisphere all the lesion treated groups had significantly higher edema then the non-lesion control group ($F = (5,41)$ 7.358, $p \leq 0.001$) (see Figure 14). In comparison to the CONTROL group, there were increased edema levels in all the treatment groups: CORT ($p \leq 0.05$), FOX ($p \leq 0.01$), RESTRAINT ($p \leq 0.01$), SHOCK ($p \leq 0.001$), and LESION ($p \leq 0.01$). There was no significant difference in edema formation between the LESION group and any of the stress treated groups. There were no significant differences between the groups in the amount of edema in the non-lesion

(right) hemisphere. Using paired t-tests, significantly higher levels of edema were noted in the lesion hemisphere when compared to the non-lesion hemisphere in the RESTRAINT group (t=6.416, p≤0.001), FOX (t=3.913, p≤0.01), LESION (t=5.686, p≤0.001), SHOCK (t=2.876, P≤0.05), and CORT group (t=4.982, p≤0.01). No significant difference in brain water content between the left and right hemisphere of the CONTROL group were found.
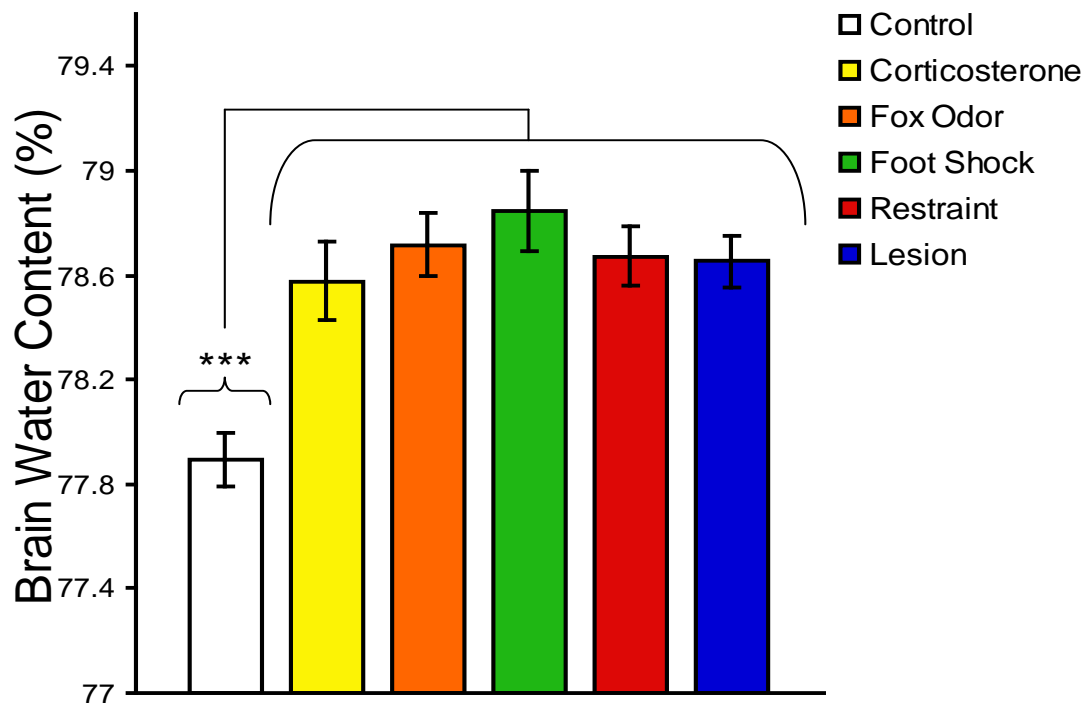
Figure 14. Percentage of brain water content (edema) in the lesion hemisphere after devascularization. Exposure to corticosterone (yellow) and stressors such as FOX ODOR (orange), FOOT SHOCK (green), and RESTRAINT (red) after the lesion had no influence on edema formation compared to the LESION (blue) group. The lesion hemisphere in all the lesion groups had significantly higher edema levels than the CONTROL (white) group. * p≤ 0.05, **p≤0.01, ***p≤0.001. Comparisons between groups were performed using ANOVA and the Scheffe's test. All data are presented as group means ± SEM.

Single Pellet Reaching Success

At baseline both the STRESS & LESION and LESION groups had a average success rate of 45%. There were no significant differences between the success rates in the STRESS & LESION and LESION groups at baseline, pre-lesion stress, and post-lesion testing. Success rates dropped in both groups as a result of the lesion (see Figure 15).

Within-group comparisons revealed no significant difference in percent success from baseline to pre-lesion testing in the LESION group. There was a significant decline in success rate from pre-lesion to post-lesion testing (t=7.785, p≤0.001) in the LESION group. In the STRESS & LESION group there was a significant decline in percent success from baseline to pre-lesion testing (t=2.151, p≤0.05). There was further significant decline in percent success during post-lesion testing compared to pre-lesion testing in the STRESS & LESION group (t=9.308, p≤0.001). There was no correlation between infarct size and success rate on 14 DPO in the LESION (R=0.133, p=0.680), and STRESS & LESION (R=-0.006, p=0.987) groups. There was no correlation between infarct volume and success rate on the final day of testing (R=0.376, p=0.077) when the groups were examined together.

Figure 15. Success rate in the single pellet reaching task. No significant differences were noted between the LESION (blue) and STRESS & LESION (red) groups at any time point. After the ischemic lesion there was a drop in success rates in both groups. Comparisons between groups was done with ANOVA. All data are presented as group means $\pm$ SEM.

Total Number of Pellets Eaten

No significant difference was found between the LESION and STRESS & LESION groups in the total percentage of pellets eaten during baseline, pre-lesion, or post-lesion testing (see Figure 16). The percentage of pellets eaten dropped in both groups after lesion in all groups.

Within-group comparisons revealed no significant differences in the number of pellets eaten from baseline to pre-lesion testing in both the LESION and STRESS & LESION groups. During post-lesion testing, the STRESS & LESION (t=10.587, p≤0.001) and the LESION (t=8.500, p≤0.001) groups ate significantly fewer pellets then

during pre-lesion testing. There was no correlation between infarct volume and number of pellets eaten on 14 DPO in the LESION (R=-0.252, p=0.430) and STRESS & LESION (R=-0.004, p=0.992) groups. There was no correlation between infarct volume and the number of pellets eaten on the final day of testing (R=0.264, p=0.224) when the groups were examined together.

Figure 16. Percentage of total pellets eaten in the single pellet reaching task. No significant differences were noted between the LESION (blue) and STRESS & LESION (red) groups at any time point. After the ischemic lesion there was a drop in the percentage of pellets eaten in both groups. Comparisons between groups were performed using ANOVA. All data are presented as group means ± SEM.

Number of Attempts

No significant differences were found in the number of attempts for the 20 pellets between the LESION and STRESS & LESION group at baseline, pre-lesion, and post-lesion testing (see Figure 17).

