Investigating the Efficacy of Novel TrkB Agonists to Augment Stroke Recovery

by

Zuha Warraich

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved July 2013 by the
Graduate Supervisory Committee:

Jeffrey Kleim, Chair
Sarah Stabenfeldt
Marco Santello
Stephen Helms Tillery

ARIZONA STATE UNIVERSITY

August 2013

ABSTRACT

Stroke remains the leading cause of adult disability in developed countries. Most survivors live with residual motor impairments that severely diminish independence and quality of life. After stroke, the only accepted treatment for these patients is motor rehabilitation. However, the amount and kind of rehabilitation required to induce clinically significant improvements in motor function is rarely given due to the constraints of our current health care system. Research reported in this dissertation contributes towards developing adjuvant therapies that may augment the impact of motor rehabilitation and improve functional outcome. These studies have demonstrated reorganization of maps within motor cortex as a function of experience in both healthy and brain-injured animals by using intracortical microstimulation technique. Furthermore, synaptic plasticity has been identified as a key neural mechanism in directing motor map plasticity, evidenced by restoration of movement representations within the spared cortical tissue accompanied by increase in synapse number translating into motor improvement after stroke. There is increasing evidence that brain-derived neurotrophic factor (BDNF) modulates synaptic and morphological plasticity in the developing and mature nervous system. Unfortunately, BDNF itself is a poor candidate because of its short half-life, low penetration through the blood brain barrier, and activating multiple receptor units, p75 and TrkB on the neuronal membrane. In order to circumvent this problem efficacy of two recently developed novel TrkB agonists, LM22A-4 and 7,8-dihydroxyflavone, that actively penetrate the blood brain barrier and enhance functional recovery. Findings from these dissertation studies indicate that administration of these

pharmacological compounds, accompanied by motor rehabilitation provide a powerful therapeutic tool for stroke recovery.

DEDICATION

I dedicate this work to my late cousin, Ali Jawad Malik, who believed in diligence, science, art, and the pursuit of academic excellence.

ACKNOWLEDGMENTS

I am incredibly grateful to have worked with everyone in the Kleim lab, I want to express my timeless gratitude to my P.I., Dr. Jeffrey A. Kleim for all his guidance, encouragement, support, and patience. His sincere interests in science and research have been a great inspiration to me. I could have never asked for a better mentor. I would like to also acknowledge members of my committee, Dr. Marco Santello, Dr. Stephen Helms Tillery, and Dr. Sarah Stabenfeldt. Their help and support was invaluable to me. A special thanks to Dr. Stabenfeldt for lending her expertise and letting me conduct countless experiments in her lab.

Nagheme, you get first. Without you I wouldn't be here. Thank you for your love and support over all of these years! I want to extensively thank all of the undergraduates in the lab for their continuous help with data collection and analyses. Their assistance was paramount to all the studies. I have enjoyed working with all of you.

I want to express my deepest gratitude to my wonderful family and friends for always being there. And above all, I would like to thank my incredible mother for always believing in me and constantly motivating me through her tremendous sacrifices.

TABLE OF CONTENTS

Page

CHAPTER Page

CHAPTER Page

CHAPTER Page

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

Chapter 1

INTRODUCTION

1.1 Overview

Every year approximately, 750,000 Americans suffer a stroke. Of the 80% who survive, most will experience upper extremity impairments, making stroke the leading cause of adult disability in the United States (AHA, update 2011). The only clinically proven treatment for motor impairment is motor rehabilitation. Unfortunately, patient response is highly variable reflecting both the heterogeneity of the disorder and our lack of understanding of the neurobiological substrates of functional improvement. Animal models of stroke have become a critical part of neurorehabilitation as they serve to guide the development of more effective rehabilitation therapies. Over the last decade there has been a concerted effort by researchers to develop better animal models that include more comprehensive measures of both motor impairment and subsequent improvement in response to rehabilitation. Second, animal research has expanded beyond studies of neuroprotection to studies directed at identifying the fundamental neural substrates that support rehabilitation-dependent functional improvement. Further, these studies have begun to reveal the key neural signaling systems that drive compensatory and restorative neural plasticity within residual brain areas that supports functional improvement. The general hypothesis guiding the studies within this dissertation research is that by combining motor rehabilitation with adjuvant, plasticity promoting interventions that drive neural plasticity can significantly enhance motor function after stroke.

1.2 Cerebral pathophysiology

A blockade in oxygen supply for a few minutes is sufficient to do irrepairable damage to the human brain often resulting in stroke. Essentially stroke refers to an umbrella of conditions caused by the occlusion or hemorrhage of blood vessels supplying the brain. Most often, blood flow is compromised within the territory of an occluded blood vessel, referred to as ischemia. 80% of human strokes are ischemic in origin (Roger VL, Heart disease and stroke statistics-2012 update). In all instances, stroke ultimately involves death and dysfunction of brain cells and neurological deficits that reflect the location and size of the compromised brain area.

Ischemic stroke is characterized by complex spatial and temporal events developing progressively over hours to days. In most clinical settings, the first phase is generally referred to as the sub-acute phase (0-1 week), followed by 90 days of acute phase and from 6 month onwards the patient is classified to be in chronic phase (Cramer & Riley, 2008). During the acute and sub-acute phases there is a cascade of neurodestructive events that occur in the brain tissue.

Brain tissue affected by a stroke can be divided into a core region where blood flow has dropped substantially resulting in necrosis of the tissue, and a surrounding penumbral region where blood flow is also reduced. Within center or core of an ischemic territory, blood flow deficits accompanied by low ATP levels and energy stores, ionic disruptions and metabolic failures are severe leading to major cell death (Ames, 1992). At least three fundamental molecular mechanisms have been identified that are inextricably linked and contribute to ischemic cell death. Excitotoxicity and ionic imbalance, oxidative/nitrosative stress and apoptotic-like cell death. These mechanisms mediate

injury primarily within neurons, glia and vascular elements. They also pose a serious risk to cell bodies, their processes and synaptic endings (Nedergaard, Gjedde, & Diemer, 1986).

The reactive oxygen radical is a key mediator of tissue damage after reperfusion of the brain. Mitochondria are strongly implicated, and this might be due to excessive superoxide production during electron transport and inhibition of mitochondrial electron transport mechanisms by free radicals, leading to even more oxygen radical generation (Fiskum, Murphy, & Beal, 1999)(Chan et al, 2001). Free radicals are also generated during the inflammatory response after ischemia. Not surprisingly, then, oxidative stress, excitotoxicity, energy failure and ionic imbalances are intricately linked, and contribute to ischemic cell death.

The end result of the cellular changes is widespread neurophysiological changes that result in significant functional impairments. However, the impairments are not the manifestation for the lost tissue but rather the ability of the residual tissue to maintain function. The capacity to maintain function is related to the restoration, recruitment and retraining of residual tissue that is mediated by neural plasticity.

1.3 Functional Impairments

Following unilateral stroke there is significant loss of function in the body side contralateral to the damage and a robust degenerative-regenerative cascade of events in both hemispheres. The most common and widely recognized impairment caused by stroke is motor impairment, which can be regarded as a loss or limitation of function in muscle control or movement or a limitation in mobility (Wade, Collen, Robb, & Warlow,

1992). Motor impairment after stroke typically affects the control of movement of the face, arm, and leg of one side of the body (Warlow, Sudlow, Dennis, Wardlaw, & Sandercock, 2003) and affects about 80% of patients. Only 5% of adult stroke survivors regain full function of the upper limb and 20% regain no functional use. Upper limb dysfunction remains an important hurdle for many stroke survivors. Frequently this loss is compensated for by overuse of the less-affected body side to accomplish everyday living tasks (e.g. brushing teeth, drinking coffee). This behavior is accompanied by a "learned disuse" (Wolf et al., 2006) of the impaired side thought to occur due to repeated experience with its ineptness.

Therefore, much of the focus of stroke rehabilitation, and in particular the work of physiotherapists and occupational therapists, is on the recovery of impaired movement and the associated functions.

1.3.1 Rehabilitation following stroke

After unilateral brain damage, some functional improvements in the impaired limb have been found following practice on a skilled reaching task (e.g. Whishaw, 2000; Gharbawie & Whishaw, 2005; Allred, Maldonado, Hsu, & Jones, 2005; Nudo et al., 1996). Exercise following unilateral lesions did not result in improvement in impaired forelimb skill (Maldonado et al., 2005), which suggests that improvements result from practice on a motor skill task, not simply from activity. As previously noted, in intact rats, experience with a skilled reaching task (reaching and grasping for food) results in an increase in pyramidal neuron dendritic arborization (Greenough et al., 1985; Withers and Greenough, 1989) as well as in changes in motor map representations, including an increase in distal forelimb (i.e. digit and wrist) representations and a decrease in proximal

forelimb (elbow/shoulder) representations (e.g. Remple, Bruneau, VandenBerg, Goertzen, & Kleim, 2001; Kleim et al., 1998). Following motor cortex damage, depending on lesion size and location, training with the impaired limb has been found to induce motor map representational changes in monkeys (Friel & Nudo, 1998; reviewed in Nudo, 2003), rats (Castro-Alamancos & Borrell, 1995) and humans (Liepert et al., 2000; Weiller, Ramsay, Friston, & Frackowiak, 1993; Cramer, Moore, Finklestein, & Rosen, 2000; Green, 2003).

Motor rehabilitation restores skilled movement, reinstates motor maps (Nudo 1996; Frost 2003; Kleim 2003) and increases synapse number. Similar effects can be seen following acute pharmacological manipulations. Transient inhibition of protein synthesis within motor cortex causes an enduring reduction in motor map area and skilled movement impairments. Within cortical areas lacking movement representations, there is also a loss of synapses but not neurons, indicating again the disruption in cortical circuitry. However, both the motor maps and skilled movement can be restored with motor training

1.4 Need for effective rehabilitation and adjuvant therapies

Currently there are no universally accepted, standardized therapies established in the clinic. The therapies employed more often reflect the personal experience of the therapists than evidence-based practice. The present therapies employed rarely engage all of the key behavioral signals known to drive plasticity. Neurorehabilitation is considered as a multidisciplinary and multimodal concept to help neurological patients improve physiological functioning, activity, and participation by creating learning situations,

inducing several means of recovery. The central nervous system of adult human beings has an astounding potential for recovery and adaptability, which can be selectively promoted. Use of drugs targeting specific neural signaling systems have been shown to enhance functional outcome in both animal studies and early clinical trials. However, the approach has some limitations. First, the cell signaling systems that drive plasticity are very complex and may change as the CNS adapts over time to the injury. So activating one cell signaling system alone may not be sufficient to drive all of the different cellular systems required to induce experience-dependent plasticity over time. Despite these limitations, pharmacology will undoubtedly play a role in neurorehabilitation. Regardless of what adjuvant therapies are discovered, they will only be effective if they are paired with the best behavioral therapies. Hence, the challenge for the researchers is to design an intervention that maximizes specific signals for an individual patient. For this purpose the role of animal models in development of novel therapies has been instrumental.

1.5 Advantages of using rat models

Modeling human neurological conditions in animals is not an easy task, primarily because the same neurological disorder may have different physical manifestations across different species. The key to successfully modeling human neurological symptoms, such as those associated with stroke, is to first identify functional rather than physical similarities in neurological impairments. No laboratory animal has been studied in more detail than the rat and this work has revealed a number of motor behaviors that can be used to study both motor impairment and recovery associated with various neurological disorders (Cenci, Whishaw, & Schallert, 2002). In addition to species-

specific motor behaviors, detailed analysis of limb movement shows very similar motor components in human upper extremity and rat forelimb movement during reaching behavior (Whishaw et al. 2002). This information has led to the development of a battery of sensorimotor tests that can measure various aspects of both motor impairment and recovery after ischemic insult. Further, investigators' extensive knowledge of the anatomical and neurophysiological organization of the rodent motor system facilitates the identification of the neural mechanisms underlying motor recovery, information that in turn allows for the development of novel adjuvant therapies that may enhance recovery or limit impairment. In addition, rat models enable the use of more complex experimental designs to examine issues such as time course of recovery and dose-response relationships that may not feasible in other animal models.

1.5.1 Rats are good a animal model

Frequently following stroke, humans lose the ability to accurately shape their paretic hand for grasping objects and show deficits in fine skilled movements (Lang, DeJong, & Beebe, 2009). Some deficits following stroke can be modeled in rats, which have very skillful use of the forepaw. Rats present a good repertoire of upper extremity motor movements that mimic human stroke like symptoms. There are established sensorimotor tests that measure deficits incurred from brain damage (Kleim, Boychuk, & Adkins, 2007; Cenci, Whishaw, & Schallert, 2002), including single pellet retrieval task (a reach to grasp task). Recovery of function can be linked with resultant changes in neural activity and connectivity. A recent review from Cramer (2008) details findings that suggest that there are similarities in the ways rats and humans recover after stroke. For example, reorganization in the peri-infarct zone in rats and humans is a major contributor

to recovery of motor function in both species. Although it is not possible to duplicate all components of human stroke condition in an animal model, but with the development of various models, researchers have been successful in identifying pathways that are involved in pathophysiology of stroke, and characterize plasticity-promoting mechanisms for functional recovery.

1.6 Anatomy of the rat sensorimotor cortex

1.6.1 General organization. The forelimb area of the rat sensorimotor cortex (SMC) is partially overlapped between primary motor (M1) and primary sensory (S1) cortices (Donoghue & Wise, 1982). S1, which is posterior and lateral to M1, receives sensory input primarily in layer IV, the granular region, and is characterized by receptive fields that respond to sensory stimuli. M1, which lacks a clearly defined granular layer, contains large pyramidal neurons in layer V and is defined based on populations of neurons that, when stimulated, elicit distinct body movements (Sanderson, Welker, & Shambes, 1984; Wise & Jones, 1977) (Donoghue & Wise, 1982). This is the basis of the motor maps revealed using ICMS (Intracortical Microstimulation) procedures. Layer V pyramidal neurons within the overlap zone synapse on spinal motor neurons to elicit forelimb movements (Hicks & D'Amato, 1977; Valverde, 1966). The caudal and rostral forelimb areas are distinct from each other and are distinguishable based on characteristics of their movement representations. The SMC lesions used in these dissertation studies are aimed at the caudal forelimb area. The caudal forelimb representation area, and not the rostral area, may be the more important contributor to skilled motor learning (e.g., Gharbawie, Karl, & Whishaw, 2007). It receives input from

S1 (Sievert & Neafsey, 1986) and is the targeted region for the lesions induced in these dissertation studies. Damage to this region in rats has been shown to result in pronounced deficits in the forelimb contralateral to the lesion (Allred & Jones, 2004; Hsu & Jones, 2005; Maldonado et al., 2005).

1.6.2 Ascending and descending projections. S1 receives thalamic input from the ventral posterior nucleus (Donaldson, Hand, & Morrison, 1975; Wise & Jones, 1978) and the posterior thalamic complex, which terminates mainly in layer IV and lower layer III (Koralek, Jensen, & Killackey, 1988; Killackey, 1973; Killackey & Sherman, 2003). M1 also receives input from the ventral posterior thalamus, which mainly terminates in layer III (Killackey, 1973; Killackey & Sherman, 2003). The overlapping region of M1/S1 receives extensive input from the ventrolateral thalamus with projections terminating in layers II/III and V (Donoghue & Parham, 1983). The ventrolateral thalamus receives input from several regions, including the spinal cord, cerebellum, and basal ganglia (Donoghue & Parham, 1983). Projections originating in S1 terminate in areas involved in motor behavior, including the striatum and pontine nuclei (Wise & Jones, 1977; Donoghue & Parham, 1983; Mihailoff, Lee, Watt, & Yates, 1985). Layer V neurons originating in M1 then project to the spinal cord (Leong, 1983; Bates & Killackey, 1984; Miller, 1987) red nucleus, and pons (Legg et al.,1989; Mihailoff et al., 1985)), bilaterally to striatum (Donogue & Kitai, 1981), and also to the reticular formation (Valverde, 1966).

The corticospinal tract is mostly a crossed pathway, but approximately 5-10% of this pathway is estimated to be ipsilateral (Brosamle & Schwab). Layer V motor neurons

project to the spinal cord via corticospinal projections. Damage to the corticospinal tract (CST) can result in deficits in motor tasks, though the medial and lateral portions of the CST appear to contribute to different aspects of motor performance. The SMC contributes to both pathways. Rats with lateral CST lesions (aimed at C5 of the spinal cord) have more enduring deficits on a skilled reaching task compared to medial CST lesion animals, though the medial CST appears to play a larger role in mediating grip strength (Anderson, Gunawan, & Steward, 2007).

1.6.3 Role of ipsilesional corticospinal tract. Most of the motor cortex contribution to the corticospinal tract (CST) is crossed in the rat, such that the contralateral cortex is responsible for moving the limb. However, there is some ipsilateral contribution (about 5-10%, Brosamle & Schwab, 1997). A question is whether the 10% of this pathway arising from the contralesional cortex mediates recovery of the impaired forelimb. If so, learning with the intact forelimb may also interfere with motor relearning of the skilled task with the impaired forelimb because it interferes with the takeover of the impaired limb by the ipsilateral CST. The likelihood of this effect, however, seems low. In animals with transections of the corpus callosum the maladaptive effect of intact forelimb training on impaired recovery was absent. If this pathway was involved in mediating recovery of the impaired forelimb, the maladaptive effect should have still been present in transected animals. Additionally, after lesion animals had recovered on a skilled reaching task, a second lesion in the contralateral (intact) hemisphere did not reinstate impaired forelimb deficits greater than deficits seen in animals with a unilateral lesion, suggesting that it is not normally responsible for its recovery, at least after these focal SMC lesions (Maldonado, Allred, Hsu, & Jones, 2006). The contribution of the ipsilateral, uncrossed

pathway has previously been shown not to contribute noticeably to skilled reaching behavior and the uncrossed pathway also does not contribute to motor recovery when the crossed pathway is severed (Whishaw & Metz, 2002). However, the ipsilateral components of the CST may at least sometimes play a larger role in mediating recovery following some types of brain damage, perhaps particularly after very large lesions, when there is little of the peri-lesion cortex remaining.

1.7 Motor Maps As A Neural Substrate for Motor Behavior

1.7.1 Motor Maps Illustrating the Somatotopic Organization of M1. In the history of work on motor function of the CNS, a variety of techniques have been used to identify brain structures projecting to motoneurons. Fritsch and Hitzig (1870) first evoked muscle contractions by applying electrical stimulation to the motor area of the cerebral cortex in the dog (Fetz, 1991). The motor map is assessed using microstimulation, whereby a microelectrode is used to excite a small population of cortical neurons. The motor responses evoked by microstimulation reflect a complex combination of activation of intracortical (Jankowska and others 1975) and spinal circuitry. Using microstimulation in developing animals and in maturity, small territories of cortex that are approximately 500 µm in diameter (Keller 1993) show a preponderance for controlling a single or small set of limb muscles (Nudo and others 1992; Martin and Ghez 1993; Chakrabarty and Martin 2000). Over the years considerable progress has been made in developing microstimulation techniques for more refined and discrete activation of descending neurons (Asanuma and Sakata, 1967). This effort has culminated in with methods capable

of detecting the excitatory and inhibitory effects of single neurons on muscle activity in the awake animal during task performance (Fetz 1976).

Motor maps adapt in response to motor learning, therefore can serve as a surrogate marker for changes in motor behavior such as those observed during learning or rehabilitation. Several studies have demonstrated that motor training can induce changes in motor map organization that reflect the nature of the acquired skill. Nudo and colleagues have shown that training squirrel monkeys on a skilled digit manipulations task causes an expansion of digit representations (Nudo, Milliken 1996). It is important to note that ICMS-evoked movements may result from both direct excitation of local neurons and polysynaptic activation of more distant neurons. It is possible that the mosaic representations of movement as defined by the ICMS technique are at least partially a result of complex excitatory and inhibitory effects on spinal motoneurons arising from a relatively wide area of cortex. Nevertheless, the relative stability of the response at a given site, the striking differences in response between adjacent sites, and the correlation of ICMS defined boundaries with cytoarchitectonic boundaries suggest that ICMS is a reliable technique for defining functional boundaries in motor cortex (Huang et al 1988; Nudo 1990). Multiple muscles may be represented in at a single site in the primary motor distal forelimb representation. Fetz demonstrated that the output of a single cortical neuron typically facilitates multiple muscles. Using anatomical techniques in cats, Shinoda et al., 1981 have identified individual corticospinal neurons that project to multiple spinal cord segments, suggesting that some corticospinal neurons may project to multiple motoneuron pools.

1.7.2 Skilled reach training drives motor cortical plasticity. In these dissertation studies a skilled learning task was used as both an outcome measure and as a behavioral/therapeutic manipulation. Motor skills training in animals induces plastic changes in the motor cortex contralateral to the trained forelimb, including increase in dendritic arborization (Greenough 1985) and synapses (Kleim et al., 2004) and an expansion of motor representations devoted to reaching movements (e.g., digit and wrist, (Kleim, Barbay, & Nudo, 1998). It has been further established that practice with a skilled motor task (and not simple bar pressing task) is necessary to induce motor map reorganization (Kleim et al., 1998). Practice with skilled reaching in humans has also been shown to be associated with changes in motor cortical movement representations, as measured using transcranial magnetic stimulation (TMS) (Ziemann, Muellbacher, Hallett, & Cohen, 2001) and functional magnetic resonance imaging (fMRI) (Perez et al., 2007).

1.7.3 Learning induced structural plasticity within M1. The functional adaptation in Motor cortex (M1) that accompanies motor skill learning depends on restructuring of motor cortex microcircuitry. In rats trained to reach, pyramidal neurons (PMN) in layers II/III and V have enlarged dendritic fields (Greenough, 1985; Greenough & Withers1989). This enlargement of dendritic surface is accompanied by an increase in the number of synapses per neuron in layer V PMNs suggesting that learning promotes synaptogenesis. This was confirmed by Kleim et al (1996) in a study showing an increase in the number of synapses/neuron occurred during the early phase of skilled reach training. Further, the increased synapse number was colocalized with an increase in distal forelimb representations (Kleim et al., 1998). Rioult-Pedotti and coworkers showed that

motor skill learning is associated with LTP like synaptic plasticity in rats. Acquisition of a reaching task induced a long-lasting increase in synaptic strength in horizontal connections of layer II/III in the M1 forelimb representation contralateral to the trained paw. Similar results were obtained in an *in vivo* animal model (rat) introduced by Monfils and Teskey (2004).

1.8 Behavioral and cortical changes following unilateral sensorimotor cortex damage

Neuronal cortical connections can be remodeled by our experience was suggested by Hebb half a century ago. Since then, many studies have demonstrated chemical and anatomic plasticity in the cerebral cortex of adult animals. Unilateral SMC damage has been shown to result in profound sensory and motor impairments in the contralesional forelimb, including deficits in skilled reaching (Gilmour, Iversen, O'Neill, & Bannerman, 2004; Whishaw, 2000), delayed responsiveness to tactile-stimulation ((Napieralski, Banks, & Chesselet, 1998), decreased use of this limb in tests of postural support (Adkins, Voorhies, & Jones, 2004; Allred & Jones, 2004) and increased errors in measures of coordinated limb use (Bury & Jones, 2002). Following SMC damage, a deficit in skilled reaching performance has been found to occur in rats (Gharbawie, Auer, & Whishaw, 2006; Gilmour, et al., 2004; Whishaw, 2000), in monkeys (Friel & Nudo, 1998; Nudo, Wise, SiFuentes, & Milliken, 1996) and in humans (Green, 2003).

1.8.1 Synaptic Plasticity in the Peri-infarct Cortex. Studies have shown that alternations in electrophysiological properties of motor cortex neurons contribute

significantly to network reorganization after stroke. Hagemann and colleagues reported enhanced long-term potentiation (LTP) in the peri-infarct zone around a photothrombotic lesion to primary somatosensory cortex (S1) (Hagemann et al., 1998). Neurons in the peri-infarct cortex (PIC) are more excitable after a stroke because NMDA-receptor expression is upregulated (Kalia et al., 2004) and GABAA- receptors are downregulated (Zilles et al., 1996). Reducing inhibition by blocking GABA-A-receptors is usually a prerequisite for the induction of LTP *in vitro*. Therefore increased cortical excitability might be a plausible explanation for facilitated LTP in the PIC.

Mechanisms involved in learning motor skills task may share some similarities with those involved in LTP, an artificial means of inducing synaptic strengthening. Motor skills training results in LTP-like effects in the cortex opposite to the trained forelimb (Monfils, VandenBerg, Kleim, & Teskey, 2004; Rioult-Pedotti, Friedman, Hess, & Donoghue, 1998). LTP can be induced in motor cortex in awake animals (e.g., (Monfils & Teskey, 2004). LTP is thought to occur via strengthening of horizontal connections in the stimulated hemisphere (Hess, 1994) and is dependent on protein synthesis (Luft et al., 2004). Kleim and colleagues (2003) have shown that, following protein synthesis inhibition reorganized motor map representations are eliminated and synapses are lost in the same region, effects which were replicated by Hsu and colleagues (2007).

1.8.2 Disrupting Cortical Circuitry. Studies in animals have shown that disrupting motor maps, without explicitly damaging the supporting cortex, causes an inability to produce skilled limb movement but does not abolish movement. For example, focal ischemic insult can cause a loss of movement representation within intact regions of the

motor cortex distal to the infarction. The disappearance of movement representations is accompanied by skilled movement impairments but not a total absence of movement (Nudo 1996). The loss of motor map is thought to be the result of neuronal dysfunction and a disruption of cortical circuitry, as there is a loss of synapses but not neurons within these regions (Kleim 2003). Effect likely mediated via changes in intracortical synaptic efficacy. Similar results have been found with pharmacological agents known to mediate synaptic plasticity within the motor cortex.

1.8.3 Disruption of Synaptic Plasticity in Peri-infarct Cortex. Motor skill training induces synaptic plasticity (Kleim et al., 2002) and an expansion of motor maps in the motor cortex opposite the trained forelimb (Nudo, 2003; Kleim et al., 1998; Monfils et al., 2005). The ability for motor maps to reorganize is related to strengthening of synaptic connections, which is experience dependent (e.g., Kleim et al., 2002). The reorganization of peri-lesion cortex after stroke, including reorganization of motor maps, has been linked with functional recovery (e.g., Nudo, 2003). Conversely, increased activity in intact cortex, evident following unilateral brain damage (Que et al., 1999; Reinecke et al., 1999; Murase et al., 2004), is related to greater inhibition and disruption of peri-lesion cortex and poorer recovery (Murase et al., 2004).

1.8.4 Interhemispheric connectivity of the SMC. There is converging evidence that the two cerebral hemispheres have a balance of activity that can be disrupted following unilateral brain damage. The SMCs of the two hemispheres interact via transcallosal inputs, which mediate interhemispheric inhibition and excitation (Lee, Gunraj, & Chen,

2007). Transcallosal inputs make excitatory connections onto pyramidal neurons of the opposite cortex (Chapin, Sadeq, & Guise, 1987; Karayannis, Huerta-Ocampo & Capogna 2007, Carr & Sesak, 1998; Cisse, Grenier, Timofeev, & Steriade, 2003) and also project onto GABAergic inhibitory interneurons (Perez & Cohen, 2008; Innocenti, Clarke, & Kraftsik, 1986). Bilateral and intercortical activity occurs as a response to unilateral sensory stimulation (Liepert, Havernick, Weiller, & Barzel, 2006) and movement (Cisek, Crammond & Kalaska, 2003; Brus-Ramer, Carmel & Martin, 2009). Previously, the SMC region damaged in these studies was shown to receive transcallosal input from the opposite cortex, as reflected in biodextrine-amine (BDA) labeled axons (Bury & Jones, 2004). Layer V pyramidal neurons also make synaptic connections with ipsilateral and contralateral striatums (Cospito & Kutlas-Ilinkshy, 1981).

In humans, following stroke there is an increased inhibitory drive (as measured using a paired pulse TMS paradigm) from the contralesional to the lesion cortex (Duque et al., 2005; Perez & Cohen, 2009). This has also been shown to be correlated with a reduction in reaction time on a finger tapping task (Murase et al., 2004). Callosal lesions and agenesis of the corpus callosum in humans results in a loss of interhemispheric inhibition (Meyer, Roricht, Grafin von Einsiedel, Kruggel, & Weindl, 1995), which strongly suggests that interhemispheric activity is mediated through the corpus callosum. A disruption of interhemishperic activity following unilateral damage may become exaggerated with behavioral experience, and it stands to reason that if this unbalance is blocked, e.g., by callosal transections, this would prevent activity in the intact hemisphere from disrupting the ability of the lesion cortex to recover.

1.9 Compensation vs. Recovery in rats following cortical ischemia

The interaction of both hemispheres after an ischemic injury may result in changes in motor performance via several mechanisms: restitution, substitution, or compensation (Carmichael, 2005). Levin and co-workers,however, distinguished motor recovery and motor compensation in accordance with the WHO International Classification of Functioning, Disability and Health framework and proposed that motor recovery relates to: restoration of function in neural tissue that was initially lost; restoration of ability to perform movement in the same way as before injury; and successful task completion as typically done by individuals who are not disabled. Types of motor compensation in these three areas include the acquisition by neural tissue of a function that it did not have before the injury; performance of a movement in a new way; and successful task completion by use of different techniques (Levin, Kleim & Wolf, 2008).

In an effort to delineate recovery vs compensatory behavior, behavioral scientists have developed a comprehensive testing battery that may be divided into two categories, based on whether they involve endpoint measures demonstrating how well are the animals doing the task, or qualitative measures indicating how the task is being done. Examples of endpoint measures are accuracy in reaching, number of strands of pasta obtained, or number of foot faults, whereas qualitative measures examine the normality of motor elements in a single reaching movement (Kleim et al, 2007). The use of these two different measures makes it possible to differentiate between true recovery of motor function as opposed to the development of compensatory motor strategies. For example,

increases in accuracy on a skilled reaching task may result from restoring the movement sequence used before the insult, developing a completely new movement sequence, or some combination of the two. The neural mechanisms that underlie restoration of the original movement sequence are likely very different from those involved in development of a completely new motor pattern. Analysis shows that improvements in reaching accuracy after stroke are due, at least in part, to adaptations in the original movement sequence (Gharbawie and Whishaw 2006). The strategy adopted in response to a given therapy may be influenced be any number of factors, including the size and location of the damage, the nature/efficacy of the rehabilitation treatment, or the timing of the treatment after insult. It is essential to understand the contribution of these factors in order to optimize the kind of rehabilitation administered to each patient.

