The Dissertation Committee for Amber Jo O'Bryant certifies that this is the approved version of the following dissertation:

Promoting restorative neural plasticity with motor cortical stimulation after stroke-like injury in rats.

Committee:

Theresa Jones, Supervisor

Timothy Schallert

Yvon Delville

Alex Huk

Andrew Dunn

Promoting restorative neural plasticity with motor cortical stimulation after stroke-like injury in rats.

by

Amber Jo O'Bryant, B.A.

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Dedication

To my wonderful husband, Collin O'Bryant. Nothing ever seems real or all that important until I can share it with you.

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Promoting restorative neural plasticity with motor cortical stimulation after stroke-like injury in rats.

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In adult rats, following unilateral stroke-like injury to the motor cortex, there is significant loss of function in the forelimb contralateral to the ischemic damage. In the remaining motor cortex, changes in neuronal activation patterns and connectivity are induced following motor learning and rehabilitation in the brains of adult animals. Rehabilitative training promotes functional recovery of the impaired forelimb following motor cortical strokes; however, its benefits are most efficacious when coupled with other rehabilitative treatments. Multiple lines of evidence suggest that focal cortical electrical stimulation (CS) enhances the effectiveness of rehabilitative training (RT) and promotes changes in neural activation and plasticity in the peri-lesion motor cortex. Specific examples of plastic events include increases in dendritic and synaptic density in the peri-lesion cortex following CS/RT compared to rehabilitative training alone.

The objective of these studies was to investigate which conditions, such as timing and method of delivery of CS, when coupled with RT, are most efficacious in promoting neuronal plasticity and functional recovery of the impaired forelimb following ischemic

cortical injury in adult animals. The central hypothesis of these dissertation studies is that, following unilateral stroke-like injury, CS improves the functional recovery of the impaired forelimb and promotes neural plasticity in remaining motor cortex when combined with RT. This hypothesis was tested in a series of experiments manipulating post-ischemic behavioral experience with the impaired forelimb. Adult rats were proficient in a motor skill (Single Pellet Retrieval Task) and received ischemic motor cortex lesion that caused impairments in the forelimb. Rats received daily rehabilitative training on a tray reaching task with or without concurrent cortical stimulation. Epidural cortical stimulation, when paired with rehabilitative training, resulted in enhanced reaching performance compared to RT alone when initiated 14 days after lesion. These results were found to be maintained well after the treatment period ended. Rats tested 9-10 months post-rehabilitative training on the single pellet retrieval task continued to have greater reaching performance compared to RT alone. However, delayed onset of rehabilitative training (3 months post-infarct) indicated that CS does not further improve forelimb function compared to RT along. It was further established that CS delivered over the intact skull (transcranial stimulation) of the lesioned motor cortex was not a beneficial adjunct to rehabilitative training.

Together these dissertation studies provide insight into the effectiveness and limitations of CS on behavioral recovery. The findings in these studies are likely to be important for understanding how post-stroke behavioral interventions and adjunct therapies could be used to optimize brain reorganization and functional outcome.

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Chapter 1

Introduction

1.1 Introduction

Stroke is the third leading cause of death in the United States, and it is estimated that 795,000 Americans have a stroke each year (American Heart Association, 2010). At the level of the brain, a stroke is caused by the lack of blood and oxygen to cortical tissue resulting in the loss of function and death of the affected brain cells. The majority of strokes are ischemic in nature, caused by an interruption of the blood supply, and a minority of strokes are hemorrhagic, caused by bleeding in the brain. The lack of blood, oxygen and nutrients to cortical tissue results in permanent neurological damage due to a cascade of metabolic changes in the affected tissue. This results in permanent neurological damage and, depending on the brain region affected, impairments in speech, vision and body movements. Loss of function in body movement includes disuse of the impaired side of the body and an increase in compensatory reliance on the less-affected side of the body (Schallert et al., 1997).

Although studies have indicated that early treatment, such as rehabilitative therapy, is beneficial following stroke damage, there are still many people with long-term impairments of affected limbs, particularly in dexterous use of the hands and fingers, which are typically very difficult to rehabilitate (Taub et al., 2003). One major focus of stroke research in animal models is the use of rehabilitative training or other motor learning manipulations that promote functional recovery of the impaired limb (reviewed in Johansson 2000; Jones et al., 1998; 2003). Coupling rehabilitative training with

cortical electrical stimulation (CS) has been shown to enhance functional recovery of the impaired forelimb and induce neuronal plasticity in the peri-lesion cortex (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003; Hsu, Adkins and Jones, 2008). The purpose of this research is to investigate potential adaptive plasticity in the peri-lesion cortex and to determine its relationship to functional recovery of the impaired forelimb following CS and rehabilitative training (CS/RT). A better understanding of the plasticity and structural effects of CS is needed to optimize its therapeutic potential.

In adult rats, focal unilateral ischemic damage to the motor cortex can result in long-lasting impairments of the contralesional forelimb. Behavioral improvements in sensorimotor performance, as assessed by a skilled reach-to-grasp task, occur in rats receiving rehabilitative training following ischemic injury. Accumulating evidence suggests that cortical stimulation (CS), delivered over the peri-infarct cortex via an implanted electrode, coupled with rehabilitative training (RT) improves functional recovery of the impaired forelimb compared to RT alone. Previous research has also found increases in dendritic and synaptic density (Adkins-Muir and Jones, 2003; Adkins et a;., 2006; 2008; Hsu and Jones, 2008) and changes in motor map representations (Kleim et al., 2003; Teskey et al., 2003) in the peri-lesion cortex following CS/RT compared to RT alone. These studies support that CS alters neural efficacy and reorganization more so than training alone. In addition, these studies suggest that specific CS parameters are more efficacious than others and that repetitive CS is necessary for functional improvements and structural changes in peri-lesion motor cortex. Together

these findings support the general hypothesis that the functional recovery of the impaired forelimb is linked to plasticity of neuronal structure in the peri-lesion cortex.

The central hypothesis of these dissertation studies is that, following unilateral stroke-like injury, CS improves the functional recovery of the impaired forelimb and promotes neural plasticity in remaining motor cortex when combined with RT. This hypothesis was tested in a series of experiments that manipulated post-lesion behavioral experience with rehabilitative training of the impaired forelimb, with or without concurrent cortical stimulation. The first study (Chapter 3) examined the role of the ipsilesional forelimb in affecting reaching performance of the contralesional forelimb. The second study (Chapter 4) tested the long-term effects of epidurally administered cortical stimulation paired with rehabilitative training. A third study (Chapter 5) looked at whether the beneficial effects of cortical stimulation and rehabilitative training would occur if initiated 3-months post-injury. A fourth study (Chapter 6) tested whether cortical stimulation is as effective when delivered through the skull (transcranially) as it is when delivered epidurally.

The remainder of this chapter focuses on background research that supports the approach adopted for these studies. First, skilled reach training is discussed as a model for outcome assessments of behavioral recovery. Next, the behavioral and neural effects of motor learning and unilateral brain damage are considered. This section is then concluded with discussions of the effects of rehabilitation and electrical cortical stimulation after brain damage. These topics set up the basis for the questions asked in

these dissertation studies.

1.2 Motor Learning Induced Behavioral and Cortical Changes in Motor Cortex

In intact animals, studies in animals indicate structural, biological and anatomical changes occur in cortex following learning and behavioral manipulations (Butefish, 2004; Kleim et al., 2003; Rioult-Pedotti et al., 2000, reviewed in Nudo, 2003). Acrobatic training, a type of motor learning, results in synaptic plasticity in the cerebellum (Black et al., 1990) and layer II/III (Kleim et al., 1996) and layer V (Jones et al., 1999) of the motor cortex compared to animals that receive repetitive exercise. Motor learning also results in an expansion of motor map representations that support, at least in part, performance of the learned task (Kleim et al., 1998;2002). The motor cortex undergoes synaptogenesis and plasticity of movement representations in response to motor learning, but not in response to repetitive exercise or motor movements that do not require motor learning (Kleim et al., 1998; 2002). Unilateral skilled reaching results in increases in dendritic arborization and synaptogenesis (Greenough et al., 1985; Kleim et al., 1996; Jones et al., 1999; Kleim et al., 2004) and motor map reorganization (Kleim et al., 1998; 2004; Nudo et al.,, 1996; Remple et al., 2001). Motor learning also results in increases in dendritic processes as assessed by microtubule associated protein-2 (MAP-2), a dendritic cytoskeletal protein (Bury and Jones, 2002; Adkins-Muir and Jones, 2003), and increases in the motor cortical expression of synaptophysin, a synaptic vesicle protein (Derksen et al., 2006). Motor learning, or the acquisition of a motor skill, appear to be important for inducing structural plasticity in the motor cortex, but not repetitive motor movements

(Plautz et al., 2000). Unilateral skilled reach training, as used in these dissertation studies, are sensitive to distal forelimb performance and result in structural and plasticity changes in the cortex opposite the trained limb. In rats and non-human primate, motor map reorganization following skilled reach training suggest that motor learning expands the wrist/digit representations and the caudal forelimb area necessary for skilled reaching (Kleim et al., 1998; Nudo et al., 1996). Motor learning-induced changes in motor cortex opposite the trained limb suggest that the motor map reorganization and the structural changes in plasticity are necessary for the integrity of skilled reach training These studies suggest that motor skill training-induced changes in structural plasticity and reorganization of motor maps contribute to the functional use of the forelimb.

1.3 Effects of Unilateral Ischemic Injury to the Motor Cortex

1.3.1 Behavioral Changes Following Sensorimotor Cortex (SMC) Lesions

Motor skill learning is an important aspect of stroke research because it has been implicated in the functional recovery of the stroke-impaired limb. Previous research has demonstrated that unilateral ischemic injury to the motor cortex results in motor impairments in the contralesional limb and an increased reliance on the unimpaired limb (Jones and Schallert, 1994). Unilateral cerebral ischemic lesions result in impairments in skilled reach-to-grasp tasks (Montoya et al., 1991) and robust, long-lasting deficits in somatosensory function (Napieralski et al., 1998; Schallert et al., 2000). Compensatory reliance on the unimpaired limb results in structural plasticity in the contralesional homotopic cortex, including increases in dendritic arborization and synapse number per

neuron (Jones et al., 1996) and the numbers of multiple synaptic boutons and synapses with perforated postsynaptic densities in layer V of the contralesional SMC (Jones, 1999). These changes are thought to be indicative of increases in synaptic efficacy and improvements in behavioral performance of the unimpaired forelimb. However, the increased reliance and enhanced behavioral function of the unimpaired forelimb results in decreased use of the impaired limb (Bury and Jones, 2002) and may interfere with functional recovery and exacerbate learned non-use of the impaired forelimb (Allred et al., 2005a; Taub et al., 2006).