Within-group comparisons revealed that there was no difference in the number of attempts from baseline to pre-lesion testing in the LESION group. The LESION group made significantly more attempts to grasp the pellet after the lesion when compared to pre-lesion performance ($t=-5.287$, $p \leq 0.001$). The STRESS & LESION group made significantly more attempts during pre-lesion testing in comparison to baseline testing ($t=-7.926$, $p \leq 0.001$). In addition, the STRESS & LESION group made more reaching attempts during post-lesion testing then during pre-lesion testing ($t=-6.965$, $p \leq 0.001$).
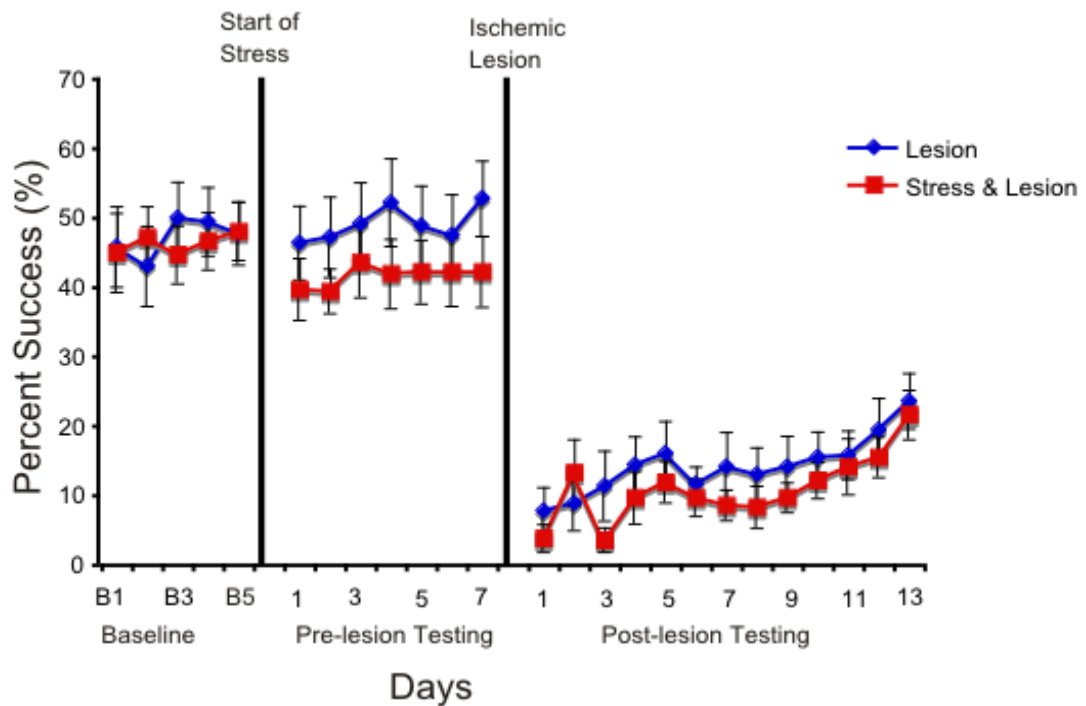

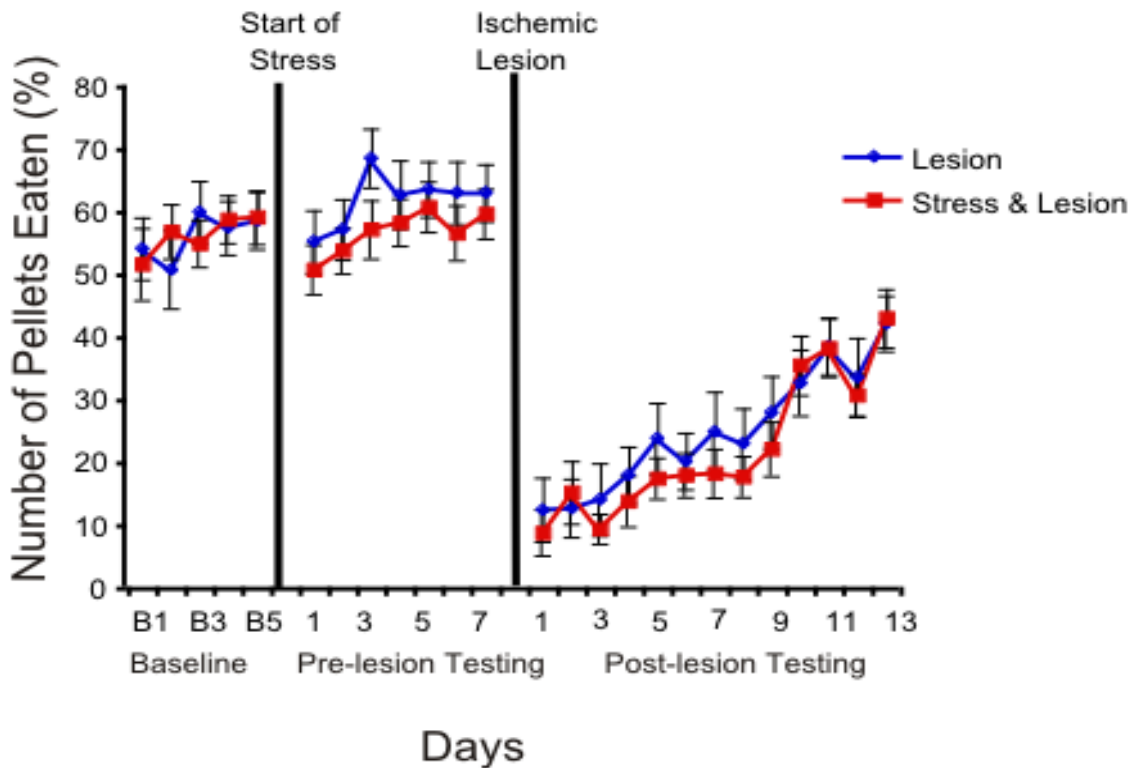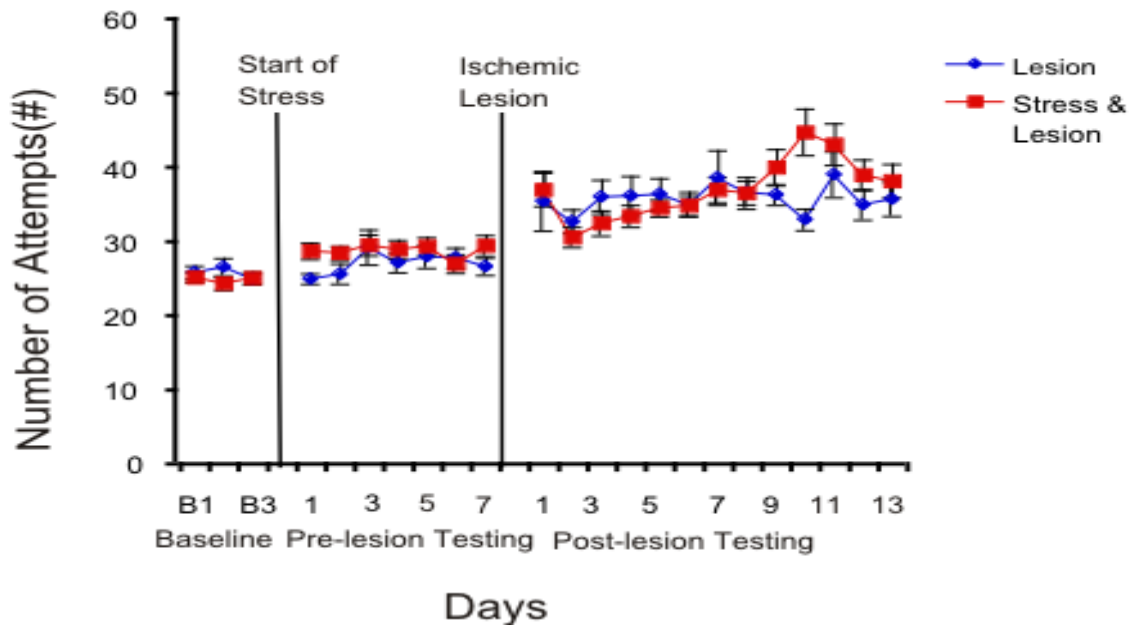*Figure 17*. Number of attempts to grasp the pellet in the single pellet reaching task. No significant differences were noted between the LESION (blue) and STRESS & LESION (red) groups at any time point. Comparisons between groups were performed using ANOVA. All data are presented as group means $\pm$ SEM.