Chapter 2

NEUROTROPHINS AND CORTICAL PLASTICITY

The cerebral cortex is a rich source of neurotrophins that regulate the function of cortical neurons and cortical afferents (Cabelli et al. 1995; Lu et al. 2001; McAllister et al. 1995). Several lines of evidence suggest that neurotrophins may play a protective role in the pathophysiology of cerebral ischemia (Endres et al, 2000; Saarelainen et al, 2000; Yanamoto et al, 2000). Neurotrophins have also been shown to have a positive impact on the recovery of cortical projections after a cortical infarct (Figueiredo et al, 1993; Garofalo et al., 1992, 1993). In support of this hypothesis, several synaptically localized proteins have been identified and linked to changes in synaptic function (Martin and others 2000). One of the key neural signals involved in this process include neurotrophic factors such as brain derived neurotrophic factor (BDNF) that have been implicated in modulating dendritic morphology (McAllister, 1996; Tolwani 2002) and cortical map organization (Rocamora 1996). There is increasing evidence that brain-derived neurotrophic factor (BDNF) modulates synaptic and morphological plasticity in the developing and mature nervous system.

2.1 Endogenous BDNF and TrkB receptors and its impact on possible downstream signaling mechanisms

BDNF influences neuronal proliferation, survival, and differentiation as a result of binding to its tyrosine kinase receptor (TrkB). Both BDNF and tyrosine kinase receptor are widely distributed throughout the brain, with highest expression in the hippocampus. BDNF is considered to be a retrograde messenger. It is secreted from the pre-synaptic

cell and has both pre-synaptic and post-synaptic targets (Manabe, 2002). Neuronal activity stimulates BDNF release from pre-synaptic sites (Yamada and Nabeshima, 2003). The secreted BDNF will bind to TrkB receptors on the pre-synaptic cell membrane and trigger an increase in pre-synaptic glutamate release. BDNF also binds to TrkB receptors on the post-synaptic membrane, which will become activated and trigger intracellular signaling cascades. Thus, increased neuronal activity enhances synaptic efficacy to increasing the transcription, secretion and binding of BDNF, which leads to enhanced glutamate release and activation of signal transduction pathways.

2.2 Role of BDNF in enhancing neuroplasticity

BDNF has several important functions in the CNS. First, it promotes the differentiation and survival of a variety of neuronal populations during development and adulthood (Maness et al., 1994; Huang and Reichardt, 2001; Lu, 2003). Second, BDNF promotes neurite extension of sensory processes to their targets within the CNS (Maness et al., 1994). Finally, BDNF plays a crucial role in mediating synaptic plasticity and transmission (Lu, 2003). These BDNF-induced changes in synaptic plasticity can occur in an acute manner. For example, application of BDNF to the neuromuscular junction elicits a rapid enhancement of neurotransmitter release (Stoop and Poo. 1995). BDNF can also play a regulatory role in synapse development and function by triggering changes in the production of synaptic proteins (Corman and Berchtold, 2002) and by modulating growth/arborization of cortical dendrites (Horch and Katz, 2002).

2.2.1 Structure and function of BDNF. BDNF is a basic protein with 119 amino acid residues in its structure (Maness et al., 1994). Although BDNF is primarily found in the

central nervous system (CNS), it is also produced in lesser quantities in Schwann cells of the peripheral nervous system (Acheson et al., 1991) and in organs such as the heart and lungs (Rosenthal et al., 1991). In the CNS, BDNF is widely distributed in a number of brain regions, including the hippocampus, striatum, cerebellum and substantia niagra (Maness et al, 1994). In the cerebral cortex, studies have shown BDNF to be primarily localized to layers III and V (Murer at al., 1999).

2.2.2 TrkB Receptors. TrkB consists of an IgG-like extracellular domain that is essential for ligand binding. There are 6 kinase domains in the intracellular part of the receptor (Klein et al, 1990a). Binding of BDNF or NT4 to TrkB homodimers results in the autophosphorylation of their kinase domains and thus starts a signaling cascade (Marsh et al, 1993).

TrkB is expressed in all neural tissues during embryonic development but shows restricted expression in specific regions and neuron types of the adult brain (Klein et al., 1990b). It is expressed in both the pre- and post-synaptic compartments and is evenly distributed in primary hippocampal and cortical neurons (Kryl et al, 1999). TrkB is enriched in postsynaptic densities (Wu et al, 1996) and colocalizes with NMDARs in the dendrites and synaptic vesicles in the axons of cortical neurons (Gomes et al., 2006). Surface TrkB is found in dendritic filopodia and axonal growth cones, structures that take part in synapse formation. In addition to the full length TrkB receptor, two C-terminally truncated splice variants of TrkB have been identified - TrkB.T1 (Klein et al, 1990) and TrkB.T2 (Barbacid, 1994). TrkB, TrkB.T1 and TrkB.T2 share the same extracellular and transmembrane domains and the first 12 amino acid sequences of the intracellular

domain. The truncated isoforms lack the kinase domain of the TrkB and instead have isoform specific C-terminal sequences (11AA for TrkB.T1 and 9AA for TrkB.T2). Although TrkB.T1 receptors form homodimers upon ligand binding, downstream signaling does not occur due to the lack of functional kinase domains. Along with TrkB, TrkB.T1 is expressed in dendrites and axons of cortical and hippocampal neurons, with moderate enrichment in postsynaptic densities (Kryl et al, 1999).

2.2.3 MAPK, AKT/ERK, PI3K. BDNF primarily supports the function of glutamatergic neurons and preferentially binds to the TrkB tyrosine kinase receptor on these neurons (Cotman and Engesser-Cesar, 2002). Following ligand binding, the TrkB receptor autophosphorylates, which leads to activation of the tyrosine kinase and thus the activation of the receptor itself (Huang and Reichardt, 2001). Once activated the receptor is capable of triggering a number of intracellular signaling cascades, including the phosphatidylinositol-3-kinase/protein kinase B (PI-3-K/PKB), the phospholipase C-γ1 pathway and the MEK/extracellular signal-regulated kinase (ERK) systems (Kaplan and Miller, 2000). The ultimate results of these pathways are both short term (enhancement of glutamate release) and long term (activation of transcription factors in the nucleus that alter gene expression) (Kaplan and Miller, 2000).

2.2.4 C-AMP Response Element-Binding Protein (CREB). One MEK/ERK-activated transcription factor that may be especially important to BDNF-induced neuroprotection is cAMP response element-binding protein (CREB). CREB is also activated by components of other intracellular signaling pathways, including protein kinase C, Ca^{2+} calmodulin

kinase and ribosomal S6 kinase 2 (Walton and Dragunow, 2000). CREB has been described as the genetic switch that regulates the expression of genes necessary for the establishment of long-term synaptic plasticity (Alberini, 1999). It is involved in other aspects of neuronal functioning, including neuronal excitation (Moore et al., 1996) and development (Imaki et al., 1994). In addition, CREB has been linked to cell survival. Several studies have shown that activated CREB promotes cell survival while inhibition of CREB phosphorylation triggers apoptosis (Bonni et al., 1999; Walton and Dragunow, 2000; Jaworski et al., 2003). CREB may also play a crucial role neuronal survival following ischemia (Walton et al., 1996; Jin et al., 2001).

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

2.2.5 Mechanism and regulation of BDNF action. The ability of BDNF to mediate synaptic modulation is dependent upon synaptic activity (Lu, 2003). Thus increased electrical activity in the cells is proposed to up-regulate BDNF, which in turn facilitates synaptic plasticity (Cotman and Berchtold, 2002). Increased neuronal activity can be initiated by several different types of stimuli, including experimentally induced seizures (Ernfors et al., 1991), tetanic stimulation (Morimoto et al., 1998) and physical activity

(Neeper et al., 1996) and each has been shown to up-regulate BDNF mRNA levels in the brain. Further, increased neuronal activity increases the number of TrkB receptors expressed on the presynaptic neuron (Meyer-Franke et al., 1998).

BDNF is considered to be a retrograde messenger. It is secreted from the pre-synaptic cell and has both pre-synaptic and post-synaptic targets (Manabe, 2002). Neuronal activity stimulates BDNF release from pre-synaptic sites (Yamada and Nabeshima, 2003). The secreted BDNF will bind to TrkB receptors on the pre-synaptic cell membrane and trigger an increase in pre-synaptic glutamate release. BDNF also binds to TrkB receptors on the post-synaptic membrane, which will become activated and trigger intracellular signaling cascades. Thus, increased neuronal activity enhances synaptic efficacy to increasing the transcription, secretion and binding of BDNF, which leads to enhanced glutamate release and activation of signal transduction pathways.

2.2.6 BDNF/TrkB and Brain Injury. Given the beneficial effects of BDNF in the brain, it has been hypothesized that BDNF could be a viable treatment to ameliorate damage from various forms of brain injury. There is an abundance of research supporting this idea. (Yanamoto et al, 2000) found that pretreatment with BDNF prior to temporary focal ischemia significantly reduced infarct volume. Schabitz et al. (2002) found that BDNF delivered intravenously following focal cerebral ischemia reduced infarct volume and neurological deficits. In addition, these authors also found that the expression of proapoptotic protein Bax was decreased, while expression of antiapoptotic protein Bcl-2 was increased, which may be one mechanism by which BDNF exerts its neuroprotective action. Finally Wu and colleagues (Wu et al, 2003) found that low BDNF mRNA levels

are related to poor recovery of spatial memory and impairments in motor performance following traumatic brain injury. BDNF is stored and released from glutamate neurons in a use-dependent fashion and has been implicated in long-term potentiation, learning, memory formation, depression, and recovery from brain injury. Administering BDNF in the acute post-ischemic period reduces cell death and delayed treatment facilitates motor recovery in rats. Finally, among survivors of hemorrhagic stroke, patients with the Met allele of the BDNF val^{66}met polymorphism show significantly worse functional outcome than patients without the allele (Siironen et al., 2007). Together these data suggest that the TrkB signaling pathway is involved in mediating motor recovery and concomitant cortical plasticity after stroke.

2.3 Protein synthesis and motor skill learning

Neurotrophins are a family of vertebrate specific growth factors that are critically involved in regulating neuronal survival and differentiation in development and continues to shape neuronal structure and function throughout life (Castrén, Zafra, Thoenen, & Lindholm, 1992; Dechant, 2001; Prakash, Cohen-Cory, & Frostig, 1996). The receptor/ligand systems used to mediate these effects involve four genes encoding four ligands (NGF, BDNF, NT3 and NT4/5) and four genes encoding four receptors (TrkA, TrkB, TrKC and p75). TrKB receptors are the most generalized, binding to three of the four ligands (BDNF, NT3 and NT4/5) and mediate intracellular signaling via a receptor tyrosine kinase pathway. One of the key functions of the TrkB receptor is to bind BDNF. TrkB-mediated signaling pathways have been tested in vivo by mutating the recruitment site, Y816, to phenylalanine (Minichiello et al., 2002). Electrophysiological experiments

show that the TrkB mutant mice have significant deficiencies in the induction of both the early and late phases of hippocampal CA1 long-term potentiation (LTP), an artificial means of inducing synaptic strengthening (Huang and Reichardt 2003). Phosphorylation of CREB is severely impaired in these neurons. Trk receptor signaling also controls the activity and localization of neurotransmitter receptors through protein phosphorylation. For example, BDNF, activation of TrkB promotes the phosphorylation and dephosphorylation of the NMDA receptor subunit NR2B with phosphorylation increasing the open probability of the NMDA receptor ion channel and thereby rapidly enhancing synaptic transmission (Levine et al., 1998; Lin et al., 1999). In addition to these acute effects of Trk activation, additional deficits are seen in mice lacking normal TrkB-mediated signaling. These include reductions in vesicles docked at release sites and reduced expression of synaptic proteins (Pozzo-Miller et al., 1999).

BDNF has been demonstrated to modulate synaptic strength and neuronal excitability. BDNF expression is regulated in an activity dependent manner at different molecular levels (Bengzon et al., 1993; Pattabiraman et al., 2005; Rossi et al., 1999). It has been demonstrated that cellular mechanisms behind learning-related plasticity in the motor cortex (M1) appear to depend on protein synthesis within this structure and may specifically involve brain-derived neurotrophic factor (Kleim et al., 2003). In both humans and animal models, BDNF influences synaptic plasticity (Akaneya et al., 1997; Lu, 2003) resulting in reorganization of cortical circuitry such that the muscles controlling trained movements are more easily evoked in response to cortical stimulation.

Mechanisms involved in learning motor skills task may share some similarities with those involved in LTP. LTP can be induced in motor cortex in awake animals (e.g.,

(Monfils & Teskey, 2004). Motor skills training results in LTP-like effects in the cortex opposite to the trained forelimb in rats (Monfils, et al., 2004; Rioult-Pedotti, et al., 1998). LTP is thought to occur via strengthening of horizontal connections in the stimulated hemisphere (Hess, 1994) and is dependent on protein synthesis (Luft et al., 2004). Kleim and colleagues (2003) have shown that, following protein synthesis inhibition reorganized motor map representations are eliminated and synapses are lost in the same region, effects which were replicated by Hsu and colleagues (2007). Moreover, training-dependent increases in motor cortical excitability (Antal et al., 2010; Cheeran et al., 2009) and Functional Magentic Resonance Imaging (fMRI) signal (McHughen et al., 2010) are reduced in healthy humans with a valine-to-methionine substitution at codon 66 (Val66Met) in the BDNF gene, when compared to subjects without this polymorphism (Kleim et al., 2006).These findings also lead to the hypothesis that BDNF is involved in mediating experience-dependent plasticity of human motor cortex and the presence of this particular polymorphism could influence motor skill learning. Relearning motor skills after stroke is multifaceted incorporating or relying on spatial context, retrieval of previously learning movement strategies, motivation, working memory, and internal and external sensory feedback (Corbett 2009). Therefore, strategies that increase BDNF, broadly within the nervous system, may enhance neuroplasticity processes in multiple neuronal systems involved in motor relearning during stroke rehabilitation.

2.4 Limitations of using BDNF as a pharmacological intervention

The beneficial role of BDNF in the brain makes it a viable therapeutic candidate. However, BDNF when administered in its native form itself exhibits a poor

pharmacokinetic profile with a short plasma life and a low permeability through the blood brain barrier. BDNF interacts with both the TrkB and with p75 receptor (Chao &Hempstead, 1995). Activation of the p75 receptor can result in many undesired side effects such as pain (Zhang, Chi, & Nicol, 2008) and apoptosis (Barrett et al. 2000). Of the two receptors, TrkB is an essential modulator of neural plasticity and activation of TrkB has been shown to be essential for the survival-promoting actions of BDNF (Reichardt, 2006). Hence, there is a critical need to develop interventions that would up regulate endogenous signaling by pharmacologically harnessing the beneficial synaptic effects of BDNF that would help restore lost functions after stroke.

2.5 Development of novel TrkB receptor agonists

In an effort to overcome the limitations presented by BDNF in its use as a pharmacological agent, several researchers have developed compounds that mimic the role of BDNF by binding to TrkB receptor and activating downstream signaling pathways through phosphorylation of catalytic sites of TrkB receptor. Further, these compounds were developed to reliably cross the blood brain barrier in an effort to make them more clinically viable.

2.5.1 7,8 Dihydroxyflavone. 7,8 Dihydroxyflavone is a synthetic flavonoid derivative, which selectively binds to TrkB receptor and stimulates receptor dimerization, a function similar to BDNF (Jang et al., 2010).

2.5.2 LM22A-4. Another recently discovered compound, LM22A-4 (N,N',N'-tris (2-hydroxyethyl)-1,3,5-benzene tricarboxamide), a small molecule ligand designed to mimic the loop II domain of BDNF (Massa et al., 2010) has been shown to 1. Selectively mimic

the cell signaling actions of BDNF on the TrkB receptor, actively cross the blood brain barrier, and enhance functional recovery in animal models of several neurological disorders (Han et al., 2012; Schmid et al., 2012). LM22A-4's effects are qualitatively and quantitatively different than the native BDNF protein (Massa et al., 2010).

Efficacy of these two compounds was tested in a rodent model of cortical ischemia, whose findings will be discussed in the following chapters.

Chapter 3

7,8 dihydroxyflavone enhances motor performance and partially restores forelimb movement representations following cortical ischemia

3.1 Introduction

Stroke remains the leading cause of adult disability with prominent functional motor deficits in surviving stroke victims. Motor recovery after stroke can be thought of as a “relearning” process whereby lost motor functions are reestablished through functional restoration and compensation (plasticity) within spared motor brain regions. Identifying and harnessing the neural signaling systems that drive both neural plasticity and motor learning can be used to guide the development of adjuvant treatments to enhance motor recovery after stroke.

Genetic, pharmacological and electrophysiological studies in a variety of organisms have demonstrated the importance neurotrophins such as Brain Derived Neurotrophic Factor (BDNF) for inducing synaptic plasticity (Lu, 2003), cortical reorganization (Maffei, 2002; Kleim et al., 2006) and learning (Yamada & Nabeshima, 2003; Rattiner et al., 2004). This has led to the hypothesis that BDNF and the tropomysin related kinase receptor (TrkB) is a key neural signal driving rehabilitation-dependent motor recovery and cortical plasticity after stroke.BDNF appears to be a powerful therapeutic tool. However, clinical trials using recombinant BDNF failed because of enzymatic degradation, poor delivery, and other limitations (Ochs et al., 2000 and Thoenen and Sendtner, 2002).

Emerging evidence suggests that dietary phytochemicals, in particular flavonoids,

may exert beneficial effects in the central nervous system presumably by protecting neurons against stress-induced injury, by suppressing neuroinflammation and enhancing existing neurocognitive performance, through changes in synaptic plasticity (Spencer, 2007). Flavonoids, found in plants and fruit, exert anti-oxidative effects and have demonstrated reactive oxygen species (ROS) scavenging abilities (Schroeter et al., 2002; Mandel et al., 2004; Williams et al. 2004).

Previous studies have highlighted that modulations of mitogen-activated protein kinase (MAPK) signaling are central to mediating the cellular effects of flavonoids (Schroeter et al. 2002; Williams et al. 2004). The role of flavonoids has also been implicated in regulating AKT and phosphorylation of extracellular signal-regulated kinase (ERK) signaling pathways (Schroeter et al. 2007). One of the major receptors through which these cellular effects are initiated in the brain has been identified to be tyrosine kinase receptors. Trks are activated by binding of mature neurotrophin dimers and multimerization, leading to phosphorylation and signaling adaptor recruitment (Huang & Reichardt, 2003).

Flavonoids are well reported to cross the blood-brain barrier (BBB), but the extent of permeation is low depending on compound's lipophilicity (Yodium et al. 2004, 2003). Many efforts have been directed at circumventing these problems and recently, an exogenous agent, so-called 7,8-DHF, was identified as a potent and selective TrkB receptor agonist, mimicking Brain Derived Neurotropic Factor (BDNF).

Mechanism: The basal structure of flavonoids consists of 15 carbon atoms with two terminal aromatic rings linked through an oxygenated heterocycle (C_6-C_3-C_6) (Beecher, 2003). Flavonoids can be divided into six main classes (flavanols, flavanones, flavones,

isoflavones, flavonols and anthocyanidins). Because of their hydroxyl groups, flavonoids stabilize ROS by interacting with the reactive compound of the radical according to the following reaction:

where FOH is flavanoid, R` is free radical, and FO` is less reactive free radical.

The purpose of this study was to investigate the efficacy of 7,8 Dihydroxyflavone for reducing behavioral and neurophysiological impairments associated with cortical ischemia in rats. Rats use their forepaws in dexterous ways that are in some capacities homologus to humans (Iwaniuk and Wishaw, 2000). And there is a growing appreciation that forepaw movements provide a useful model for probing aspects of hand function and dysfunction. Rats skillfully manipulate the food with the movements of the forepaws, including fine digit movements. It has long been known that CNS damage can influence the way rodents grasp and handle food (Peterson & Mc, 1951) and (Castro, 1972). Motor learning and plasticity have been associated with TrkB and/or BDNF expression in the cortex (Klintsova, Dickson, Yoshida, & Greenough, 2004). BDNF has also been reported to improve motor recovery and increase measures of neuronal remodeling after cortical ischemia (Schabitz et al., 2004). Because BDNF possesses a broad spectrum of physiological activities, and its dysregulation is involved in numerous neurological disorders, flavonoid-based TrkB agonists have the potential to be developed into a powerful class of therapeutic drugs (Jang et al., 2010). 7,8-DHF (Figure 3.1) is a synthetic compound, slightly modified from a naturally occurring version and is highly selective compared to BDNF with a dissociation constant of approximately 320nM. Therefore, it could possibly serve as an alternative to BDNF.

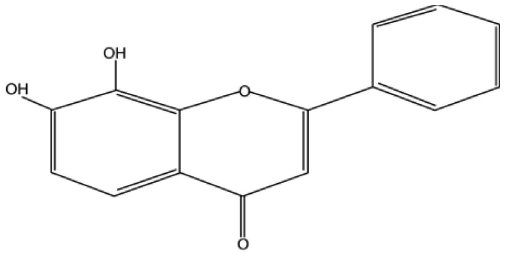

Figure 3.1 Structure of 7, 8-Dihydroxyflavone (Adapted from Jang et al., 2010).

3.2 Methods

3.2.1 Experiment 1. Twenty four, male adult (100-120 days of age) Long Evans rats were assigned to one of three conditions: 7, 8 DHF, (N=8); Vehicle, (N=8) and Healthy controls (N=8). Animals in the 7,8 DHF condition received 5mg/kg (i.p.) of 7,8 DHF, 2 hours prior to inducing ischemia by topical application of endothelin-1 (ET-1) onto the frontalcortex. These animals then received 7,8 DHF daily for the next twenty-one days. Animals in the Vehicle condition were given similar cortical infarctions but received vehicle (30% DMSO) two hours prior and for the same duration of time. Controls animals did not receive infarctionsor the drug treatment. All animals were given 7 days to recover after surgery and motor impairments were assessed on a battery of forelimb motor tasks including cylinder paw placement, sunflower seed opening, and vermicelli handling for the next two weeks. Intracortical microstimulation (ICMS) was then used to map forelimb movement representations within the rostral and caudal forelimb areas of the ipsilesional motor cortex. Rats were euthanized using pentobarbatol, perfused and fixed in 4% paraformaldehyde. Histological analysis on brain sections was conducted to assess infarct volume.

3.2.2 7, 8 Dihydroxyflavone administration. For intraperitoneal administration 7,8-DHF was dissolved in dimethyl sulfoxide ($DMSO_4$) and phosphate buffer saline (PBS) was gradually added into the DMSO solution, to bring the final drug concentration to 0.5mg/ml with 30% DMSO V/V. Rats were injected with 5mg/kg of 7,8-DHF.

3.2.3 Cortical Ischemia. Animals were anesthetized under 4% isoflorane gas, at 40psi and maintained 1.5% isoflourane during the procedure and secured in a stereotaxic frame. The skull and dura were removed between 0.5 mm posterior and 1.5 mm anterior to bregma and 3.0-4.5 mm lateral to midline. Ischemia was then induced unilaterally in the proximity of middle cerebral artery by topical application of 4 µl vasoconstrictor, endothelin-1 (ET-1). ET-1 (80pmol) was aimed at the overlapping primary somatosensory and motor cortical representation regions of the forelimb. The skull was left undisturbed for ten minutes after ET-1 administration and Cerebral perfusion in the distribution of the middle cerebral artery was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc.), and only animals with a >80% decrease in cerebral perfusion were included in this study. ET-1application. It provides continuous and real time measures of regional cerebral blood flow changes. The exposed cortex was then covered with kwik-sil, a translucent, medium viscosity silicone adhesive (37 °C). The scalp was sutured and rats were allowed to recover in their respective home cages.

3.2.4 Behavioral Testing. Animals were given 7 days to recover after surgery and then assessed on forelimb behavioral testing battery. The tasks ranged from measures of gross motor performance to fine object manipulation. Forelimb tasks included sunflower seed

opening, vermicelli pasta handling, and cylinder paw placement. These tests are especially sensitive to detecting upper extremity dysfunction (Table 3.1).

Table 3.1: Motor Test Battery		
		
Sunflower Seed Opening	Cylinder	Vermicelli Handling
Time (secs) # Pieces	# Wall touches # Landings	# Adjustments # Time

Table 3.1 Motor test battery of tasks assessing upper extremity function

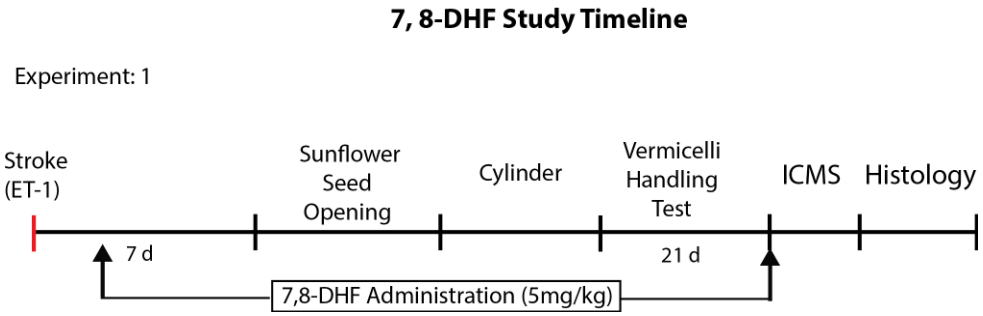

Figure 3.2 Schematic drawing of the experimental plan

Sunflower seed opening test

Rats are inherently adept at opening shelled seeds to obtain food, and sunflower seed opening is an effective measure of bilateral object manipulation (Whishaw, Sarna, & Pellis, 1998) as well as a good detector of motor impairments after stroke (Gonzalez & Kolb, 2003). Each rat was placed in a rectangular Plexiglas chamber and given five sunflower seeds. Rats start by manipulating the seed into a preferred position before

shelling. They then chew away a corner of the seed in order to facilitate splitting it longitudinally and finally the seed is split open into two or more pieces. In this task the total amount of time it took the animal to manipulate, open and consume all five seeds as well as the number of pieces of shell the animal had to break to retrieve the seed was measured.

Vermicelli handling task

This test consisted of 4 trials with 7 cm long pasta pieces given one at a time per each trial. The experimental set up was similar to the one described in (Allred et al., 2008). Data were collected with the rat facing the experimenter such that the digits and joints of the metacarpals and phalanges of both forepaws could be seen. The rats exhibited typical holding patterns, as previously described by (Whishaw & Coles, 1996).

Eating time was recorded, beginning when the pasta piece was grasped and placed in the mouth and ending when the piece was released by the paws and disappeared into the mouth. Total number of paw adjustments made while consuming pasta for each trial were also recorded.

Forelimb use asymmetry

The Schallert cylinder test was used to examine asymmetries in forelimb use for postural support during explorative activity by placing each rat in a transparent cylinder 20cm in diameter and 30 cm high for 3 min (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). This test encourages upright support against the cylinder wall, which sensitively reveals forelimb asymmetries. Rats were videotaped in the cylinder and then, during slow-motion video play back, instances of the sole use of the ipsilateral (to the

lesion) or contralateral forelimb or the simultaneous bilateral use of both forelimbs for upright support was recorded.

Behavior was quantified by determining the occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use observations on the wall; the occasions when the impaired forelimb (contralateral to the blood injection site) was used as a percentage of total number of limb use observations on the wall; and the occasions when both forelimbs were used simultaneously (or nearly simultaneously during lateral side-stepping movements) as a percentage of total number of limb use observations on the wall . The ipsilateral asymmetry score was computed using the formula: % (ipsilateral forelimb support+1/2 bilateral forelimb support)/(ipsilateral+contralateral+bilateral forelimb support). Forelimb placement during rearing, wall exploration and landings were recorded to determine forelimb asymmetry.

3.2.5 Mapping Procedure. Intracortical microstimulation (ICMS) techniques were used to generate detailed ipsilesional maps of forelimb regions of the motor cortex. Prior to surgery animals were anesthetized with ke7,8 DHF tamine hydrochloride (70 mg/kg intraperitoneally [i.p.]) and xylazine (5 mg/kg i.p.). Animals received supplemental doses of ketamine (20 mg/kg i.p.) and xylazine (0.02 mg/kg i.p.]) as needed. A digital image of the cortical surface was taken and a 500-μm grid was superimposed onto the image. A glass microelectrode (controlled by a hydraulic microdrive) was used to make systematic penetrations across the cortex using the cortical surface image and grid as a guide. At each penetration site, the electrode was lowered to approximately 1550 μm (corresponding to cortical layer V). Animals were maintained in a prone position with the

limb consistently supported. Sites where no movement was detected at ≤60 μA were recorded as unresponsive. Forelimb movements were classified as either distal (wrist/digit) or proximal (elbow/ shoulder) and representational maps were generated from the pattern of electrode penetrations (Fig. 5a). An image analysis program (CANVAS v. 3.5) was used to calculate the areal extent of the CFA. Briefly, each map was imported into CANVAS and calibrated to magnification. Individual areas were traced using the marquis tool to produce a measure of area (mm^2).

3.2.6 Histology and lesion verification. Rats were given an overdose of sodium pentobarbital and intra-cardially perfused with 0.9% saline followed by 4% paraformaldehyde solution immediately after mapping. The brains were removed and post fixed in 4% paraformaldehyde solution for 24-48 hrs and then finally placed in a 30% sucrose-formalin solution for 3 days before being cut frozen on a microtome. The brains were sectioned coronally at a thickness of 40μm. Sections were collected in a chilled 0.1M phosphate buffer solution and one series of every seventh section from the cortex and the sub-cortex was mounted on subbed slides and stained with cresyl violet to demonstrate Nissl substance (Morecraft, Geula, & Mesulam, 1992). Myelin staining was also carried out in the same sections. The mounted sections represented every 240-μm intervals in an individual series of tissue sections on the slides. Infarct size was determined by measuring areas of noninfarcted tissue in both the damaged and undamaged hemispheres. Lesion volumes were indirectly estimated based on interhemispheric volume differences by tracing whole cerebral hemispheres in sections 440 μm apart using Image J software.

3.2.7 Experiment 2:

2A. The potential effects of 7,8 DHF on vasodilation were investigated through measures of cortical blood perfusion using PeriCam PSI system (PeriMed). Four animals received 7,8-DHF (5mg/kg; i.p.) and four received vehicle (30% DMSO; i.p.) 2 hours prior to ischemic injury. Cortical blood flow measures were recorded in anesthetized rats starting 10 mins pre topical ET-1 through 90 mins post ET-1.

2B: The effects of 7,8 DHF treatment on pTrkB protein expression was assessed using Western blot analysis. Healthy Long Evans rats (120 days old) were used in this experiment. 5 rats received 7,8-DHF (5mg/kg; i.p.) and 5 received vehicle (30% DMSO; i.p.) once a day for 7 consecutive days up to 2 hours prior to being sacrificed.

Western Blotting

Rats were killed by rapid decapitation, and the brains were removed from the skull. Frontal cortices and hippocampi were removed. Protein extracts were subjected to Western blot analysis using standard techniques with slight modifications (Han et al. 2012). Blots were probed with antibodies recognizing pTrkB-Y817 (rabbit mAb 1:30,000, Epitomics), TrkB (rabbit polyclonal antibody1:2500, Millipore), and HRP goat anti-rabbit (1:10,000, Pierce Biotechnology). GAPDH (14C10) (rabbit mAb 1:10,000) was used a loading control. Optical densities were measured using LiCor Odyssey Infrared imaging system. Ratios were calculated as pTrkB over full length TrkB receptor and data was normalized to saline-treated stroked rats.