Behaviorally-induced changes in the peri-infarct cortex are important for functional improvements of the impaired forelimb or paretic hand. In humans, after stroke, functional activation of the peri-infarct motor cortex is linked with improved motor recovery of the affected hand (Chollet et al., Weillar et al., 1993; Cramer et al., 2000). In rats and monkeys, functional recovery occurs following reorganization of motor maps and movement representations in the peri-infarct area (Castro-Alamancos and Borrel, 1995; Nudo et al., 1996). Following unilateral ischemic damage to the SMC, training on the Acrobatic task (motor training) reduced tissue loss in the peri-infarct area and an increase in synaptic plasticity in contralesional cortex (Chu & Jones, 2000; Jones et al., 1999). In addition, reach training improved fine digit movements and spared the loss of the peri-infarct forelimb representation areas (Biernaskie and Corbett, 2001; Glimour et al.,2005; VandenBerg and Kleim, 2001; Whishaw, 2000).

A recent study found that administration of bilateral repetitive transcranial magnetic stimulation, which inhibits the unaffected cortex and stimulates the lesioned

cortex, resulted in improved performance of the paretic hand following motor training (Takeuchi et al., 2009). Inhibition of cortical activity by low-frequency repetitive transcranial magnetic stimulation (rTMS) of the contralesional motor cortex improved performance of the impaired hand (Nowak et al., 2008). In addition, constraint induced movement therapy (CIMT), which limits the use of the less-affected hand, resulted in functional improvements of the affected hand (Taub et al., 1993; Ro et al., 2006; Wolf et al., 2006) and a reorganization of motor movements in ipsilesional motor cortex (Ro et al., 2006). This seems important in light of recent findings indicating competitive interactions between the hemispheres that become unbalanced by unilateral brain damage and which might be targeted for therapeutic interventions (Rushmore et al., 2006; Takeuchi et al., 2005; Voller et al., 2006; Ward and Cohen, 2004).

These studies suggest that unilateral ischemic injury to the motor cortex results in permanent deficits in behavior and motor performance. In human and animals studies, the peri-infarct area appears to be important for mediating behavioral recovery and functional improvement of the impaired forelimb or paretic hand.

1.3.2 SMC Lesions Induce Structural Changes in Motor Cortex

Unilateral injury to the SMC results in plasticity in the ipsilesional and contralesional cortex. In the ipsi-lesional cortex there are cellular and structural changes in the peri-infarct area (Keyvani and Schallert, 2002). For example, increases in immediate early genes (reviewed in Keyvani and Schallert, 2002) and the expression of growth-promoting proteins and dendritic growth (Li et al., 1998) have all been found in the peri-infarct region. Immediately following SMC damage, the tissue surrounding or

connected to the ischemic injury site undergoes excitotoxic and neurotoxic degeneration (e.g., Doyle, Simon, & Stenzel-Poore, 2008). Cortical stroke results in both growth inhibitory and growth promoting changes in the peri-infarct area (Carmichael, 2006). Previous research indicates that dendritic and synaptic plasticity occur in the contralesional cortex following SMC damage. For example, increases in dendritic (Adkins et al., 2004; Jones and Schallert, 1992; Jones et al., 1996) and synaptic plasticity (Jones et al., 1996, Jones, 1999; Luke et al., 2003) and increases in the density of dendritic processes immunoreactive for microtubule associated protein, MAP2 (Bury and Jones, 2002; Allred and Jones, 2004) have been found in contralateral cortex (Cheng, Tong, and McNeil, 1998; Jones and Schallert, 1992; Jones, Kleim and Greenough, 1996; Jones et al., 1996; Jones, 1999; Luke et al., 2003; McNeil et al., 1999; Stroemer et al., 1995).

SMC damage results in cellular and structural changes in the contralesional, homotopic motor cortex and the ipsilesional, peri-infarct area. A remaining question is whether focused motor rehabilitative training of the impaired forelimb in rats can result in behavioral recovery and induce beneficial structural changes in the peri-infarct motor cortex.

1.4 Rehabilitative Training

Previous research has shown that rehabilitative training results in neuroplastic changes in the cortex and contributes to behavioral improvements. After unilateral middle cerebral artery occlusion (MCAO) or more focused stroke-like damage, complex

rehabilitative training (enriched environment, acrobatic training) enhances synaptic and dendritic plasticity in the intact cortex (Jones et al.,1999; Biernaskie and Corbett, 2001) and decreases the loss of remaining tissue in the peri-lesion cortex (Chu and Jones, 2000). In monkeys, focused rehabilitation of the impaired forelimb after small ischemic lesions promotes motor map reorganization within adjacent cortical tissue and prevented further loss of hand territory in the undamaged cortical tissue adjacent to the infarct, suggesting that rehabilitative training may contribute to reorganization of this tissue and to motor recovery (Nudo et al., 1996).

The effectiveness of rehabilitative training is, at least in part, dependent on its initiation after stroke-like injury. A study by Biernaskie et al. (2004) demonstrated that animals receiving early (beginning post-ischemic injury day 5) rehabilitative training combined with enriched environment have greater improvements in behavioral assays compared to animals that receive it at a later time point (post-ischemic day 30). Additionally, Barbay et al. (2006) found that although delayed rehabilitative training (30 days post-ischemia) resulted in recovery of motor skills, there was a diminished capacity to reorganize peri-infarct cortex compared to the early onset training.

Although motor rehabilitation improves some aspects of motor performance, there can be limitations to the behavioral improvements. As mentioned, the effectiveness of motor rehabilitative training appears to be time-dependent. Initiation of rehabilitative training early after stroke-like damage results in structural changes and functional recovery of the impaired limb (Biernaskie et al., 2004; Barbay et al., 2006). Following unilateral stroke-like damage, rehabilitative training does improve function of the

impaired forelimb, however, these improvements are limited and do not approach pre-operative reaching performance (Adkins-Muir and Jones, 2003; Chapters 3, 4 & 5). Thus, the use of adjunct therapies may be particularly important for maximizing the rehabilitative training efforts.

1.5 Rehabilitative Training and Cortical Stimulation (CS)

Studies in human and animal models suggest that electrical stimulation of the motor cortex facilitates recovery of function and improved performance. CS combined with RT has been shown to be safe and efficacious. CS enhances both the cortical and behavioral effects of RT. In rats and monkeys, following stroke-like injury, CS of the motor cortex combined with rehabilitative training changes functional activity patterns and neuronal structure in the peri-infarct area and improves behavioral performance of the impaired limb (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Adkins et al., 2008; Kleim et al., 2003; Plautz et al., 2003; Plautz and Nudo, 2005; Boychuk et al., 2010; Zhou et al., 2010). CS results in increases in the size of movement representations (Kleim et al., 2003; Plautz et al., 2003), increases in synaptic density (Adkins, Hsu and Jones, 2008; Adkins et al., 2006) and increases in dendritic processes in the peri-infarct area (Adkins-Muir and Jones, 2003; Zhou et al., 2010). CS concurrent with rehabilitative training also results in a decrease in movement thresholds (the amount of electrical current needed to elicit a movement) and enhances motor cortical evoked potentials (Teskey et al., 2003). Human clinical trials have found that epidural cortical stimulation combined with motor training is safe and, at least sometimes, efficacious in improving

motor function after stroke (Brown et al., 2006; Harvey and Winstein, 2009; Huang et al., 2008; Levy et al., 2008a,b).

Recently, a study investigating the protective effects of repetitive transcranial magnetic stimulation (rTMS) following ischemic damage found that rats receiving rTMS had less apoptosis and infarct volume compared to control animals (Gao et al., 2010). Non-invasive stimulation approaches, such as repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS), have also shown significant potential for improving motor function following stroke in humans (Hummel and Cohen, 2006; Khedr et al., 2005; Nair et al., 2007; Pascual-Leone, 2006; Yozbatiran et al., 2008). Noninvasive stimulation may also be a beneficial adjunct when paired with rehabilitative treatments. In humans, the combination of tDCS and occupational therapy (Nair et al., 2007), or rTMS and physical therapy (Khedr et al., 2005), has been shown to result in more enduring functional benefits than therapy alone. In addition, tDCS delivered to the motor cortex improved motor function of the paretic hand in tasks relevant to daily activities (Hummel et al., 2005). Recently, higher frequencies of rTMS applied to the ipsilesional motor cortex were found to be safe and resulted in modest improvements in behavioral function of the impaired hand (Yozbatiran et al., 2008). Furthermore, high frequency rTMS applied to the injured motor cortex, when combined with a complex, sequential motor task, was found to improve motor learning in human stroke survivors during the chronic period (Kim et al., 2006). This convergence of findings points to potential commonalities in the neural mechanisms of epidural CS and those of tDCS and rTMS (Hummel and Cohen, 2006).

In humans, CS stimulation can also be used to improve performance of the paretic hand on a motor skill task by inhibiting or disrupting neuronal activity in the contralesional hemisphere. As previously mentioned, following ischemic damage, there is an increased reliance on the intact side and structural changes in contralesional cortex. In humans, rTMS stimulation to the contralesional hemisphere results in a decrease in overactivity of the contralesional cortex and motor improvements of the affected hand (Liepert et al., 2007; Nowak et al., 2008). Bihemispheric tDCS, which increases excitability in ipsilesional motor cortex and decreases excitability in the contralesional motor cortex, results in significant motor improvements (Lindenberg et al., 2010). These studies, along with previous findings of increased behavioral reliance on the intact forelimb concurrent with plasticity in the contralesional cortex, suggest that inhibition of the contralesional hemisphere and intact hand or forelimb can allow for greater improvements in motor performance and ipsilesional plasticity.

Although research suggests that CS coupled with rehabilitative training after cortical injury improves performance and increases plasticity, little is known about how cortical stimulation induces structural reorganization. Previous research indicates CS is neuroprotective during ischemic damage (Glickstein et al., 2003; Maesawa et al., 2004). In addition, CS may enhance neuronal activation by increasing the likelihood that neurons will fire. Cortical and hippocampal neurons stimulated with low-amplitude electrical current resulted in lower membrane potentials and an increase in spontaneous depolarizations (Bikson et al., 2004; Purpura and McMurtry, 1965; Nowak and Bullier, 1998). Therefore, CS may contribute to the activity of neuronal networks in the peri-

infarct area, thus enhancing the likelihood that depolarization will occur. When CS is paired with motor rehabilitative training, the enhancement of neuronal activity may be further strengthened, resulting in greater ability of the motor cortex to elicit movement, thus increasing functional recovery of the impaired forelimb compared to RT alone. As previously mentioned, CS combined with rehabilitative training alters functional activity patterns, increases the size of motor map representations in peri-infarct motor cortex, increases synaptic density and dendritic processes in peri-infarct area, decreases movement thresholds and enhances motor cortical evoked potentials (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). These studies suggest that CS induces activity-dependent structural changes in plasticity and a reorganization of neural networks important for functional improvements of the impaired forelimb.