Qualitative Reaching Movement Analysis

Baseline comparisons in qualitative reaching analysis found no significant difference between any of the ten reaching components (limb lift, digits close, aim, advance, digits open, pronation, grasp, supination 1, supination 2, and release). There was no significant difference in the total score between the two groups at baseline. During the pre-lesion week of stress there were significant differences in release (Z=-2.294, p≤0.05) and the total score (Z=-2.120, p≤0.05) between the STRESS & LESION and LESION groups with the STRESS & LESION group having a lower score in both categories. During the post-lesion stress period there was no significant difference between any of the components and the total score between the two groups (see Figure 18). Using within-group comparisons, the LESION group showed no significant difference between component scores at baseline and pre-lesion stress time points. The score for aim (Z=-2.344, p≤0.05) and the total score (Z=-2.367, p≤0.05) dropped significantly in the STRESS & LESION group from the baseline period to one week of pre-lesion stress. From the period of pre-lesion stress to fourteen DPO, the LESION group showed significant impairments in limb lift (Z=-3.170, p≤0.01), aim (Z=-3.040, p≤0.01), advance (Z=-2.840, p≤0.01), digits open (Z=-2.950, p≤0.01), pronation (Z=-3.479, p≤0.001), grasp (Z=-3.621, p≤0.001), supination 1 (Z=-3.464, p≤0.001), supination 2 (Z=-3.550, P≤0.001), release (Z=-3.206, p≤0.01) and total score (Z=-3.621, p≤0.001). Within-group comparisons of the STRESS & LESION group from pre-lesion stress to fourteen DPO found impairments in limb lift (Z=-3.209, p≤0.01), digits close (Z=-2.191, p≤0.05), advance (Z=-3.045, p≤0.01), digits open (Z=-2.376, p≤0.05), pronation (Z=-2.698, p≤0.01), grasp (Z=-3.170, p≤0.01), supination 1 (Z=-3.516, p≤0.001), supination 2 (Z=-

3.408, p≤0.001), release (Z=-2.158, p≤0.05), and total score (Z=-3.631, p≤0.001) (see Figure 8).
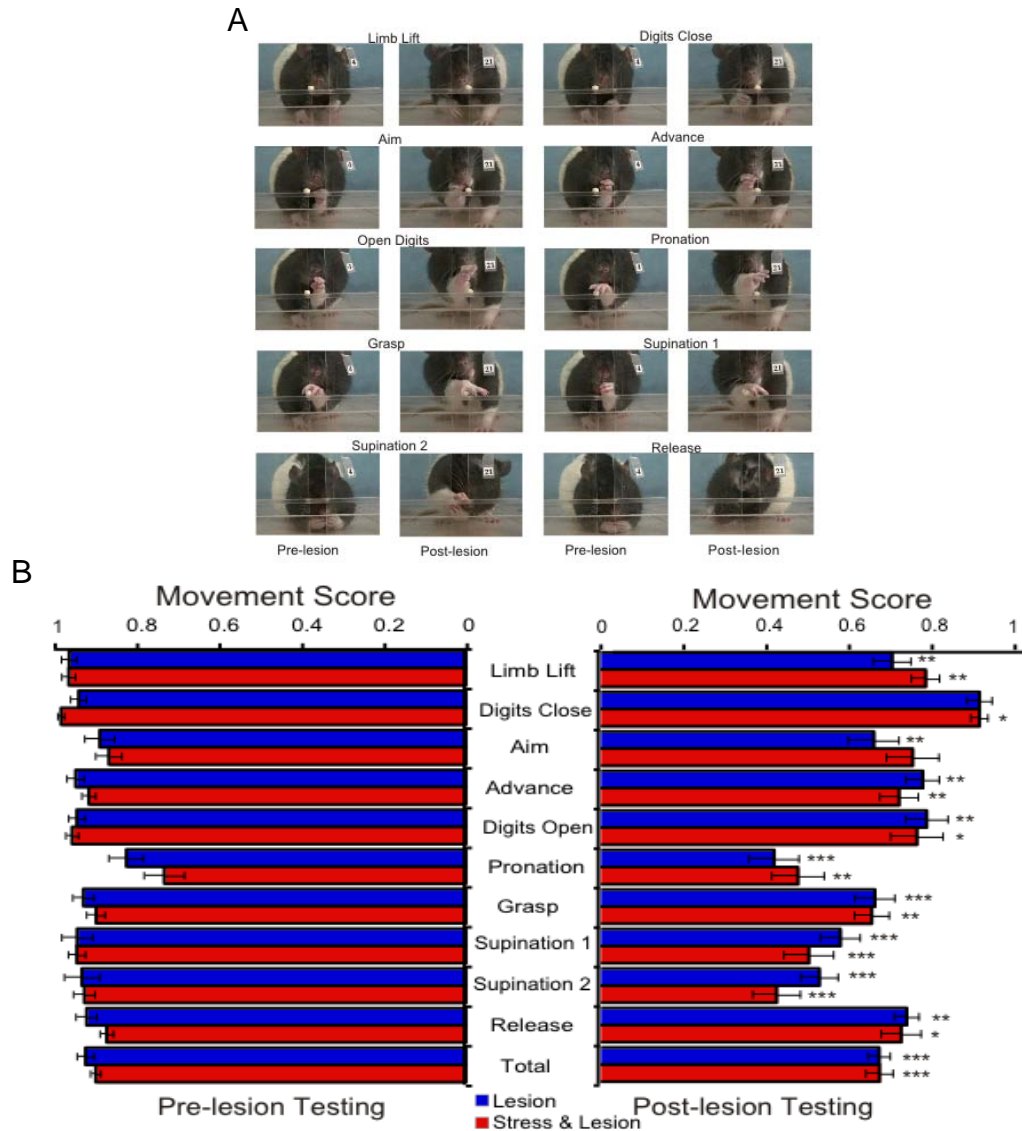
Figure 18. Qualitative analysis of reaching movement performance in the single pellet reaching task (A). After lesion, rats were compared to pre-lesion testing. (B) After lesion the LESION (blue) and STRESS & LESION (red) groups showed impairments in limb lift, advance, digits open, pronation, grasp, supination 1, supination 2, release, and total score. There were no significant differences between the groups after the lesion. * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001. Comparisons between groups using Mann-Whitney U test. Within group comparisons using Wilcoxon Signed Rank test. All data are presented as group means ± SEM.

Ladder Rung Walking Task

There were no group differences between the LESION and STRESS & LESION groups in average limb score of any of the limbs at any time point (see Figure 19). In the LESION group there were no differences between the contralateral limb and the ipsilateral limb between baseline and pre-lesion testing. At post-lesion testing there were significant impairments in the contralateral forelimb compared to the ipsilateral forelimb ($Z=-2.911$, $p \leq 0.01$) and significant impairments between the contralateral hind limb and the ipsilateral hind limb ($Z=-2.669$, $p \leq 0.01$) in the LESION group. There were no changes in the average score of the ipsilateral forelimb and hind limb at any time point in the LESION group. There was a significant decrease in contralateral forelimb ($Z=-2.911$, $p \leq 0.01$) and hind limb ($Z=-2.107$, $p \leq 0.05$) scores from pre-lesion to post-lesion testing.

In the STRESS & LESION group there were no impairments between the contralateral and ipsilateral limbs at any time point. There were no changes in the average score of the ipsilateral or contralateral limbs from baseline, pre-lesion, to post-lesion testing.