3.3 Results

3.3.1 Statistical analysis. Behavioral data from lesion studies were analyzed using univariate analyses of variance (ANOVA) for all measurements. Fisher's protected least

significant difference (LSD) ($p < 0.05$) *post hoc* statistical measures were computed to determine group differences in behavioral results by treatment. A modified LSD was used because Fisher's LSD does not account for multiple comparison and severely inflates Type 1 error (i.e., finding a difference when it does not actually exist). Protected Fisher's LSD allows all pairwise comparison among means and if the analysis of variance is significant, then each mean is compared with each other using a t-test. All descriptive statistics are reported as means ± S.E.M. unless otherwise indicated. The significance levels were * $p< 0.05$,

** $p< 0.03$.

3.3.2 7,8 DHF treated rats showed reduced motor impairments in comparison to vehicle injected rats on sunflower seed opening task, but not on cylinder or vermicelli handling tests

Sunflower Seed Test. In order to assess fine digit manipulation the sunflower seed opening test was conducted for two consecutive days a week after the injury. The mean time to consume the seeds in healthy control rats was 55.31± 3.92 s, averaged over the population examined. 7, 8 DHF treated group took 65.18 ± 6.9 s to complete the task whereas the vehicle treated group took the longest, 152 ± 47.25 s to consume all five seeds in the chamber (Figure 3.3). A univariate ANOVA comparison of multiple groups showed a significant main effect of the drug between 7,8 DHF and the vehicle group (F=3.74, P = 0.037), control group and vehicle injected groups also differed significantly (p = 0.021).

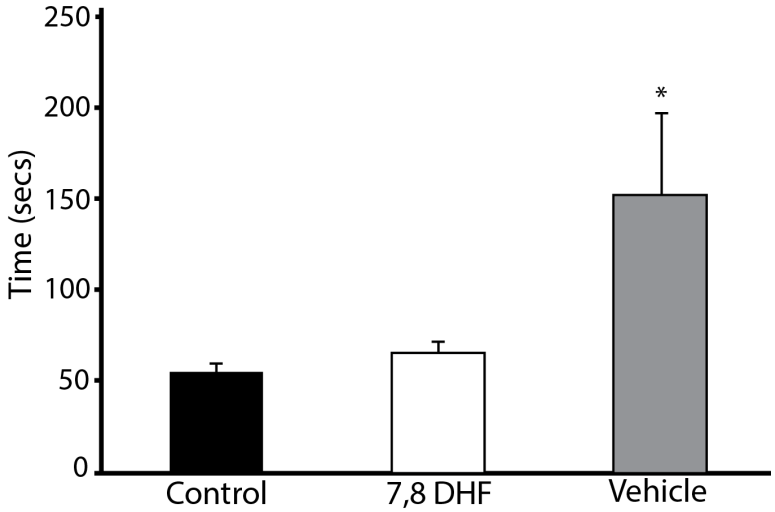

Figure 3.3 Sunflower seed opening test. Average time taken to consume five sunflower seeds: (Healthy control rats = 55.31 ± 3.92 s; 7,8 DHF group = 65.18 ± 6.9 s; vehicle group = 152 ± 47.25). Healthy control rats were better at fine digit manipulation during sunflower seed opening task and took the least amount of time in consuming the seeds, but 7,8-DHF treated rats showed less impairments by finishing the task significantly faster than the vehicle injected rats, ($p < .05$)

Vermicelli Handling Test. In vermicelli handling, animals in the control group were faster taking 28.68 ± 3.71 seconds to consume 4 pieces of vermicelli pasta. 7,8-DHF treated group had an average of 52.7 ± 4.94 s, and vehicle injected rats consumed their pasta in 46.18 ± 6.47 s (Figure 3.4). A univariate ANOVA comparison of multiple groups showed a significant difference between lesioned and non-lesioned animals ((F=5.866, P = 0.03) Vehicle injected group did not differ from 7,8 DHF receiving group.

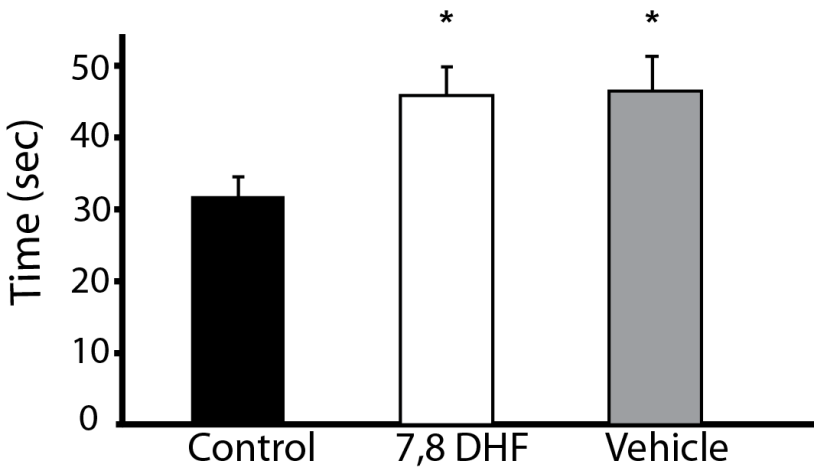

Fig 3.4 Vermicelli Handling Test. Average time to consume four pieces of vermicelli pasta: (Control=28.68 ± 3.71; 7,8-DHF=52.7 ± 4.94; Vehicle= 46.18 ± 6.47). A statistically significant difference ($p < 0.05$) was observed between stroke rats and the controls, no significant difference was observed between 7,8-DHF or vehicle treated groups during post-lesion testing.

Cylinder Test. Compared with controls, stroke rats had marked forelimb use asymmetry. The forelimb use asymmetry score was not significantly different between7,8-DHF treated and vehicle receiving rats. Stroke animals preferred to use their non-impaired limb with a higher frequency than their impaired paw (Figure 3.5). Further, vehicle treated rats used their impaired limb to a lesser degree than 7,8-DHF administered rats, but the difference was not significant.

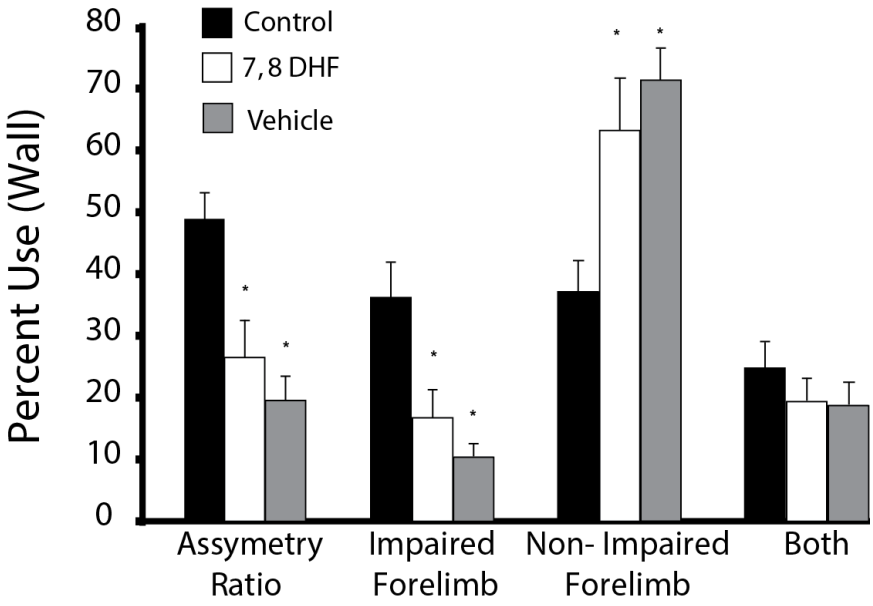

Figure 3.5 Cylinder test. Forelimb use asymmetries during cylinder wall touches. 7,8-DHF and Vehicle treated rats showed significantly reduced asymmetries compared to controls ($p < 0.05$). 7,8-DHF and Vehicle receiving rats use non-impaired limb significantly more than controls for weight bearing during wall explorations. No statistically significant differences were observed between 7,8-DHF and Vehicle receiving animals.

3.3.3 7,8-DHF treated rats showed greater forelimb motor map areas than vehicle treated animals

Total forelimb area expressed in mm^2 consisted of caudal and the rostral forelimb representations. Over all, healthy controls (N=7) had the largest forelimb area of 7.01 ± 0.865 mm^2. 7,8 DHF group (N= 5) had an average total forelimb representation of 2.48 ± 0.81 mm^2 and vehicle group (N=6) had 0.189 ± 1.4 mm^2 area representing the forelimb function (Figure 3.6). A univariate ANOVA comparison revealed a significant effect of

group ((*F*= 25.9, *P* = 0.000). Follow-up tests (LSD), showed that 7,8 DHF treated group had larger map area than the vehicle injected group ($p = 0.04$). Motor maps of the ipsilesional sensorimotor cortex displaying proximal and distal forelimb representations are shown in Figure 3.7.

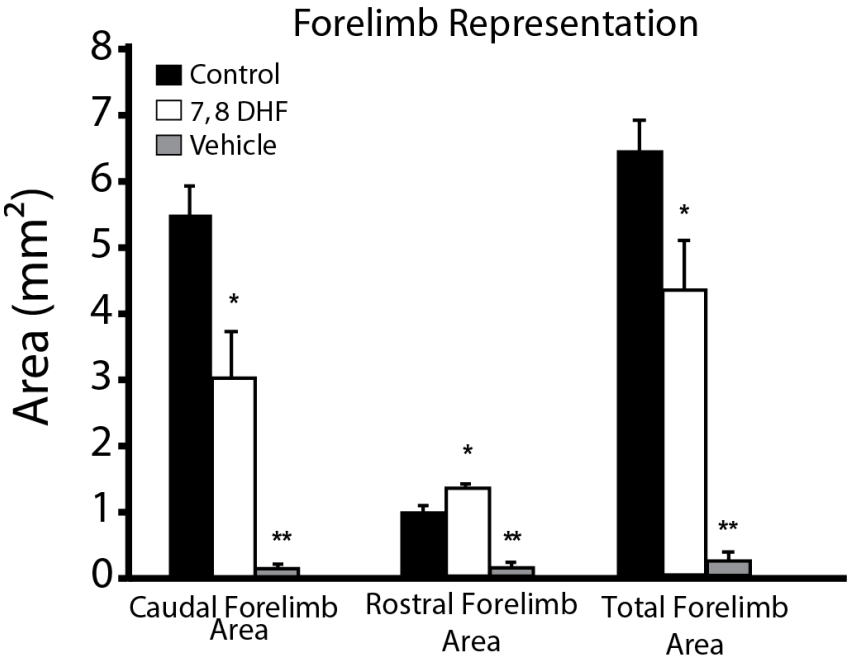

Figure 3.6 Forelimb area determined by ICMS. Area of caudal and rostral forelimb regions on the sensorimotor cortex in vehicle, 7,8-DHF and control rats. Forelimb motor maps are significantly reduced in vehicle receiving rats compared to 7,8-DHF receiving rats ($p < .05$).

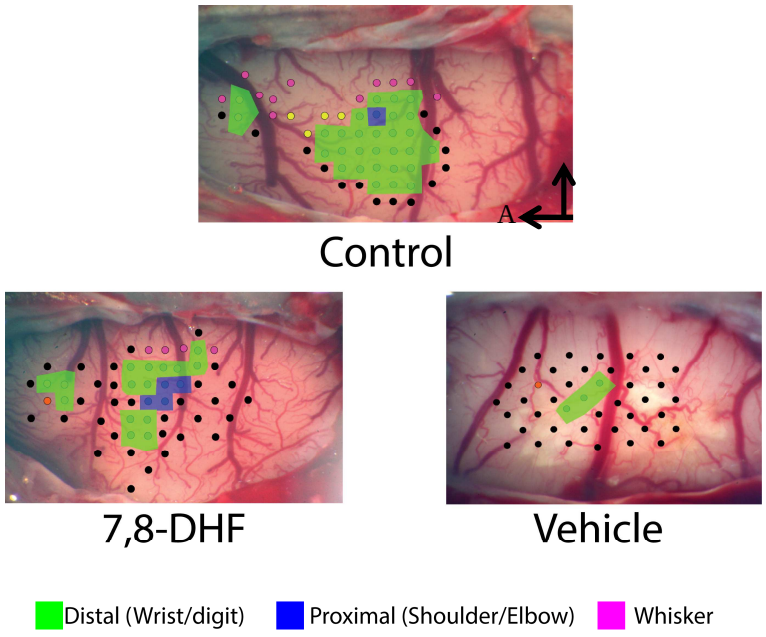

Figure 3.7 Total forelimb representation. Representative ipsi-lesion motor maps of caudal and rostral forelimb areas from a Control, Vehicle, and 7,8-DHF and Vehicle treated rats. Forelimb motor map representations are shown in green (wrist/digit) and blue (elbow/shoulder).

3.3.4 7,8 DHF treated animals had significantly smaller infarctions than vehicle treated animals

Lesions were verified in histological coronal sections, as described previously. Sections were stained with myelin and cresyl-violet stain. Infarct size was determined by measuring areas of non-infarcted tissue in both the damaged and undamaged hemispheres. Infarct size was calculated as a ratio of ipsi/contra volume (7,8-DHF=0.932 ± 0.0144; Veh=0.864 ± 0.025). A Student's t-test revealed a significant difference

between % tissue loss in 7,8- DHF group (DHF=8%) and the vehicle injected group (Veh=14%) $p<0.03$ (Figure 3.8).

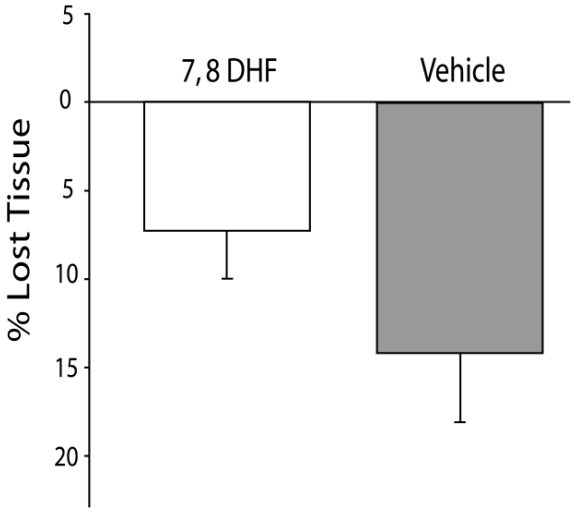

Figure 3.8 Volumetric analysis of the infarct size. 7,8-DHF treated rats had reduced tissue loss than vehicle treated rats.

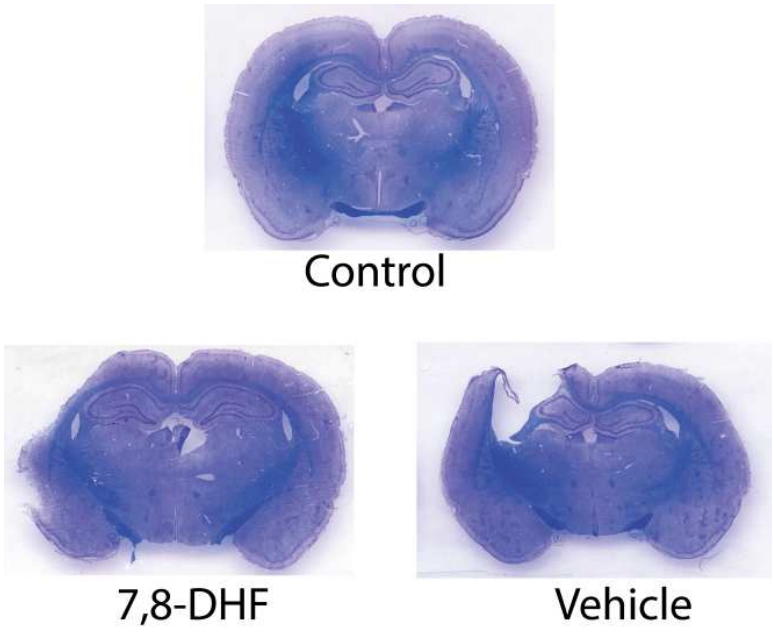

Figure 3.9 Lesion Representation. Representative **c**oronal sections of 7,8-DHF, Vehicle and Control rats stained with cresyl-violet and myelin.

3.3.5 Vasodilatory effects of 7,8-DHF

To investigate the impact of 7,8-DHF on arterial endothelial cells of the cortex, rats were systemically given 7,8 DHF or Vehicle two hours prior to stroke induction. Baseline blood flow measures, using a laser Doppler, were recorded for a duration 10 mins before ET-1 injection and recorded up to 90 mins post ET-1 application. There was >80% reduction in the cortical blood flow around MCA and its branches within 10 minutes of stroke induction. The perfusion units were normalized to baseline measures. No significant differences were observed in the mean blood flow measures between vehicle injected and 7,8 DHF rats after stroke suggesting that the effects of 7,8 DHF were not due to increased vasodilation post ET-1 treatment (Figure 3.10).

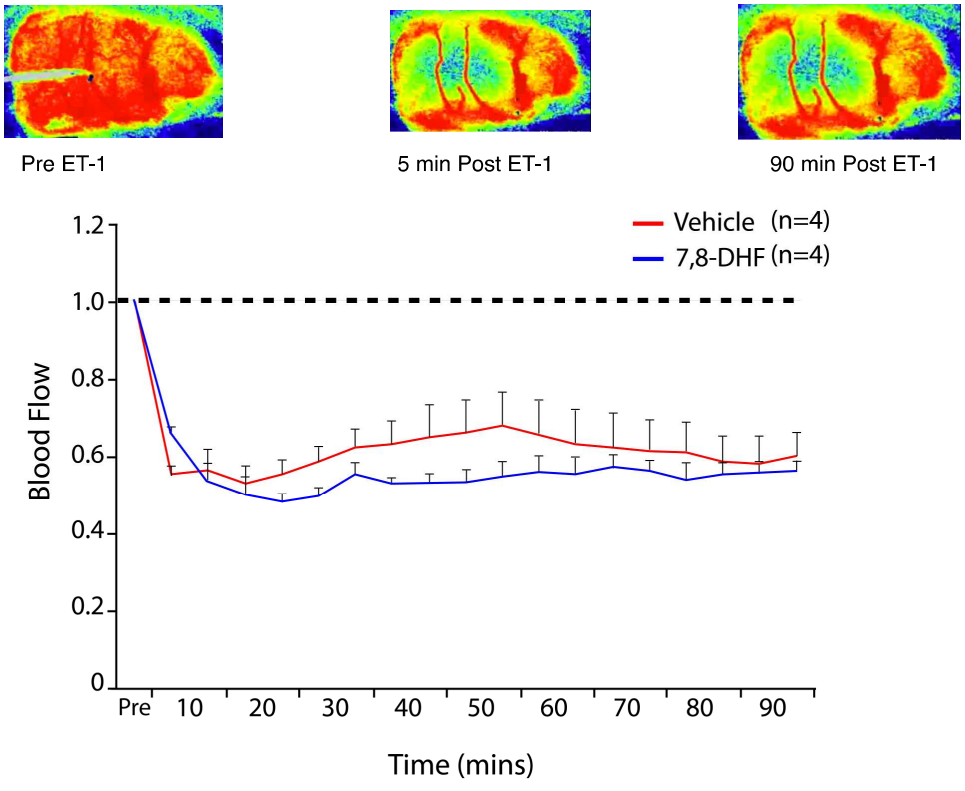

Figure 3.10 Blood flow measures post ET-1 using laser Doppler in 7,8-DHF and vehicle injected rats. Images of the cortical surface at

various time points showing reduction in the blood flow in the region of ET-1 injection.

3.3.6 7,8 DHF did not enhance p-TrkB expression in motor cortex

To determine the ability of systemic 7,8-DHF to activate p-TrkB, *in vivo*, in healthy rats, TrkB and p-TrkB (at Tyr816) levels in the motor cortex and hippocampus were examined (Figure 3.11). Western blot analysis did not reveal a significant increase in the levels of p-TrkB in cortical homogenates compared to vehicle-administered rats. TrkB levels in neither 7,8-DHF nor vehicle receiving rats were changed. Over all, there was a higher expression of p-TrkB in the hippocampus, but no significant group differences were detected (Figure 3.12A,B).

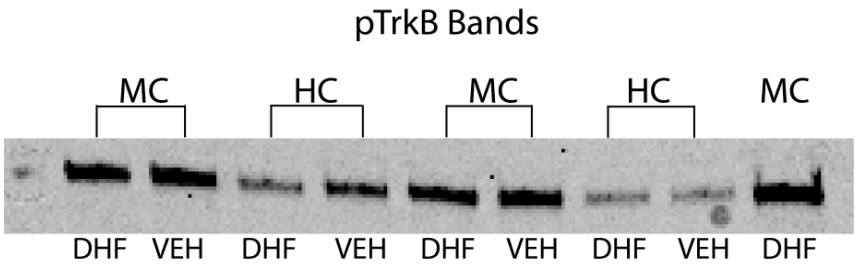

Figure 3.11 Representative bands generated after western blotting showing the density of pTrkB levels in 7,8-DHF and Vehicle treated rat brains in the motor cortex and hippocampus.

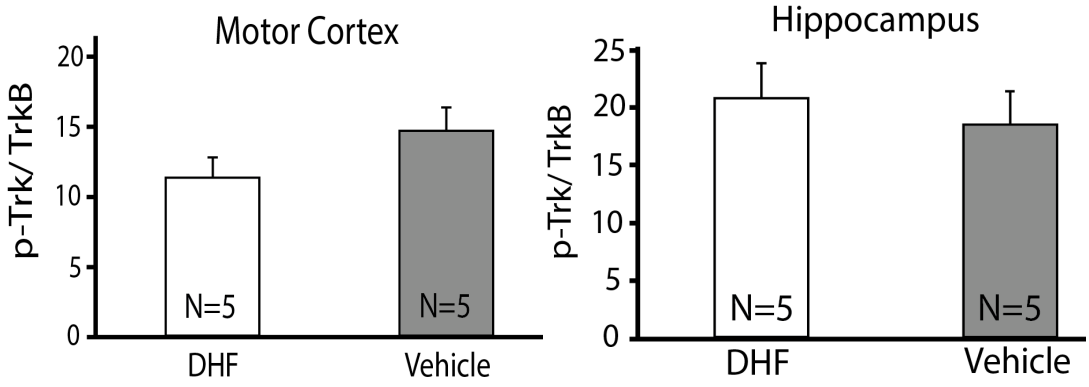

Figure 3.12 Western blot quantitative analysis. Protein levels quantified and reported as a ratio of pTrkB/TrkB in motor cortex and hippocampal homogenates. No enhanced expression of TrkB phosphorylation was detected in 7,8-DHF rats compared to vehicle treated rats.

3.4 Discussion

The aim of this study was to investigate the efficacy of 7,8 DHF and determine its impact on motor recovery post cortical ischemia. A comprehensive behavioral testing paradigm was used, which proved to be effective in measuring impairment levels and detecting spontaneous behavioral improvements after injury.

It was found that animals receiving 7,8 DHF prior to and after ischemia demonstrated milder impairments in their fine motor skills compared to vehicle treated animals. Data analysis revealed that in sunflower seed opening task, 7,8 DHF treated rats were significantly faster at consuming seeds than vehicle rats after stroke. During the vermicelli handling task, test measuring forepaw dysfunction, 7,8 DHF treated animals

did not differ from vehicle injected rats, nor were there any differences in forelimb asymmetries between the two groups in the cylinder task. Uluc and colleagues (2013) have shown that 7,8-DHF reduces white matter injury. Perhaps, it is due to his effect that the preservation of fine fractionated movements using digits was observed as opposed to improvement in gross motor performance. This pattern of functional recovery indicates that 7,8 DHF may be impacting the pyramidal neurons, which receive direct inputs from the sensorimotor cortex and are critical in driving upper extremity motor movements in the distal musculature requiring fine dexterity (Lemon, 1992). There is an enhanced expression of TrkB in dendrites of pyramidal neurons in layer V of neocortex in rats after ischemia (Narumiya, 1998). Therefore, these observation also suggests the importance of dendrites, as well as cell soma, for signal transduction of the TrkB receptors for its potential contribution to neurobehavioral outcome.

In agreement with the behavioral findings, detailed forelimb motor maps generated from these animals showed that 7, 8 DHF treated animals had larger cortical forelimb representation than those of the vehicle injected animals, indicating a substantial amount of functional sparing in the flavone receiving group. This is in part due to the smaller lesion size in the DHF animals. However, while the DHF animal's lesions were half the size of the vehicle animals, the motor maps were several times larger suggesting that the differences in motor map area cannot be explained by lesion size alone. The enlargement of motor maps can be attributed to axonal sprouting due to upregulation of TrkB downstream signaling cascade influencing growth promoting genes (Carmichael, 2005), hence facilitating the formation of new axonal projections.

It is hypothesized that an ischemic insult causes a functional and anatomical

disruption of intracortical circuitry within peri-infarct regions of cortex. The disruption is manifested as a loss of movement representations and causes motor impairments. In support of this idea, regions of peri-infarct motor cortexthat exhibit a loss of movement representations alsoexhibit a reduction in synapse number (Kleim, 2001). The loss of motor map is thought to be the result of neuronal dysfunction and a disruption of cortical circuitry, as there is a loss of synapses but not neurons within these regions (Kleim 2003). Recent evidence from studies in mice suggests that following stroke there is a decrease in spine density by 38% in peri-infarct cortex at 24 hours (Murphy et al., 2008). Previous experiments have demonstrated that *in vitro* 7,8-DHF rescues long-term synaptic plasticity in the hippocampus (Zeng et al. 2011). This finding may offer a plausible explanation for the better behavioral outcome and relatively bigger forelimb maps in the 7,8-DHF group due to the strengthening of synapses and/or minimizing loss of synapses as a result of insufficient availability of BDNF to activate TrkB receptors, after an ischemic event.

It is interesting to note that the proposed mechanism of 7,8-DHF functioning in the brain is via its binding to TrkB receptors, which then causes activation through phosphorylation of the protein (Jang et al. 2010). Surprisingly, administration of 7,8-DHF in healthy rats did not yield increased levels of TrkB phosphorylation in the sensorimotor cortex in comparison to vehicle receiving rats. It is noteworthy that the protein analysis was conducted in healthy and not ischemic animals where the differences may have been detected.

Vasodilatory mechanism of flavonoids

In the experiments conducted by Jang and colleagues (2010), they showed that by injecting 7,8-DHF (5mg/kg) in rats 2 h prior to MCAO induction reduces infarct volume by 50% and apoptosis at 48 h. Previous studies have highlighted the effects of flavonoids in modulating endothelial function and dysfunction extensively. Endothelin-1 is a potent vasoconstrictor released by endothelial cells. Endothelial dysfunction is associated with elevation of endothelin-1 and in this model ET-1 was used to induce ischemia. Endothelium and nitrous oxide (NO) dependent relaxation has been reported for several isolated flavonoids, especially the anthocyanin delphinidin (Andriambeloson et al., 1998) and flavone chrysin (Duarte et al., 2001)of capillary endothelium(Tiurenkov, Voronkov, Slientsans, Petrova, & Dorkina, 2010). Certain flavonoids derived from cocoa extracts have also shown to have potent effects on endothelium relaxation (Karim, McCormick, & Kappagoda, 2000). It has been reported that 4 carbonyl group in flavones is required for the vasodilatory activity (Duarte et al., 1993). Duarte and colleagues were able to show that the main vasodilatory mechanism of flavonoids seems to be the inhibition of protein kinase C (PKC). An inhibitory effect on cyclic nucleotide PDE and Ca^{2+} uptake is also known to contribute to the vasodilatory action of flavones. Based on these findings, it was hypothesized that 7,8-DHF, due to its structural similarities to above mentioned compounds may cause vasodilation of the vessels. Blood flow changes were measured in anesthetized rats, treated with 7,8-DHF or vehicle 2 h prior to MCAO induction. There were no significant differences between the two groups. These findings lead to the conclusion that neuroprotective effects of 7,8-DHF do not appear to be due to changes in

vasodilation. Presumably 7,8-DHF is facilitating synaptic strengthening due to increased levels of TrkB in dendrites after ischemia.

7,8-DHF acting through different signaling pathways

Considerable evidence has accumulated to suggest that cellular effects of flavonoids may be mediated by their interactions with specific proteins central to intracellular signaling cascades, such as mitogen activated protein kinase (MAP kinase) signaling pathway and the phosphoinositide 3-kinase (P13K/Akt) signaling cascade. Treatment with quercetin after spinal cord injury has been correlated with recovery of motor function in a rat model (Schultke, Griebel, & Juurlink, 2010). Due to structural similarity it is believed that 7,8 DHF administration is potentially inducing motor recovery after ischemia by similar mechanisms.

Neuroprotective effects of 7,8-DHF were evident through significantly reduced infarctions in 7,8 DHF treated animals compared to vehicle treated animals. For this compound to be considered neuroprotective, it is imperative that it effectively crosses the blood-brain barrier and provoke TrkB activation. Activation of the kinase receptor interferes with a large number of biochemical signaling pathways and, therefore, impacts physiological and pathological processes. One of the consequences of this activation is the stimulation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) signaling cascade. Active ERK and Akt regulate the expression of specific genes that contribute to cell survival in an event of an injury (Almeida et al., 2005). However, it can only be speculated that these events took place in 7,8-DHF treated animals based on previous findings, since mass spectrometry was not conducted on this brain tissue to verify the penetration of the compound. Also, western analysis of downstream signaling

molecules was not performed to measure levels of downstream growth promoting proteins induced by TrkB receptor.

It is important to note that 7,8-DHF is a poorly soluble compound and requires at least 30% $DMSO_4$ to dissolve. Unfortunately, toxicity of $DMSO_4$ as a solvent has been reported to be considerably high (Trivedi, 1990), consequently damaging a considerable number of neuronal population and repressing cell growth in the brain. Hence, this property of 7,8-DHF makes it less desirable of a compound to be administered after an ischemic injury.

The improvement in motor function, even though not as robust as was predicted to be, is presumably by protecting vulnerable neurons or enhancing existing neuronal function by synapse strengthening (Zeng et al, 2011). Although specific pathways through which 7,8-DHF results in mediating neural plasticity still require further investigation, the results of these studies suggest that administration of 7,8 DHF after stroke supports partial functional recovery.