1.5 Conclusions

Unilateral cortical injury results in impairments in reaching performance on a motor skill task. Changes in neuronal activation patterns are induced following motor learning and rehabilitation in the brains of adult animals. Rehabilitative training promotes functional recovery following motor cortical strokes. However, its benefits are most efficacious when coupled with other rehabilitative treatments. Multiple lines of evidence suggest that focal cortical electrical stimulation (CS) enhances the effectiveness of rehabilitative training (RT) and promotes changes in neural activation and plasticity in the peri-lesion cortex. The purpose of these dissertation studies was to use a model of focal unilateral ischemic damage to the sensorimotor cortex (SMC) in adult rats to

investigate changes in neuronal structure in the peri-lesion cortex following CS/RT. More specifically, these studies investigated (1) whether CS/RT increases dendritic and synaptic density in the peri-lesion cortex in the chronic stage of recovery following focal ischemic lesions of the SMC, (2) whether CS/RT facilitates motor learning induced neuronal activation and structural plasticity in the peri-lesion motor cortex, and (3) whether transcranial CS/RT facilitates motor learning induced behavioral changes following ischemic SMC damage compared with RT alone.

Chapter 2

Abnormalities in skilled reaching movements are improved by peripheral anesthetization of the less-affected forelimb after sensorimotor cortical infarcts in rats.

2.1 Abstract

Unilateral damage to sensorimotor cortical (SMC) regions can profoundly impair skilled reaching function in the contralesional forelimb. Such damage also results in impairments and compensatory changes in the less-affected/ipsilesional forelimb, but these effects remain poorly understood. Furthermore, anesthetization of the ipsilesional hand in humans with cerebral infarcts has been reported to produce transient functional improvements in the paretic hand (Floel et al., 2004; Voller et al., 2006). One aim of this study was to sensitively assay the bilateral effects of unilateral ischemic SMC damage on performance of a unimanual skilled reaching task (the single pellet retrieval task) that rats had acquired pre-operatively with each forelimb. The second aim was to determine whether partially recovered contralesional reaching function is influenced by anesthetization of the ipsilesional forelimb. Unilateral SMC lesions were found to result in transient ipsilesional impairments in reaching success and significant ipsilesional abnormalities in reaching movements compared with sham-operates. There were major contralesional reaching impairments which improved during a 4 week training period, but movements remained significantly abnormal. Anesthetization of the ipsilesional forelimb

with lidocaine at this time attenuated the contralesional movement abnormalities. These findings indicate that unilateral ischemic SMC lesions impair skilled reaching behavior in both forelimbs. Furthermore, after partial recovery in the contralesional forelimb, additional improvements can be induced by transient anesthetization of the ipsilesional forelimb. This is consistent with the effects of unilateral anesthetization in humans which have been attributed to the modulation of competitive interhemispheric interactions. The present findings suggest that such interactions are also likely to influence skilled reaching function in rats.

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2.2 Introduction

Unilateral lesions to sensorimotor cortical (SMC) regions in rats result in sensory and motor impairments in the contralesional forelimb, including impairments in the fine movements used in reaching and grasping (Whishaw et al., 1991; Whishaw and Coles, 1996). Such damage also results in complex behavioral changes in the "less-affected", ipsilesional forelimb. Subtle deficits in the ipsilesional forelimb have been documented using sensitive measures of skilled reaching movements (Gonzalez et al., 2004; Marston et al., 1995; Montoya et al., 1991). Animals also increase reliance on the ipsilesional forelimb for many behaviors including postural support (Jones and Schallert, 1992),

coordinated limb placement during locomotion (Schallert et al., 2000), reaching and food handling (Whishaw and Coles, 1996; Whishaw, 2000) in a manner which compensates for impairments in the contralesional forelimb. Because skilled reaching tests in rodents are thought to assay impairments homologous to upper extremity impairments in humans with cerebral strokes, it seems important to understand the nature of the functional impairments and improvements that occur in this task in rats. Whishaw (2000) found that recovery of reaching ability in the contralesional forelimb is dependent upon whole-body compensatory changes and this includes compensatory ways of using the ipsilesional forelimb to assist in movements of the impaired forelimb. However, it is unknown whether compensation with the ipsilesional forelimb is a necessary chronic contributor to improvements in reaching performance with the contralesional forelimb. Furthermore, recent findings in humans suggest that sensory and motor function of one hand can be improved by ischemic nerve block of the opposite hand (Voller et al., 2006; Werhahn et al., 2002). This effect is thought to be due to modulation of interhemispheric inhibition and it points to a potential target for therapeutic interventions (Ward and Cohen, 2004), but it is has not been established to occur in rats. Thus, one purpose of the present study was to test whether partially recovered reaching performance with the contralesional forelimb after unilateral SMC lesions is enhanced or impaired by peripheral anesthetization of the ipsilesional limb.

Another purpose of the present study was to sensitively analyze bilateral changes in reaching performance after unilateral SMC lesions. Focal SMC lesions can *enhance* skilled reaching in a task that animals learn to perform with the ipsilesional forelimb after

the lesion compared to intact animals learning the same task (Allred and Jones, 2004; Bury and Jones, 2002; Hsu and Jones, 2005; Luke et al., 2004). This effect co-exists with subtle ipsilesional abnormalities in the movements used for reaching (Hsu and Jones, 2006) and may be linked to neuronal growth promoting effects of denervation and enhanced excitability in the contralesional motor cortex (Adkins et al., 2004; Allred and Jones, 2004; Bury and Jones, 2002; Hsu and Jones, 2005; 2006; Luke et al., 2004). In humans, transient virtual lesions of the motor cortex created using repetitive transcranial magnetic stimulation also enhance the performance of a motor task with the ipsilesional hand (Kobayashi et al., 2004; Takeuchi et al., 2005). However, Gonzalez et al. (2004) found reductions in skilled reaching success rates in the forelimb ipsilateral to relatively large unilateral frontal cortex lesions using a task that the animals had acquired prior to the injury (see also Montoya et al., 1991; Whishaw, 2000). It may be that enhancement in ipsilesional forelimb reaching function can only be detected after small lesions that do not produce very major ipsilesional deficits (Hsu and Jones, 2006). It is also possible that lesion-induced facilitation in skill learning does not generalize to performance of skills that were well learned prior to the injury, even after relatively small cortical lesions. This latter possibility was tested in the present study.

In this study, rats were trained to be proficient in a unimanual skilled reaching with each forelimb. Endothelin-1, a vasoconstricting peptide, was then used to induce ischemic SMC lesions of the forelimb representation region of one hemisphere. Quantitative and qualitative changes in reaching performance were then assessed in each forelimb. To promote improvements in the reaching function of the contralesional

forelimb, rats received a period of rehabilitative reach training of this limb, an approach that has previously been found to be effective in improving function (Biernaskie and Corbett, 2001; DeBow et al., 2003; Nudo, 2003; Riolobos et al., 2001) and promoting reorganization of movement representations in the peri-lesion cortex (Liepert et al., 2004; Nudo and Milliken, 1996). The ipsilesional forelimb was then anesthetized to test its effects on reaching performance of the impaired forelimb. Figure 1 summarizes the experimental design.

2.3 Materials and Methods

2.3.1 Animals

Seventeen male Long-Evans hooded rats were used in this study. Rats were obtained at 1 month of age from a breeding colony of the Animal Resources Center at the University of Texas at Austin (n=7) or from Charles Rivers Laboratories (n=10). Rats were housed in clear Plexiglas cages in pairs or triplets, were on a 12:12 h light:dark cycle and were given water *ad libitum*. Rats were ~ 100 days old at the onset of training. Beginning a few days before training, rats were restricted to 14-15 grams of chow per animal given at the end of each day to ensure that they were not sated at the time of the reach training. Animals were assigned to two groups: one group received unilateral ischemic lesions in the forelimb representation area of the motor cortex (n=9) and the second group received a sham operation (n=8). The mean ± SEM weekly weight change during the experiment was -0.27 ± 0.33 % in the lesion group and 1.58 ± 1.52% in the sham group. Assignments were random with the exception that groups were matched as

closely as possible for pre-operative reaching performance and for breeding colony source. The protocol for this study was approved by The University of Texas at Austin Animal Care and Use Committee.

2.3.2 Surgeries

Unilateral ischemic lesions of the forelimb area of the SMC were made using endothelin-1 (ET-1), a vasoconstricting peptide, using methodology adapted from Fuxe et al. (1997; see also Adkins-Muir and Jones, 2003; Adkins et al., 2004). Lesions were made contralateral to the forelimb rats preferred to use for reaching. Prior to surgery, rats were anesthetized using Equithesin (approximately 140 mg/kg chloral hydrate and 35 mg/kg sodium pentobarbital). Atropine (0.25 mg/kg) was also administered to negate the depressive respiratory effects of Equithesin. The skull was removed between 1.5 mm anterior and 2.5 mm posterior to bregma and 1.5 mm medial and 4.5 lateral to bregma. The dura underlying the craniectomy was carefully removed and 80µM ET-1 (Peninsula Laboratories, Inc.) was administered to the cortical surface with a Hamilton microsyringe. The total amount of ET-1 administered was 4.0 µl, delivered in two 2.0 µl applications separated by 2 min and followed by an 8 minute wait time, after which the skin was

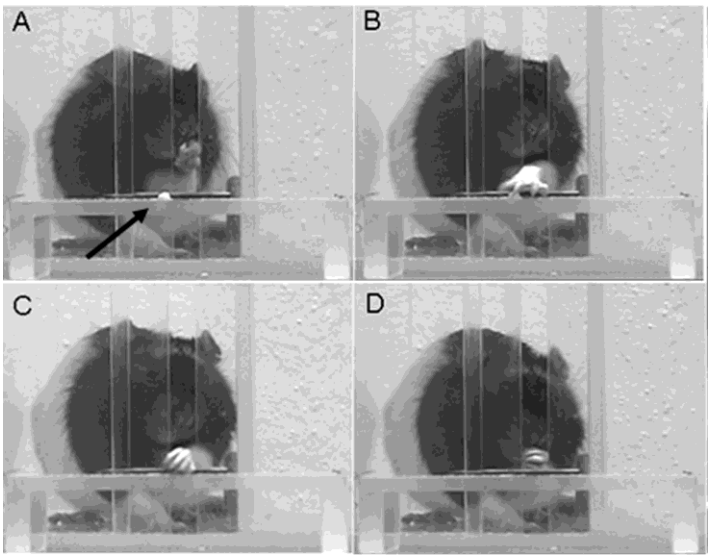


Figure 2.1: Single pellet retrieval task. Sequential photographs of a rat A) aiming, B) grasping, C) retrieving and D) eating a palatable food pellet (arrow).

sutured. Sham animals were treated to the same conditions up to the removal of skull. Craniectomies were not performed in sham-operates because this procedure has been found to cause behavioral and neurochemical asymmetries (Adams et al., 2004) and it was important in this study for this group to serve as intact controls.