There were no significant differences between the groups in the percentage of errors made at any time point (see Figure 10). Analysis of limb performance over time revealed a significant increase in the number of errors made by the contralateral forelimb from pre-lesion to post-lesion testing ($Z=-3.385$, $p \leq 0.001$) in the LESION group. \
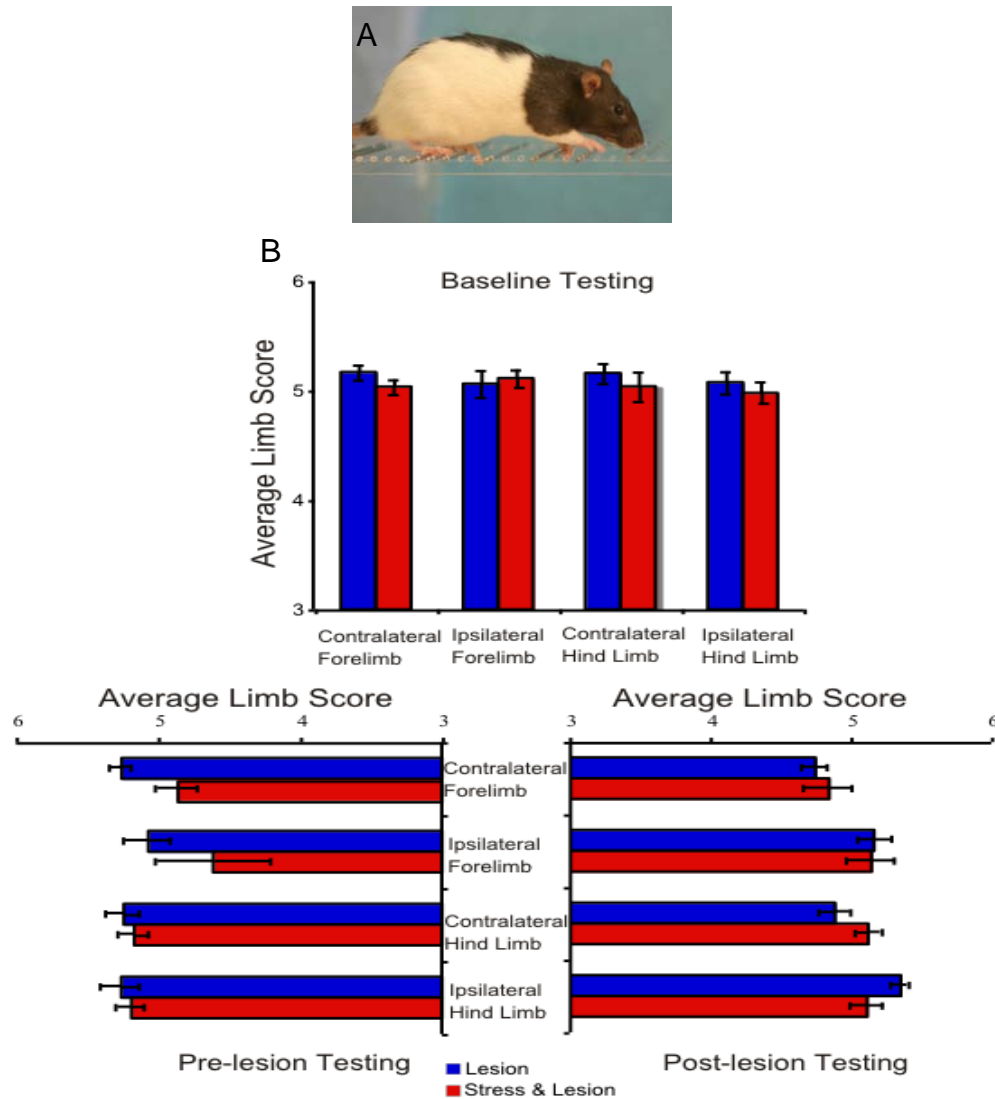
Figure 19. Average limb score in the ladder rung walking task (A). There were no significant differences between the LESION (blue) and STRESS & LESION (red) groups at baseline, pre-lesion, or post-lesion testing (B). Between-group comparisons used Mann-Whitney U test. All data are presented as group means $\pm$ SEM.

There were no significant differences between the groups in the percentage of errors made at any time point (see Figure 20). Analysis of limb performance over time revealed a significant increase in the number of errors made by the contralateral forelimb from pre-lesion to post-lesion testing (Z=-3.385, p$\leq$0.001) in the LESION group.

The LESION group made significantly more errors with the contralateral forelimb compared to the ipsilateral forelimb post-lesion (Z=-2.947, p≤0.01). The contralateral hind limb also made more errors than the ipsilateral hind limb post-lesion (Z=-2.669, p≤0.01). There were no other significant differences between the contralateral and ipsilateral limbs at any other time point. There were no significant differences between contralateral and ipsilateral limbs at any time point in the STRESS & LESION group. There was a significant decrease in percent errors in the ipsilateral hind limb from baseline to pre-lesion testing (Z=-2.698, p≤0.01) in the STRESS & LESION group. There was no other difference in percent errors of the ipsilateral or contralateral limbs from baseline, pre-lesion, to post-lesion testing.
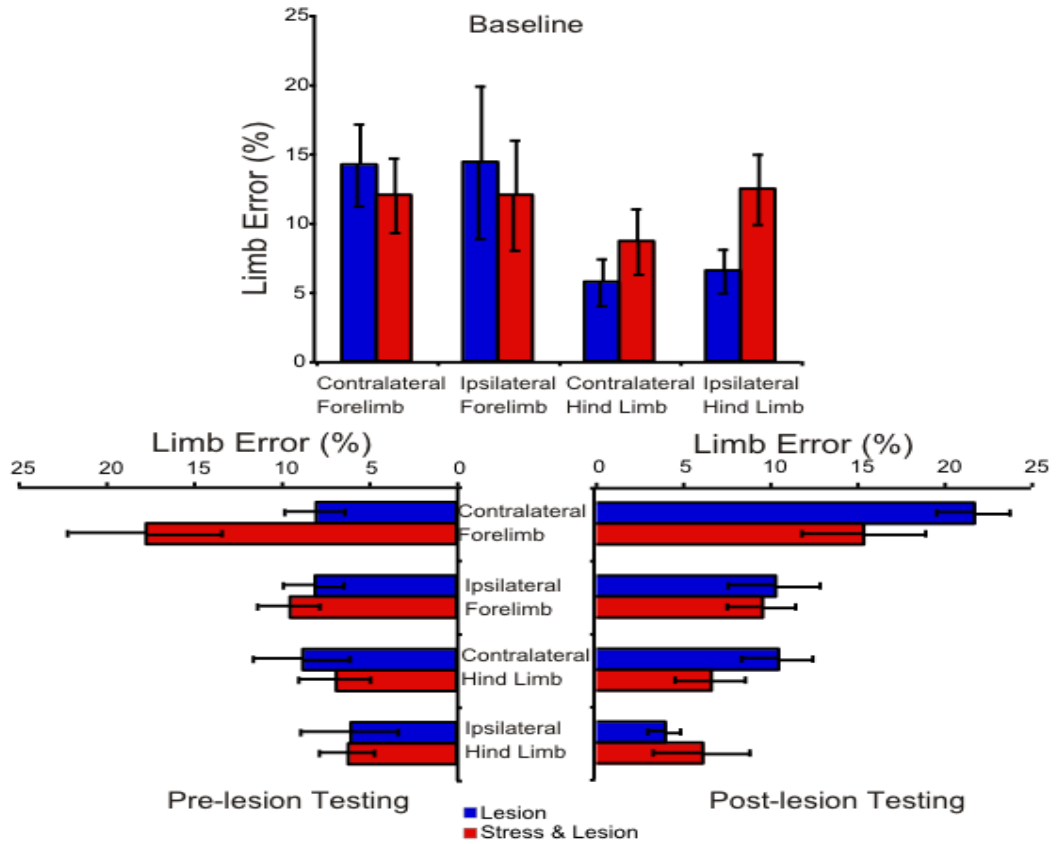
Figure 20. Percentage of limb errors in the ladder rung walking task. There were no significant differences between the LESION (blue) and STRESS & LESION (red) groups at baseline, pre-lesion or post-lesion testing. Comparisons between groups using Mann-Whitney U test. All data are presented as group means $\pm$ SEM.