Chapter 4

A novel TrkB agonist LM22A-4 enhances motor recovery and motor map plasticity following stroke

4.1 Introduction

Stroke remains the leading cause of adult disability in developed countries (AHA statistical update, 2012). Most survivors live with residual motor impairments that severely diminish independence and quality of life. After stroke, the only accepted treatment for these patients is motor rehabilitation. However, the amount and kind of rehabilitation required to induce clinically significant improvements in motor function is rarely given due to the constraints of our current health care system. In an effort to maximize recovery after stroke, another proposed method for improving rehabilitation is the combined use of drug therapy together with the physical therapy. Pharmacological intervention can alter plastic changes and the best documented evidence is with amphetamine and related noradrenergic agents (Feeney et al, 1993 & 1997), but these studies have produced some conflicting results. Therefore, developing adjuvant therapies that might augment the impact of motor rehabilitation, improve functional outcome and increase quality of life would be of great benefit.

Neurorehabilitation is used to promote relearning of original movements as well as learning compensatory movement patterns. Various studies in animals and humans have demonstrated reorganization of maps within motor cortex as a function of experience in both healthy and brain-injured animals/patients. Synaptic plasticity is the neural mechanism mediating motor learning and therefore motor relearning as evidenced by

restoration of movement representations within the spared cortical tissue accompanied by increase in synapse number translating into motor improvement after stroke (Wittenberg et al., 2003; Taub et al., 2003; MacDonald et al., 2007).Neurotrophic factors have been associated with physiological and anatomical plasticity to enhance motor recovery after stroke. Brain Derived Neurotrophic factor (BDNF) is one such neurotrophin and is known to modulate synaptic and morphological plasticity in the developing and mature nervous system (Lu et al., 2003; Reichardt et al., 2006). The most well characterized of these systems is the BDNF/TrkB receptor signaling pathway and have been implicated in neuronal survival, synaptic plasticity, and cortical reorganization after brain injury.

The beneficial role of BDNF in the brain makes it a viable therapeutic candidate. Unfortunately, BDNF itself is a poor candidate because of its short half life, low penetration through the blood brain barrier, and activating multiple receptor units, p75 and TrkB on the neuronal membrane (Chao & Hempstead, 1995). Activation of the p75 receptor can result in many undesired side effects such as pain (Zhang, Chi, & Nicol, 2008) and apoptosis (Barrett et al. 2000).

A recently discovered compound, LM22A-4 (N,N',N'-tris (2-hydroxyethyl)-1,3,5-benzene tricarboxamide), is a small molecule ligand designed to mimic the loop II domain of BDNF (Massa, et al., 2010) has been shown to selectively mimic the cell signaling actions of BDNF on the TrkB receptor, actively cross the blood brain barrier, and enhances functional recovery in animal models of several neurological disorders (Han, et al., 2012; Schmid, et al., 2012). LM22A-4's effects are qualitatively and quantitatively different than the native BDNF protein (Massa et al., 2010).

LM22A-4 compound interacts with and act through TrkB phosphorylation

To verify that the compound works through activation of TrkB, the discoverers were able to block them by inhibiting receptor activation. Hippocampal neuron cultures were treated with Trk inhibitor K252a, which demonstrated a reduction in neurotrophic activity of the test compound and BDNF. As an alternative approach, the effects of an antibody directed against the extracellular domain (ECD) of TrkB that is known to inhibit BDNF function (Balkowiec et al., 2000) were examined. This resulted in a reduction in cell survival and increased numbers of TUNEL-positive cells in the presence of LM22A-4 (Massa et al., 2010). Also, LM22A-4 specifically activated TrkB and this was verified by the addition of LM22A-4 to 3T3-TrkB and NGF cultures, which resulted in the activation of TrkB only. In another set of experiments LM22A-4 increased survival in 3T3-TrkB cells by 56%, while no significant increase in survival was detected in 3T3-TrkA, 3Te-trkC or 3T3-$p75^{NTR}$ cells.

LM22A-4 can ameliorate behavioral impairments after TBI, Stroke and Rett's syndrome

Previous studies have demonstrated beneficial effects of LM22A-4 in other neurological disorders and injuries. LM22A-4 treated rats subjected to parietal controlled cortical impact injury showed increased rotarod performance, indistinguishable from sham-operated controls, 2-3 weeks after injury. Thus suggesting that LM22A-4 is able to reverse deficits in motor task learning caused by TBI. Schmid and colleagues developed respiratory dysfunction in heterozygous *Mecp2* Het mice. LM22A-4 administration restored wild type breathing frequency in *Mecp2* Het mice, therefore providing a validation of TrkB as a therapeutic target in mouse models of RTT (Schmid et al., 2012).

This study was designed to investigate the influence of LM22A-4 when combined with rehabilitative training in the form of single pellet reaching in facilitating motor recovery after stroke in a rat model. LM22A-4 in conjunction with motor rehabilitation, begun three days after middle cerebral artery occlusion significantly improved forepaw skilled reaching accompanied by an expansion of the secondary motor areas in severely injured rats.

This study demonstrates that TrkB agonist treatment when paired with rehabilitation over an extended period of time is capable of improving functional recovery in severely injured rats. These results provide proof-of-concept evidence that activation of TrkB alone is a potential therapeutic approach for accelerating stroke recovery in patients who have suffered a stroke.

4.2 Experiment 1: Validating The Efficacy Of LM22A-4 For Activating The TrkB Receptor

In this experiment a dose of 5mg/kg i.p. LM22A-4 (N=5) and saline as Vehicle (N=5) was administered in healthy naïve Long Evans rats for seven consecutive days and in a separate experiment 25mg/kg i.p. of LM22A-4 (N=8) and Saline (N=7) was delivered in Long Evans rats from day 3-10 post stroke. Rats were sacrificed at 1 hour after the last dose of LM22A-4 or vehicle. Ipsilateral sensorimotor cortex was isolated, homogenized in cell lysis buffer, sonicated, and equalized as described in the methods. Protein extracts were subjected to Western blot analysis using standard techniques. Blots were probed with antibodies recognizing pTrkB-Y817 (rabbit mAb 1:30,000, Epitomics), TrkB (rabbit polyclonal antibody1:2500, Millipore), and HRP goat anti-rabbit (1:10,000, Pierce Biotechnology). Optical densities were measured using LiCor Odyssey Infrared

imaging system and Image J analysis. Ratios were calculated as pTrkB over full-length TrkB receptor and data was normalized to saline-treated stroked mice.

4.2.1 LM22A-4 penetrates the brain after 7 day administration and phosphorylates the target receptor

LM22A-4 did not increase p-TrkB levels in motor cortex in healthy rats

To determine the ability of systemic LM22A-4 to activate p-TrkB *in vivo* in healthy naïve Long Evans rats were examined trkB and p-TrkB (at Tyr816) levels in the motor cortex. 5mg/kg (i.p) of LM22A-4 (N=5) or saline as Vehicle (N=5) was administered for 7 consecutive days. Western blot results did not show a significant increase in the levels of pTrkB in animals receiving LM22A-4 compared to Vehicle treated group at this dosage. Mean pTrkB ratio of healthy LM22A-4 receiving rats was 0.132 ± 0.016 and Vehicle receiving animals 0.136 ± 0.02 (Fig 4.1A).

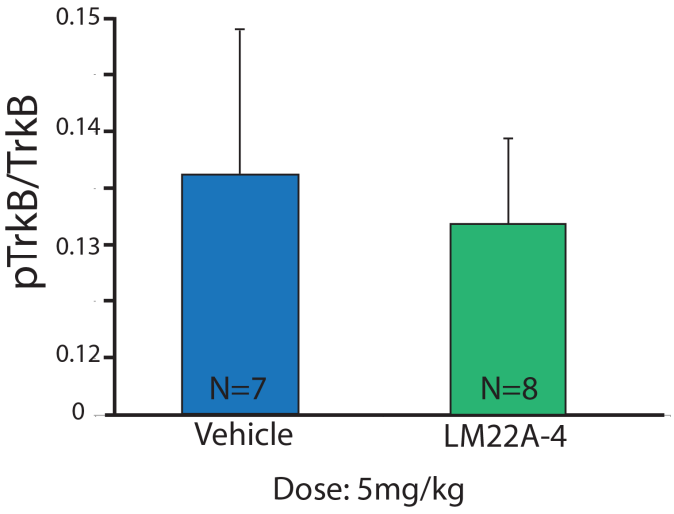

Figure 4.1 A) Western blot quantitative analysis (5mg/kg). pTrkB /TrkB ratio in the motor cortex after 7 days of LM22A-4 (5mg/kg) in healthy rats. A low dose of LM22A-4 (5mg/kg) does not enhance TrkB phosphorylation in healthy rats.

LM22A-4 enhances p-TrkB expression in motor cortex after stroke

In this experiment 25mg/kg of LM22A-4 (N=7) and Saline (N=7) was

administered in Long Evans rats for seven consecutive days from days 3 to 10 after stroke. Rats were then sacrificed at 1 hour after the last dose of LM22A-4 or vehicle. Ipsilateral cortex was isolated and processed for western blotting analysis.

LM22A-4 induced TrkB tyrosine phosphorylation in cortical neurons. There was a 27.7% ($p < 0.03$) increase in the levels of pTrkB in LM22A treated rats when normalized to total TrkB (Fig 4.1B).

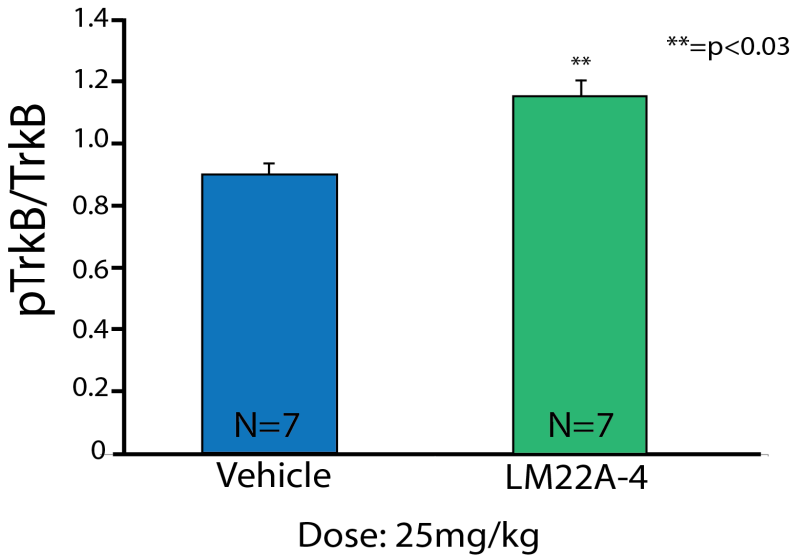

Figure 4.1 B) Western blot quantitative analysis (25mg/kg).

pTrkB /TrkB ratio in the motor cortex after 7 day administration (i.p.) of LM22A-4 (25mg/kg) and saline in stroke rats. LM22A provokes TrkB phosphorylation at a higher dose (25mg/kg) in stroke rats compared to vehicle treated stroke rats ($p < .03$).

LM22A-4 crosses the blood-brain barrier

The brain concentration of LM22A-4 was evaluated in rats treated daily with 25mg/kg i.p. from days 3-10 after stroke, then sacrificed at 1 hour after the last dose of

LM22A-4 or vehicle. Ipsilateral hemispheres were obtained and immediately frozen on dry ice. The tissue extraction and LC-MS/MS analysis were performed by Absorption Systems (Exton, PA). Brain homogenate from an untreated Long Evans rat was used for the standard curve.

A concentration of 53.98 ± 17.01 nM of LM22A-4 in brain tissue of drug administered rats was reported. The concentration in plasma was much higher 7291.82 ± 4209.93 nM. Atenolol, a drug that does not cross the blood-brain barrier, was administered with the last dose of LM22A-4 as a control to correct for contamination by blood present in the brain vascular space.

4.3 Experiment 2: Motor Rehabilitation and drug treatment

Using the results from experiment 1 showing that 25mg/kg of LM22-A was effective for increasing pTrkB, the efficacy of a novel TrkB agonist (LM22A-4) was investigated for reducing behavioral and neurophysiological impairments associated with cortical ischemia in rats. Animals were tested on a comprehensive battery of motor tasks before and after stroke and treated with LM22A (25mg/kg; i.p.) or saline along with receiving motor rehabilitation in the form of skilled reaching movement. Intracortical microstimulation (ICMS) was used to assess neurophysiological correlates of enhanced motor performance within the motor cortex. Histological analysis was performed to verify the extent and size of the lesion.

Figure. 4.2 Schematic drawing of the experimental design

Forty Long Evans hooded male rats were used in this experiment. Immediately following handling, all animals completed a comprehensive behavioral testing battery to assess forelimb motor function from gross motor movements to performing fine object manipulations. These tasks included: single pellet reaching, cylinder forepaw placement testing, vermicelli pasta handling and sunflower seed opening across approximately two weeks.

Single Pellet Reaching Task

The single pellet-retrieval test (McKenna & Whishaw, 1999; Miklyaeva & Whishaw, 1996; Peterson & Devine, 1963; Withers & Greenough, 1989) was performed in a Plexiglas chamber with a tall narrow window in the center of the front wall. Animals were placed on scheduled feeding (13-17g rat chow/one time per day) beginning two days before experiments began to motivate reaching behavior. All animals were given banana flavored food pellets (45 mg, Bioserve, Inc.) in their home cages for approximately two weeks before the start of reaching behavior to reduce neophobic responses to unknown food. Animals were shaped over several days on the tray reaching multiple pellets reaching task whereby a limb of preference (dominant limb for the task) was established (more than 15 reach attempts with same limb over a 20 minute period).

After a dominant limb was established, animals were trained on the single pellet

retrieval task to a proficient level (> 40% success/reach attempt) with this forelimb. Pre-operative training consisted of 15 min sessions in which, on each trial, rats could make up to 5 reach attempts for a banana pellet located in a shallow well (1 cm from the window). A trial ended when greater than 5 reach attempts (extension of the forepaw through the window) were made or the pellet was knocked from its well (failures), the pellet was dropped inside the chamber before consumption (drop), or when the pellet was successfully retrieved from its well and eaten. A short pause preceded each trial such that the animal was distracted from, or turned away from, the center window (by tapping on the side of the chamber, or by dropping a pellet in the chamber) while a new pellet was placed in the well.

This task is exceptionally sensitive to cortical damage given the level of difficulty required to perform successful reaches. The impairment level of each animal was calculated by (Post stroke accuracy/Baseline accuracy) X100.

Following collection of baseline data, animals were randomized to either 1. Control; 2. Stroke+LM; 3. Stroke+Veh. The assignment before inducing ischemia was done in a manner that counterbalanced reaching baseline performance such that all groups had equivalent mean baseline reaching accuracies. Animals then received a focal cortical infarct via topical application of endothelin 1 onto forelimb motor cortex and were allowed to recover for four days before they were again tested on the same battery of motor tests (approximately one week). Animals were placed into additional conditions for analysis based on the level of impairment shown on the single pellet reaching task. The impairment level of each animal was calculated by (Post stroke accuracy/Baseline accuracy) X100. Stroke animals performing at 20% or less of baseline levels were

considered severely impaired while animals performing above 25% of baseline levels were considered mildly impaired. This resulted in five groups of animals 1. Healthy Control (N=12); 2. Mild Stroke+LM (N=5); 3. Severe Stroke+LM (N=5); 4. Mild Stroke+Veh (N=5); 5. Severe Stroke+Vehicle (N=5). Animals were placed into additional conditions for analysis based on the level of impairment shown on the single pellet reaching task (Table 4.1).

Stroke animals performing at 20% or less of baseline levels were considered severely impaired while animals performing above25% of baseline levels were considered mildly impaired. This resulted in five groups of animals.

Table 4.1 Stratification of rats based on impairment levels after stroke

Condition	# Animals	% Baseline (Reach Accuracy)
Control	9	110.8%
Veh-Mild	5	59.8%
Veh-Severe	5	5.5%
LM-Mild	5	52.9%
LM-Severe	5	9.1%

LM22A-4 treatment (25mg/kg i.p.) and motor rehabilitation was initiated 3 days after stroke and continued for approximately seven weeks. The drug was not administered during week six, the administration was reinstated during week seven. Periodic testing on the motor test battery was performed to assess both impairment and recovery profiles. ICMS was then used to derive topographical maps of the forelimb representations in both the lesioned and intact hemispheres. 40□m tissue sections were then taken through the frontal cortex and stained for nissl substance and myelin to assess lesion volume (Figure 4.2).

4.3.1 Differential Effects of LM22A-4 on Severely vs Mildly impaired rats

A repeated measures ANOVA with CONDITION as a between subject factor and TIME as a within subjects factor revealed a significant CONDITION X TIME interaction on mean reaching accuracy [$F(180,810)=1.54$; $p<0.001$]. Subsequent multiple comparisons (Fisher's PLSD; $p<0.05$) revealed that all animals in all four stroke conditions exhibited significant reductions in reaching accuracies in comparison to Controls.

LM-Mild and Veh-Mild conditions showed transient impairments that lasted for the first four days of motor rehabilitation after which they performed at control levels. They showed a progressive increase during the Off-Drug phase that continued on after drug treatment was resumed to the point where they were performing at level significantly higher than controls on the last few days of training (Days: 36-45). A different pattern of results was observed in the severely impaired animals. Both the LM-Severe and Veh-Severe animals showed profound decreases in reaching accuracy after insult. However, animals in the LM-Severe condition showed a gradual increase in reaching accuracy and were performing at significantly higher levels than Veh-Severe animals by the third week of rehabilitation. During the Off-Drug phase they showed an initial decrement in performance that was followed by a progressive increase in reaching accuracy back to and on two days out performed the Controls (Figure 4.3).

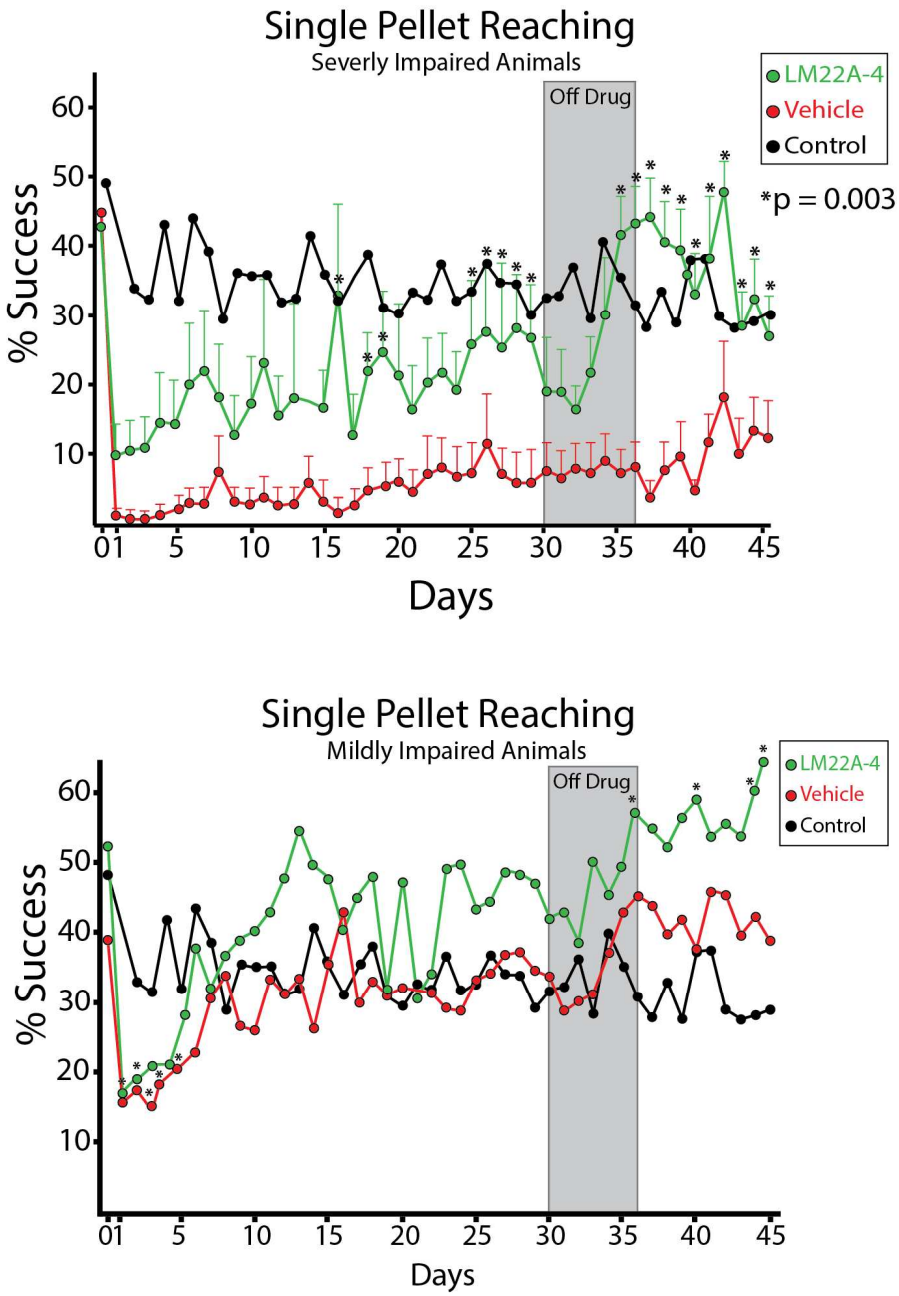

Figure 4.3 Single-pellet retrieval after stroke.

Single pellet reaching performance in severely and mildly impaired rats.

Depicts daily data of all five groups during rehabilitation training period.

4.3.2 Enhanced Reaching performance observed during 'off drug' phase post stroke

An interesting pattern was observed in the behavior during one week off drug phase after 5 weeks of continuous daily administration. Initially there was a drop in the performance on skilled reaching task, but after 3 days there was a sudden upward spike in their performance accuracy (Figure 4.3).

4.3.3 Sunflower Seed Opening Test

To measure fine digit manipulation, animals were tested every two weeks for the first six weeks of training on sunflower seed opening task. The average time to consume 5 seeds in intact animals is approximately 45 seconds. A repeated measures ANOVA with CONDITION as a between subject factor and TIME as a within subjects factor revealed a significant CONDITION X TIME interaction on mean reaching accuracy [$F(3,29)=3.14$; $p<0.01$]. In comparison to Baseline performance, all animals in the stroke conditions showed a significant increase in time to open the seeds two weeks post stroke. Initially, LM-severe group showed the most impairment by taking the longest time to complete the task (66.67 ± 11.39s) after two weeks post stroke. All animals showed significant improvement in the task (LM-Severe = 41 ± 2.16s) on week four and six, except for the Veh-Severe animals which failed to show any significant recovery (Figure 4.4).

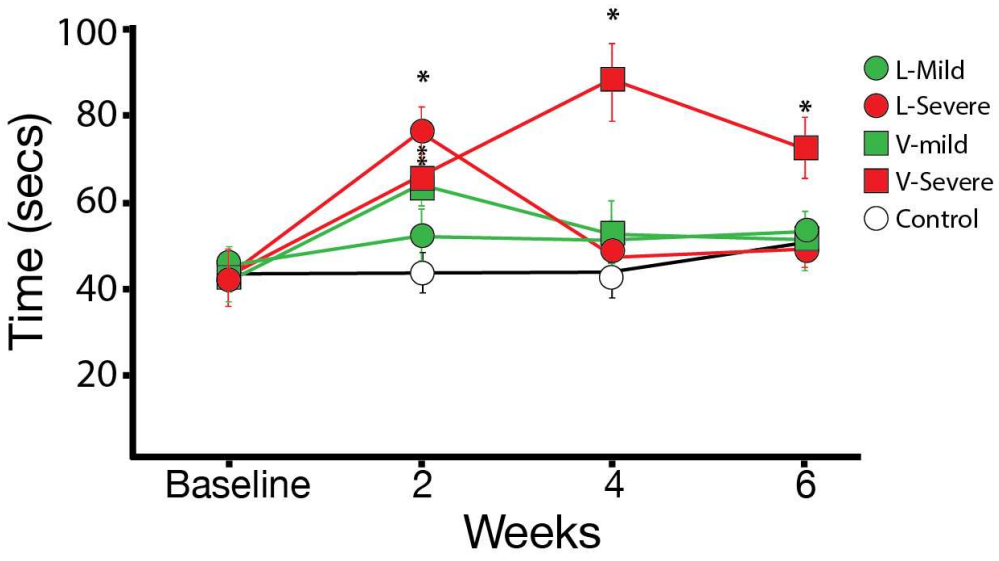

Figure 4.4 Time to consume 5 seeds on sunflower seed opening task

LM22A-4 rats start showing reduced times in consuming sunflower seeds after 4 weeks of drug administration compared to vehicle treated rats.

4.3.4 Pasta Handling behavior

Time to eat. A repeated measures ANOVA with CONDITION as a between subject factor and TIME as a within subjects factor revealed a significant CONDITION X TIME interaction on mean reaching accuracy [$F(3,29)=3.74$; $p<0.01$].All rats took significantly longer to consume the pasta pieces after stroke. There were no significant differences between LM receiving and vehicle treated animals post injury, except that rats with severe stroke took longer than mildly impaired rats to eat the pasta pieces at week 2, 4 and 6. Further, LM-Mild took significantly less amount of time than Vehicle receiving rats when compared to their baseline measures. Difference between pre and post injury times for Veh-Mild=-37.22%; LM-Mild=-9.8%; Veh-Sev=-97%; LM-Sev=-83.2%; Control=-49.67%.

4.3.5 LM22A-4 induces expansion of rostral forelimb area

Ipsilesional Maps: A one-way ANOVA with CONDITION as a between subject factor was conducted on cortical surface area occupied by the total forelimb area, CFA and RFA. Results showed a significant main effect of CONDITION on total forelimb area [$F(4,24)=10.79$; $p<0.001$], CFA [$F(4,24)=11.32$; $p<0.001$], and RFA [$F(4,24)=3.72$; $p<0.01$]. Subsequent multiple comparisons (Fisher's PLSD; $p<0.05$) revealed that all animals in the stroke conditions had significantly smaller forelimb motor maps in comparison to controls (LM-Mild=3.58 ± 0.5 mm^2, LM-Severe=3.05 ± 0.2mm^2, Veh-Mild= 3.7 ± 0.4 mm^2, Control=5.2 ± 0.3 mm^2). Further, Veh-Severe animals had significantly smaller maps than all other conditions (Veh-Severe=0.8 ± 0.2 mm^2). This same pattern of results was observed for measures of CFA with animals in the LM-Severe group (LM-Severe=1.9 ± 0.3 mm^2) having smaller total CFA areas than both the LM-Mild (2.8 ± 0.4mm^2) and Veh-Mild (3.2 ± 0.3 mm^2). Finally, the Veh-Severe animals again had significantly smaller mean CFA area than all of the conditions (0.7 ± mm^2). Interestingly, the RFA showed a different pattern of results. The RFA of LM-Severe animals was significantly larger than all other conditions including the controls (LM-Severe=1.15 ± 0.08 mm^2, LM-Mild=0.65 ± 0.1 mm^2, Veh-Mild= 0.5 ± 0.1 mm^2, Control=0.6 ± 0.13 mm^2) Conversely, the Veh-Severe animals had significantly smaller maps than all other conditions (Veh-Severe= 0.02 mm^2) (Figure 4.5).

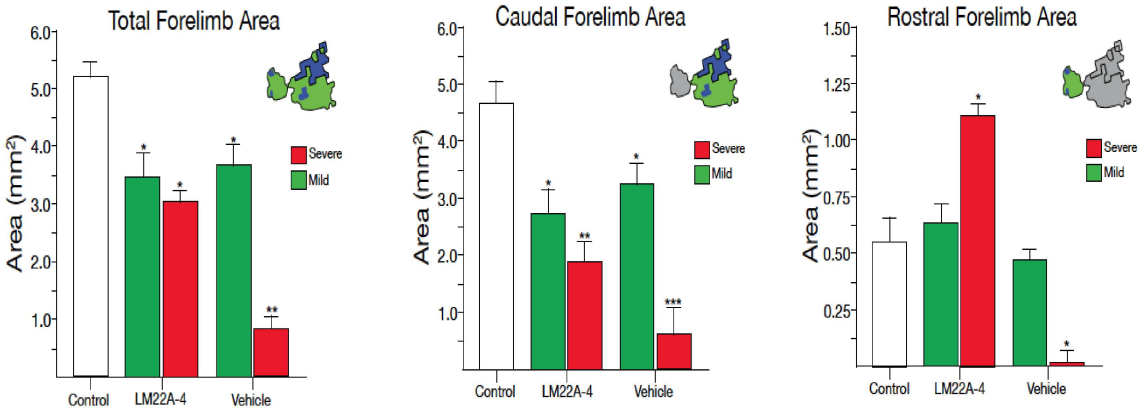

Figure 4.5 Forelimb motor map area (ipsilesional). Forelimb motor map area (ipsilesional) in control, LM22A and vehicle receiving rats. Caudal and rostral forelimb maps are severely reduced in Veh-severe rats, LM-severe rats have the largest rostral forelimb area compared to all other groups, including controls.

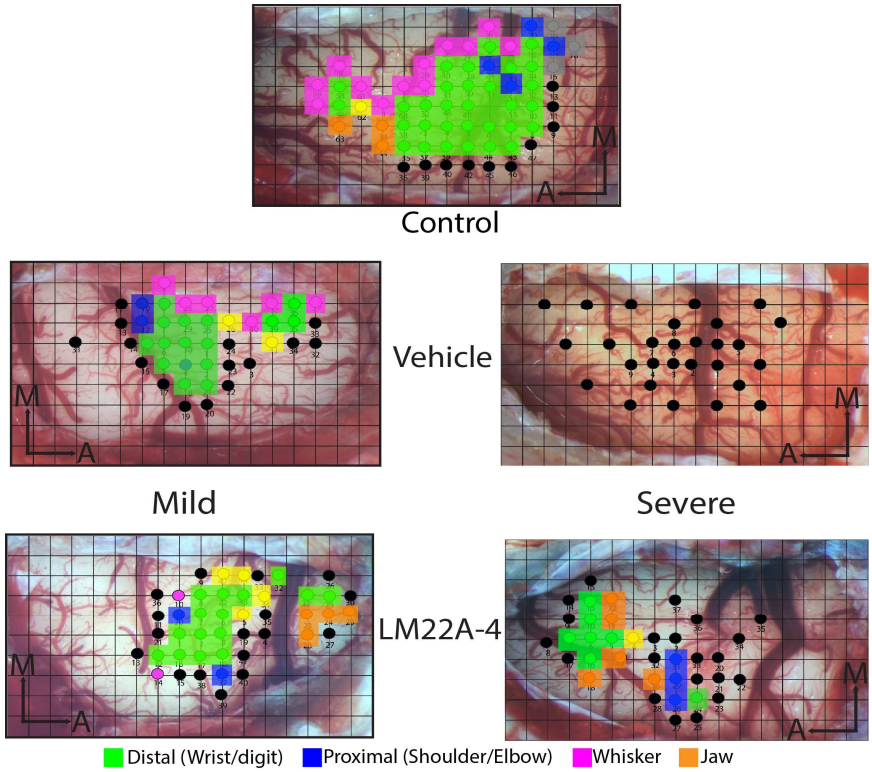

Figure 4.6 Representative forelimb motor maps (Ipsilesional)

Motor maps from a control, LM-Mild, LM-Severe, Veh-Mild and Veh-Severe rat showing caudal and rostral forelimb areas consisting of distal (wrist/digit) and proximal (elbow/shoulder) representations on the sensory motor cortex. Increased representation of wrist and digits is seen in rostral forelimb area in LM-Severe rats.