2.3.3 Reach Training and Testing on the Single Pellet Retrieval Task

Animals were trained on the single pellet retrieval task, adapted from Whishaw (Miklyaeva and Whishaw, 1996; Whishaw, 1992) and others (Greenough et al., 1985; Peterson and Devine, 1963), as previously described by Bury and Jones (2002). The apparatus was a Plexiglas reaching chamber (26cm long X 34 cm high X 16 cm wide) with a tall narrow window (1 cm wide X 23 cm high) that was located in the middle of the 16 cm wide wall. The animals were trained to reach with one forelimb through the window in order to retrieve a banana flavored pellet (Bio-Serve, Inc., Frenchtown, NJ) placed in a well on a 3cm high shelf (Figure 2.1). The well was 1 cm from the window opening. In order to prevent the animal from scraping the food pellet into the reaching chamber, a lip consisting of a 2mm diameter metal rod was placed on the shelf in front of the window. Animals were trained pre-operatively to reach with each forelimb. Previous research has shown that most rats develop a preferred limb for performing unilateral reaching tasks (Whishaw, 1992). The preferred limb was determined using shaping trials in which rats were permitted to use either forelimb to retrieve centrally placed pellets. Once the animal made 15 out of 20 reaches with one forelimb and, on the following day,

made 10 consecutive reaches with the same forelimb, this limb was designated as the animals' preferred limb. Shaping typically required 3-5 raining sessions.

Once a preferred limb was established, rats began training on this limb. A Plexiglas wall (34 cm high) was inserted into the reaching chamber ipsilateral to the preferred limb and pellets were placed, one at a time, in a well positioned contralateral to this limb (aligned with the edge of the reaching window). This configuration effectively enforced the use of the preferred forelimb for reaching. Rats then received 16

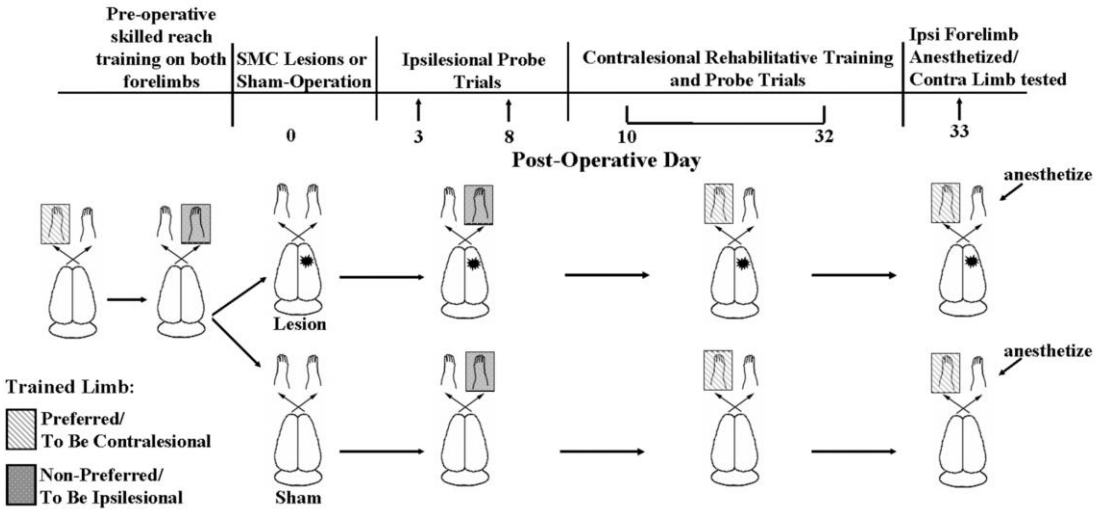


Figure 2.2: Schematic diagram illustrating the experimental design and the time course of behavioral training.

consecutive days of training on the preferred forelimb. They were then required to switch to using the non-preferred limb, by switching the position of the wall and pellet, and were trained for 9 days. Each day's training session consisted of 60 trials or 15 minutes, whichever came first. For each trial, a pellet was placed in the well and rats were permitted to make up to 5 attempts to retrieve it. Animals were prompted to turn

around before starting each new trial by placing a pellet at the back of the cage. Performance was based on the percentage of successful retrievals per reach attempt. A successful reach was one in which the animal grasped the pellet and ate it without dropping it. Unsuccessful reaches included ones in which the pellet was missed, knocked from the well or dropped.

Post-operatively, performance in both the ipsilesional and contralesional forelimb was assessed. Animals were tested in probe trials of reaching performance with the less-affected, ipsilesional limb (in sham-operates, the non-preferred limb) on day 3 and day 8 post-lesion. More extensive testing of the ipsilesional limb was avoided because this has been found to exacerbate functional deficits in the contralesional forelimb (Allred et al., 2005). Rats were tested with the contralesional (pre-operatively preferred) limb on day 10, prior to the onset of rehabilitative training, three times during the rehabilitative training period (Days 15-17, 20-22 and 25-27 post-operatively) and then after the rehabilitative training period without (Days 29-32) and with (Days 32-34) anesthetization of the ipsilesional forelimb (described below). To simplify the presentation, the testing sessions are referred to as post-operative Days 16, 21, 26, 30 and 33. The post-operative data are presented as the difference in performance compared with the last pre-operative test day for each limb.

2.3.4 Post-Lesion Training on a Tray Reaching Task

To promote improved function of the impaired forelimb, rats under went 12 consecutive days of rehabilitative training on a tray-reaching task beginning on post-

lesion Day 11. This task was used because it has previously been found to be effective in improving performance on the single-pellet retrieval task of the forelimb contralateral to SMC lesions in comparison to untrained controls (Maldonado et al., 2004). In this task, 100 pellets were placed on an inclined tray and rats were permitted to reach using the impaired (lesion group) or pre-operatively preferred (sham group) forelimb for them for 10 minutes. The metal tray was 7.5 cm long X 5.2 cm high X 6 cm wide and had a 25 degree incline toward the reaching window. The task uses the same Plexiglas chamber with an inner chamber wall as used for the single-pellet retrieval task. Rats in both groups were able to retrieve all 100 pellets in all but the first 2 days of tray reaching, when the lesion group retrieved slightly less than this (98.2 ± 1.0, 99.9 ± 0.06 mean ± S.E.M pellets retrieved on day 1 and 2 of training, respectively).

2.3.5 Anesthetization of the Ipsilesional Forelimb

Rats were assessed on the single pellet retrieval task (40 trials), using their contralesional/pre-operatively preferred limb, after a subcutaneous injection of lidocaine (~0.2cc, 10 mg/ml) into the proximal ipsilesional forelimb. The lidocaine test is based on the one used by Schallert et al. (1997) and the dosage was based on a previous study by Bury and Jones (2002). The lidocaine injections made the forelimb limp. Immediately following the lidocaine administration animals were observed in their home cage to confirm that the injection sufficiently anesthetized the ipsilesional forelimb to make it appear limp. Approximately 10 minutes after the lidocaine injection, rats were observed in a transparent cylinder to verify that they were either completely disusing or slipping

when attempting to use the ipsilesional limb for upright postural support. Animals then received 40 trials on the single-pellet retrieval task. Postural support with the anesthetized forelimb appeared highly abnormal in all animals during these trials.

2.3.6 Analysis of reaching movements

An adaptation of the Whishaw rating scale of reaching movements (Gonzalez et al., 2004; Whishaw et al., 1993; Whishaw and Metz, 2002) was used to analyze the movements used to perform the single pellet retrieval task. A Canon 3CCD digital camera was used to videotape animals as they were reaching with the ipsilesional forelimb on post-lesion Day 9 and with the contralesional forelimb during post-lesion week 4 (with and without the non-trained/ipsilesional forelimb anesthetized). Animals' reaching behavior was assessed during 5 trials in which the animal successfully retrieved the pellet. The 10 components assessed were 1) Digits semi-flexed: the paw is aligned with the midline of the body and digits are together, 2) Aim: the elbow is adducted and the forelimb is aligned with the reaching window, 3) Advance: the head is lifted and the limb is advanced directly toward the pellet, 4) Digits Open: the digits are extended and opened at the end of the advance, 5) Pronation: the digits are open and the paw is directly over the pellet, 6) Grasp: digits are closed to secure the pellet, 7) Withdrawal: the paw is withdrawn at a slight elevation through the reaching window, 8) Supination I: the paw is dorsiflexed and supinated 90° as the limb is withdrawn, 9) Supination II: the paw is supinated again by approximately 45° to bring the pellet to the mouth, 10) Release: the digits are opened and the pellet is released into mouth. The use of the inner

chamber wall in the present study invalidates one movement component (“digits to midline”) of the original Whishaw Rating Scale and this component was omitted from the analysis. In addition "withdrawal" is an addition to the rating scale because observations in other studies indicated that this is a movement in which the ipsilesional forelimb sometimes provides assistance. Each movement was rated on a scale of 0 (normal), 0.5 (mildly abnormal), or 1.0 (absent or highly abnormal). The mean score of the 5 trials was recorded for each movement component. A cumulative score was also calculated by summing the 10 components into a score between 0 (no abnormalities) and 10 (severe abnormalities). In other studies, we have seen the ipsilesional forelimb assist the contralesional forelimb in the movements needed to withdraw the forelimb, supinate the wrist and to open the digits and release the food pellet into the mouth (unpublished data). Therefore, during the movement analysis of the contralesional forelimb, any movements made by the ipsilesional forelimb to assist in withdrawal, supination I and II and release were also recorded.

2.3.7 Measurement of Forelimb Asymmetry

The Schallert cylinder test (Schallert et al., 2000) was used to measure asymmetrical forelimb use for postural support to verify that the lesions resulted in increased reliance on the ipsilesional forelimb. Animals were placed in the Plexiglas cylinder (19 cm diameter) and digitally video-recorded for 2 minutes. The cylinder encourages animals to explore the environment in an upright position in which they support themselves and move along the cylinder wall using the forepaws. This

sensitively reveals asymmetries in the use of the forepaws for postural-support. In slow-motion playbacks, each instance of forelimb use was recorded. The asymmetry score was calculated by the formula: (ipsilateral + 1/2 simultaneous bilateral use) / total limb use X 100.

2.3.8 Histology and Lesion Analysis

On post-lesion day 46-51, rats were given a lethal overdose of sodium pentobarbital and transcardially perfused with 0.1M sodium phosphate buffer, followed by a fixative solution containing 4% paraformaldehyde in the same buffer. Brains were removed and placed in the fixative solution and were sliced on a vibratome within 24 hours of removal. Six rostral to caudal tissue sections were taken and stored in 0.1M sodium phosphate buffer and saline. One set of 50-μm coronal sections was Nissl stained with Toluidine Blue and used to determine lesion placement and size.