Adrenal Weights

The adrenal weights of five animals were excluded from analysis due to damage caused by their removal. The STRESS & LESION (n=15) had significantly larger adrenal weights ($F(1,29) = 4.495$, $p \leq 0.05$) then the LESION (n=16) group (see Figure 21).
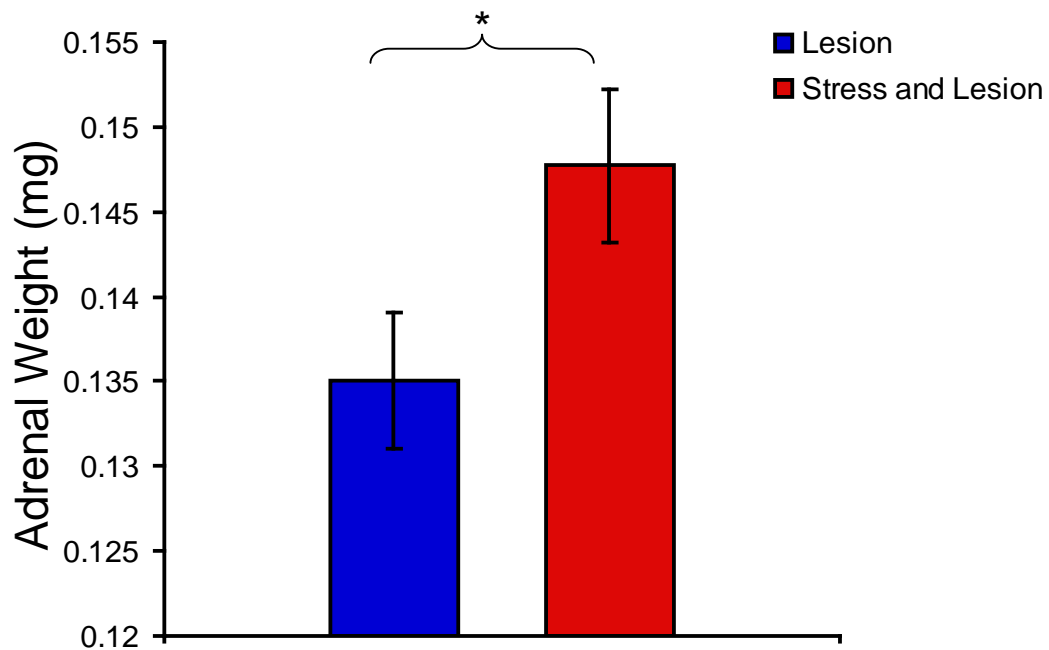
Figure 21. Adrenal gland weights corrected for body weight. Adrenal glands were removed on 15 DPO. The STRESS & LESION group (red) had significantly larger adrenal glands than the LESION group (blue). * p≤ 0.05. Comparisons between groups using ANOVA. All data are presented as group means ± SEM.

Discussion

Summary

Chapter 3 investigated the effects of chronic multiple stress on recovery of skilled motor function after devascularization lesion. The data show that chronic multiple stress did not induce motor impairments in skilled reaching after ischemic injury. Chronic multiple stress did not increase the size of the infarct, nor did stress influence edema formation. The findings of this study did not support the hypothesis that chronic stress impedes motor recovery after ischemic injury in rats.

Multiple Stress Does Not Modulate Recovery of Skilled Reaching

The results from Experiment 3 is in contrast to Experiment 2 and Kirkland et al. (submitted) which found that chronic restraint stress impairs motor function after devascularization. Experiment 2 found that chronic restraint stress was capable of inducing impairments in success rate, total pellets eaten, increase the number of attempts to grasp the pellet, and influence compensatory motor strategies during skilled reaching after ischemic infarct. It was thought that because the rats would be unable to habituate to the multiple stress paradigm, that the deficits induced by chronic multiple stress would be more severe. This was not correct, as no significant difference between stress and non-stress animals were noted in the single pellet reaching task in quantitative and qualitative parameters after lesion. There did appear to be minor deficits induced by exposure to chronic multiple stress during pre-lesion testing. While there were no significant differences between the groups during pre-lesion testing, exposure to chronic multiple stress did decrease percent success and increase the number of attempts to grasp the pellet compared to previous baseline performance in the stress group. It seems that exposure to chronic multiple stress slightly increased the number of attempts to grasp the pellet, thus it slightly reduced the occurrences of the rats grasping the pellet in one distinct reach, thus reducing success rate, but not reducing the number of pellets eaten. This suggests that chronic multiple stress can induce minor changes in motor function in skilled reaching tasks in "healthy" animals but these changes were not as pronounced as the changes induced by chronic restraint stress as seen in Metz et al. (2005b) and Experiment 1.

During pre-lesion testing, stress induced changes in aim, release and total score. Due to the lack of impaired reaching success in the stress animals during pre-lesion testing, it is questionable whether these qualitative changes had any influence on reaching success. In contrast to expectations, chronic multiple stress did not induce impairments in quantitative analysis of skilled reaching in the week prior to lesion. In Experiment 1 and in a study by Metz et al. (2005b) chronic restraint stress in normal rats induced deficits in reaching success. In the present study, there were some minor differences in the qualitative analysis of reaching during pre-lesion stress. Experiment 1 found no differences in qualitative analysis of skilled reaching during pre-lesion stress despite lower success rate in the stress group. Chronic restraint stress-induced impairments in qualitative reaching analysis in Metz et al. (2005b), however, these impairments were small, and could also be due to the use of females in that study. A pilot study found that chronic restraint stress in a cloth bag also failed to induce deficits in skilled reaching in normal animals and after devascularization lesion (Coma et al., unpublished observations). It may appear that chronic stress induced impairments in skilled reaching is limited to chronic restraint stress in the Plexiglass tubes. While more work in the effects of chronic stress on skilled motor function is needed, Experiment 3 does not support the hypothesis that chronic stress induces motor impairments in normal rats or after devascularization.

Multiple Stress Does Not Modulate Skilled Walking

Chronic multiple stress did not modulate limb function in the ladder rung walking task. It was also found in Experiment 1 that chronic restraint stress was unable to induce

motor deficits in the ladder rung walking task after devascularization lesion. While the stressed rats tended to show fewer impairments in the contralateral limbs then the ispilateral limbs after lesion in comparison to the non-stressed rats, there was no difference between the groups, meaning that for better or worse, stress did not modulate limb recovery after devascularization.

Multiple Stress Does Not Exacerbate Infarct Size

It is surprising that chronic multiple stress did not exacerbate infarct size. Seven days of restraint stress prior to MCAO increased infarct size (Madrigal et al., 2003). In the current study, eight days of multiple stress prior to the devascularization lesion did not exacerbate infarct volume. This could be due to differences in the stress paradigm used or differences between devascularization lesion and MCAO. In Experiment 1, chronic restraint stress after devascularization lesion increased infarct volume while chronic restraint stress prior to lesion had no influence on infarct size.