Contralesional Maps. Contralesion map analysis revealed Veh-mild having the largest (Veh-mild= 6.75 ± 0.99 mm^2) and LM-Severe having the smallest total forelimb area amongst all groups (LM-Severe= 3.4 ± 0.99 mm^2). Control rats had an average area of 3.7 ± 0.7 mm^2, Veh-severe= 4.5 ± 0.99 mm^2 and LM-mild = 4.87± 0.99 mm^2 (Fig 4.7). There were no significant differences between any of the groups.

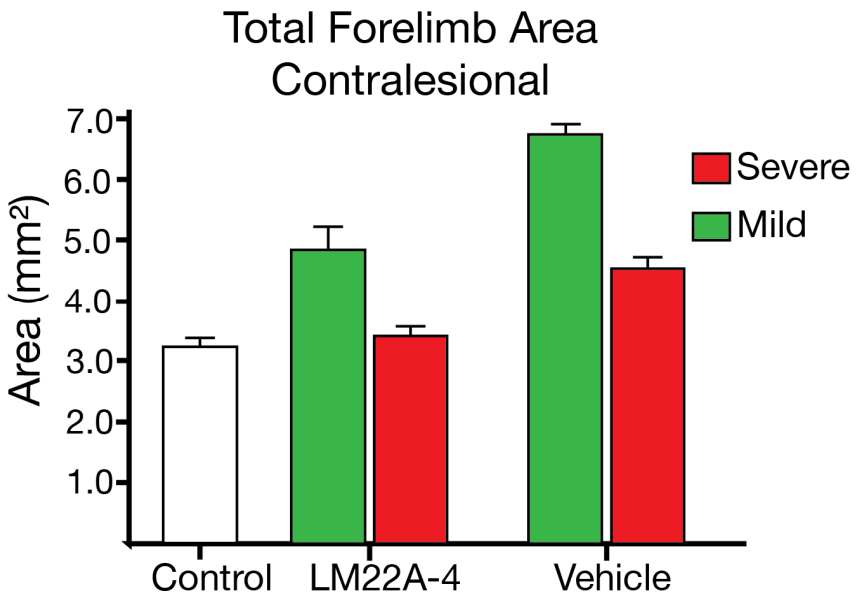

Figure 4.7 Total forelimb area in contralesional hemisphere. Forelimb motor map (contralesional) area in control, LM22A-4 and vehicle receiving animals.

4.3.6 Infarct Size

To assess potential neuroprotective effecs of LM22A on infarct size, the volume of the remaining non-infarcted tissue was measured. The ischemic infarct typically included lateral regions of cortex. (Fig 4.8) Represents the topographic location of the ischemic infarct in vehicle receiving severely impaired rat. Infarct size was determined by measuring areas of non-infarcted tissue in both the damaged and undamaged hemispheres by using Image J software. These area measures were then used to calculate values corresponding to the volume of remaining hemispheric, cortical and subcortical tissue. Data is represented as ratio of mean volumetric values of ipsilateral over contralateral hemisphere in Fig 4.10. Severely impaired vehicle receiving rats has significantly larger infarcts than all other stroke animals. Ratio measured in Veh-Sev=0.77 ± 0.07 and Veh-Mild=0.99 ±0.01) ($p < .05$). Lesion size of LM-Severe animals was not significantly different from LM-Mild rats (LM-Sev=0.90 ±0.03; LM-Mild=0.934 ± 0.02)

Analysis revealed that all four stroke groups had significantly reduced spared tissue than the controls, but no significant difference in infarct size was found between Veh-mild and LM-mild animals. Veh-severe animals had significantly reduced volume than all other conditions. Volumetric analysis shows that LM22A-4 may have contributed towards tissue sparing in severely stroke animals by inducing its

neuroprotective effects as both LM-mild and LM-severe animals have no significant differences in lesion size (Figure 4.9).

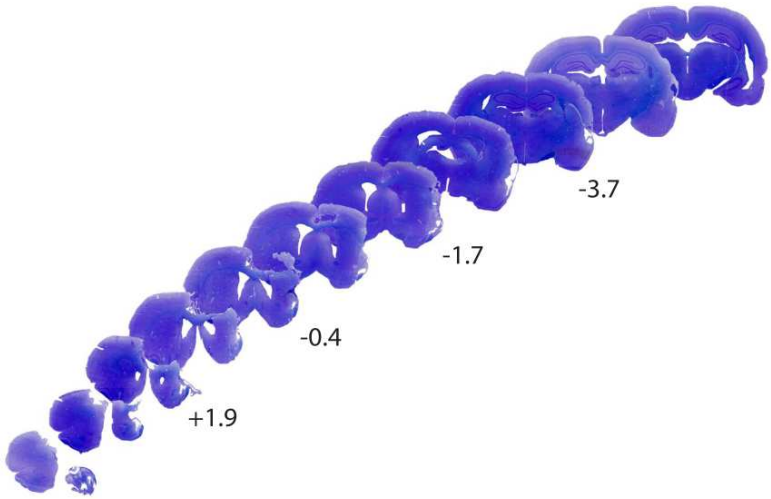

Figure 4.8 Coronal sections of representative ischemic infarct in Veh-severe rat.

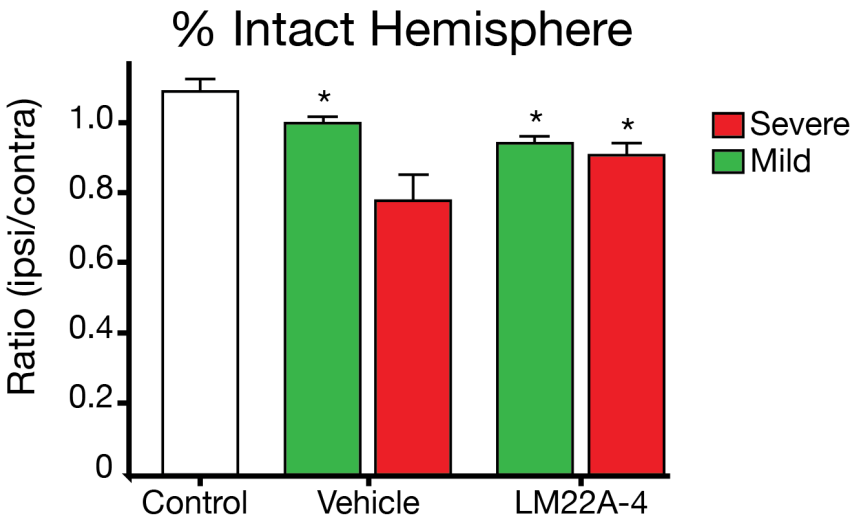

Figure 4.9 Volumetric analysis of lesion size. Data is represented as ratio of mean volumetric values of ipsilateral over contralateral hemisphere.

4.4 Discussion

The goal of this study was to test the viability of LM22A-4 on motor recovery in a clinically relevant model. Cortical ischemia was induced by injecting a vasoactive peptide Endothelin-1, which causes a temporary occlusion of the branches of the middle cerebral artery supplying blood to the sensorimotor cortex. The damage caused by this method results in a significant neurological injury similar to that seen in disabling of upper extremity in human stroke. Stroke animals demonstrated substantial motor deficits allowing behavioral testing to be performed to measure the impairment and recovery profiles across multiple time point. Further, the drug was administered systemically rather than directly into the brain to mimic how it could be used clinically. Finally, changes in motor map area were measured to allow for potential translation to clinical studies using transcranial magnetic stimulation to monitor motor cortex plasticity. The key findings of this study are that stimulation of TrkB signaling with a small molecule partial agonist can improve functional recovery and enhance motor map plasticity in animals with severe but not mild motor impairments.

LM22A-4 Is Not Neuroprotective

In this study, the rats were trained prior to stroke to evaluate functional recovery rather than learning, and LM22A-4 administration was initiated three days after stroke to minimize any potential neuroprotective effects. No significant differences in lesion size between vehicle and LM22A-4 treated animals were observed suggesting that the observed differences in motor performance and motor map topography were not due to the sparing of neural tissue.

It is speculated that the dose of 5mg/kg is insufficient to activate TrkB receptors

significantly in a healthy brain. Absorption of material delivered intraperitoneally is typically much slower than for intravenous injection, because the primary route of absorption is into the mesenteric vessels, which drain into the portal vein and pass through the liver (Lukas G., Greengard P., 1971). Therefore a higher dose may have greater chances of passing the blood brain barrier. Also, it has been reported that after an ischemic injury, there is an increased availability of TrkB receptors in the peri-infarct region (Gordon et al, 2009), a higher dose of 25mg/kg was effective in penetrating through the brain and enhance p-TrkB levels when administered after stroke.

LM22A-4 Differentially Affected Severe vs. Mildly Impaired Animals

LM22A-4 produced dramatic effects on motor recovery after an extended period of rehabilitation training in the form of single pellet reaching task. LM-Mild and Veh-Mild conditions showed transient impairments that lasted for the first four days of motor rehabilitation after which their scores returned to the normal range. LM-Mild continued showing a progressive increase in their scores and eventually out performed the controls and Veh-mild rats, which was sustained till the end of the training period.

Similarly, rats demonstrating severe impairments after injury when treated with LM22A started showing a progressive increase in their reaching performance compared to vehicle receiving severely impaired rats, which failed to show any improvements in single pellet reaching. However, most marked behavioral improvements in LM-severe group were seen from week 3 onwards (after 22 days of consecutive i.p. administration). These results indicate that LM22A-4 treatment is effective when administered for an extended period of time, where it's able to penetrate into the brain and augment

pro-recovery mechanisms presumably by inducing axonal sprouting, increased dendritic density and strengthen synapse formation. This pattern of recovery highlights a critical aspect of rehabilitation training that improvements can take weeks, which is a novel finding because most animal models show mild impairments that are restored quickly. This is encouraging when thinking of translating it to the clinic. It can be assumed that extensive behavioral training may have had a pro-neurogenic effect. However, since control and vehicle treated rats received identical training, the observed increase in neurogenesis occurred on top of this background. It has been established in previous studies that LM22A-4 increases neurogenesis in the absence of stroke and training (Han et al, 2012).

It is important to point out that while viewing the recorded sessions in slow motion, LM-Sev animals displayed several sensory errors while retrieving the pellet. Some rats would hold the pellet for a brief period of time before consuming it, which implies that these rats were relying on cutaneous sensory feedback for successful completion of the trial. Also, over all there were fewer trials per each session.

LM22A-4 Induces An Expansion Of RFA

In correlation with motor performance, neurophysiological evidence revealed that LM22A-4 mediates cortical plasticity by facilitating recovery through expansion of the secondary motor areas. In many animals, including non-human primates rostral and caudal forelimb motor cortex contain different proportions of digit, wrist/forearm, and proximal representational area (Nudo et al., 2003). The target lesion was the primary sensorymotor cortex. Motor maps of mildly impaired rats remained unchanged, and this

is because mild deficits may only require minor post-lesion learning (or compensatory motor strategies) for which the intact, adjacent neuronal network would be sufficient. LM-severe rats showed a relative preservation of reaching due to expansion of secondary motor. A plausible explanation offered for this is that after a cortical lesion, cortical areas distant from the site of injury are known to undergo physiological and anatomical changes. Motor learning has been found to be essential for the expansion of cortical motor representations (Plautz et al., 2000). However, the mechanisms through which reorganization of distant cortical areas is initiated are poorly understood.

This data underlines the complex interactions between various components of the cortical motor system. Compensatory changes that occur in regions distant from the site of an injury are not exclusively based on rules of cortical connectivity. The ability for motor maps to reorganize is related to strengthening of synaptic connections, which is experience dependent (Kleim et al., 2002). The reorganization of peri-lesion cortex after stroke, including reorganization of motor maps, has been linked with functional recovery (Nudo, 2003). Furthermore, direct demonstration of axonal sprouting in the peri-infarct cortex following focal stroke has been shown by Carmichael and colleagues (Carmichael., 2001).

Nudo and colleagues (2003) have shown that increase in the PMV hand representational area was directly proportional to the relative size of the primary motor cortex infarct. It appears that reorganization of the secondary cortical areas is a general feature of injury-induced plasticity. Another proposed theory is that remote reorganization is directly related to the reciprocal connectivity of the various motor areas. The greater the damage to reciprocal intracortical pathways, the greater the plasticity in

the secondary intact area (Nudo, 2006). Evidence for the contribution of premotor cortex to recovery has come from both human (Fridman et al. 2002; Miyai et al. 1999) and animal (Castro-Alamancos and Borrel 1995; Liu and Rouiller 1999) studies. Results from a transcranial magnetic stimulation study suggest that premotor cortex contributes to functional motor recovery in human stroke patients (Fridman et al., 2002). Miyai et al. (1999) reported that following middle cerebral artery occlusion, recovery in human stroke survivors was improved in those with intact premotor cortex compared with those that had premotor cortex damage. Hence it's feasible to assume that in LM22A-4 treated severe animals, non-primary motor areas contribute to functional recovery following injury in primary motor cortex.

This study provides an understanding of modulatory effect of combined behavioral and pharmacological interventions on neurophysiologic reorganization. The plastic changes observed here have been magnified in RFA with appropriate physiotherapeutic and/or pharmacotherapeuric intervention. Further study of sprouting patterns and synaptic structure and function in stroke models that can be identified and quantified (Carmichael et al., 2010; Carmichael et al., 2011) will be needed to conclusively determine if LM22A-4 has an effect on post-stroke plasticity.

Stopping Drug Treatment May Enhance Motor Improvement

The initial decrement and then increase in performance of LM-severe animals during "Off Drug" phase is intriguing. Multiple studies have indicated that for optimal BDNF/TrkB signaling events, the physiological range must be maintained (Lu and Gottschalk, 2000; King 2001). It is possible that daily administration of the drug prior to

suspension may have saturated the available TrkB receptors with functional catalytic domain, which led to aninitial decline in their motor skills. An enhancement in performance during “off drug” week suggests that continued administration of the drug could set up an ongoing increment in synaptic function due to the elevation of transcription factors induced by neurotrophin agonist binding that might last days or weeks.

The subsequent improvement in their reaching abilities suggests the possibility TrkB receptor signaling cascade in setting up an ongoing increment in synaptic function off drug that might last for days or weeks. Hence, intermittent treatment of the drug may be considered when translating into a clinical setting.

Potential Mechanism

Two types of cellular events have been implicated in functional recovery after stroke, neurogenesis and neuronal plasticity. BDNF may promote both by binding its two receptors, TrkB and p75NTR. These expereiments tested whether LM22A-4, as a small molecule ligand selective for the TrkB receptor, would be able to induce these effects. Neurogenesis occurs in the weeks and months after stroke and may augment damaged neuronal networks (Ohab and Carmichael, 2006; Lichtenwalner et al, 2006; Jiang et al, 2007). BDNF may increase neurogenesis by increasing survival of new neurons (Bath & Lee, 2010; Goldman et al, 2001; Chen et al, 2008) perhaps by strengthening new synapses. Plasticity likely contributes to functional recovery via rewiring of neuronal circuits, as reviewed recently (Carmichael, 2010; Benowitz. 2010; Murphy & Corbett, 2009). BDNF and TrkB are critical for activity-dependent plasticity and LTP (Mattson et

al, 2004; Cunha et al, 2010). A selective TrkB ligand such as LM22A-4, which does not activate p75NTR, may be more beneficial after stroke than BDNF.

It is proposed that improvements on skilled reaching and sunflower seed opening tasks may be mediated at least in part via an effect on the synaptogenesis of layer V pyramidal neurons of sensorimotor cortex, which coordinate forepaw reaching and fine digit manipulation and are sensitive to the neurotrophic effects of BDNF. BDNF is normally delivered to these neurons via inputs from primary motor cortex (Hiebert GW, 2002), which is injured in this stroke model and pharmacological TrkB activation may compensate for this loss. Increases in TrkB phosphorylation in cortical homogenates after 7 days of LM22A-4 treatment are consistent with this hypothesis. This suggests that LM22A-4 will facilitate recovery from stroke, and may improve motor function in patients after potentially disabling strokes.

It is clear from both preclinical and clinical studies that post-injury training is an important element in promoting recovery. The quality of the post-injury experience is crucial to the rate and extent of recovery. LM22A-4 specifically binds and activates the BDNF receptor TrkB, and results from this study indicate that this alone is sufficient to promote recovery. These findings show that LM22A-4 when paired with behavioral experience appears to facilitate recovery in an additive or interactive way. Neuromodulation of plasticity is helpful to enhance plasticity, creating a permissive state for learning. LM22A-4 may enhance neural signals to maximize sensorimotor integration. Despite these promising findings many factors related to the optimal design of clinical trials pairing LM22A-4 and behavioral experience, such as timing of treatment relative to injury onset, and the timing, quality and quantity of the behavioral experience

have yet to be established. This particular study design in an animal model outlines the importance of active ingredients such as repetitive and progressive training paradigms that will yield better functional motor outcome after stroke.

Chapter 5

Middle cerebral artery occlusion induces limb motor deficits and reduces forelimb motor maps, but does not affect cranial motor function or oral motor maps

5.1 Introduction

Among stroke subtypes, ischemic etiology is the most frequent cause of stroke, survivors of acute stroke often experience oral motor impairments, such as dysphagia, dysarthria and apnea that significantly reduces the quality of life in stroke patients (Martin & Corlew, 1990; Trapl, Eckhardt, Bosak, & Brainin, 2004). Despite a wealth of clinical and preclinical research investigating novel treatments to enhance recovery of upper and lower extremity impairments, the treatment of dysphagia has received comparatively little attention. There are no currently no animal models of post stroke dysphagia. The current study attempted to create an animal model of post stroke oral motor impairment.

Respiratory disorders and swallowing dysfunction after stroke

Dysphagia is a symptom of difficulty in swallowing. Normal swallowing involves a complex sequence of carefully timed muscular contractions that transport food from the mouth to the stomach whilst ensuring protection of the airway. The central regulation of swallowing depends on swallowing centres in the brainstem, which receive sensory input from pharynx and oesophagus and, together with local peristaltic mechanisms, control much of the swallowing sequence (Miller, 1982; Jean, 1990). However, the initiation of swallowing is a voluntary action that requires the integrity of motor areas of the cerebral

cortex. If these higher centers, or their connections to the brainstem, are damaged, then patients have severe difficulty in starting a swallow without choking (dysphagia) (Horner, 1988; Alberts, 1992). Dysphagia is often accompanied with dysarthria, which is a motor speech disorder resulting from neurological injury of the motor component of the motor-speech system (Joseph D, 2005), which can eventually lead to abnormal patterns. Apnea is referred to suspension of external breathing. Under normal breathing conditions gas exchange primarily controls the rate of respiration. During apnea there is no movement of the muscles of respiration and the volume of the lungs initially remains unchanged. Depending on the patency of the airways there may or may not be a flow of gas between the lungs and the environment (Cohen, 1959).

Second to hemiparesis, these impairments are the most frequent neurological deficits in patients with first-ever acute ischemic stroke (Lubart et al., 2005). The incidence of dysphagia after stroke approximates 55% in the acute stage (Guyomardet al., 2009; Martino et al., 2005), while the incidence of dysarthria ranges between 25% (Lubart et al., 2005) and 42% (Lawrence et al., 2001). Many studies have reported the co-occurrence of these impairments after stroke. Lapointe and McFarland (2004) documented that 79% of their acute stroke patients with dysphagia had concomitant communication impairments, such as dysarthria, aphasia, and respiratory impairments. Trapl et al. (2004) reported that 10% of their acute stroke patients had both dysarthria and aphasia. Currently, there are no known clinical predictors of these co-occurring impairments after acute stroke.

These impairments have been associated with significant reductions in patient quality of life, social interactions and mental well-being. Dysphagia can lead to malnutrition

(Crary et al., 2013), dehydration (Crary et al., 2013), aspiration pneumonia (Martino et al., 2005) and death (Altman,Yu, & Schaefer, 2010). Given these potentially detrimental outcomes, it is important to identify precursors of dysphagia and respiratory dysfunction after stroke and understand the underlying neural mechanisms leading to malfunctioning of these systems. These efforts will aid in developing measures to assess clinical practice behaviors and reduce oral motor complications in stroke patients.

Cortical reorganization of swallowing musculature after stroke

In an effort to develop neurobiologically informed therapies that can enhance oral motor function in clinical population affected by stroke, it's essential to identify the neuroanatomical substrates that facilitate the coordination of various muscles associated with these functions. Considerable evidence has accumulated to establish that lateral precentral cortex contains the motor representations for the tongue and face (Sessle et al., 1997; Murray et al, 1992). It is believed that cortical bulbar pathways receives inputs from cortical motor regions, including supplementary motor, primary motor, and the subcortical motor connections, which are more commonly involved with muscle coordination during swallowing than posterior cortical regions (Daniels, 1999). Given that bilateral sensorimotor systems facilitate normal swallowing behaviors (Conklin et al, 1997), consequently allowing great potential for recovery after damage: the majority of stroke patients recover within weeks of the insult (Barer, 1989). Interestingly though, mapping these projections have demonstrated that various swallowing muscles are arranged somatotopically, with the oral muscles, laterally and that in the majority of individuals, the projection from one hemisphere tends to be larger than the other. This reveals that there is an asymmetric representation for swallowing between the two

hemispheres, independent of handedness (Hamdy, 1998). This fact about the organization of cortical control of swallowing in humans highlights aspects of its reorganization, which are important for compensation and recovery after damage. Hence, swallowing could turn out to be an excellent model for studying central nervous plasticity.

Hamdy and colleagues have shown that damage to the hemisphere that has the greater swallowing output appears to predispose that individual to swallowing problems, while damage to the hemisphere with the smaller swallowing output will not affect swallowing (Hamdy, 1997). However, when there is dysphagia because there is additional substrate for swallowing in the undamaged hemisphere, the capacity for compensatory reorganization in the contralateral motor cortex can be increased, leading to a greater likelihood of recovery. Thus it would be desirable to devise techniques that might be useful in speeding up the process of recovery.

Breathing control centers in the brain

Ventilation is normally controlled by the autonomic nervous system with only limited voluntary override. The pattern of motor stimuli during breathing can be divided into inspiratory and expiratory phases. Inspiration shows a sudden, ramped increase in motor discharge to the inspiratory muscles (including pharyngeal dilator muscles). Before the end of inspiration, there is a decline in motor discharge. Exhalation is usually silent, except at high minute ventilation rates (Thibodeau et al, 2009). The mechanism of generation of the ventilatory pattern is not completely understood, but involves the integration of neural signals by respiratory control centers in the medulla and pons. Ventilation is achieved through combinations of tidal volume and breathing frequency and is an integrated homeostatic response designed to help maintain cellular metabolism

and acid base balance in the face of environment and metabolic changes in CO_2 and O_2 levels (Raven et al., 2007).

Swallowing and aspiration pneumonia are highly coordinated and integrated systems. Cranial nerves that control the muscles involved in planning and programming aspect of the motor-speech system include trigeminal nerve's motor branch, the facial nerve, the glossopharyngeal nerve, the vagus nerve, and the hypoglossal nerve. Dysphagia, dysarthria and malfunctioning in normal breathing control are common sequelae of both cortical and subcortical stroke. Individuals suffering from dysarthria also experience challenges in various speech sub systems and diaphragm dysfunction is one of them (MacKenzie, 2011). Some preliminary evidence exists that cortical pathways (voluntary) contribute to the control of breathing during states of respiratory challenge/stress (Davenport, 2009).

Despite a number of animal models for examining the neural substrates of general motor symptoms of stroke, there is a paucity of models for investigating neural mechanisms underlying cranial motor impairments. This study was designed to establish a rodent model of oral motor and respiratory dysfunction after stroke.

Specifically for this purpose a comprehensive battery of motor tasks was employed that dissociates cranial motor and upper extremity impairments after unilateral cortical ischemia. Further, intracortical microstimulation (ICMS) technique was used to generate detailed motor maps of cranial motor and upper extremity movement representations within motor cortex. The underlying hypothesis was that middle cerebral artery occlusion disrupts corticospinal tract and corticobulbar projections from the motor cortex. ICMS provides a method for measuring the integrity of corticospinal (upper

extremity) and corticobulbar (cranial motor) circuits after middle cerebral artery occlusion.

5.2 Methods

Adult male Long Evans rats were used in this study. Nine were healthy controls and five animals received a unilateral middle cerebral artery occlusion (MCAO). All rats were trained on a variety of behavioral tasks used to determine baseline measures for forelimb and oral motor tasks. Forelimb tasks included the single pellet reaching task (Whishaw et al., 1992), cylinder paw placement task (Schallert et al, 2000), sunflower seed opening task (Kleim et al., 2007), and the pasta handling task (K. A. Tennant et al., 2010). Subjects were trained for single pellet reaching for twenty-one days, and the remaining motor tasks for three days each before induction of ischemia (Figure 5.1).

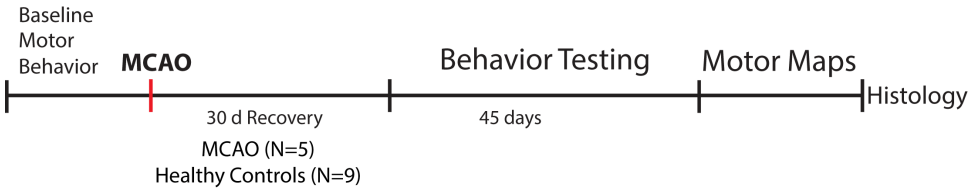

Figure 5.1 Schematic drawing of the experimental design

Forelimb Motor Testing

Single pellet reaching task. The single pellet-retrieval test (McKenna & Whishaw, 1999; Miklyaeva & Whishaw, 1996; Peterson & Devine, 1963; Withers & Greenough, 1989) was performed in a Plexiglas chamber with a tall narrow window in the center of the front wall. Animals were placed on scheduled feeding (13-17g rat chow/one time per

day) beginning two days before experiments began to motivate reaching behavior. All animals were given banana flavored food pellets (45 mg, Bioserve, Inc.) in their home cages for approximately two weeks before the start of reaching behavior to reduce neophobic responses to unknown food. Animals were shaped over several days on the tray reaching multiple pellets reaching task whereby a limb of preference (dominant limb for the task) was established (more than 15 reach attempts with same limb over a 20 minute period).

After a dominant limb was established, animals were trained on the single pellet retrieval task to a proficient level (> 40% success/reach attempt) with this forelimb. Pre-operative training consisted of 15 min sessions in which, on each trial, rats could make up to 5 reach attempts for a banana pellet located in a shallow well (1 cm from the window). A trial ended when greater than 5 reach attempts (extension of the forepaw through the window) were made or the pellet was knocked from its well (failures), the pellet was dropped inside the chamber before consumption (drop), or when the pellet was successfully retrieved from its well and eaten. A short pause preceded each trial such that the animal was distracted from, or turned away from the center window (by tapping on the side of the chamber, or by dropping a pellet in the chamber) while a new pellet was placed in the well to begin the nest trial.

Sunflower seed opening test. Rats are inherently adept at opening shelled seeds to obtain food, and sunflower seed opening is an effective measure of bilateral object manipulation (Whishaw, et al., 1998) as well as a good detector of motor impairments after stroke (Gonzalez & Kolb, 2003). Each rat was placed in a rectangular Plexiglas chamber and given five sunflower seeds. Rats start by manipulating the seed into a

preferred position before shelling. They then chew away a corner of the seed in order to facilitate splitting it longitudinally and finally the seed is split open into two or more pieces. In this task the total amount of time it took the animal to manipulate, open and consume all five seeds as well as the number of pieces of shell the animal had to break to retrieve the seed was measured.

Vermicelli handling trials. A test consisted of 4 trials with 7 cm long pasta pieces given one at a time per each trial. The experimental set up was similar to the one described in (Allred, et al., 2008). Data were collected with the rat facing the experimenter such that the digits and joints of the metacarpals and phalanges of both forepaws could be seen. The rats exhibited typical holding patterns, as previously described by (Whishaw & Coles, 1996). Time to eat was recorded, beginning when the pasta piece was grasped and placed in the mouth and ending when the piece was released by the paws and disappeared into the mouth. Total number of paw adjustments made while consuming the pasta for each trial were also recorded.

Forelimb use asymmetry. The Schallert cylinder test was used to examine asymmetries in forelimb use for postural support during explorative activity by placing each rat in a transparent cylinder 20cm in diameter and 30 cm high for 3 min (Schallert, et al., 2000). This test encourages upright support against the cylinder wall, which sensitively reveals forelimb asymmetries. Rats were videotaped in the cylinder and then, during slow-motion video play back, instances of the sole use of the ipsilateral (to the lesion) or contralateral forelimb or the simultaneous bilateral use of both forelimbs for upright support was recorded.

Behavior was quantified by determining the occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use observations on the wall; the occasions when the impaired forelimb (contralateral to the blood injection site) was used as a percentage of total number of limb use observations on the wall; and the occasions when both forelimbs were used simultaneously (or nearly simultaneously during lateral side-stepping movements) as a percentage of total number of limb use observations on the wall . The ipsilateral asymmetry score was computed using the formula: % (ipsilateral forelimb support+1/2 bilateral forelimb support)/(ipsilateral+contralateral+bilateral forelimb support). Forelimb placement during rearing, wall exploration and landings were recorded to determine forelimb asymmetry.

Oral Motor Testing

Plethysmography. Respiratory behavior in unanesthesized rats was characterized by utilizing a plethysmography chamber. In Plethysmography rat's functional lung capacity is measured by exposing them to normal air to obtain a baseline followed by intermittent periods of hypercapnia. Ventilatory parameters measured in this task are breathing frequency, tidal volume and minute ventilation. This chamber utilizes physical principles established by the Boyle-Mariotte law to determine volume and pressure changes for data collection. Theoretical basis for this method may be presented by considering the conditions influencing air temperature and vapor pressure within a closed chamber, the temperature, and vapor pressure of the air contained within the lungs of an animal within the chamber. Drorbaugh and Fenn equation is used to calculate respiratory volumes including minute ventilation (ml/min/100 g) and tidal volume (ml/100 g). Overall breathing frequency (breaths/min), the duration of inspiration and expiration, and

peak airflow rates were calculated from the airflow traces. During the experiments, pressurized gas mixtures flowed through the chamber at a rate of 2 L/min to enable control of inspired gases (DRORBAUGH & FENN, 1955). Baseline recordings lasted 1–1.5 h and were made while the chamber was flushed with 21% O2 (balance N2) (i.e. eucapnic normoxia). Rats were then exposed to a 10 min period of hypercapnic gas (7% CO2, 21% O2, balance N2). Rectal temperature was measured immediately before rats were placed in the chamber, and again immediately after the hypercapnic exposure.