The volume of remaining cortex in the SMC region was measured to indirectly determine lesion size. The area of the remaining cortex from sections within the SMC region were obtained using Neurolucida perimeter tracing software (Microbrightfield, Inc.) at a final magnification of X 17. A total of six sections, 400 μm apart, moving in a caudal direction were measured beginning with the appearance of the head of the caudate. The Cavalieri method (Gundersen and Jensen, 1987) was used to calculate volume as the product of the summed areas and the distance between section planes (400 μm). The extent and placement of each lesion was also reconstructed onto schematic cortical coronal sections adapted from Paxinos and Watson (1986).

2.3.9 Statistical Analyses

To analyze the reaching and cylinder test data, SPSS (SPSS, Inc.) program for general linear models for repeated measures analysis of variance (ANOVA) was used to determine the effects of Day, Group and Group by Day interactions. Post hoc T tests were used to further analyze reaching data. The *apriori* α level for all comparisons was 0.05. To analyze the reaching movement analysis data, SPSS general linear model multivariate analysis was used. Bivariate correlations were used to assess the relationship between reaching performance and cortical volume.

2. 4 Results

2.4.1 Lesion Reconstruction and Volume

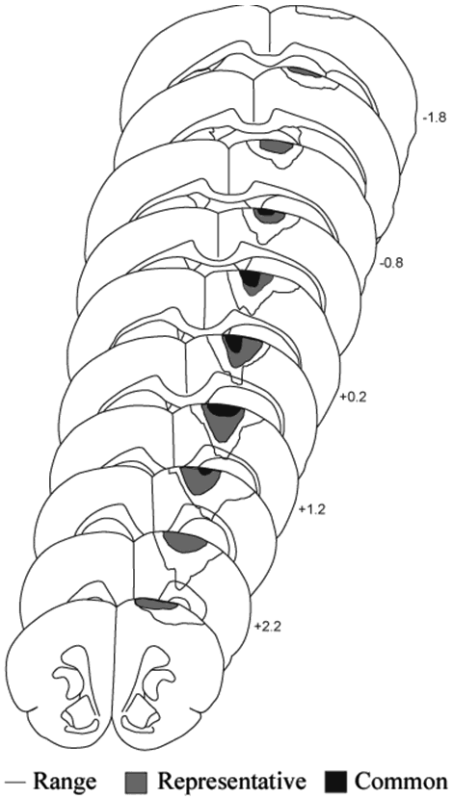


Figure 2.3 shows the reconstruction of the unilateral endothelin-1 induced lesions aimed at the forelimb area of the sensorimotor cortex. All lesions appeared to result in major damage in the forelimb area of the SMC. Most of the animals had cortical

Figure 2.3: Reconstruction of the extent and placement of focal unilateral forelimb region SMC lesions. The areas in black (common) are regions of damage common to all FLsmc lesions, areas in dark gray (representative) is a representative lesion and the areas in light gray show the largest extent of all SMC lesions combined (range). The numbers on the right indicates the approximate coordinate in mm relative to bregma.

damage that extended deep into layer V. Two of the animals had damage that extended into the corpus callosum. It appeared that no animals had any direct damage to the striatum.

Volume measurements of the remaining SMC revealed a loss of volume in the infarcted hemisphere compared with sham-operates. For the lesion group, the mean ± SEM remaining SMC region volume in mm^3 was 90.60 ± 1.96 in the damaged hemisphere and the interhemispheric difference in volume (contra - ipsi) in the SMC region was -13.15 ± 3.20, which was significantly reduced compared with sham operates (p =.001). In the sham-control group, the volume of this region of sensorimotor cortex was 103.06 ± 1.87 in the hemisphere contralateral to the preferred forelimb and the interhemispheric cortical volume difference in the SMC region was 0.71 ± 1.80.

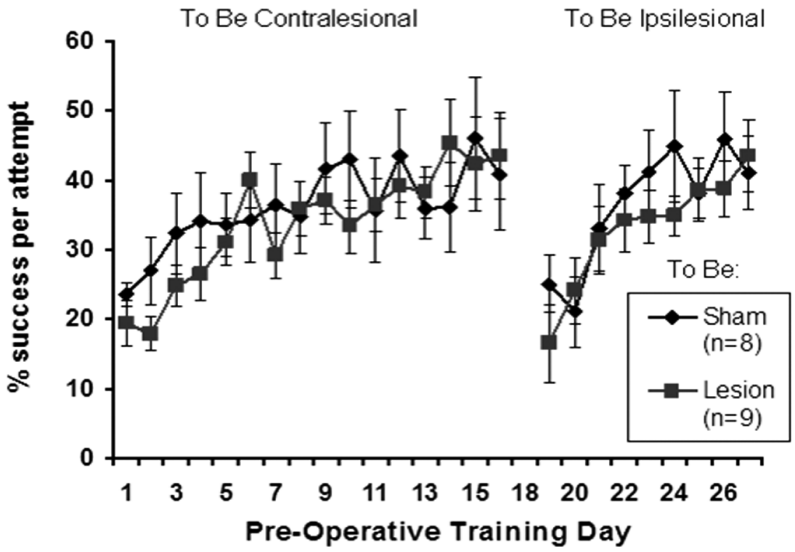


Figure 2.4: Pre-operative performance on the single pellet retrieval task on the to-be-contralesional and to-be-ipsilesional forelimbs. Rats were trained to criterion using each forelimb and groups were matched for reaching performance prior to surgery. Data are means ± S.E.M.

2.4.2 Pre-Lesion Skilled Reaching Performance

Rats in the lesion and sham groups were matched as closely as possible for pre-lesion success rates with both forelimbs on the skilled reaching task, as shown in Figure 2.4. Using repeated measures ANOVAs, there were significant effects of Day for both the preferred ($F(15,225) = 6.37$, $p = .001$) and non-preferred ($F(8,120) = 7.99$, $p<.00000001$) forelimbs reflecting that, over time, rats improved in their reaching performance. There was no significant Group or Group by Day interaction effects in pre-operative reaching performance with either forelimb between the pre-sham and pre-lesion groups ($p's > 0.05$).

2.4.3 Lesion Effects on Reaching with the Ipsilesional Forelimb

As shown on Figure 2.5A, after unilateral

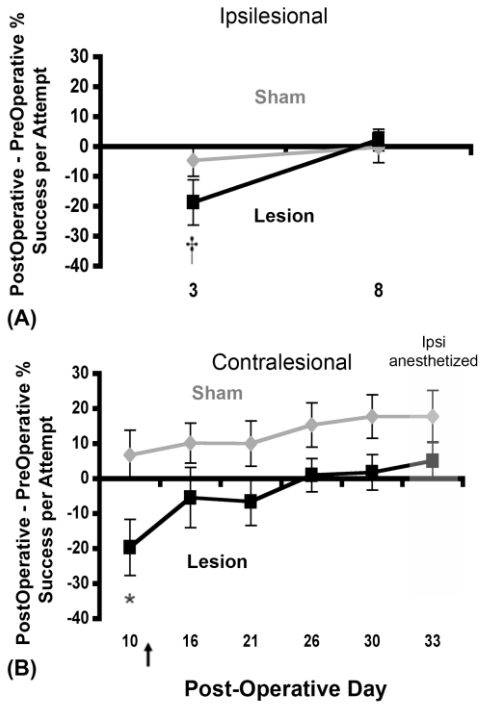


Figure 2.5: Post-operative reaching performance on the single pellet retrieval task using the ipsilesional (A) and contralesional (B) forelimb. There was a significant decrease in reaching performance of the ipsilesional forelimb on day 3 († p <.05), but not day 8, after SMC lesions compared to pre-operative performance. The contralesional forelimb was also impaired compared to shams as assessed on post-lesion day 10 (* p <.05). Contralesional performance improved over days of rehabilitative training. Anesthetization of the ipsilesional forelimb (the non-trained limb of shams) did not significantly change reaching success. Black arrow indicates onset of rehabilitative training. Data are means ± S.E.M.

SMC lesions, there was a transient decrease in reaching success rates with the ipsilesional forelimb compared with pre-lesion performance. Using a repeated measures ANOVA, there were significant effects of Group by Day ($F(1,15)=4.82$, $p = .044$) and Day ($F(1,15) = 11.64$, $p = .004$) but no significant main effect of Group ($F(1,15) = .676$, $p = .424$). In post hoc analyses, the difference in reaching performance between the sham-operated and lesion groups on post-lesion day 3 failed to reach significance ($p = .16$). However, in the lesion group, there was a significant difference between post-operative day 3 and both day 0 (pre-operative, $p = .038$) and day 8 ($p = .005$), whereas the sham-operated group did not show a significant difference in reaching performance between days.

As shown on Figure 2.6 and Table 1, the SMC lesions also resulted in significant abnormalities in movements of the ipsilesional forelimb. As assessed on day 9 post-lesion, lesion animals had an increase in the cumulative movement abnormality score based on an adaptation of Whishaw's Rating Scale. A one-way ANOVA revealed a significant increase in the movement abnormality score in the lesion group compared to the sham group in the ipsilesional limb ($F(1,16) = 8.25$, $p = .012$). Multivariate analyses including each movement component as a dependent variable revealed a significant increase in abnormalities in digits semi-flexed ($p = .041$) and pronation ($p = .011$), but not the other movement components, in the lesion group compared to the sham group.

2.4.4 Lesion Effects on Reaching with the Contralesional Forelimb

As first assessed on post-lesion Day 10, rats with a unilateral lesion had significant impairments in contralesional reaching performance in the single pellet retrieval task compared to the sham-operated group (Fig. 2.5B). The greatest severity of impairment was evident on Day 10. In repeated measures ANOVA, there was a significant effect of Group (F (4,60) = 5.02, p = .041) but no significant Group by Day interaction effect (F (1,15) = .73, p = .576). There was also a significant effect of Day (F (4,60) = 5.05, p = .001), which reflects that both the sham-operated and the lesion animals improved in the reaching performance over the days of testing (during which time they were receiving training on the tray reaching task).

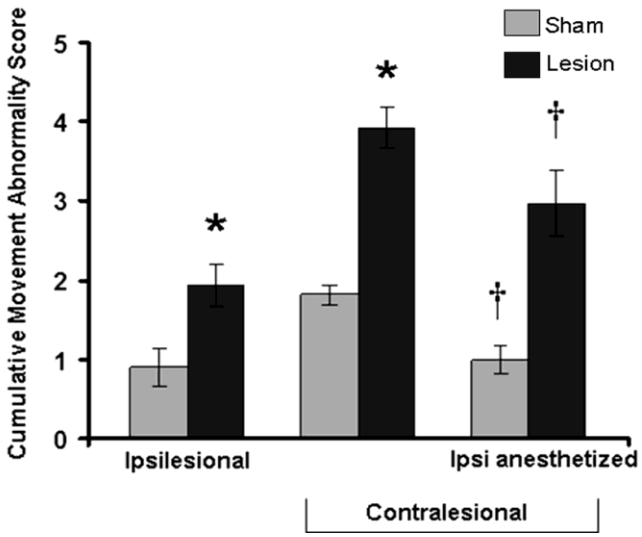


Figure 2.6: Cumulative movement abnormality scores in the ipsilesional and contralesional forelimbs. In the ipsilesional limb the cumulative abnormality score was significantly different between lesion and sham groups (* p = .012). As expected, unilateral lesions resulted in significant movement abnormalities in the contralesional forelimb compared with the sham group (*p < .002). Anesthetization of the ipsilesional/non-trained forelimb significantly reduced the abnormality score of the unanesthetized forelimb in both groups (†P<.05 pre- vs. post-lidocaine).