In Experiment 1, chronic restraint stress after the lesion increased infarct size. In the present study, chronic multiple stress did not increase infarct volume after lesion. A possible reason for the discrepancy between restraint stress and multiple stress is that chronic restraint stress may be more stressful than chronic multiple stress and as such is more salient in exaggerating cell death after ischemic lesion. This may be due to the rats inability to interact with its environment to attempt to cope with the stress while in the Plexiglass tube, which may induce a higher state of learned helplessness. This is not the case with stressors used in the multiple stress paradigm such as fox odor, overcrowding, social isolation, shaking, and saline injection as they are able to interact with the

environment while the stressor is occurring. Thus, chronic restraint stress may further increase corticosterone levels, resulting in increased cell death. However, chronic multiple stress after ischemic lesion has been shown previously to increase cell death. Ritchie and colleagues (2004) found that a chronic mild stress paradigm beginning seven DPO and lasting for six weeks promoted cell death after the 2-VO model of global ischemia in the CA1 layer of the hippocampus in the rat. Potentially, the chronic multiple stress paradigm used by Ritchie et al. (2004) was more stressful than the chronic multiple stress paradigm used in the current study which could explain the inability of the multiple stress paradigm in Experiment 3 to increase infarct size. Additionally, since each stressor in the current multiple stress paradigm occurred at relatively the same time each morning (9:00 AM to 11:00 AM) and afternoon (3:00 PM to 6:00 PM), that could have increased the predictability of the stress paradigm. Additionally, Ritchie et al. (2004) examined cell death within the hippocampus, which is the most vulnerable area of the brain to ischemic injury (Pulsinelli et al., 1982). Since the hippocampus is more vulnerable to corticosterone-induced cell death than the motor cortex, this could also explain the discrepancy between Ritchie et al. (2004) and the present thesis. Regardless, Experiment 3 does not support the hypothesis that chronic stress exacerbates infarct size.

Stress Does Not Modulate Edema after Devascularization Lesion

Experiment 4 did not find a relation between stress and corticosterone treatment with edema after devascularization lesion. This study replicates previous findings in Experiment 2 which found that restraint stress failed to increase edema levels after ischemic lesion. Experiment 4 confirmed that devascularization lesion increases water

content in the lesion hemisphere using the wet/dry method of edema calculation. It is important for animal models of stroke to exhibit increases in edema after lesion because it is an important part of the pathology of stroke in humans. The current study and Experiment 2 do not support the potential role of corticosterone or stress on edema formation after ischemic injury. More work is needed on the subject preferably using a model of stroke that does not involve a craniotomy.

Increased Stress and Stress Hormones after Exposure to Multiple Stress

A potential weakness of the current study is that the chronic multiple stress paradigm used was not salient enough to induce motor impairments after lesion. If chronic restraint stress is experienced as more stressful to the rats than chronic multiple stress then it could explain the differences between the results in Experiment 1 and 3. Each stressor used in the current study, however, has been shown to induce stress and corticosterone release in the previous literature. It is possible that different stressors do not activate the HPA axis to the same degree. Activation of ACTH and corticosterone release after exposure to heat stress (38°C) was higher than overcrowding, cold stress, and food deprivation as measured by corticosterone levels (Djordjevic et al., 2003). This suggests that stressors will activate the HPA axis to different degrees. Blood samples were not collected after exposure to each stressor and comparisons between the effects of each stressor on corticosterone release was not performed. Adrenal weights were increased in rats exposed to stress suggesting that the animals did experience the stress (Westenbroek et al., 2005). Additionally, each stressor used in the present study has each

been used in numerous studies to induce stress in rats and should have been adequate to induce stress in the current study.

Conclusion

In summary, *Chapter 3* found no evidence for a role of chronic stress on motor recovery after lesion. Chronic multiple stress had no influence on infarct volume or edema formation. Thus, the present data does not support the hypothesis that chronic stress can influence motor recovery after ischemic injury in rats.

Chapter Four

General Discussion

Summary

The present thesis describes the effects of chronic stress on motor recovery after focal ischemic injury in rats. In Experiment 1, chronic restraint stress induced temporary deficits in skilled reaching while altering compensatory reaching strategies, resulting in a slowed rate of recovery. Chronic restraint stress prior to lesion induced the most behavioural changes and impairments in skilled reaching compared to chronic restraint stress given after the lesion. No chronic stress-induced impairments in skilled walking were noted. Only post-lesion chronic restraint stress after lesion increased infarct size. Experiment 3 examined whether impairments seen in chronic restraint stress generalizes to other stress paradigms. Using a chronic unpredictable multiple stress paradigm, Experiment 3 found no evidence of stress-induced impairments in skilled reaching or skilled walking after ischemic injury in rats. Chronic multiple stress also had no influence on infarct size. Experiments 2 and 4 suggest that stress has no influence on edema formation after devascularization lesion.

Pre- versus Post-Lesion Exposure to Stress

The objective of this thesis was to examine if an intrinsic factor such as stress can influence the course of recovery and the development of compensatory motor strategies. The present thesis found that chronic restraint stress can influence the rate of functional improvement after devascularization lesion. The present thesis suggests that in cases

where chronic stress does modulate recovery and compensation of skilled reaching after ischemic lesion, such as chronic restraint stress the timing of the stress can differentially modulate outcome. Chronic stress prior to the lesion has a larger influence on behavioural impairments seen after lesion, the post-lesion chronic stress. Both pre-lesion and post-lesion chronic stress can impair motor function to the same degree, however, pre-lesion stress seems to have additional effects, such as decreasing success rate and increasing the number of attempts to grasp food pellets after lesion in rats. This may be due to behavioural and neuroplastic changes that are allowed to take place prior to the lesion, and are further modified by the infarct. Post-lesion chronic stress is able to increase infarct size, while pre-lesion chronic stress is not. This might be due to the increased vulnerability of the ischemic tissue to further metabolic injury. Interestingly, post-lesion stress does not further increase behavioural deficits despite the increase in infarct volume. Potentially, the devascularization lesion induced a sort of floor effect on motor recovery, and behavioural processes, in that additional stress was unable to further alter behavioural function.

Restraint versus Multiple Stress: Possible Mechanisms

The present thesis suggests that the effects of chronic stress on motor recovery after ischemic lesion in rats is limited by the type of stress. It was found in *Chapter 2* that chronic restraint stress can influence functional impairments in skilled reaching after devascularization lesion in rats. In *Chapter 3*, however, it was found that chronic multiple stress does not modulate functional impairments in skilled reaching after

devascularization lesion in rats, suggesting that not all chronic stressors are capable of modulating recovery and compensation after lesion in rats.

Potentially, chronic multiple stress was experienced as not salient enough to induce impairments in motor function before and after the lesion. Moderate to severe psychological stress such as restraint stress may be more easily able to impact the rate of motor recovery after lesion because it is experienced as a more salient stressor by the rats. Chronic restraint stress may induce impairments via stress-induced hyperactivity and an increase in anxiety and depression-like behaviour. Based on the current thesis and previous literature, it seems that if chronic stress is able to increase anxiety/depression-like behaviours, then it may prohibit the rats' ability to cope with the deficits after the ischemic infarct. Thus rats' ability to recover and compensate would be altered. Unfortunately, it is difficult to assess the degree of coping in rats, but future studies should assess anxiety and learned helplessness in rats to get a measure if emotionality of the rat can influence motor recovery after devascularization lesion in the rat. Chronic restraint stress altered the rate of recovery and compensation, while a multiple stress paradigm did not. Previous literature has shown that chronic restraint stress is more effective at increasing anxiety-like behaviours (Vyas et al., 2002; Vyas and Chatarji, 2004; Richardson et al., 2006) and inducing hyperplasticity within the amygdala (Vyas et al., 2002) then chronic multiple stress. Patients with anxiety disorders or post-stroke depression show impaired recovery after stroke (Sinyor et al., 1986; Shimoda and Robinson, 1998; Paolucci et al., 1999; Lewis et al., 2001). The present thesis suggest that stress might not be capable of modulating behavioural recovery if it does not induce feelings of anxiety/helplessness.