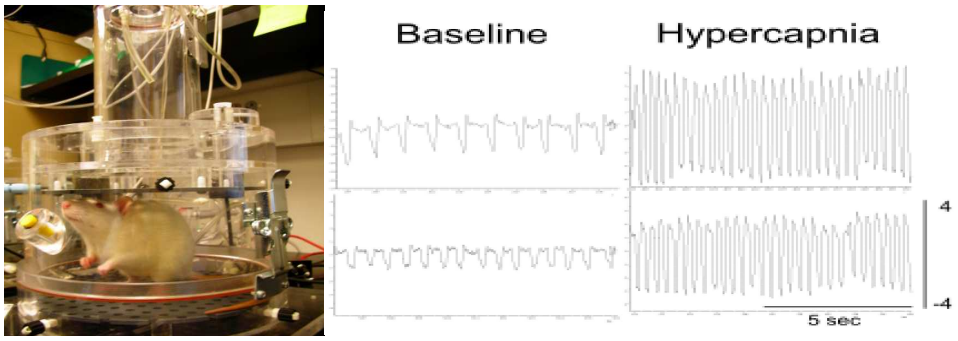

Figure 5.2A Plethysmography apparatus; 5.1B Examples of respiratory behavior recorded with barometric plethysmography in awake rats

Licking Task. Lick apparatus is used in rats to quantify behavior expressed by the tongue by measuring lick frequency (breaths per second), force (g), and rhythm (cycles per second). This enclosure contains a lick disk (18mm in diameter) attached to the shaft of a force transducer with a computer controlled peristaltic pump that delivers water to the center of the lick disk through a 0.5mm diameter hole. The force transducer is capable of resolving force measurements to 0.2g equivalent weights. Through a Labmaster

interface (Scientific Solutions, Mentor, OH) a computer program records session force-time data at a rate of 100 samples per second allowing for force-time waveforms of each individual lick. During training, the force requirement for a water bolus reward is set at 2g and continuous licking behavior is reinforced by delivery of 0.06ml of water to the lick disk surface after every 12th lick (fixed-ratio of FR12 schedule) (Figure 5.3).

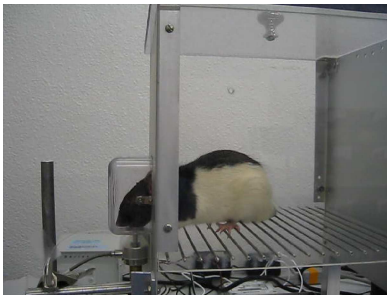

Figure 5.3 Lick Apparatus

Ischemic Insult

Unilateral ischemia was induced by slowly injecting endothelin-1 (80 pmol) via Hamilton syringe in the proximity of the middle cerebral artery contralateral to the animal's preferred reaching paw (Windle et al., 2006), (Adkins, Voorhies, & Jones, 2004).

Mapping Procedure

Intracortical microstimulation (ICMS) techniques were used to generate detailed ipsilesional maps of forelimb regions of the motor cortex. Prior to surgery animals were anesthetized with ke7,8 DHF tamine hydrochloride (70 mg/kg intraperitoneally [i.p.]) and xylazine (5 mg/kg i.p.). Animals received supplemental doses of ketamine (20 mg/kg i.p.) and xylazine (0.02 mg/kg i.p.]) as needed. A digital image of the cortical surface was taken and a 500-μm grid was superimposed onto the image. A glass microelectrode

(controlled by a hydraulic microdrive) was used to make systematic penetrations across the cortex using the cortical surface image and grid as a guide. At each penetration site, the electrode was lowered to approximately 1550 μm (corresponding to cortical layer V). Animals were maintained in a prone position with the limb consistently supported. Sites where no movement was detected at ≤60 μA were recorded as unresponsive. Forelimb movements were classified as either distal (wrist/digit) or proximal (elbow/ shoulder) and representational maps were generated from the pattern of electrode penetrations (Fig. 5a). An image analysis program (CANVAS v. 3.5) was used to calculate the areal extent of the CFA. Briefly, each map was imported into CANVAS and calibrated to magnification. Individual areas were traced using the marquis tool to produce a measure of area (mm^2).

Histology and lesion verification

Rats were given an overdose of sodium pentobarbital and intra-cardially perfused with 0.9% saline followed by 4% paraformaldehyde solution immediately after mapping. The brains were removed and post fixed in 4% paraformaldehyde solution for 24-48 hrs and then finally placed in a 30% sucrose-formalin solution for 3 days before being cut frozen on a microtome. The brains were sectioned coronally at a thickness of 40μm. Sections were collected in a chilled 0.1M phosphate buffer solution and one series of every seventh section from the cortex and the sub-cortex was mounted on subbed slides and stained with cresyl violet to demonstrate Nissl substance (Morecraft, et al., 1992). Myelin staining was also carried out in the same sections. The mounted sections represented every 240-μm intervals in an individual series of tissue sections on the slides. Infarct size was determined by measuring areas of noninfarcted tissue in both the damaged and undamaged hemispheres. Lesion volumes were indirectly estimated based

on interhemispheric volume differences by tracing whole cerebral hemispheres in sections 440 μm apart using Image J software.

Statistical analyses

One-way ANOVA and t-test statistical analysis was performed using SPSS software, with results displaying a p value less than or equal to 0.05 were considered significant. All descriptive statistics are reported as means ± S.E.M. unless otherwise indicated.

5.3 Results

5.3.1 Unilateral cortical ischemia causes forelimb motor deficits

Single Pellet Reaching. In the skilled reaching task, both groups had about the same amount of successful retrieval percentage in pre-injury trials (MCAO = 44.1%, Control = 42.66%), but the MCAO group showed a significant decrease in successful pellet retrieval percentage during post-injury trials. Control animals displayed an average of 44.11% ± 5.7% during post-lesion tests, whereas MCAO animals averaged only a 14.6± 7.98% successful retrieval rate. These results were highly significant ($p < 0.02$) and demonstrated a marked difference between the two groups (Figure 5.4).

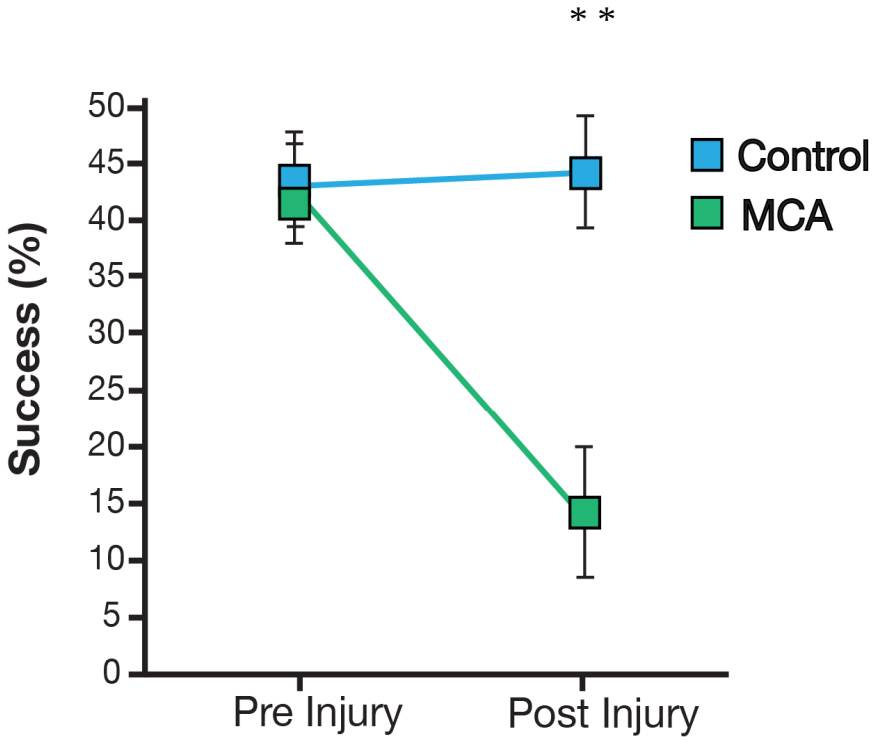

Figure 5.4 Single Pellet Reaching. Skilled reaching data showing the percent decrease in successful reaching rate between pre-injury and post-injury testing. A statistically significant difference was observed between the MCAO and control groups during post-injury testing ($p \leq 0.03$).

Sunflower Seed Opening Test. This test revealed deficits in fine motor skills in MCAO rats. There was a significant difference between the two test groups during post-injury testing. During pre-injury tests, the two groups showed no significant differences in average time taken to complete the task (control = 52.6 ± 2.5 sec, MCAO = 58.2 ± 5.7sec). However, during post-injury trials MCAO animals spent an average of 83.9 seconds to eat the five sunflower seeds, while the control group spent only 55.5 ± 3.2

seconds completing the task. The differences between the MCAO and control animals during post-injury testing were highly significant ($p = 0.0004$). Also, it took MCAO rats much longer to complete this task between pre and post-lesion trials. This group displayed a 30.6% increase in the average completion time between pre and post-lesion testing (Figure 5.5). There were no significant differences in the number of pieces the shell was broken into pre (12 ± 1.2 pcs) and post (11.59 ± 1.1 pcs) injury.

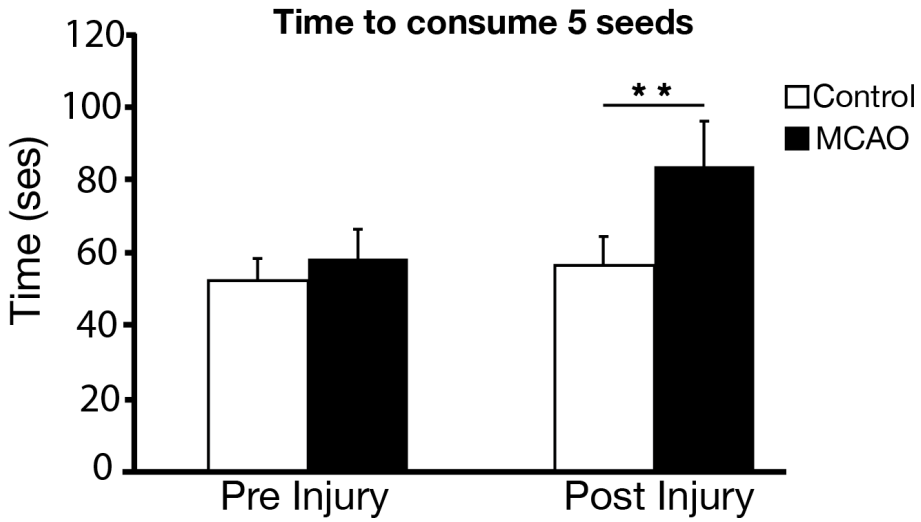

Figure 5.5 Sunflower seed opening test. Sunflower seed data showing MCAO rats taking more time to consume seeds. Statistically significant difference ($p = 0.0004$) in task completion time was observed between the two groups during post-lesion testing.

Vermicelli Handling Test. In the Vermicelli pasta handling task forepaw dysfunction was measured. MCAO rats demonstrated a marked increase in the number of adjustments made with the non-affected pas between pre-lesion and post-lesion trials.

MCAO rats displayed a 22.3% increase in the number of non-affected paw adjustments made during the past handling task between pre-lesion and post-lesion trials (Figure 5.6). MCAO animals had an average number of 14.8 ± 2.33 adjustments/trial with the non-affected limb in post-lesion trials whereas the control animals averaged only 6.42 ± 0.74 adjustments/trial during post-lesion testing. There was a significant difference between the two test groups ($p \leq 0.03$). No significant difference was found between pre and post-lesion trials in the affected paws of either group (Figure 5.6).

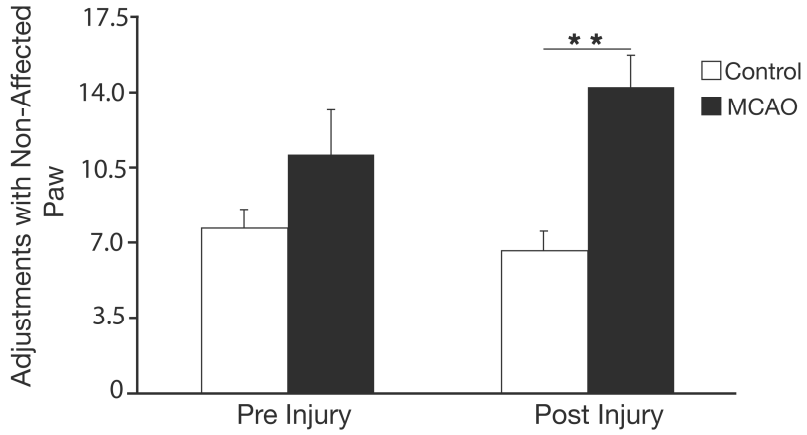

Figure 5.6 Vermicelli handling task: No. of adjustments with Non-affected paw/trial. MCAO rats made considerably more adjustments with their non-impaired paw per trial. A statically significant difference ($p \leq 0.03$) was observed between control and MCAO rats during post-lesion testing.

Cylinder Paw Placement Test. In the cylinder paw placement task, no statistically significant differences were revealed in forelimb asymmetries between the MCAO group and the control group during the pre-lesion and post-lesion testing. During pre-injury

testing, the average asymmetry ratio between the two groups was almost identical (control = 49%, MCAO = 50%), as well as the average wall contacts made by the dominant paw (control =35%, MCAO = 36%) and average non-dominant paw (control = 38%, and 37%). During post-injury testing, no statistically significant differences were seen between testing groups during pre-injury and post-injury cylinder task testing. During post injury testing, the average asymmetry ratio (control = 55 ± 7.6 %, MCAO = 42 ± 10.24 %), average dominant paw use (control = 41%, MCAO = 33%), and average non-dominant paw use (control = 31%, MCAO = 48%) was almost identical.

5.3.2 Unilateral MCAO did not induce significant deficits in breathing or licking behavior

Plethysmography. No significant irregularities were observed in breathing patterns using the plethysmography apparatus. During post-injury testing, MCAO animals averaged 163 ± 44 breaths per minute during the challenge portion of this test while control animals averaged 173 ± 70 breaths per minute. These numbers varied from their base line measures of challenge trials where MCAO and control animals averaged about the same breathing frequency (MCAO = 143 ± 53 bpm, control = 132 ± 61 bpm) (Figure 5.7).

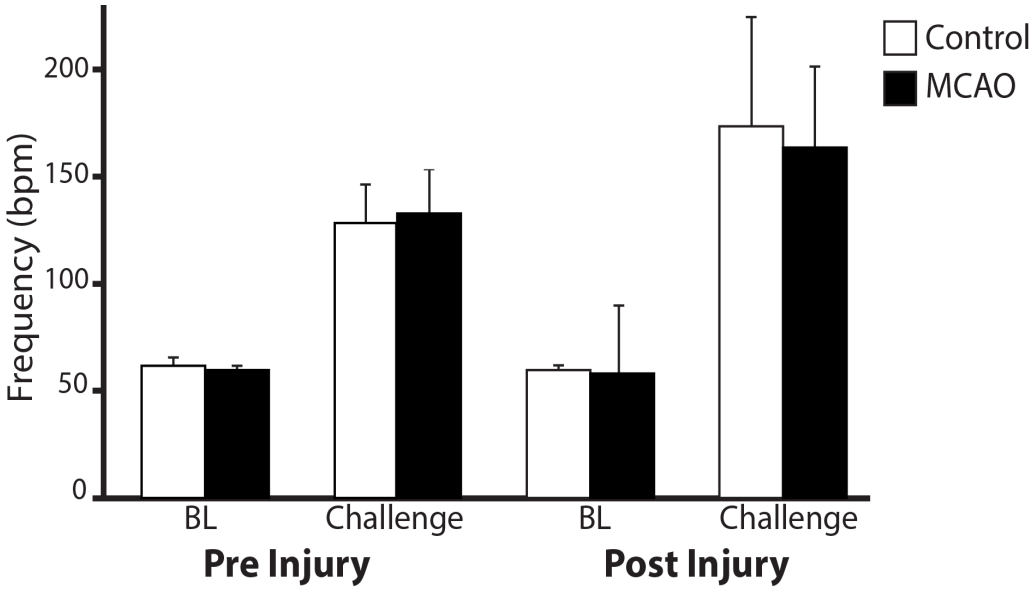

Figure 5.7 Breathing frequency measured viaPlethysmography. Bar graphs representing the ventilator parameter frequency (breaths per min) in MCAO and control animals. No statistical significant differences were observed in any pre-lesion or post-lesion testing.

Lick Task. During the lick force testing no statistically significant difference was found between groups in pre-lesion or post-lesion testing. Average peak force (g) during pre-lesion testing was 45.9 ± 5.1g for the control group, and 42 ± 6.9g for the MCAO animals. Average peak force during post-lesion testing was 40 ± 3.71g for the control group, and 37 ± 4.9g for the MCAO group (Figure 5.8).

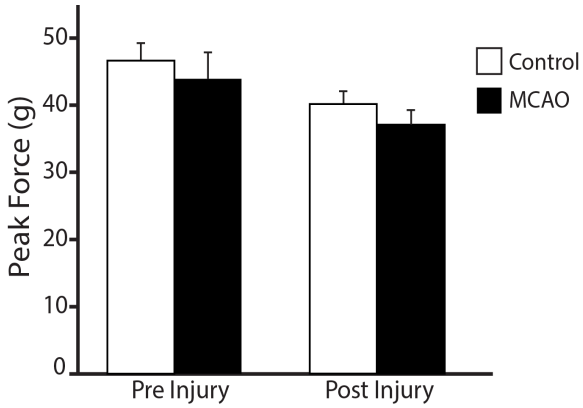

Figure 5.8 Lick Force (g). No statistically significant differences were observed between the groups during pre or post-lesion testing.

No statistically significant differences were observed between the MCAO and control groups during pre-injury or post-injury licking rhythm values. The average licking rhythm for the control group during pre-lesion trials was 7 ± 0.3 Hz, and the average rhythm for MCAO animals was 6 ± 0.4Hz. The average licking rhythm for the control groups during post-lesion trials was 6.5 ± 0.21Hz for the control group, and 5.8 ± 0.29Hz for the MCAO group (Figure 5.9).

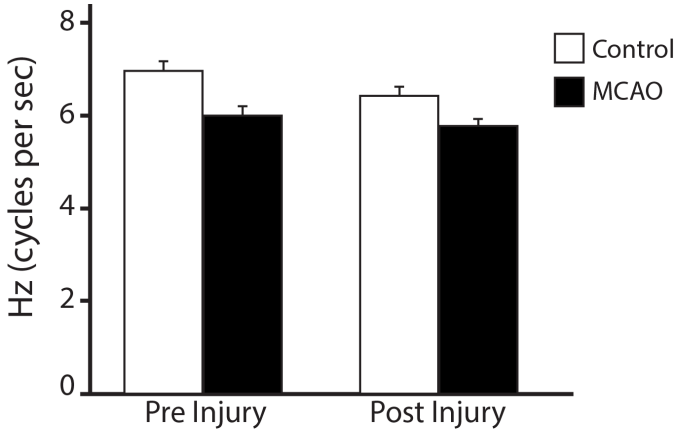

Figure 5.9 Lick Rhythm (Hz)

5.3.3 MCAO animals had significantly smaller forelimb motor maps compared to controls

Consistent with the behavioral findings, overall forelimb motor maps of MCAO animals were significantly smaller in area than those of control rats (1.7 ± 0.69mm^2 and 6.3 ± 0.8mm^2 respectively) (p=0.007). The most pronounced differences between the two groups were seen in distal rostral and caudal forelimb representations. For total caudal forelimb representations, control animals had an average motor map area of 5.34 ± 0.76 mm², while MCAO rats had an average map size of 1.4 ± 0.65 mm² (p=0.01). Most notable differences were seen between MCAO and control groups for distal caudal forelimb representations. Control animals had an average distal caudal forelimb area of 4.22 ± 0.4 mm^2 while MCAO animals had significantly smaller map size of 1.01 ± 0.43 mm^2 (Figure 5.10).

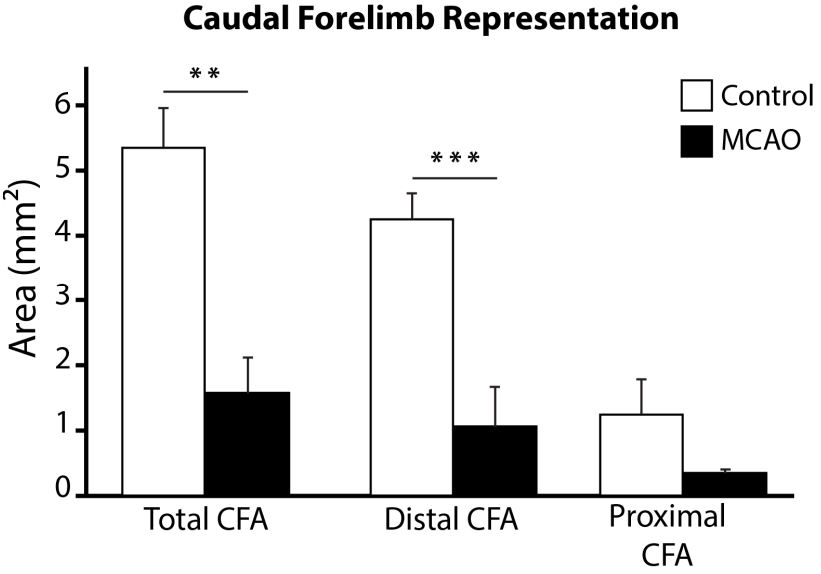

Figure 5.10 Caudal forelimb area. Bar graphs comparing average caudal forelimb motor map area. Control animals had a larger motor map size in proximal, distal, and total caudal forelimb area representations, with

statistically significant differences between the two groups observed in the total and distal caudal forelimb area.

In rostral forelimb representations, total rostral forelimb area in controls was significantly larger than MCAO rats (0.98 ± 0.15 mm^2) only the distal forelimb representations displayed significant difference between the control and MCAO groups (Figure 5.11). Control rats had an average distal rostral forelimb map size of 0.98 ± 0.15 mm^2 while MCAO rats had an average size of 0.32 ± 0.13 mm^2.

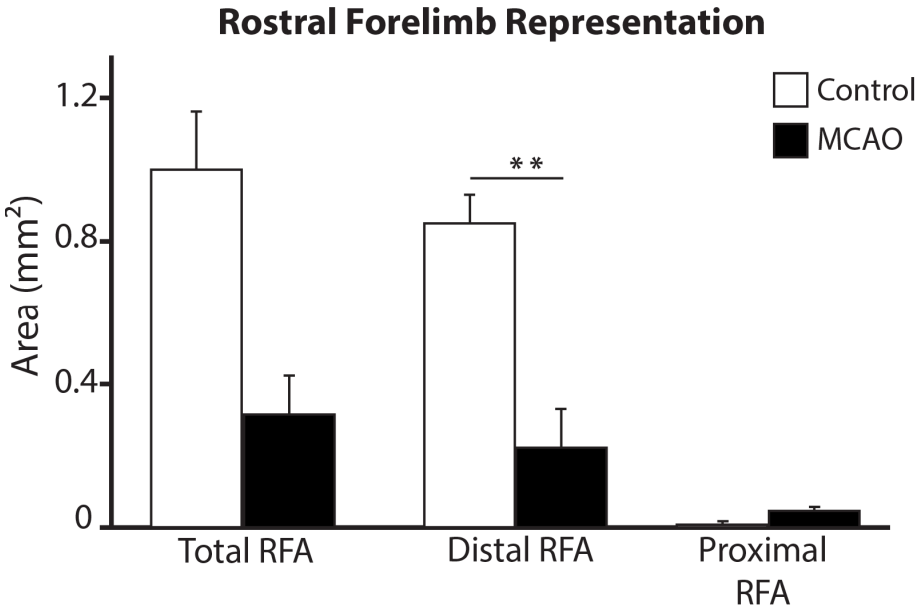

Figure 5.11 Rostral Forelimb Area. Control animals had larger motor maps in proximal, distal, and total rostral forelimb area representations, with a statistically significant difference between the two groups in the distal forelimb area. There was no significant difference between proximal caudal forelimb areas in the two groups.

5.3.4 MCAO had no impact on oral motor representations

No significant difference in oral motor (jaw and tongue) cortical representations was found between MCAO and control rats (Figure 5.12).

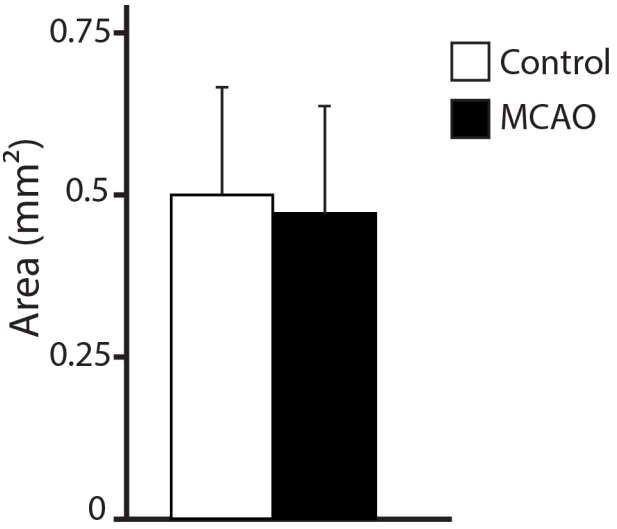

Figure 5.12 Oral motor map area of tongue and jaw representation.

No statistically significant difference was observed between the MCAO and control groups.

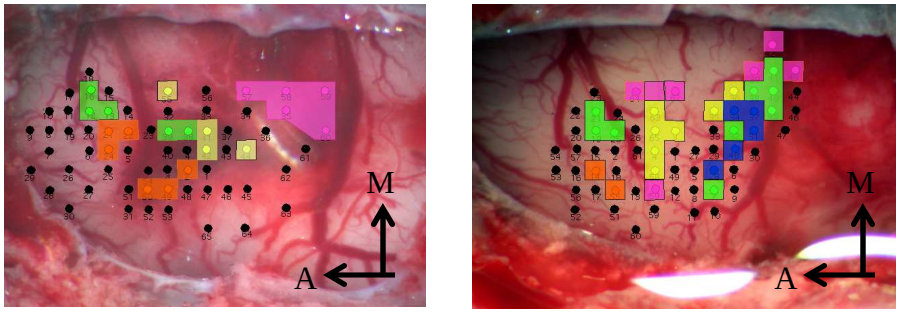

Figure 5.13 MCAO representative motor maps

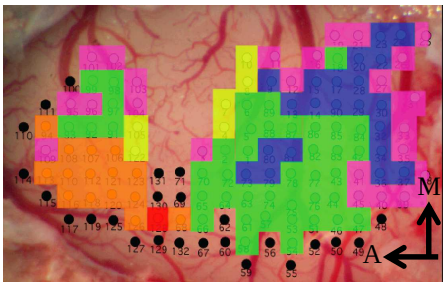

Figure 5.14 Control rat representative motor maps

5.4 Discussion

Animal models of cortical ischemia have predominantly focused on upper extremity impairments after stroke. However, there is some evidence that the same manipulation can induce deficits in licking (Whishaw et al., 1987; Skitek et al., 1999), chewing (Whishaw et al., 1997) and breathing parameters. The aim of this study was to investigate whether middle cerebral artery occlusion can induce changes in tongue dynamics with noted reductions in lick force, frequency and rhythm (Ciucci & Connor, 2009; Fowler & Mortell, 1992; Fowler & Wang, 1998). Unfortunately, there has been little investigation into the differential neural mechanisms underlying cranial motor versus upper extremity impairments in these models. To address this issue a behavioral paradigm in an animal model was established that directly compared upper extremity and cranial motor impairments and measured concomitant changes in corticospinal and corticobulbar circuitry.

Results from this study show that corticospinal system, which is predominantly involved in upper extremity movements and more fractionated fine digit manipulations is affected by middle cerebral artery occlusion, whereas cranial motor function involved in mouth, tongue, jaw and to some extent diaphragm movements remained unaffected by MCAO. Post injury behavior testing began a month after stroke induction in order to assess enduring impairments in chronic stage and minimizing the impact of spontaneous recovery influencing the behavioral outcome. Rats with MCAO displayed significant impairments in the Vermicelli pasta handling task, single pellet reaching, and sunflower seed opening task compared to control animals. However, no significant differences

were seen between MCAO rats and controls in the cylinder task, measuring forelimb asymmetries, and tests assessing tongue or respiratory behavior.

In vermicelli pasta handling task more adjustments were made with the non-affected limb by the MCAO group, and in skilled reaching task MCAO animals showed a significant decline in successful retrieval rate. In the sunflower seed opening task, MCAO rats took much longer compared to the control group to consume all five sunflower seeds. This correlates with the differences seen in motor map areas in the MCAO group. In caudal and rostral forelimb motor map representations, the distal forelimb area differed significantly between the two groups. In both instances, MCAO rats had a much smaller motor map size for distal forelimb representations. The decreased representation of distal forelimb area in both caudal and rostral forelimb area in the MCAO group explains the sunflower seed, skilled reaching task, and Vermicelli handling task results. Distal forelimb area representations correspond to motor movement in the upper extremity, involving wrist and digits. The noticeable decrease in distal forelimb area map size implies that a considerable region of the sensorimotor cortex sends descending axonal projections through the corticospinal tract controlling paw and fine digit movements. The decrease in area explains why the tasks most oriented to test distal upper extremity movement displayed the most significant differences between control and MCAO groups.

The corticospinal system, responsible for upper extremity movement, is largely under unilateral control, with 90% of axonal fibers terminating on the contralateral side of the spinal cord (Lemon & Griffiths, 2005). A unilateral injury due to middle cerebral artery occlusion results in profound motor impairments in the limb contralateral to the

site of the lesion, as was evident from these results. For this purpose, single pellet reaching task is considerably sensitive in detecting deficits occurring from such injuries. MCAO rats were less successful in retrieving pellets after injury compared to controls. In the sunflower seed task, MCAO rats took longer for task completion and were unable to carry out fine digit manipulation in the same manner as in pre-lesion testing. The Vermicelli pasta handling task data reveals compensatory behavior adopted by the animals after stroke. Injured rats made more adjustments per trial with their unaffected paw in order to compensate for the lack of fine motor ability in the affected paw.