As shown in Figure 2.6 and Table 1, SMC lesions also resulted in major abnormalities in reaching movements in the contralesional forelimb as assessed using the Whishaw Rating Scale during post-lesion week 4. In One-Way ANOVA, lesion animals had a significantly higher cumulative movement abnormality score than the sham group

(F (1,16) = 45.69, p = .000006). Multivariate analysis revealed significantly greater abnormalities in the lesion group compared to the sham group in the following individual movement components: digits semi-flexed (p = .007), aim (p = .047), grasp (p = .003), withdrawal (p = .004), and release (p = .000000002). In rats with lesions, there was a significantly greater cumulative abnormality score during performance with the contralesional forelimb compared to the ipsilesional forelimb (p = .001). However, there was also a significant difference in the cumulative abnormality score in sham-operates reaching with the preferred versus non-preferred forelimb (p = .004). Nevertheless, the magnitude of the difference between the contralesional and the ipsilesional cumulative movement abnormality score in the lesion group was significantly greater than the interlimb difference in sham-operates (p = .031). In lesion rats, the mean ± SEM percentage of trials in which the ipsilesional forelimb was observed assisting the contralesional forelimb in the retrieval and/or consumption of the banana pellet was 23.33 ± 11.13. In sham-operates the percentage of trials in which the non-trained limb was observed assisting the trained limb was 16.67 ± 10.04. There was no significant difference between lesion and sham operates in the frequency of non-trained forelimb assistance (p = .46).

2.4.5 Effects of Anesthetizing the Ipsilesional Forelimb on Reaching with the Impaired Forelimb

Lidocaine was peripherally administered to the ipsilesional limb to anesthetize it and then the reaching performance of the contralesional limb was assessed. As shown in

Figure 4B, neither group had a significant change in reaching performance as a result of lidocaine injection into the ipsilesional and/or non-reaching forelimb compared with the prior test of performance (t-test for Day 30 versus 33: $p = .474$ lesion group, $p = .998$ sham group).

As shown on Figure 2.6, following peripheral administration of lidocaine into the ipsilesional/non-reaching forelimb, both the lesion ($p = .032$) and sham ($p = .005$) group had significantly reduced cumulative movement abnormality scores with the unanesthetized forelimb compared to the pre-lidocaine assessment of this limb. There was no difference in the magnitude of the change in the cumulative movement abnormality scores in the lesion versus sham-operate group ($p = .926$). However, there were some group difference in the specific movements most affected (Table 1). Multivariate analyses revealed a significant decrease in abnormalities in the individual movement components pre-lidocaine versus post-lidocaine. This was found in the sham group in advance ($p = .025$) and supination II ($p = .002$) and in the lesion group in withdrawal ($p = .012$). As with the pre-lidocaine test, ANOVA revealed a significant difference between the sham and lesion group in the cumulative movement abnormality score during the lidocaine test ($F(1,16) = 19.19$, $p = .001$).

2.4.6 Increased Reliance on the Ipsilesional Forelimb for Postural Support Behavior

Unilateral SMC lesions resulted in an increased reliance on the ipsilesional forelimb in the Schallert cylinder test. Pre-operatively, the asymmetry score (%ipsi + ½ bilateral use/ total use observations) was 48.89 ± 4.58 in the lesion group and 46.67 ±

2.76 in the sham group. Post-operatively (Day 4), the asymmetry score was 73.52 ± 4.43 in the lesion group and 51.67 ± 2.64 in the sham group. A repeated measures ANOVA revealed a significant Group ($F(1,15) = 26.64$, $p < .001$) and Group by Day effect ($F(2,30) = 3.40$, $p = .047$) and post-hoc analysis indicated significantly increased asymmetry in the lesion group compared with the sham-operates at the post-operative ($p = .001$), but not pre-operative time point.

2.5 Discussion

In summary, unilateral ischemic lesions of the forelimb representation region of the SMC resulted in major reaching impairments and movement abnormalities in the contralesional forelimb and less major, but significant, impairments in the ipsilesional limb on a skilled reaching task that had been well-learned with both forelimbs prior to the injury. Peripheral anesthetization of the ipsilesional/non-preferred forelimb failed to have a significant effect on reaching success rates with the impaired forelimb, but it significantly decreased movement abnormalities in the unanesthetized forelimb in both the sham and lesion group.

The ipsilesional effects of the SMC lesions were revealed as a minor, transient, reduction in reaching success rates and more notable abnormalities in reaching movements. These ipsilesional effects are consistent with the findings of Gonzalez et al. (2004) in which rats that pre-operatively learned a skilled reaching task had reduced success rates and movement abnormalities in the ipsilesional forelimb following unilateral ischemic lesions of the motor cortex produced by pial strip or of the

laterofrontal cortex following distal middle cerebral artery occlusion (MCAO). The impairments in the present study appear to be more minor and transient compared to the findings in Gonzalez et al. (2004) which might be attributed to the differences in methods of lesion induction, lesion size and the reaching task. Nevertheless, both of these studies suggest that a previously acquired motor skill is disrupted in the ipsilesional forelimb following unilateral cerebral infarcts. Lidocaine injections into the motor cortex have also been found to impair reaching function in the ipsilateral forelimb of intact animals and to reinstate deficits in an impaired forelimb when injected contralateral to unilateral MCAO (Biernaskie et al., 2005).

The ipsilateral ventral corticospinal tract provides about 5% of the corticospinal tract and may contribute to movements of the ipsilateral body side. However, this pathway seems unlikely to be a major contributor to the ipsilesional impairments found in the present study because Whishaw and Metz (1996) found that unilateral corticospinal tract lesions at the medullary level, rostral to the decussation, produced no impairments in reaching with the ipsilateral forelimb. Another potential explanation is that unilateral lesions result in degenerative effects in the contralateral cortex that disrupt the neural circuits contributing to the performance of pre-learned motor movements and/or movement sequences. The forelimb representation regions of either hemisphere are interconnected via transcallosal fibers (e.g., Akers and Killackey, 1978; Henry and Catania, 2006) and denervation of transcallosal fibers is associated with reactive astrocytic changes (Bury et al., 2000; Gomide and Chadi, 1999; Moumdjian et al., 1991) and loss of axonal processes (Jones, 1999) in the cortex. Partial deafferentation causes

cellular and structural changes, such as increases in neurotrophic factors (Isackson, 1985) and reactive synaptogenesis (Cotman et al., 1981). Unilateral cortical lesions also result in increased excitability in the contralateral cortex (Que et al., 1999; Reinecke et al., 1999; Shimizu et al., 2002; Witte et al., 2000). It is possible that these relatively subtle degenerative effects and excitability changes disrupt previously established motor skills while facilitating acquisition of new skills. Compared to intact animals, unilateral lesions of the SMC also result in better performance of a skilled reaching task that rats learned after the lesion (Allred and Jones, 2004; Bury and Jones, 2002; Hsu and Jones, 2005; Luke et al., 2004). This effect was muted in animals with larger lesions and greater ipsilesional movement abnormalities (Hsu and Jones, 2006).

Consistent with many previous studies (Montoya et al., 1991; Whishaw et al., 1991; Whishaw and Coles, 1996), unilateral SMC lesions resulted in reaching impairments of the contralesional forelimb. By Day 32, rats had returned to pre-lesion reaching success levels with the contralesional forelimb. However, analysis using Whishaw's rating scale of reaching movements revealed movement abnormalities compared to the sham-operated group at this time point, also consistent with previous findings (Gharbawie et al., 2005; Whishaw, 2000; Whishaw et al., 2004). Anesthetization of the ipsilesional/non-preferred forelimb at this stage partially normalized movements in the other limb on this rating scale in both groups of rats. It did not interfere with reaching performance of the contralesional limb as would be expected if animals had been significantly relying on the anesthetized limb for compensation. The overt use of this limb to assist in movements made with the contralesional forelimb, such

as assistance in limb withdrawal and release of the pellet into the mouth, was not significantly increased in frequency relative to sham operates at this time point (Day 32-34 post infarct). The ipsilesional/non-reaching forelimb continued to be used for postural support in both lesion and sham-operates, as animals stand on this limb while reaching with the other forelimb. This postural support was highly abnormal after lidocaine administration indicating that it is unlikely to be an important contributor to successful reaching (at least in this task design, see Miklyaeva et al. 1996). It cannot be ruled out that any detriment of having the non-reaching forelimb anesthetized is masked by the enhancing effect of unilateral anesthetization or that there is a rapid development of alternative compensatory strategies. However, the failure to observe increased use of the ipsilesional forelimb to aid the contralesional limb in movements used during reaching provides further support that the recovered contralesional reaching ability is likely to be largely independent of the contribution of ongoing ipsilesional compensation.

The functional improvements resulting from peripheral anesthetization are consistent with research in humans in which anesthetization of one hand in healthy subjects (Werhahn et al., 2002) or of the non-paretic hand of human stroke survivors (Floel et al., 2004; Voller et al., 2006) using ischemic nerve block is found to result in transient sensory and motor functional improvements of the non-anesthetized hand. Furthermore, this effect is associated with increased somatosensory evoked potentials contralateral to the non-anesthetized hand (Werhahn et al., 2002). These authors have suggested that these effects may occur because the loss of sensory input from one hand decreases interhemispheric competition and inhibitory interactions (Murase et al., 2004;

Ward and Cohen, 2004; see also Clarey et al. 1996). In the present study, although the magnitude of reduction in the cumulative movement abnormality score was similar in the lesion versus sham group, there were differences in the specific movements most affected by the anesthetization. Furthermore, in both groups some movements were relatively unaffected by the lidocaine injection, such as the abnormalities in grasp and release in the lesion group. These differences cannot be easily attributed to the magnitude of the pre-anesthesia abnormality score, to distal versus proximal movements or to phases of the reach. A better understanding of the neural mechanisms of these effects may shed light on why some movement abnormalities are most sensitive to the effects of peripheral anesthetization.