Another possible mechanism of why chronic multiple stress had no impact on recovery after lesion in rats may be due to neuroplasticity. Chronic multiple stress may be less efficient at altering neural processes within the motor system then chronic restraint stress. Previous literature has found that chronic multiple stress is less capable of inducing neuroplasticity within the hippocampus or amygdala compared to chronic restraint stress in rats (Vyas et al., 2002). Potentially chronic restraint stress is able to induce long-term changes in neuroplasticity within the motor system of the brain and as such is able to modulate motor recovery after lesion, while chronic multiple stress cannot. High levels of GR are found within the motor cortex, striatum, and cerebellum (Ahima et al., 1990; Ahima et al., 1991) suggesting that stress hormones may regulate cell function within the motor system. *Chapter 2* found evidence of altered behavioural function, such as increases in the attempts to grasp the pellet in rats, lasting well after the extinction of the stress. This suggests that stress may alter long-term neural processing within the motor system. More work is needed to further understand the mechanism of how stress modulates motor recovery after lesion. Unfortunately, in the present thesis no mechanism for how chronic restraint stress increased motor impairments while chronic multiple stress did not.

Stress Fails to Influence Skilled Walking

None of the chronic stress paradigms influenced recovery of skilled walking after stroke. This may be due to rats being more easily able to compensate with their ipsilateral limbs while crossing the ladder rung task, which could have masked any deficits in

skilled walking induced by chronic stress. Additionally, the rats could have improved by the time rats were tested after the lesion.

Stress and Compensation after Devascularization Lesion

The ability of chronic restraint stress to potentially alter compensation after ischemic injury is intriguing, and warrants replication and further study. Few studies have examined whether neuroprotective drugs or rehabilitative therapies assist or impair in the development of compensation after lesion. One study found that motor training influences the development of compensatory reaching strategies. A study by Fouad et al., (unpublished data) found that rats that underwent daily training in skilled reaching after spinal cord lesion adopted compensatory reaching strategies. Rats that were not trained daily after spinal cord lesion maintained a similar reaching pattern before the spinal cord lesion. In addition, delayed treatment of fetal transplants and neurotrophin-3 or brain-derived neurotrophic factors after spinal cord lesion promoted recovery of skilled forelimb function rather then compensatory movements (Lysnkey et al., 2006). It would seem that therapeutic treatments and chronic stress may be able to influence recovery and the development of compensatory motor movements in skilled reaching after neurological injury in rats.

The current thesis suggests that chronic restraint stress may alter behavioural and neural processes of reaching which may influence the development of compensation after ischemic infarct. This may be due to chronic restraint stress-induced changes in anxiety and depression pathways in the brain. The current thesis suggests that feelings of anxiety/helplessness will impair the ability of rats and patients to cope with their motor

deficits after stroke. Coping is defined as managing internal and external demands on the individual via cognitive or behavioral methods. Individuals will use different coping styles after brain injury (Anson and Ponsford, 2006). Problem-focused coping is adaptative and focuses on dealing with the situation by trying to obtain new information or skills to deal with the situation such as developing compensatory reaching strategies (Anson and Ponsford, 2006). Emotional-focused coping involves the patient altering how they attend to the situation (such as denial or avoidance) or how the patient views the situation (such as acceptance) (Anson and Ponsford, 2006). Emotional-focused coping is seen as more maladaptive and associated with poor self-esteem, and high levels of depression, and anxiety.

Chronic restraint stress in rats may alter the rate of upper limb recovery and the development of compensation after stroke is by altering coping styles. In the clinical setting, if chronic stress increases feelings of anxiety and depression, it may modulate coping style and modulating functional improvement after the lesion. Patients with a more positive attitude about their prospects of recovery may develop more active problem focused coping strategies which may result in trying to develop compensatory movement strategies to regain function. Chronic stress that does not increase anxiety/depressive like behaviours may not alter coping strategies, potentially explaining why chronic multiple stress did not modulate functional impairments after devascularization lesion. Chronically stressed patients who develop high levels of anxiety/depression may become pessimistic about their chances of recovery, thus develop emotional-focused coping techniques. These patients may focus more on how they view the problem rather than actively trying to improve their function. Therefore reducing the

likely-hood of developing compensatory movement strategies. More studies need to be done to examine whether neural or behavioural processes can alter the development compensatory motor strategies after stroke.

It is important to note that chronic restraint stress induced impairments in skilled reaching after ischemic lesion was only temporary. By the second week of testing after the lesion, rats exposed to chronic stress returned to control levels in success rate and the percentage of total pellets eaten. Chronic restraint stress only temporarily induced impairments in skilled reaching after ischemic infarct. The potential mechanisms for impairments in reaching proposed in the present thesis occur during a short time window after the lesion. As such, regardless of the paradigm used, chronic stress did not result in permanent impairments in skilled reaching after devascularization lesion in rats in terms of success rates and percentage of total pellets eaten. While chronic stress is able to modulate motor recovery after lesion in rats, chronic stress does not induce permanent impairments in skilled reaching.

Chronic Stress and Infarct Growth

Chronic stress generally had little effect on infarct size after ischemic lesion in the present thesis. Previous work has suggested that exposure to acute stress prior to ischemic lesion exacerbates infarct size in rodents (Sugo et al., 2002; Madrigal et al., 2003). In the present thesis, chronic restraint stress prior to lesion and chronic multiple stress failed to increase infarct size. Only chronic restraint stress after the lesion increased infarct size. The discrepancy between stress prior to or after lesion on infarct size may be due to

timing of the stress. After ischemic injury, cells within the penumbra are most vulnerable to injury within the first few days, meaning that stress after lesion will have the largest effect on infarct volume. There is, however, currently no evidence of the existence of a penumbra in the devascularization model of stroke. Potential mechanisms of stress-induced cell death include increased excitotoxicity (Madrigal et al., 2003), hyperglycemia (Payne et al., 2003), inability to use glucose leading to further metabolic insult (Sapolsky, 1985), suppressing Bcl-2 expression (DeVries et al., 2001), stress-induced hyperthermia (Kitatkin and Wise; 2001, Clarke et al., 2003). Another possible mechanism of stress-induced injury after devascularization is increased blood pressure. Future studies should use MCAO or Et-1 models of stroke when studying the effects of stress on infarct size, as the blood vessels are not severed in those animal models.

The lack of infarct growth after ischemic infarct during exposure to chronic multiple stress is surprising, since the animals should not have habituated to the stressors due to the limited exposure of each stressor to the rat. It is possible that exposure to chronic restraint stress after the lesion is more stressful then chronic multiple stress and is more capable of inducing cell death. There may be additional cellular pathways that are activated only during chronic restraint stress and not during chronic multiple stress. Chronic restraint stress produces increased behaviours and neural plastic changes related to anxiety that are not seen in chronic multiple stress. It is possible that chronic restraint stress activates cellular pathways capable of inducing cell death after ischemic injury that are not activated during chronic multiple stress.