The relatively unaffected cranial motor function and no significant size differences observed for oral motor maps between control and MCAO groups may be explained by the fact that the infarct produced in the stroke model used appeared to spare the oral motor areas of the motor map. Furthermore, the corticobulbar tract innervates cranial muscles bilaterally. Since both brain hemispheres can control cranial motor function, unilateral injury is associated with less prominent functional deficits due to compensation by the intact hemisphere. In addition, breathing is primarily controlled by phrenic motoneuron pools located in the mid cervical spinal cord between C3-C6, innervating muscles of the diaphragm and phrenic motoneuron pools receive projections from bulbospinal fibers (Fuller, 2003). However, voluntary control of breathing is driven from the cerebral cortex (i.e. speaking, breath holding) (Davenport & Vovk, 2009; Redline et al., 2007). Typically after a spinal cord injury altered respiratory patterns are generally characterized by increased breathing frequency (Fuller, Johnson, Olson, & Mitchell, 2003). Interestingly, MCAO rats in this study showed decreased frequency in breaths during challenge. Although this effect was not significant, it allowed for the

speculation that as a result of a unilateral cortical injury the system has reduced ability to engage cortical inputs due to loss of connections and the spinal cord is augmenting output during the respiratory challenge as a compensatory mechanism.

Thus, motor cortex stroke affects corticospinal versus corticobulbar circuits resulting in different patterns of impairment in upper extremity versus cranial motor function. This may reflect differences in functional relationships between the primary motor cortex and the corticospinal versus corticobulbar system. The results may also reflect inherent differences in the functional anatomy of the two systems. Corticospinal projections have a greater laterality bias than corticobulbar projections, potentially affording a greater opportunity for contralesional compensation. For future studies it would be feasible to create a more robust injury model in order to observe the impact on bilaterally innervated cranial motor systems.

Chapter 6

General Discussion

6.1 Summary

In these dissertation studies, principles of neural plasticity and their promotion by means of neurorehabilitation and adjuvant therapies have been investigated with the aim of enhancing functional outcome after stroke. The term ‘‘neural plasticity’’ might refer to transiently achieved functional changes in the context of learning and recovery, as well as structural changes manifesting as functional changes in synaptic efficacy, modifying protein synthesis and proteinase activity in nerve cells, creation of new anatomical connections or by altering synapses morphologically, and by specific apoptosis (Møller, 2006). Pharmacological interventions can address several brain mechanisms that have been identified to be related to motor learning. Drugs have been studied in animal models, healthy volunteers, and stroke patients in single or multiple dosages, with and without additional therapeutic tasks. No single medication evaluated for its beneficial effect of modulating plasticity in the human M1 in stroke patients has reached class I evidence so far.

The primary goal of these dissertations studies was to test the efficacy of TrkB agonists for enhancing cortical plasticity and augmenting motor recovery associated with cortical ischemia in rats. Characterization and measurement oral motor dysfunction in a rodent model after a unilateral middle cerebral artery occlusion was also studied.

7,8 Dihydroxyflavone As An Adjuvant Therapy

In Chapter 3 significant effects on positive behavioral outcome were seen within a few days of administration of a synthetic flavonoid derivative compound 7,8-DHF. The

improved behavior was more pronounced in tasks requiring fine digit manipulation, hence implying the involvement corticospinal tract. The integrity of cortcospinal tract is essential in successfully executing fractionated individualized movements. However, tasks employing gross motor movements failed to show any significant improvements as a result of the treatment. This finding is noteworthy because the targeted lesion is in the primary sensory motor cortex which projects axons directly to corticospinal tract, indicating that the compound is inducing factors that are keeping the tissue viable. It is difficult to say that these effects are a result of TrkB stimulation, because protein quantification did not reveal significant increase of the activated receptor. This may reflect either the failure of the compound to drive changes in TrkB signaling proteins or possibly the lack of sensitivity of the protein assays used. It is possible that the drug was driving other signaling pathways know to enhance neural plasticity outside of the TrkB receptor. For example, flavonoids have been shown to up regulate the NMDA receptor (Wang et al., 2011; Cao et al., 2009).

7,8-DHF treated rats showed greater forelimb motor map areas than those of the vehicle injected animals and upon lesion verification it was seen that this was primarily due to the smaller lesion size in the DHF animals. It has long been established that ischemic strokes cause reorganization in M1 networks of the peri-infarct cortex and beyond. But interestingly the pattern of reorganization in these animals did not follow a linear correlation, as the expansion of motor maps was several times greater than the proportion of spared tissue in DHF receiving animals. This finding is contrary to what Nudo and colleagues have demonstrated in non-human primates where after large lesions destroying >50% of the M1 distal forelimb, the PMv distal forelimb invariably increased

in size, and the increased area positively correlated with the size of lesion (Nudo, 1996). It may be the case that 7,8-DHF is facilitating synaptic strengthening and promoting axonal sprouting in these brain regions.

It is speculated that 7,8-DHF was not able to induce relaxation of the arteries because of the structural modification of 7,8-DHF for its reactivity. Naturally occurring flavonoids impart vasodilatory effects due to the presence of 4 carbonyl group (Duarte et al., 1993). In 7,8-DHF the 8-position hydroxyl group is essential for the compound to activate TrkB receptor. The neuroprotective effects of 7,8-DHF were evident by reduced infarct volume, but the underlying mechanism behind this finding remains somewhat elusive partly due to the fact that 5mg/kg dose administered was unable to enhance pTrkB expression in the sensory-motor cortex of healthy rats. Due to the absence of injury, it could be proposed that 7,8-DHF was ineffective in provoking TrkB receptors and the presence of endogenous BDNF was sufficient to carry out activation of its target receptors. In an event of an ischemic stroke the normal physiological range of BDNF and TrkB is disrupted, hence presenting a possibility for 7,8-DHF to take on the role of BDNF.

LM22A-4 As An Adjuvant Therapy

The experiments conducted to study the role of LM22A-4 (chapter 4) provide substantial support for how adjunct therapies following unilateral stroke like damage can influence behavioral recovery of the impaired forelimb and mediate peri-lesion neural plasticity. The use of animal model in these experiments expanded beyond the study of neuroprotection to studies directed at identifying the fundamental neural substrates that support rehabilitation-dependent functional improvement.

First, an effective dose was established by measuring the increase in protein levels of pTrkB using two varying dosages of LM22A-4. pTrkB levels in the ipsilesional motor cortex were increased by 27.7% in LM22A-4 administered (7d) post stroke. Further steps were taken to verify successful penetration of the compound into the brain by the help of mass spectrometry. Although the amount detected in the brain was low, due to intraperitoneal route of administration it was sufficient to induce increased in p-TrkB/TrkB ratios after one week. Further, the drug was administered for a considerably longer period of time in experiment 2, which was designed to measure behavioral and neurophysiological correlates of LM22A-4.

Evidence from clinical studies indicates that early start and high intensity of therapies are decisive for favorable long-term outcome. On the basis of pathophysiological data, the first 3 weeks after stroke are considered a particularly promising period. In animal models, active training leads to better functional recovery and sprouting, whereas inactivity results in additional loss of ability (Nudo 2006). Hence based on this evidence, a rigorous rehabilitation regime, in conjunction with pharmacological therapy, was enforced starting no later than three days post stroke to maximize functional recovery

In Experiment 2 of Chapter 4, single pellet reaching task was utilized as the rehabilitation-training paradigm and also to analyze changes in the performance of the reaching movements. This method for qualitative reaching movement analysis is sensitive to compensatory forelimb movements that reveal enduring impairments or compensatory strategies in reaching and grasping motor action patterns after brain injury (e.g., Gharbawie et al. 2005a; Metz and Whishaw 2000; Whishaw et al. 1993). Several

other motor tasks measuring impairment profiles in upper extremity movement coordination, such as sunflower seed opening, vermicelli handling and cylinder test were also employed. After a unilateral stroke injury, animals appear to develop an exaggerated compensatory reliance on the intact forelimb. For example there is a tendency to increase reliance on intact limb is a measure of postural support movements, or rats using the aid of intact forelimb in successful retrieval of food pellets in skilled reaching. Therefore, these tasks were instrumental in delineating recovery vs. compensatory patterns adopted by animals towards functional outcomes.

The most encouraging finding from the study of LM22A-4 was the proficiency in reaching attained by severely impaired LM treated animals after three weeks of daily drug administration. Also, these rats demonstrated greater efficiency in bilateral manipulation skills while consuming sunflower seeds. This indicates that the drug preserves integrity of white matter tracts, which are essential in coordinating fine skilled movements. The neurophysiological manifestation of this behavior was seen in the expansion of secondary motor areas located distal to the site of injury. Based on previous studies in non-human primates, it is suggested that interruption of projections from the primary motor cortex (M1) leads to increased recruitment of secondary motor areas such as the dorsolateral premotor cortex and supplementary motor areas. Basic underlying mechanisms of these findings include both different functional use of existing networks and synapses, but also structural changes (Nudo, 2003, Kleim 2002). This effect of LM22A-4 was only seen in severely impaired animals which is indeed promising when translating these findings to the clinic.

LM22A-4 administration provides compelling evidence of mediating synaptic

plasticity in peri-lesion cortex, which is usually disrupted as a result of ischemic injury. The ability for motor maps to reorganize is related to strengthening of synaptic connections, which is experience dependent (e.g., Kleim et al., 2002). The reorganization of peri-lesion cortex after stroke, including reorganization of motor maps, has been linked with functional recovery (e.g., Nudo, 2003).

The finding that pTrkB expression is enhanced in peri-lesion cortex following LM22A-4 treatment indicates an increased capacity for receptor binding after stroke. Phosphorylation of TrkB through BDNF has been shown to both down regulate (in an acute stage) and up regulate (at a more chronic time point) various genes, including pCREB, which has been implicated in plasticity promoting mechanisms. Administering BDNF in the acute post-ischemic period reduces cell death and delayed treatment facilitates motor recovery in rats. These dissertation studies were able to provide proof-of-principle that LM22A-4 is indeed mimicking BDNF by inducing similar cellular and behavioral outcomes in the effected systems.

In the early course of ischemic stroke, pathophysiological mechanisms in the perilesional region are initiated, which include enhanced expression of plasticity-related proteins, neurotrophic factors (e.g., brain-derived neurotrophic factor, synapsin I), and certain neurotransmitters, but also expression of inhibitory factors occurs in the central nervous system. These modifications probably lead to morphological changes, e.g., synaptic plasticity and sprouting (Biernaskie, Chernenko, & Corbett, 2004). Off drug increase in performance indicates activation of TrkB receptor elevates levels of transcription factors that set up ongoing increment in synaptic function that may be sustained for weeks.

Animal Model Of Oral Motor Impairments After Stroke

The final study of the dissertation examined at the impact of unilateral MCAO on limb motor and oral motor function provided important insights regarding the function of bilaterally innervated systems. Since dysphagia, dysarthria and malfunctioning in normal breathing control are common sequelae of stroke (Cameron D, 2000). Swallowing and food intake are important for quality of life and autonomy of patients and will for many patients be considered an important goal of rehabilitation. This study was an attempt to create an animal model of post stroke oral impairment.

Rats with MCAO displayed a typical profile of upper extremity impairments, with enduring reduction in their single pellet reaching scores, exhibiting forelimb use asymmetries and forepaw dysfunction There were no prominent differences observed in the breathing patterns of MCAO and control rats, neither were there any tongue disorders detected trough the licking task. The relatively unaffected cranial motor function and no significant size differences observed for oral motor maps between control and MCAO groups may be explained by the fact that the infarcts produced in the stroke model used appeared to spare the oral motor areas of the motor map. Furthermore, the corticobulbar tract innervates cranial muscles bilaterally. Since both brain hemispheres can control cranial motor function, unilateral injury is associated with less prominent functional deficits due to compensation by the intact hemisphere

6.2 Translation To The Human Stroke Population

The ultimate goal of these studies is to provide basic information that can guide

the development of effective clinical therapies. Every attempt was made to develop an animal model that facilitates translation to human stroke patients. In these experiments upper extremity function was studied at several levels ranging from simple forelimb use to skilled reaching. ICMS techniques were used to measure changes in motor cortex organization similar to how TMS is used in human patients. While rats make good models of stroke-induced behavioral deficits that mimic, in some fashion, deficits seen after cortical strokes in humans (Whishaw et al., 1992; Sacrey et al., 2009) there are several key differences in these animal models that must be recognized when attempting to translate the results to the clinic.

First, these stroke animals are readily engaging key behavioral signals known to drive neural plasticity (intensity, timing and salience) that is not commonly observed in human patients. Enforcing food restriction prior to training makes the task more salient for the rats. Food pellets used as positive reinforcement makes them highly motivated and focused on the task at hand. Also, the animals make hundreds of attempts during each session in very short intervals of time therefore successfully incorporating repetition and intensity factors. Rats relearn or adapt combination of finger flexions and wrist extensions that were used normally prior to the stroke, hence reinforcing the timing element. Simultaneous delivery of LM22A-4 as an adjunct therapy to stimulate pro-recovery molecular mechanisms facilitating the formation and reorganization of neural processes contributed a great deal towards attaining functional recovery. It may be possible that the beneficial effects of LM22A-4 may require such intense therapy and this must be recognized if the drug is to be moved forward into human clinical trials.

A major concern following stroke is the loss of functionality and subsequent

learned disuse and overuse of the less-affected body side. While this may be advantageous especially during the acute post-stroke phase, in the long-term this experience is detrimental to impaired forelimb recovery and may mask the extent of actual impairment. This is especially problematic for human stroke patients but less so for rats. Rats are quadrupeds and therefore begin using their impaired forelimb immediately after stroke.

A major limitation of these studies is the inability to directly apply this knowledge to human stroke populations, which are inherently diverse. Stroke incidence increases with age. These dissertation studies used young adult rats (approximately 5-6 months of age at stroke onset). In general, in clinical populations there are a variety of strokes, both subcortical and cortical, and recovery is dependent upon a multitude of factors, including size and location of the infarct, various comorbidities such as high blood pressure and obesity (Kleim, 2006). Animal studies are very well controlled, and the lesions produced in these dissertation studies were focal and highly conserved across animals and across studies. This control restricts data variability, making it feasible to ask these questions in an experimental model, but it also makes it more difficult to generalize to clinical populations.

The single pellet reaching task was primarily used to assess impairment of the affected limb, but it is not a truly lateralized task because the non-reaching forelimb is used to help aid in reaching behaviors. It is possible that even greater impaired forelimb deficits would be revealed after training on a more unilateral task like the Montoya staircase task (Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991). This task requires animals to reach for pellets placed on descending stairs in an apparatus

configured to allow reaches with only one forelimb (the side ipsilateral to placed pellets).

Recently, some rehabilitation facilities have started incorporating these principles as part of the therapy session in the clinic and their preliminary results demonstrate promising outcomes. Catherine Lang's group is one such example (Birkenmeier et al., 2010). They worked with individual patients and let them determine specific tasks based on their interest and preference (salience). The researchers then established ways to progressively increase the difficulty level while providing feedback on performance. The intervention paradigm was rather intense including three one-hour sessions per week for six weeks during which the patients were challenged to perform 100 repetitions on each of the three different tasks they chose, thereby enforcing repetition and intensity. As a result, the patients showed a significant improvement in their active arm test scores. Thus it is possible to adapt standard rehabilitation interventions to more optimally drive the key behavioral signals and produce meaningful functional gains

6.3 Over all conclusions:

Stroke is the most common cause of long-term disability in adults. There are many parallels between postlesional neuroplasticity (relearning) and learning in the development of individuals as well as task learning of healthy persons. One key principle of neurorehabilitation is the repetitive creation of specific learning situations to promote mechanisms of neural plasticity in stroke recovery. Preliminary data from these studies suggests that therapies targeting neural signals will undoubtedly play a major role in neurorehabilitation in the near future. Exploiting behavioral signals with early initiation

of treatment, high intensity active therapies and use of potential enhancers of neural plasticity e.g., pharmacological augmentation, have proven to be quite promising.

6.4 Future directions:

There remain many unanswered questions and possibilities for future studies. It would be feasible to test the efficacy of the novel TrkB agonists used in these dissertation studies in non-human primates conjunction with intense physical therapy (for example, constraint induced movement therapy). However in order to effectively translate it to clinic, these findings need to be replicated, large controlled trials need to be conducted, and patient selection criteria reapproved before pharmacological augmentation can be generally recommended.

The two novel TrkB agonists investigated in the studies have shown to mimic BDNF's role in the brain. BDNF mechanisms have been implicated in learning and memory and higher cognitive functioning paradigms, hence these compounds have great potential to enhance cognitive abilities. It would be encouraging to design experiments with the goal to study ameliorative effects of these compounds on stroke dementia and higher cognitive disorders. Regardless of what studies may be done in the future, this dissertation demonstrates that adjuvant pharmacological therapies that drive key neural signals orchestrating neural plasticity can significantly enhance motor recovery and cortical plasticity after stroke.

REFERENCES

Acheson, A., Barker, P. A., Alderson, R. F., Miller, F. D., & Murphy, R. A. (1991). Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. *Neuron, 7*(2), 265-275.

Akaneya, Y., Tsumoto, T., Kinoshita, S., & Hatanaka, H. (1997). Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J Neurosci, 17*(17), 6707-6716.

Alberini, C. M. (1999). Genes to remember. *J Exp Biol, 202*(Pt 21), 2887-2891.

Allred, R. P., Adkins, D. L., Woodlee, M. T., Husbands, L. C., Maldonado, M. A., Kane, J. R., et al. (2008). The vermicelli handling test: a simple quantitative measure of dexterous forepaw function in rats. *J Neurosci Methods, 170*(2), 229-244.

Almeida, R. D., Manadas, B. J., Melo, C. V., Gomes, J. R., Mendes, C. S., Graos, M. M., et al. (2005). Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ, 12*(10), 1329-1343.

Altman, K. W., Yu, G. P., & Schaefer, S. D. (2010). Consequence of dysphagia in the hospitalized patient: impact on prognosis and hospital resources. *Arch Otolaryngol Head Neck Surg, 136*(8), 784-789.

Ames, A., 3rd. (1992). Energy requirements of CNS cells as related to their function and to their vulnerability to ischemia: a commentary based on studies on retina. *Can J Physiol Pharmacol, 70 Suppl*, S158-164.

Anderson, K. D., Gunawan, A., & Steward, O. (2007). Spinal pathways involved in the control of forelimb motor function in rats. *Exp Neurol, 206*(2), 318-331.

Andriambeloson, E., Magnier, C., Haan-Archipoff, G., Lobstein, A., Anton, R., Beretz, A., et al. (1998). Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J Nutr, 128*(12), 2324-2333.

Antal, A., Chaieb, L., Moliadze, V., Monte-Silva, K., Poreisz, C., Thirugnanasambandam, N., et al. (2010). Brain-derived neurotrophic factor (BDNF) gene polymorphisms shape cortical plasticity in humans. *Brain Stimul, 3*(4), 230-237.

Barbacid, M. (1994). The Trk family of neurotrophin receptors. *J Neurobiol, 25*(11), 1386-1403.

Barker, A. T., Jalinous, R., & Freeston, I. L. (1985).Non-invasive magnetic stimulation of human motor cortex.*Lancet, 1*(8437), 1106-1107.

Barrett, G. L. (2000). The p75 neurotrophin receptor and neuronal apoptosis.*Prog Neurobiol, 61*(2), 205-229.

Basmajian, J. V., Gowland, C. A., Finlayson, M. A., Hall, A. L., Swanson, L. R., Stratford, P. W., et al. (1987). Stroke treatment: comparison of integrated behavioral-physical therapy vs traditional physical therapy programs. *Arch Phys Med Rehabil, 68*(5 Pt 1), 267-272.

Beecher, G. R. (2003). Overview of dietary flavonoids: nomenclature, occurrence and intake. *J Nutr, 133*(10), 3248S-3254S.

Bengzon, J., Kokaia, Z., Ernfors, P., Kokaia, M., Leanza, G., Nilsson, O. G., et al. (1993). Regulation of neurotrophin and trkA, trkB and trkC tyrosine kinase receptor messenger RNA expression in kindling. *Neuroscience, 53*(2), 433-446.

Berglund, K., & Fugl-Meyer, A. R. (1986). Upper extremity function in hemiplegia.A cross-validation study of two assessment methods.*Scand J Rehabil Med, 18*(4), 155-157.

Biernaskie, J., & Corbett, D. (2001). Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. *J Neurosci, 21*(14), 5272-5280.

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. *J Neurosci, 24*(5), 1245-1254.

Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., & Greenberg, M. E. (1999). Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science, 286*(5443), 1358-1362.

Brus-Ramer, M., Carmel, J. B., & Martin, J. H. (2009). Motor cortex bilateral motor representation depends on subcortical and interhemispheric interactions. *J Neurosci, 29*(19), 6196-6206.

Bury, S. D., & Jones, T. A. (2002). Unilateral sensorimotor cortex lesions in adult rats

facilitate motor skill learning with the "unaffected" forelimb and training-induced dendritic structural plasticity in the motor cortex. *J Neurosci, 22*(19), 8597-8606.

Cabelli, R. J., Hohn, A., & Shatz, C. J. (1995). Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF.*Science, 267*(5204), 1662-1666.

Carmichael, S. T. (2006). Cellular and molecular mechanisms of neural repair after stroke: making waves. *Ann Neurol, 59*(5), 735-742.

Carter, A. R., Connor, L. T., & Dromerick, A. W. (2010). Rehabilitation after stroke: current state of the science. *Curr Neurol Neurosci Rep, 10*(3), 158-166.

Castro, A. J. (1972). Motor performance in rats. The effects of pyramidal tract section. *Brain Res, 44*(2), 313-323.

Castrén, E., Zafra, F., Thoenen, H., & Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proc Natl Acad Sci U S A, 89*(20), 9444-9448.

Castro-Alamancos, M. A., & Borrell, J. (1995). Contribution of NMDA and nonNMDA glutamate receptors to synchronized excitation and cortical output in the primary motor cortex of the rat. *Brain Res Bull, 37*(5), 539-543.

Cenci, M. A., Whishaw, I. Q., & Schallert, T. (2002). Animal models of neurological deficits: how relevant is the rat? *Nat Rev Neurosci, 3*(7), 574-579.

Chao, M. V., & Hempstead, B. L. (1995). p75 and Trk: a two-receptor system. *Trends Neurosci, 18*(7), 321-326.

Chapin, J. K., Sadeq, M., & Guise, J. L. (1987). Corticocortical connections within the primary somatosensory cortex of the rat. *J Comp Neurol, 263*(3), 326-346.

Cheatwood, J. L., Emerick, A. J., & Kartje, G. L. (2008). Neuronal plasticity and functional recovery after ischemic stroke.*Top Stroke Rehabil, 15*(1), 42-50.

Cheeran, B. J., Ritter, C., Rothwell, J. C., & Siebner, H. R. (2009). Mapping genetic influences on the corticospinal motor system in humans. *Neuroscience, 164*(1), 156-163.

Cho, S., Savas, S., & Ozcelik, H. (2006). Genetic variation and the mitogen-activated protein kinase (MAPK) signaling pathway. *OMICS, 10*(1), 66-81.

Cirstea, M. C., & Levin, M. F. (2000).Compensatory strategies for reaching in stroke.*Brain, 123 (Pt 5)*, 940-953.

Ciucci, M. R., & Connor, N. P. (2009). Dopaminergic influence on rat tongue function and limb movement initiation. *Exp Brain Res, 194*(4), 587-596.

Cospito, J. A., & Kultas-Ilinsky, K. (1981). Synaptic organization of motor corticostriatal projections in the rat. *Exp Neurol, 72*(2), 257-266.

Cotman, C. W., & Engesser-Cesar, C. (2002). Exercise enhances and protects brain function. *Exerc Sport Sci Rev, 30*(2), 75-79.

Cramer, S., Rempe, R., & Galla, H. J. (2012). Exploiting the properties of biomolecules for brain targeting of nanoparticulate systems. *Curr Med Chem, 19*(19), 3163-3187.

Cramer, S. C. (2008). Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. *Ann Neurol, 63*(3), 272-287.

Cramer, S. C., Procaccio, V., Americas, G., & Investigators, G. I. S. (2012). Correlation between genetic polymorphisms and stroke recovery: analysis of the GAIN Americas and GAIN International Studies. *Eur J Neurol, 19*(5), 718-724.

Cramer, S. C., & Riley, J. D. (2008). Neuroplasticity and brain repair after stroke. *Curr Opin Neurol, 21*(1), 76-82.

Crary, M. A., Humphrey, J. L., Carnaby-Mann, G., Sambandam, R., Miller, L., & Silliman, S. (2013). Dysphagia, nutrition, and hydration in ischemic stroke patients at admission and discharge from acute care. *Dysphagia, 28*(1), 69-76.

Davenport, P. W., & Vovk, A. (2009). Cortical and subcortical central neural pathways in respiratory sensations. *Respir Physiol Neurobiol, 167*(1), 72-86.

Dechant, G. (2001). Molecular interactions between neurotrophin receptors. *Cell Tissue Res, 305*(2), 229-238.

De Souza, L. H., Hewer, R. L., & Miller, S. (1980). Assessment of recovery of arm control in hemiplegic stroke patients. 1. Arm function tests. *Int Rehabil Med, 2*(1), 3-9.

DENNY-BROWN, D. (1960).Diseases of the basal ganglia.Their relation to disorders of movement.*Lancet, 2*(7160), 1099-1105.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26*(22), 3279-3290.

Donoghue, J. P., & Wise, S. P. (1982). The motor cortex of the rat: cytoarchitecture and microstimulation mapping. *J Comp Neurol, 212*(1), 76-88.

Dromerick, A. W., Edwards, D. F., & Hahn, M. (2000). Does the application of constraint-induced movement therapy during acute rehabilitation reduce arm impairment after ischemic stroke? *Stroke, 31*(12), 2984-2988.

DRORBAUGH, J. E., & FENN, W. O. (1955). A barometric method for measuring ventilation in newborn infants. *Pediatrics, 16*(1), 81-87.

Duarte, J., Jimenez, R., Villar, I. C., Perez-Vizcaino, F., Jimenez, J., & Tamargo, J. (2001). Vasorelaxant effects of the bioflavonoid chrysin in isolated rat aorta. *Planta Med, 67*(6), 567-569.

Duarte, J., Perez Vizcaino, F., Utrilla, P., Jimenez, J., Tamargo, J., & Zarzuelo, A. (1993). Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. *Gen Pharmacol, 24*(4), 857-862.

Duncan, P. W., Propst, M., & Nelson, S. G. (1983).Reliability of the Fugl-Meyer assessment of sensorimotor recovery following cerebrovascular accident.*Phys Ther, 63*(10), 1606-1610.

Endres, M., Fan, G. P., Hirt, L., Fujii, M., Matsushita, K., Liu, X., et al. (2000).Ischemic brain damage in mice after selectively modifying BDNF or NT4 gene expression.*Journal of Cerebral Blood Flow and Metabolism, 20*(1), 139-144.

Ernfors, P., Bengzon, J., Kokaia, Z., Persson, H., & Lindvall, O. (1991). Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis.*Neuron, 7*(1), 165-176.

Fetz, E. E., Finocchio, D. V., Baker, M. A., & Soso, M. J. (1980). Sensory and motor responses of precentral cortex cells during comparable passive and active joint movements. *J Neurophysiol, 43*(4), 1070-1089.

Fiskum, G., Murphy, A. N., & Beal, M. F. (1999). Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. *J Cereb Blood Flow Metab, 19*(4), 351-369.

Fowler, S. C., & Mortell, C. (1992). Low doses of haloperidol interfere with rat tongue extensions during licking: a quantitative analysis. *Behav Neurosci, 106*(2), 386-395.

Fowler, S. C., & Wang, G. (1998). Chronic haloperidol produces a time- and dose-related slowing of lick rhythm in rats: implications for rodent models of tardive dyskinesia and neuroleptic-induced parkinsonism. *Psychopharmacology (Berl), 137*(1), 50-60.

Fridman, E. A., Hanakawa, T., Chung, M., Hummel, F., Leiguarda, R. C., & Cohen, L. G. (2004).Reorganization of the human ipsilesional premotor cortex after stroke.*Brain, 127*(Pt 4), 747-758.

Fugl-Meyer, A. R., Jääskö, L., Leyman, I., Olsson, S., & Steglind, S. (1975). The post-stroke hemiplegic patient. 1. a method for evaluation of physical performance. *Scand J Rehabil Med, 7*(1), 13-31.

Fuller, D. D., Johnson, S. M., Olson, E. B., & Mitchell, G. S. (2003). Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. *J Neurosci, 23*(7), 2993-3000.

Galli, R. L., Shukitt-Hale, B., Youdim, K. A., & Joseph, J. A. (2002). Fruit polyphenolics and brain aging: nutritional interventions targeting age-related neuronal and behavioral deficits. *Ann N Y Acad Sci, 959*, 128-132.

Garofalo, L., Ribeiro-da-Silva, A., & Cuello, A. C. (1992).Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals.*Proc Natl Acad Sci U S A, 89*(7), 2639-2643.

Gharbawie, O. A., Auer, R. N., & Whishaw, I. Q. (2006). Subcortical middle cerebral artery ischemia abolishes the digit flexion and closing used for grasping in rat skilled reaching. *Neuroscience, 137*(4), 1107-1118.

Gilmour, G., Iversen, S. D., O'Neill, M. F., & Bannerman, D. M. (2004). The effects of intracortical endothelin-1 injections on skilled forelimb use: implications for modelling recovery of function after stroke. *Behav Brain Res, 150*(1-2), 171-183.

Gonzalez, C. L., & Kolb, B. (2003). A comparison of different models of stroke on behaviour and brain morphology.*Eur J Neurosci, 18*(7), 1950-1962.

Greenough, W. T., & Maier, S. F. (1972). Molecular changes during learning: behavioral strategy--a comment on Gaito and Bonnet. *Psychol Bull, 78*(6), 480-482.

Greenough, W. T., Wood, W. E., & Madden, T. C. (1972). Possible memory storage differences among mice reared in environments varying in complexity. *Behav Biol, 7*(5), 717-722.

Gundersen, H. J., Bendtsen, T. F., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., et al. (1988).Some new, simple and efficient stereological methods and their use in pathological research and diagnosis.*APMIS, 96*(5), 379-394.

Hamdy, S., Aziz, Q., Rothwell, J. C., Power, M., Singh, K. D., Nicholson, D. A., et al. (1998). Recovery of swallowing after dysphagic stroke relates to functional reorganization in the intact motor cortex. *Gastroenterology, 115*(5), 1104-1112.

Han, J., Pollak, J., Yang, T., Siddiqui, M. R., Doyle, K. P., Taravosh-Lahn, K., et al. (2012). Delayed administration of a small molecule tropomyosin-related kinase B ligand promotes recovery after hypoxic-ischemic stroke. *Stroke, 43*(7), 1918-1924.

HEBB, D. O. (1949). Temperament in chimpanzees; method of analysis.*J Comp Physiol Psychol, 42*(3), 192-206.

Heller, A., Wade, D. T., Wood, V. A., Sunderland, A., Hewer, R. L., & Ward, E. (1987). Arm function after stroke: measurement and recovery over the first three months. *J Neurol Neurosurg Psychiatry, 50*(6), 714-719.

Hicks, S. P., & D'Amato, C. J. (1977). Locating corticospinal neurons by retrograde axonal transport of horseradish peroxidase. *Exp Neurol, 56*(2), 410-420.