The failure to finding reductions in reaching success following anesthetization of the ipsilesional forelimb should not be taken to suggest that compensatory use of the ipsilesional forelimb is not an important contributor during earlier phases of recovery on this task. Previously, rats have been reported to use this forelimb in compensatory ways to assist the contralesional/reaching forelimb in earlier post-lesion periods (Whishaw, 2000). It is also likely that larger lesions than produced in the present study promote greater reliance on the ipsilesional forelimb. Restraining the ipsilesional forelimb in a sling early after large (but not small) lesions of the motor cortex during practice of the reaching task was been found to impair reaching performance (Whishaw, 2000). Thus, anesthetization of the ipsilesional forelimb at an earlier time point might have impaired skilled reaching function, especially in animals with more severe contralesional impairments. Thus, if there are facilitating effects of forelimb anesthetization at earlier

time points post-injury, these may require alternative measures to detect. It is also possible that the extensive pre-operative training, or the 2 post-injury probe sessions of performance with the ipsilesional limb, tended to suppress the rats' tendency to use this forelimb in a different way in the reaching apparatus, i.e., to assist movements of the contralesional forelimb.

In conclusion, unilateral ischemic SMC lesions of the forelimb representation region resulted in minor ipsilesional and more considerable contralesional impairments in skilled reaching performance that had been established prior to the injury. Anesthetization the ipsilesional forelimb did not interfere with the reaching performance of the contralesional forelimb suggesting that, at least at later stages of recovery after relatively small cortical infarcts, it is possible for reaching movements in the contralesional forelimb to recover in this task without ongoing compensatory reliance on the ipsilesional forelimb. The finding that skilled reaching movements were, instead, partially normalized by anesthetization of the other forelimb indicates that the interhemispheric and bimanual effects of peripheral anesthetization reported in humans most likely extend to rat reaching function. This seems important in light of recent findings indicating competitive interactions between the hemispheres that become unbalanced by unilateral brain damage and which might be targeted for therapeutic interventions (Rushmore et al., 2006; Takeuchi et al., 2005; Voller et al., 2006; Ward and Cohen, 2004). The present results suggest that rat models of skilled reaching may be useful for studying the role of interhemispheric interactions in recovery of somatomotor function after unilateral brain damage.

Chapter 3

Motor Cortex Stimulation in Rats with Cortical Infarcts: Persistent Behavioral Gains and Cortical Volume Increases

3.1 Abstract

Motor cortical stimulation (CS) is emerging as a promising treatment for motor impairments after stroke. In animal models of sensorimotor cortical (SMC) stroke, CS delivered with a motor training regime enhances impaired forelimb function and increases dendrites, synapses and movement representation area in peri-infarct motor cortex compared with training alone. These effects have been assessed during and shortly after CS treatment. This study investigated (1) the persistence of behavioral improvements resulting from CS with motor training, (2) whether CS-promotes long-term cortical morphological changes and (3) whether forced forelimb use enhances CS effects. Adult rats were trained on a skilled forelimb reaching task, then received unilateral ischemic SMC lesions and epidural implantation of electrodes over remaining motor cortex. Subgroups were placed in vests that forced use of the impaired forelimb or control vests for 2 weeks. Rats received three weeks of daily practice in reaching with the impaired forelimb with or without concurrent 100Hz cathodal CS delivered at 50% of movement thresholds. Reaching function was assessed weekly for 9-10 months post-treatment. Stereology and cytoarchitectonics were used to measure the volumes of motor cortical subregions. CS-treated rats had significantly improved forelimb function during the chronic period compared to rats receiving training alone. There was no additional improvement with forced forelimb use. CS-treated rats also had the greatest volume of

some motor cortical subregions. These findings support the usefulness of CS in chronically enhancing function after stroke and suggest that its lasting effects may be related to promotion of cortical plasticity.

3.2 Introduction

In rats and squirrel monkeys with unilateral infarcts of the sensorimotor cortex (SMC), delivering a low intensity electrical current to the surface of the peri-infarct motor cortex (cortical stimulation; CS) while animals undergo daily practice in skilled forelimb reaching improves recovery of forelimb function compared to motor training alone (Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008; Kleim et al., 2003; Teskey et al., 2003; see also Brown et al., 2006). The resultant behavioral improvements coincide with functional and structural plasticity in the stimulated region of motor cortex, including increased dendritic (Adkins-Muir and Jones, 2003) and synaptic density (Adkins, Hsu & Jones, 2008), larger forelimb movement representation areas (Kleim et al., 2003; Plautz et al., 2003) and enhanced motor cortical evoked potentials (Teskey et al., 2003). Human clinical trials have found that epidural cortical stimulation combined with motor training is safe and, at least sometimes, efficacious in improving motor function after stroke (Brown et al., 2006; Harvey and Winstein, 2009; Huang et al., 2008; Levy et al., 2008a,b). Non-invasive stimulation approaches, such as repetitive transcranial magnetic stimulation (rTMS) and direct current stimulation (tDCS), have also shown significant potential for improving motor function following stroke (Hummel and Cohen, 2006; Khedr et al., 2005; Nair et al., 2007; Pascual-Leone, 2006; Yozbatiran

et al., 2008).

Most studies have focused on CS effects during and shortly after treatment, and it was unknown whether it can be used to induce persistent functional improvements. Therefore, the major aim of the present study was to investigate whether CS chronically improves function, as assessed in rats with cortical infarcts surviving 9-10 months after the end of motor rehabilitative training. We also asked whether forced use of the contralesional forelimb is a beneficial adjunct to CS. After unilateral cerebral injury in humans and rats, constraint of the less-affected upper extremity during rehabilitative training improves motor function in the impaired limb (DeBow et al., 2003; Taub et al., 2006; Wolf et al., 2006). It thus seemed possible that adding a constraint-like treatment to the regimen of CS and skill training would result in an even greater enhancement of functional outcome.

When assessed soon after treatment, CS increases synaptic (Akdins, Hsu and Jones, 2008) and dendritic (Adkins-Muir and Jones, 2003) density in the stimulated motor cortex, and spares a loss of movement representations (Kleim et al., 2003; Plautz et al., 2003) compared with animals receiving training alone. These effects may reflect a greater functional integrity of the surviving peri-infarct cortex that, if persistent, could preserve and enhance neuronal structure in this region over the lifespan of the animal. If so, we expected this to be evident in measures of cortical volume because increased cortical volume is associated with experience-dependent neuronal growth in intact cortex (Black et al., 1997; Churchill et al., 2002; Green et al., 1983; Grossman et al., 2002) and in cortex denervated by a lesion (Jones & Schallert, 1992). In addition, long-term

electrical stimulation of the suprasylvian gyrus in cats increases dendritic branching and length of neocortical pyramidal neurons (Rutledge et al., 1974). Thus, an additional aim of this study was to investigate whether there were CS effects on the volumes of remaining motor cortical subregions of the infarcted hemisphere, as assessed 9-10 months after the end of treatment.

3.3. Materials and Methods

3.3.1 Experimental Design Overview. Figure 3.1 summarizes the experimental design. The Single Pellet Retrieval Task was used as the primary outcome measure because it is a highly sensitive assay of skilled forelimb function in rats (Miklyaeva and Whishaw,

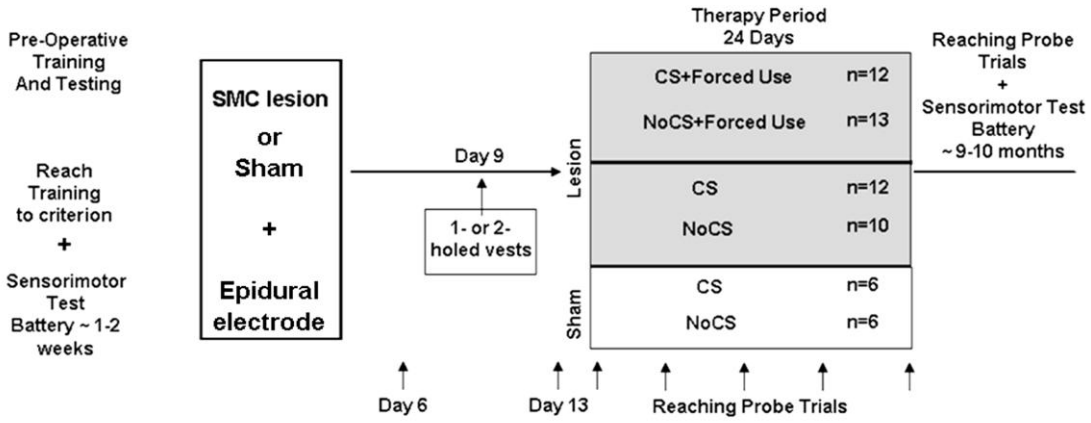


Figure 3.1. Experimental design and the time course of behavioral testing.

1996; Whishaw, 1992). After training to criterion on this task, all animals received either an ischemic lesion to the forelimb representation area of the SMC contralateral to the trained limb (Fig. 3) or a sham operation. During the same surgery, each rat also received

an electrode implantation over the motor cortex of the peri-infarct or sham-operated region. Group assignments to treatment conditions were random except that groups were matched for initial lesion-induced reaching impairments, as assessed on post-lesion Day 6. This was done because early behavioral deficits are predictive of functional outcome (Binkofski et al., 2001; Hendricks et al., 2002; Prabhakaran et al., 2008; Rey et al., 2007; see also Adkins et al., 2008). On Day 9, rats receiving forced use were placed into vests that restricted use of the ipsilesional forelimb for 14 days. All other rats were placed into control vests permitting use of both forelimbs during this period. After testing on the single pellet retrieval task on Day 13, rats began 24 days of daily rehabilitative training on a tray reaching task (Fig.4, Ch.2) with or without CS. Probe trials on the single pellet retrieval task were given periodically during training and weekly for 9-10 months after rehabilitative training ended. Rats were also tested on a sensorimotor test battery preoperatively, weekly for the first month post-rehabilitation and then monthly for the remainder of the experiment.

3.3.2 Animals

Seventy-nine male Long-Evans hooded rats were obtained from Charles Rivers Laboratories at one month of age and housed in polycarbonate cages in pairs or triplets on a 12:12 h light:dark cycle with water *ad libitum*. Housing included standardized cage supplements (a PVC tube, small wooden objects to gnaw and manipulate and 6g twice weekly complex food mixture consisting of in-shell nuts, in-hull seeds, macaroni and dried fruit pieces). Rats were tamed, placed on scheduled feeding (15g chow/day, gradually increased to permit age-related weight gain) and began training at 2.5-3

months. They received lesions at 3-3.5 months of age. A small group of rats (n=2 NoCS, n=2 CS, excluded from the behavioral analyses) were sacrificed to assay infarcts at the end of the rehabilitation period. Six rats were euthanized because their electrodes came off. Ten were removed because they did not show evidence of impaired reaching performance on post-lesion Day 6 or 13. The protocol for all procedures was approved by the University of Texas Animal Care and Use Committee.