Stress Does Not Modulate Edema after Devascularization Lesion

The present thesis investigated the effects of stress on edema after devascularization lesion in rats. Edema is the leading cause of mortality after stroke in humans (Heo et al., 2005), so it is important to determine if stress is capable of modulating edema. Dexamethasone, a synthetic GR agonist, reduces edema in animal models of stroke (Betz and Coester, 1990; Shimauchi and Yamamoto, 1992). It was expected that stress and corticosterone treatment in rats, which also activates GR, may reduce edema levels after ischemic lesion.

Experiment 2 examined the effects of chronic and acute restraint stress prior to or after the lesion on edema in rats. It was found that chronic restraint stress prior to or after lesion did not influence edema measurements in rats. Experiment 4 examined acute exposure of various stressors and corticosterone treatment after lesion on edema in rats. Neither corticosterone treatment nor exposure to fox odor, restraint, or foot shock increased edema in the lesion hemisphere of rats. The current thesis suggests that regardless of duration or type, stress did not influence edema formation after devascularization. Experiments 2 and 4 did confirm the presence of edema in the lesion hemisphere after devascularization lesion, which indicates a clinical relevance of the devascularization rat model of stroke.

As described earlier, devascularization is not the ideal rodent model of stroke to use for measuring edema. An open craniotomy, or the size of the lesion may have been a limiting factor on the effects of stress on edema. It may be that GR activation does not influence edema formation after ischemic lesion, and that dexamethasone reduces edema independently of its activation of GR through other mechanisms, or does not influence edema formation at all. Dexamethasone may not influence edema after stroke, as clinical

trials of the drug have failed. Other studies have failed to find any benefit of dexamethasone on improving recovery in patients after stroke (Ito et al., 1980; Ogun and Odusote, 2001). Thus, the current thesis does not show any evidence of stress influencing edema after devascularization in rats.

Important Points to Consider: Future Research, and Clinical Relevance

Future research needs to be done to further understand the role of chronic stress on recovery and compensation of skilled reaching after ischemic lesion in rats. It is imperative that future studies investigate the effects of chronic stress on motor recovery and compensation, or the effects of stress on edema formation in other animal models of stroke, including Et-1 and MCAO, so to improve the clinical relevance of the research. Future work needs to be careful as to the stressors used in future studies and their clinical relevance. An interesting study would be to block the release of corticosterone either by ADX or by chemical ADX prior to ischemic lesion to investigate whether blocking the corticosterone response after lesion modulates motor recovery in rats. Additionally future studies should use a longer time course then the two weeks after lesion used in the present thesis. Future studies should use a survival time of at least a month after lesion to examine the long-term effects chronic stress on motor recovery.

The present thesis suggests that the capability of chronic stress to modulate motor recovery after ischemic lesion is limited by the type of stressor or the degree of stress in which the stressor causes. Exposure to chronic stress does not necessarily result in impairments in motor recovery after lesion in rats. It is important for researchers to not generalize the effects of a stressor used in experimental research. While one stressor is

capable of influencing a particular behaviour, it does not mean all stressors will. For example, the current thesis and studies by Metz et al. 2005b, and Coma et al. (unpublished data) suggests that only chronic restraint stress in a Plexiglass tube is capable of inducing extensive motor impairments in skilled reaching in healthy rats with chronic multiple stress only inducing minor changes. This would suggest that only specific types of stress are capable of inducing pronounced motor impairments after ischemic injury in rats. Future studies need to investigate which type of stressor can modulate functional impairments in skilled reaching after ischemic injury. Whether physical vs. psychological stressors are more capable of inducing impairments after ischemic lesion in rats needs to be investigated. Future work should also investigate the role of anxiety, depression, and learned helplessness on chronic restraint stress induced motor impairments. Anxiety levels in rats can be measured via elevated plus-maze (Vyas et al., 2002) and shock-probe burying (Treit, 1990). Learned-helplessness in rats can be measured using inescapable shock (Kademian et al., 2005). A limitation to this thesis is the lack of a clear mechanism as to how chronic restraint stress impairs the rate of functional forelimb recovery after ischemic injury in rats, but chronic multiple stress does not. Previous literature suggests that impairments seen after chronic restraint stress may be due to stress-induced anxiety/depression-related behaviours but more work in the future is needed.

Another important note concerns the clinical applicability of the stressor. The ultimate goal of basic research in the field of stroke research is to translate the results from bench to bedside. It is important to use stress paradigms that are of high clinical relevance. While chronic restraint stress is a good method of inducing HPA activation in

rats, it has little clinical relevance because patients do not experience stress by being physically restrained. It is unknown which type of psychological stress is the clinical equivalent to restraint stress in rats. It is imperative that future studies compare the effects of exposure to different chronic stressors so to understand what type of stress can influence motor recovery after lesion. While it is tempting to use animal models of stress that result in robust behavioural changes such as restraint stress, the lack of clinical relevance of the model limit the its application to the clinical realm. If future research reveals that other chronic stress paradigms do not induce impairments after ischemic lesion in rats, as seen in Experiment 3 and Coma et al. (unpublished observations), then the effects of chronic stress on motor recovery after stroke in patients may be limited. What exactly is a clinically relevant model of stress is hard to measure as is any animal model, though social stressor used by Sugo et al. (2002) is a good start. Chronic multiple stress is also a useful clinical model of stress, as people often are chronically exposed to different stressors on a daily basis.

One potential problem with the current thesis is the consistency of the lesion between Experiment 1 and Experiment 3. It can be noted that the lesion volume of the control non-stress lesion group in Experiment 1 is smaller then the lesion noted in the control non-stress lesion group in Experiment 3. The lesion in the control group in Experiment 3 is twice as big as the lesion in the control group in Experiment 1. A smaller lesion in the control group in Experiment 1 could explain the improved rate of motor recovery when compared to the stress treated group that had similar lesions to the groups in Experiment 3. It has been shown previously that rats with smaller devascularization lesions show a quicker and higher initial rate of recovery then rats with larger lesions

(Whishaw, 2000). As such, the improved rate of recovery seen in the control non-stress group in Experiment 1 could be a result of the size of the lesion induced, rather then chronic restraint stress-induced impairments in the treatment groups. Potentially, chronic restraint stress may be similar to chronic multiple stress in that it does not induce impairments in skilled reaching after devascularization. Further replications of Experiment 1 and 3 should be conducted to examine in lesion size in the control non-stress rats are influencing the results seen in the current thesis.

Conclusion

In conclusion, the present thesis suggests that chronic stress is capable of slowing the rate of functional improvement and the development of compensatory motor strategies in skilled reaching after devascularization in rats. Chronic stress appears incapable of inducing permanent functional impairments in forelimb reaching success. The detrimental effects of chronic stress on motor function after ischemic lesion in rats are limited by the type of stress. In cases where stress is capable of modulated recovery and compensation, the timing of the chronic stress whether it occurs prior to or after the lesion can differentially affect outcome in rats. Chronic stress itself is not sufficient to induce motor impairments after lesion in rats and the most important factor influencing motor recovery may potentially be increased levels of stress-anxiety/depression induced by chronic stress. Exposure to chronic stress that increases feelings of anxiety and depression potentially alters coping strategies, and modulates recovery and compensation in skilled reaching after ischemic injury in rats.

While not every stressor is capable of altering behaviour, basic research should be aware of experimental stressors that could confound the results of pre-clinical research. Clinicians should still be weary of patients being exposed to or have already been exposed to moderate-severe psychological stress which may induce anxiety disorders, or depression. Attempts to improve stroke wards in hospitals could include stress management techniques and ways of improving the patient's emotional states, to help them cope better with their disabilities after stroke. Individuals who are at risk for stroke should be instructed on ways to manage stress. Improving the way in which patients deal with chronic stress may help patients ameliorate the rate of upper limb recovery after stroke.

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