Hosp, J. A., & Luft, A. R. (2011). Cortical plasticity during motor learning and recovery after ischemic stroke.*Neural Plast, 2011*, 871296.

Huang, C. S., Sirisko, M. A., Hiraba, H., Murray, G. M., & Sessle, B. J. (1988). Organization of the primate face motor cortex as revealed by intracortical microstimulation and electrophysiological identification of afferent inputs and corticobulbar projections. *J Neurophysiol, 59*(3), 796-818.

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci, 24*, 677-736.

Huang, E. J., & Reichardt, L. F. (2003). Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem, 72*, 609-642.

Imaki, J., Yoshida, K., & Yamashita, K. (1994). A developmental study of cyclic AMP-response element binding protein (CREB) by in situ hybridization histochemistry and immunocytochemistry in the rat neocortex. *Brain Res, 651*(1-2), 269-274.

Iwaniuk, A. N., Pellis, S. M., & Whishaw, I. Q. (1999). Is digital dexterity really related to corticospinal projections?: a re-analysis of the Heffner and Masterton data set using modern comparative statistics. *Behav Brain Res, 101*(2), 173-187.

Jacobs, K. M., & Donoghue, J. P. (1991). Reshaping the cortical motor map by unmasking latent intracortical connections. *Science, 251*(4996), 944-947.

Jang, S. W., Liu, X., Yepes, M., Shepherd, K. R., Miller, G. W., Liu, Y., et al. (2010). A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc Natl Acad Sci U S A, 107*(6), 2687-2692.

Jaworski, J., Mioduszewska, B., Sánchez-Capelo, A., Figiel, I., Habas, A., Gozdz, A., et al. (2003). Inducible cAMP early repressor, an endogenous antagonist of cAMP responsive element-binding protein, evokes neuronal apoptosis in vitro. *J Neurosci, 23*(11), 4519-4526.

Jørgensen, H. S., Nakayama, H., Raaschou, H. O., Vive-Larsen, J., Støier, M., & Olsen, T. S. (1995).Outcome and time course of recovery in stroke. Part II: Time course of recovery. The Copenhagen Stroke Study.*Arch Phys Med Rehabil, 76*(5), 406-412.

Kalia, L. V., Gingrich, J. R., & Salter, M. W. (2004). Src in synaptic transmission and plasticity. *Oncogene, 23*(48), 8007-8016.

Kaplan, D. R., & Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol, 10*(3), 381-391.

Karayannis, T., Huerta-Ocampo, I., & Capogna, M. (2007). GABAergic and pyramidal neurons of deep cortical layers directly receive and differently integrate callosal input. *Cereb Cortex, 17*(5), 1213-1226.

Karim, M., McCormick, K., & Kappagoda, C. T. (2000). Effects of cocoa extracts on endothelium-dependent relaxation. *J Nutr, 130*(8S Suppl), 2105S-2108S.

Keyvani, K., & Schallert, T. (2002). Plasticity-associated molecular and structural events in the injured brain.*J Neuropathol Exp Neurol, 61*(10), 831-840.

Killackey, H. P. (1973). Anatomical evidence for cortical subdivisions based on vertically discrete thalamic projections from the ventral posterior nucleus to cortical barrels in the rat. *Brain Res, 51*, 326-331.

Killackey, H. P., & Sherman, S. M. (2003). Corticothalamic projections from the rat primary somatosensory cortex. *J Neurosci, 23*(19), 7381-7384.

Kleim, J. A., Barbay, S., & Nudo, R. J. (1998). Functional reorganization of the rat motor cortex following motor skill learning.*J Neurophysiol, 80*(6), 3321-3325.

Kleim, J. A., Hogg, T. M., VandenBerg, P. M., Cooper, N. R., Bruneau, R., & Remple, M. (2004). Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. *J Neurosci, 24*(3), 628-633.

Kleim, J. A., Chan, S., Pringle, E., Schallert, K., Procaccio, V., Jimenez, R., et al. (2006). BDNF val66met polymorphism is associated with modified experience-dependent plasticity in human motor cortex. *Nat Neurosci, 9*(6), 735-737.

Kleim, J. A., & Jones, T. A. (2008). Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. *J Speech Lang Hear Res, 51*(1), S225-239.

Kleim, J. A., Jones, T. A., & Schallert, T. (2003). Motor enrichment and the induction of plasticity before or after brain injury. *Neurochem Res, 28*(11), 1757-1769.

Kleim, J. A., Lussnig, E., Schwarz, E. R., Comery, T. A., & Greenough, W. T. (1996). Synaptogenesis and Fos expression in the motor cortex of the adult rat after motor skill learning.*J Neurosci, 16*(14), 4529-4535.

Kleim, J. A., (2012). Neural Plasticity: Foundation for Neurorehabilitation. *Tanas Publishing,* Scottsdale, USA.

Klein, R., Conway, D., Parada, L. F., & Barbacid, M. (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell, 61*(4), 647-656.

Klintsova, A. Y., Dickson, E., Yoshida, R., & Greenough, W. T. (2004). Altered expression of BDNF and its high-affinity receptor TrkB in response to complex motor learning and moderate exercise. *Brain Res, 1028*(1), 92-104.

Koralek, K. A., Jensen, K. F., & Killackey, H. P. (1988). Evidence for two complementary patterns of thalamic input to the rat somatosensory cortex. *Brain Res, 463*(2), 346-351.

Krakauer, J. W. (2006). Motor learning: its relevance to stroke recovery and neurorehabilitation. *Curr Opin Neurol, 19*(1), 84-90.

Kryl, D., Yacoubian, T., Haapasalo, A., Castren, E., Lo, D., & Barker, P. A. (1999). Subcellular localization of full-length and truncated Trk receptor isoforms in polarized neurons and epithelial cells. *J Neurosci, 19*(14), 5823-5833.

Lang, C. E., DeJong, S. L., & Beebe, J. A. (2009). Recovery of thumb and finger extension and its relation to grasp performance after stroke. *J Neurophysiol, 102*(1), 451-459.

Lee, H., Gunraj, C., & Chen, R. (2007). The effects of inhibitory and facilitatory intracortical circuits on interhemispheric inhibition in the human motor cortex. *J Physiol, 580*(Pt.3), 1021-1032.

Legg, C. R., Mercier, B., & Glickstein, M. (1989). Corticopontine projection in the rat: the distribution of labelled cortical cells after large injections of horseradish peroxidase in the pontine nuclei. *J Comp Neurol, 286*(4), 427-441.

Lemon, R. N., & Griffiths, J. (2005). Comparing the function of the corticospinal system in different species: organizational differences for motor specialization? *Muscle Nerve, 32*(3), 261-279.

Levine, E. S., Crozier, R. A., Black, I. B., & Plummer, M. R. (1998). Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. *Proc Natl Acad Sci U S A, 95*(17), 10235-10239.

Liepert, J. (2010). Evidence-based therapies for upper extremity dysfunction.*Curr Opin Neurol, 23*(6), 678-682.

Liepert, J., Miltner, W. H., Bauder, H., Sommer, M., Dettmers, C., Taub, E., et al. (1998).Motor cortex plasticity during constraint-induced movement therapy in stroke patients.*Neurosci Lett, 250*(1), 5-8.

Lin, S. Y., Wu, K., Len, G. W., Xu, J. L., Levine, E. S., Suen, P. C., et al. (1999). Brain-derived neurotrophic factor enhances association of protein tyrosine phosphatase

PTP1D with the NMDA receptor subunit NR2B in the cortical postsynaptic density. *Brain Res Mol Brain Res, 70*(1), 18-25.

Lu, B. (2003). BDNF and activity-dependent synaptic modulation.*Learn Mem, 10*(2), 86-98.

Lu, B., & Gottschalk, W. (2000). Modulation of hippocampal synaptic transmission and plasticity by neurotrophins.*Prog Brain Res, 128*, 231-241.

Lubart, E., Leibovitz, A., Baumoehl, Y., Klein, C., Gil, I., Abramovitz, I., et al. (2005). Progressing stroke with neurological deterioration in a group of Israeli elderly. *Arch Gerontol Geriatr, 41*(1), 95-100.

Lukas, G., Brindle, S. D., & Greengard, P. (1971). The route of absorption of intraperitoneally administered compounds.*J Pharmacol Exp Ther, 178*(3), 562-564.

Lum, P. S., Mulroy, S., Amdur, R. L., Requejo, P., Prilutsky, B. I., & Dromerick, A. W. (2009). Gains in upper extremity function after stroke via recovery or compensation: Potential differential effects on amount of real-world limb use. *Top Stroke Rehabil, 16*(4), 237-253.

MacDonald, E., Van der Lee, H., Pocock, D., Cole, C., Thomas, N., VandenBerg, P. M., et al. (2007). A novel phosphodiesterase type 4 inhibitor, HT-0712, enhances rehabilitation-dependent motor recovery and cortical reorganization after focal cortical ischemia. *Neurorehabil Neural Repair, 21*(6), 486-496.

Mackenzie, C. (2011). Dysarthria in stroke: a narrative review of its description and the outcome of intervention. *Int J Speech Lang Pathol, 13*(2), 125-136.

Manabe, T. (2002). Does BDNF have pre- or postsynaptic targets? *Science, 295*(5560), 1651-1653.

Maness, L. M., Kastin, A. J., Weber, J. T., Banks, W. A., Beckman, B. S., & Zadina, J. E. (1994). The neurotrophins and their receptors: structure, function, and neuropathology. *Neurosci Biobehav Rev, 18*(1), 143-159.

Marsh, H. N., Scholz, W. K., Lamballe, F., Klein, R., Nanduri, V., Barbacid, M., et al. (1993). Signal transduction events mediated by the BDNF receptor gp 145trkB in primary hippocampal pyramidal cell culture. *J Neurosci, 13*(10), 4281-4292.

Martin, S. J., Grimwood, P. D., & Morris, R. G. (2000). Synaptic plasticity and memory:

an evaluation of the hypothesis. *Annu Rev Neurosci, 23*, 649-711.

Martino, R., Foley, N., Bhogal, S., Diamant, N., Speechley, M., & Teasell, R. (2005). Dysphagia after stroke: incidence, diagnosis, and pulmonary complications. *Stroke, 36*(12), 2756-2763.

Massa, S. M., Yang, T., Xie, Y., Shi, J., Bilgen, M., Joyce, J. N., et al. (2010). Small molecule BDNF mimetics activate TrkB signaling and prevent neuronal degeneration in rodents. *J Clin Invest, 120*(5), 1774-1785.

McAllister, A. K., Katz, L. C., & Lo, D. C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron, 17*(6), 1057-1064.

McHughen, S. A., Rodriguez, P. F., Kleim, J. A., Kleim, E. D., Marchal Crespo, L., Procaccio, V., et al. (2010). BDNF val66met polymorphism influences motor system function in the human brain. *Cereb Cortex, 20*(5), 1254-1262.

Metz, G. A., Antonow-Schlorke, I., & Witte, O. W. (2005). Motor improvements after focal cortical ischemia in adult rats are mediated by compensatory mechanisms. *Behav Brain Res, 162*(1), 71-82.

Meyer-Franke, A., Wilkinson, G. A., Kruttgen, A., Hu, M., Munro, E., Hanson, M. G., et al. (1998). Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron, 21*(4), 681-693.

Meyer, B. U., Röricht, S., Gräfin von Einsiedel, H., Kruggel, F., & Weindl, A. (1995). Inhibitory and excitatory interhemispheric transfers between motor cortical areas in normal humans and patients with abnormalities of the corpus callosum. *Brain, 118 (Pt 2)*, 429-440.

Minichiello, L., Klein, R., & McNamara, J. O. (2002). Immunohistochemical evidence of seizure-induced activation of trkB receptors in the mossy fiber pathway of adult mouse hippocampus. *J Neurosci, 22*(17), 7502-7508.

Monfils, M. H., & Teskey, G. C. (2004). Skilled-learning-induced potentiation in rat sensorimotor cortex: a transient form of behavioural long-term potentiation. *Neuroscience, 125*(2), 329-336.

Monfils, M. H., VandenBerg, P. M., Kleim, J. A., & Teskey, G. C. (2004). Long-term potentiation induces expanded movement representations and dendritic hypertrophy in layer V of rat sensorimotor neocortex. *Cereb Cortex, 14*(5), 586-593.

Montoya, C. P., Campbell-Hope, L. J., Pemberton, K. D., & Dunnett, S. B. (1991). The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. *J Neurosci Methods, 36*(2-3), 219-228.

Moore, A. N., Waxham, M. N., & Dash, P. K. (1996). Neuronal activity increases the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in rat hippocampus and cortex. *J Biol Chem, 271*(24), 14214-14220.

Moore, T. L., Killiany, R. J., Pessina, M. A., Moss, M. B., Finklestein, S. P., & Rosene, D. L. (2012). Recovery from ischemia in the middle-aged brain: a nonhuman primate model. *Neurobiol Aging, 33*(3), 619.e619-619.e624.

Morecraft, R. J., Geula, C., & Mesulam, M. M. (1992). Cytoarchitecture and neural afferents of orbitofrontal cortex in the brain of the monkey. *J Comp Neurol, 323*(3), 341-358.

Morimoto, K., Sato, K., Sato, S., Yamada, N., & Hayabara, T. (1998). Time-dependent changes in neurotrophic factor mRNA expression after kindling and long-term potentiation in rats. *Brain Res Bull, 45*(6), 599-605.

Morse, J. K., Wiegand, S. J., Anderson, K., You, Y., Cai, N., Carnahan, J., et al. (1993). Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. *J Neurosci, 13*(10), 4146-4156.

Murase, N., Duque, J., Mazzocchio, R., & Cohen, L. G. (2004). Influence of interhemispheric interactions on motor function in chronic stroke. *Ann Neurol, 55*(3), 400-409.

Murer, M. G., Raisman-Vozari, R., Yan, Q., Ruberg, M., Agid, Y., & Michel, P. P. (1999). Survival factors promote BDNF protein expression in mesencephalic dopaminergic neurons. *Neuroreport, 10*(4), 801-805.

Møller, A. R. (2006). Neural plasticity in tinnitus. *Prog Brain Res, 157*, 365-372.

Napieralski, J. A., Banks, R. J., & Chesselet, M. F. (1998). Motor and somatosensory deficits following uni- and bilateral lesions of the cortex induced by aspiration or thermocoagulation in the adult rat. *Exp Neurol, 154*(1), 80-88.

Neafsey, E. J., Bold, E. L., Haas, G., Hurley-Gius, K. M., Quirk, G., Sievert, C. F., et al. (1986). The organization of the rat motor cortex: a microstimulation mapping study. *Brain Res, 396*(1), 77-96.

Nedergaard, M., Vorstrup, S., & Astrup, J. (1986). Cell density in the border zone around old small human brain infarcts. *Stroke, 17*(6), 1129-1137.

Nedergaard, M., Gjedde, A., & Diemer, N. H. (1986). Focal ischemia of the rat brain: autoradiographic determination of cerebral glucose utilization, glucose content, and blood flow. *J Cereb Blood Flow Metab, 6*(4), 414-424.

Neeper, S. A., Gómez-Pinilla, F., Choi, J., & Cotman, C. W. (1996). Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. *Brain Res, 726*(1-2), 49-56.

Nemoto, K., Ohnishi, T., Mori, T., Moriguchi, Y., Hashimoto, R., Asada, T., et al. (2006). The Val66Met polymorphism of the brain-derived neurotrophic factor gene affects age-related brain morphology. *Neurosci Lett, 397*(1-2), 25-29.

Noskin, O., Krakauer, J. W., Lazar, R. M., Festa, J. R., Handy, C., O'Brien, K. A., et al. (2008). Ipsilateral motor dysfunction from unilateral stroke: implications for the functional neuroanatomy of hemiparesis. *J Neurol Neurosurg Psychiatry, 79*(4), 401-406.

Nudo, R. J. (2007). Postinfarct cortical plasticity and behavioral recovery.*Stroke, 38*(2 Suppl), 840-845.

Nudo, R. J., Jenkins, W. M., & Merzenich, M. M. (1990). Repetitive microstimulation alters the cortical representation of movements in adult rats. *Somatosens Mot Res, 7*(4), 463-483.

Nudo, R. J., & Milliken, G. W. (1996). Reorganization of movement representations in primary motor cortex following focal ischemic infarcts in adult squirrel monkeys.*J Neurophysiol, 75*(5), 2144-2149.

Nudo, R. J., Wise, B. M., SiFuentes, F., & Milliken, G. W. (1996).Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct.*Science, 272*(5269), 1791-1794.

O'Dell, M. W., Lin, C. C., & Harrison, V. (2009). Stroke rehabilitation: strategies to enhance motor recovery. *Annu Rev Med, 60*, 55-68.

Oujamaa, L., Relave, I., Froger, J., Mottet, D., & Pelissier, J. Y. (2009). Rehabilitation of arm function after stroke. Literature review. *Ann Phys Rehabil Med, 52*(3), 269-293.

Perez, M. A., Tanaka, S., Wise, S. P., Sadato, N., Tanabe, H. C., Willingham, D. T., et al.

(2007).Neural substrates of intermanual transfer of a newly acquired motor skill.*Curr Biol, 17*(21), 1896-1902.

Peterson, G. M., & Mc, G. D., Jr. (1951). Reeducation of handedness in the rat following cerebral injuries. *J Comp Physiol Psychol, 44*(2), 191-196.

Phillips, C. G., & Porter, R. (1977). Corticospinal neurones. Their role in movement.*Monogr Physiol Soc*(34), v-xii, 1-450.

Plautz, E. J., Milliken, G. W., & Nudo, R. J. (2000). Effects of repetitive motor training on movement representations in adult squirrel monkeys: role of use versus learning. *Neurobiol Learn Mem, 74*(1), 27-55.

Poduslo, J. F., & Curran, G. L. (1996). Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. *Brain Res Mol Brain Res, 36*(2), 280-286.

Pozzo-Miller, L. D., Gottschalk, W., Zhang, L., McDermott, K., Du, J., Gopalakrishnan, R., et al. (1999). Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci, 19*(12), 4972-4983.

Prakash, N., Cohen-Cory, S., & Frostig, R. D. (1996). RAPID and opposite effects of BDNF and NGF on the functional organization of the adult cortex in vivo. *Nature, 381*(6584), 702-706.

Raghavan, P., Santello, M., Gordon, A. M., & Krakauer, J. W. (2010). Compensatory motor control after stroke: an alternative joint strategy for object-dependent shaping of hand posture. *J Neurophysiol, 103*(6), 3034-3043.

Redline, S., Budhiraja, R., Kapur, V., Marcus, C. L., Mateika, J. H., Mehra, R., et al. (2007). The scoring of respiratory events in sleep: reliability and validity. *J Clin Sleep Med, 3*(2), 169-200.

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways.*Philos Trans R Soc Lond B Biol Sci, 361*(1473), 1545-1564.

Remple, M. S., Bruneau, R. M., VandenBerg, P. M., Goertzen, C., & Kleim, J. A. (2001). Sensitivity of cortical movement representations to motor experience: evidence that skill learning but not strength training induces cortical reorganization. *Behav Brain Res, 123*(2), 133-141.

Rioult-Pedotti, M. S., Friedman, D., Hess, G., & Donoghue, J. P. (1998). Strengthening of horizontal cortical connections following skill learning. *Nat Neurosci, 1*(3), 230-234.

Rocamora, N., Welker, E., Pascual, M., & Soriano, E. (1996).Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation.*J Neurosci, 16*(14), 4411-4419.

Rosene, D. L., Roy, N. J., & Davis, B. J. (1986). A cryoprotection method that facilitates cutting frozen sections of whole monkey brains for histological and histochemical processing without freezing artifact. *J Histochem Cytochem, 34*(10), 1301-1315.

Rosenthal, A., Goeddel, D. V., Nguyen, T., Martin, E., Burton, L. E., Shih, A., et al. (1991). Primary structure and biological activity of human brain-derived neurotrophic factor. *Endocrinology, 129*(3), 1289-1294.

Saarelainen, T., Lukkarinen, J. A., Koponen, S., Grohn, O. H. J., Jolkkonen, J., Koponen, E., et al. (2000). Transgenic mice overexpressing truncated trkB neurotrophin receptors in neurons show increased susceptibility to cortical injury after focal cerebral ischemia. *Molecular and Cellular Neuroscience, 16*(2), 87-96.

Sanderson, K. J., Welker, W., & Shambes, G. M. (1984). Reevaluation of motor cortex and of sensorimotor overlap in cerebral cortex of albino rats. *Brain Res, 292*(2), 251-260.

Schabitz, W. R., Berger, C., Kollmar, R., Seitz, M., Tanay, E., Kiessling, M., et al. (2004). Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. *Stroke, 35*(4), 992-997.

Schaechter, J. D. (2004). Motor rehabilitation and brain plasticity after hemiparetic stroke. *Prog Neurobiol, 73*(1), 61-72.

Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology, 39*(5), 777-787.

Schallert, T., Kozlowski, D. A., Humm, J. L., & Cocke, R. R. (1997).Use-dependent structural events in recovery of function.*Adv Neurol, 73*, 229-238.

Schiene, K., Bruehl, C., Zilles, K., Qü, M., Hagemann, G., Kraemer, M., et al. (1996). Neuronal hyperexcitability and reduction of GABAA-receptor expression in the

surround of cerebral photothrombosis. *J Cereb Blood Flow Metab, 16*(5), 906-914.

Schmid, D. A., Yang, T., Ogier, M., Adams, I., Mirakhur, Y., Wang, Q., et al. (2012). A TrkB small molecule partial agonist rescues TrkB phosphorylation deficits and improves respiratory function in a mouse model of Rett syndrome. *J Neurosci, 32*(5), 1803-1810.

Schäbitz, W. R., Sommer, C., Zoder, W., Kiessling, M., Schwaninger, M., & Schwab, S. (2000). Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. *Stroke, 31*(9), 2212-2217.

Schultke, E., Griebel, R. W., & Juurlink, B. H. (2010). Quercetin attenuates inflammatory processes after spinal cord injury in an animal model. *Spinal Cord, 48*(12), 857-861.

Seo, N. J., Rymer, W. Z., & Kamper, D. G. (2010). Altered digit force direction during pinch grip following stroke. *Exp Brain Res, 202*(4), 891-901.

Serradimigni, A., Jouve, R., & Spriet, A. (1985). [Evaluation of the effectiveness of medical treatment of intermittent claudication].*J Mal Vasc, 10*(2), 108-116.

Shieh, P. B., Hu, S. C., Bobb, K., Timmusk, T., & Ghosh, A. (1998). Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron, 20*(4), 727-740.

Siironen, J., Juvela, S., Kanarek, K., Vilkki, J., Hernesniemi, J., & Lappalainen, J. (2007). The Met allele of the BDNF Val66Met polymorphism predicts poor outcome among survivors of aneurysmal subarachnoid hemorrhage. *Stroke, 38*(10), 2858-2860.

Sirtori, V., Corbetta, D., Moja, L., & Gatti, R. (2009).Constraint-Induced Movement Therapy for Upper Extremities in Patients With Stroke.*Stroke*.

Spencer, J. P. (2007). The interactions of flavonoids within neuronal signalling pathways. *Genes Nutr, 2*(3), 257-273.

Spencer, J. P. (2008). Flavonoids: modulators of brain function? *Br J Nutr, 99 E Suppl 1*, ES60-77.

Ste-Marie, L., Vachon, P., Vachon, L., Bemeur, C., Guertin, M. C., & Montgomery, J. (2000). Hydroxyl radical production in the cortex and striatum in a rat model of focal cerebral ischemia. *Can J Neurol Sci, 27*(2), 152-159.

Stoilov, P., Castren, E., & Stamm, S. (2002). Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. *Biochem Biophys Res Commun, 290*(3), 1054-1065.

Stoop, R., & Poo, M. M. (1995). Potentiation of transmitter release by ciliary neurotrophic factor requires somatic signaling. *Science, 267*(5198), 695-699.

Sunderland, A. (2000). Recovery of ipsilateral dexterity after stroke.*Stroke, 31*(2), 430-433.

Szeszko, P. R., Lipsky, R., Mentschel, C., Robinson, D., Gunduz-Bruce, H., Sevy, S., et al. (2005). Brain-derived neurotrophic factor val66met polymorphism and volume of the hippocampal formation. *Mol Psychiatry, 10*(7), 631-636.

Tao, X., Finkbeiner, S., Arnold, D. B., Shaywitz, A. J., & Greenberg, M. E. (1998). Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron, 20*(4), 709-726.

Tiurenkov, I. N., Voronkov, A. V., Slientsans, A. A., Petrova, E. V., & Dorkina, E. G. (2010). [Relationship between the antioxidant effect of flavonoids and their effect on the vasodilating function of endothelium under endothelial dysfunction conditions]. *Eksp Klin Farmakol, 73*(10), 14-16.

Tolwani, R. J., Buckmaster, P. S., Varma, S., Cosgaya, J. M., Wu, Y., Suri, C., et al. (2002). BDNF overexpression increases dendrite complexity in hippocampal dentate gyrus. *Neuroscience, 114*(3), 795-805.

Trapl, M., Eckhardt, R., Bosak, P., & Brainin, M. (2004). [Early recognition of speech and speech-associated disorders after acute stroke]. *Wien Med Wochenschr, 154*(23-24), 571-576.

Valverde, F. (1966). The pyramidal tract in rodents. A study of its relations with the posterior column nuclei, dorsolateral reticular formation of the medulla oblongata, and cervical spinal cord. (Golgi and electron microscopic observations). *Z Zellforsch Mikrosk Anat, 71*(3), 298-363.

Van der Lee, J. H., Beckerman, H., Lankhorst, G. J., & Bouter, L. M. (1999). Constraint-induced movement therapy. *Arch Phys Med Rehabil, 80*(12), 1606-1607.

Velozo, C. A., & Woodbury, M. L. (2011). Translating measurement findings into rehabilitation practice: an example using Fugl-Meyer Assessment-Upper Extremity with patients following stroke. *J Rehabil Res Dev, 48*(10), 1211-1222.

Wade, D. T. (1989).Measuring arm impairment and disability after stroke.*Int Disabil Stud, 11*(2), 89-92.

Wade, D. T., & Hewer, R. L. (1983). Why admit stroke patients to hospital? *Lancet, 1*(8328), 807-809.

Walton, M. R., & Dragunow, I. (2000). Is CREB a key to neuronal survival? *Trends Neurosci, 23*(2), 48-53.

Warlow, C., Sudlow, C., Dennis, M., Wardlaw, J., & Sandercock, P. (2003). Stroke. *Lancet, 362*(9391), 1211-1224.

Whishaw, I. Q., Pellis, S. M., Gorny, B. P., & Pellis, V. C. (1991). The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. *Behav Brain Res, 42*(1), 77-91.

Whishaw, I. Q., Sarna, J. R., & Pellis, S. M. (1998). Evidence for rodent-common and species-typical limb and digit use in eating, derived from a comparative analysis of ten rodent species.*Behav Brain Res, 96*(1-2), 79-91.

Whishaw, I. Q., & Coles, B. L. (1996). Varieties of paw and digit movement during spontaneous food handling in rats: postures, bimanual coordination, preferences, and the effect of forelimb cortex lesions. *Behav Brain Res, 77*(1-2), 135-148.

Whishaw, I. Q., Suchowersky, O., Davis, L., Sarna, J., Metz, G. A., & Pellis, S. M. (2002). Impairment of pronation, supination, and body co-ordination in reach-to-grasp tasks in human Parkinson's disease (PD) reveals homology to deficits in animal models. *Behav Brain Res, 133*(2), 165-176.

Winstein, C. J., Merians, A. S., & Sullivan, K. J. (1999). Motor learning after unilateral brain damage. *Neuropsychologia, 37*(8), 975-987.

Wise, S. P., & Jones, E. G. (1977). Cells of origin and terminal distribution of descending projections of the rat somatic sensory cortex. *J Comp Neurol, 175*(2), 129-157.

Wittenberg, G. F., Chen, R., Ishii, K., Bushara, K. O., Eckloff, S., Croarkin, E., et al. (2003). Constraint-induced therapy in stroke: magnetic-stimulation motor maps and cerebral activation. *Neurorehabil Neural Repair, 17*(1), 48-57.

Wittenberg, G. F., & Schaechter, J. D. (2009). The neural basis of constraint-induced movement therapy.*Curr Opin Neurol, 22*(6), 582-588.

Wolf, S. L., LeCraw, D. E., & Barton, L. A. (1989). Comparison of motor copy and targeted biofeedback training techniques for restitution of upper extremity function among patients with neurologic disorders.*Phys Ther, 69*(9), 719-735.

Wolf, S. L., Winstein, C. J., Miller, J. P., Taub, E., Uswatte, G., Morris, D., et al. (2006). Effect of constraint-induced movement therapy on upper extremity function 3 to 9 months after stroke: the EXCITE randomized clinical trial. *JAMA, 296*(17), 2095-2104.

Wu, A., Molteni, R., Ying, Z., & Gomez-Pinilla, F. (2003). A saturated-fat diet aggravates the outcome of traumatic brain injury on hippocampal plasticity and cognitive function by reducing brain-derived neurotrophic factor. *Neuroscience, 119*(2), 365-375.

Yamada, K., & Nabeshima, T. (2003). Brain-derived neurotrophic factor/TrkB signaling in memory processes. *J Pharmacol Sci, 91*(4), 267-270.

Yanamoto, H., Mizuta, I., Nagata, I., Xue, J. H., Zhang, Z., & Kikuchi, H. (2000). Infarct tolerance accompanied enhanced BDNF-like immunoreactivity in neuronal nuclei. *Brain Research, 877*(2), 331-344.

Yang, T., Bernabeu, R., Xie, Y., Zhang, J. S., Massa, S. M., Rempel, H. C., et al. (2003). Leukocyte antigen-related protein tyrosine phosphatase receptor: a small ectodomain isoform functions as a homophilic ligand and promotes neurite outgrowth. *J Neurosci, 23*(8), 3353-3363.

Yeo, G. S., Connie Hung, C. C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., et al. (2004). A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. *Nat Neurosci, 7*(11), 1187-1189.

Zhang, Y. H., Chi, X. X., & Nicol, G. D. (2008). Brain-derived neurotrophic factor enhances the excitability of rat sensory neurons through activation of the p75 neurotrophin receptor and the sphingomyelin pathway. *J Physiol, 586*(13), 3113-3127.

Zhao, L. R., Risedal, A., Wojcik, A., Hejzlar, J., Johansson, B. B., & Kokaia, Z. (2001). Enriched environment influences brain-derived neurotrophic factor levels in rat forebrain after focal stroke. *Neurosci Lett, 305*(3), 169-172.

Ziemann, U. (2005). Improving disability in stroke with RTMS. *Lancet Neurol, 4*(8), 454-455.

Ziemann, U., Muellbacher, W., Hallett, M., & Cohen, L. G. (2001).Modulation of practice-dependent plasticity in human motor cortex.*Brain, 124*(Pt 6), 1171-1181