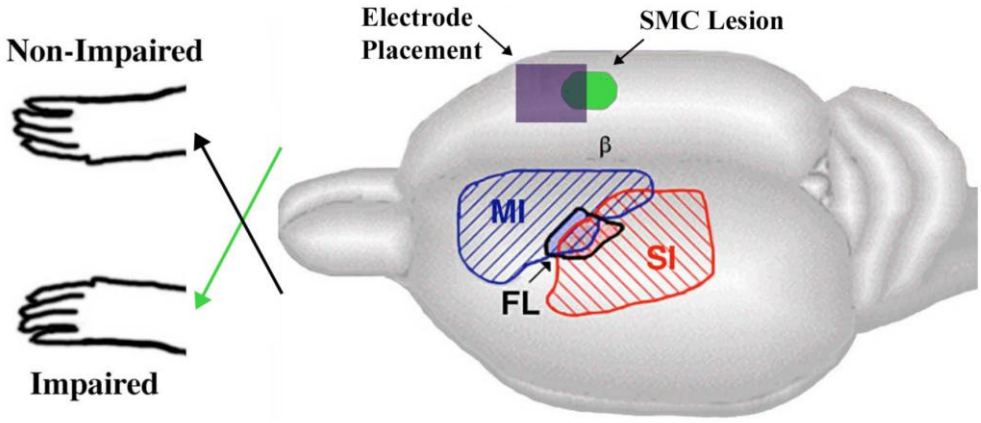


Figure 3.2: Focal unilateral ischemic lesions were made in the MC using endothelin-1. Epidural electrodes were implanted over remaining motor cortex.

3.3.3 Surgical Procedures

3.3.3.1 Cortical Infarcts. Ischemic damage was made using topical application of the vasoconstrictor, endothelin-1 (ET-1), to the SMC (Adkins-Muir and Jones, 2003; Fuxe et al., 1997). Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and the skull was removed between 0.5 mm posterior, 2.5 mm anterior and 3-5 mm lateral to Bregma. The dura was removed and 2.2 µl (200 pmol) of ET-1 (Peninsula Laboratories Inc.) were administered to the pial surface. Sham-operates were treated the

same as lesion-operated rats excluding the application of ET-1.

3.3.3.2 Electrode Implantation. Ten minutes after ET-1 application or skull opening in sham-operates, the craniectomy was enlarged ~1 mm both rostrally and medially (Figure 3.3) to expose perilesion motor cortex. The electrode (Northstar Neuroscience Inc., Seattle, WA) consisted of parallel platinum wire contacts (0.4 X 2 mm) mounted on a 3 X 3 mm supporting plate extending from a connector pedestal (Plastics One Inc., Roanoke, VA). The contacts were positioned parallel to midline above dura, which placed the electrode over the remaining motor cortex, including the remaining forelimb representation area and surrounding areas. This position was chosen because it has previously been found to reliably elicit movements of the contralateral forelimb, shoulder and/or face when current is applied (Adkins-Muir and Jones, 2003; Adkins et al., 2006). A metal disk inserted subcutaneously near Lambda served as a ground.

Figure 3.3: CS electrode

3.3.4 Cortical Stimulation (CS) Procedures

3.3.4.1 Movement Threshold Testing. A movement threshold was defined as the minimum current needed to evoke an observable forelimb or shoulder movement. Thresholds were assessed weekly in all rats during the rehabilitation period. Rats were

placed in a Plexiglas cylinder and observed during delivery of cathodal stimulation consisting of 100 µs pulses delivered at 100 Hz frequency in 3 s trains of increasing amplitude. Stimulation was delivered using a Sunrise PSS2 stimulator (Northstar Neuroscience, Inc., Seattle).

3.3.4.2 CS during Rehabilitative Training. In CS treated rats, 100 Hz cathodal current was delivered at 50% of movement threshold for the entire 10 minutes of each day's training on the tray reaching task. These parameters where chosen because they have been found in previous studies to be particularly effective with epi- or subdural electrodes in acutely improving function (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003). On probe trials of performance on the single pellet retrieval task during the rehabilitation period, the CS groups received stimulation for half of the trials, with the order alternated weekly. NoCS rats were connected to stimulator cables but received no stimulation during these training and testing sessions.

3.3.5 Forced Use Vests. On post-lesion Day 9, animals were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) and placed into customized vests that allowed free movement of both forelimbs (control vests) or forced reliance on the impaired, contralesional forelimb. Initially, 27 animals (n=13 forced use, n=14 control) were placed in vests made using veterinary Fiberglass casting tape lined with felt, as described previously (Bury and Jones, 2004). Subsequent rats (n=15 forced use, n=17 control) were

placed in commercially available Lycra/spandex rat jackets (Lomir Biomedical Inc.) that were sewn together in the back and, for the forced use condition, supplemented with elastotape (3M) to restrict the less-affected forelimb. Both forced use vest designs permitted limited movements of the ipsilesional forelimb, but largely prevented its use for postural support. This was verified using the Schallert Cylinder test (described below). Vests were removed on post-lesion Day 25.

3.3.6 Testing and Training in Skilled Reaching

3.3.6.1 Single Pellet Retrieval Task. The single pellet retrieval task (Ch.2, Fig.1) has been described in detail previously (Bury and Jones, 2002). Animals in a chamber were trained to reach through a tall narrow window to retrieve a banana flavored pellet (45mg, Bio-Serve, Frenchtown, NJ) placed in a well 1 cm from the opening. After shaping to determine endogenous limb preference (Whishaw, 1992), a removable wall was inserted ipsilateral to the preferred limb and pellets were placed, one at a time, in a well opposite this limb to ensure that it was the trained limb. Rats were permitted to make up to five attempts to retrieve each pellet. Performance was measured as the number of successful reaches (grasping, retrieving and eating the food pellet)/reach attempt. Preoperatively, animals received 60 trials/day or 15 min (whichever came first) until they reached a criterion of 45% successful retrievals/attempt (~12 days). This criterion was chosen because it is close to asymptote performance using these task parameters in this substrain of rats (O'Bryant et al., 2007a). Post-operative performance (40 trials/session) was calculated as the percentage change in reaching success relative to the average of the last

3 days of pre-operative reaching: %(success - pre-operative success)/pre-operative success. The initial lesion-induced loss in reaching success was matched as closely as possible between lesion groups.

An adaptation of the Whishaw rating scale (Gonzalez et al., 2004; Whishaw et al., 1993; Whishaw and Metz, 2002) was also used to analyze ten specific reaching movements in frame-by-frame video playback, as previously described (O'Bryant et al., 2007b). Each of the 10 movements was classified as being normal, mildly abnormal or severely abnormal. The number of mildly and severely abnormal movements per 5 successful trials was analyzed.

3.3.7 Other Behavioral Measures. The sensorimotor test battery consisted of three sensitive measures of sensorimotor impairments. The cylinder test was used to measure forelimb use for postural support behaviors during upright exploratory movements inside a clear Plexiglas cylinder (Schallert et al., 2000). Normally, animals use both forelimbs approximately equally but, following unilateral SMC lesions, rats typically rely more on the ipsilesional forelimb (Adkins et al., 2004; Schallert et al., 2000). An asymmetry score was calculated by the formula: ((ipsilateral+1/2 simultaneous bilateral)/total limb use) X 100. The footfault test was used to measure coordinated forelimb placement during locomotion across a grid floor (Barth et al., 1990). Following unilateral SMC lesions, the contralesional forelimb is frequently incorrectly placed and slips through the grid openings (Barth et al., 1990; Jones et al., 1999). The vibrissae-stimulated forelimb placing test was used to measure sensorimotor integration (Barth et al., 1990). When an

intact animal has its vibrissae (whiskers) brushed against a table edge, it places its paw on the tabletop. Following unilateral SMC lesions, animals often show fewer placing responses with the contralesional limb (Barth et al., 1990; Hoane et al., 2000; Hsu & Jones, 2006).

3.3.8 Neuroanatomical Methods

3.3.8.1 Histology On post-rehabilitation weeks 39-45, when rats were 12-13.5 months old, they were overdosed with sodium pentobarbital and transcardially perfused with buffer and fixative solutions. A small set of animals (n=4) were perfused at post-rehabilitation week 1 to assay infarcts at this time point. Coronal sections (50μm) were taken throughout the cerebrum and stained with toluidine blue (a Nissl stain). All anatomical data were collected from digital images captured or visualized in real time using an Olympus BX61 microscope. All anatomical data were collected blinded to experimental condition.

For electron microscopic analysis of synaptic density, non-necrotic/non-gliotic tissue in the peri-lesion motor cortical region underlying the electrode was sampled and included the medial and lateral agranular region, medial to the ischemic injury. Using a stereomicroscope, this region was identified, using macrostructural landmarks and unique cytoarchitectural characteristics that are evident in unstained tissue (Jones et al.,1999), and was removed in the 200 μm sections. All samples were placed in cacodylate-buffered osmium tetroxide, and en bloc stained with 2% uranyl acetate for 45 min. Samples were then dehydrated and sandwich-embedded in Eponate-12 resin. Semithin sections (0.8 μm

thickness) were then extracted, stained with Toluidine Blue and used to estimate neuronal density and to more precisely localize the region for electron microscopic sampling. To measure synaptic density, serial ribbons of ultrathin sections (70 nm) of layer V were obtained from the osmicated samples using a Leica Ultracut R microtome, mounted onto slotted copper grids coated with formvar, and stained with lead citrate to be used for electron microscopic measures. All histological data were collected blinded to the experimental condition. All anatomical data were collected from digital images captured or visualized in real time using an Olympus BX61 microscope. All anatomical data were collected blinded to experimental condition.

In the first batch of rats, there were signs of subcutaneous infection around the sites of implantation (n=11/15). The infections appeared encapsulated by skin and dura mater, did not invade brain tissue and there was no indication of poor health in these animals. Therefore, these animals were kept in the behavioral analysis but omitted from the neural morphological measures. This was not found subsequently and therefore this was potentially due to faulty electrode sterilization techniques specific to the initial batch of implantation surgeries.

3.3.8.2 Volume Measures of Cortical Subregions Because CS has been found to increase dendrites and synapses in remaining motor cortex early after treatment (Adkins et al., 2003; 2008), and because neocortical volume often changes with its quantities of dendrites and synapses (e.g., Black et al., 1997; Green et al., 1983; Grossman et al., 2002), we questioned whether CS would influence the volumes of specific subregions of the SMC as assessed long after treatment. Using cytoarchitectural characteristics to

identify subregions, we estimated the volumes of the medial agranular cortex (AGm), lateral agranular cortex (AGl), and the overlap zones (Figure X). The overlap zones are areas with characteristics of both the primary motor (MI) and primary sensory (SI) cortices, that receive specific thalamic input and have corticospinal projections (Wise and Donoghue, 1986). This includes two subregions, the forelimb overlap zone (FlOL) and hindlimb overlap zone (HlOL). The boundaries of the four subregions of interest, AGm, AGl, FlOL and HlOL were made based on their specific cytoarchitectural characteristics, which have been previously described in detail (Donoghue and Wise, 1982; Neafsey et al., 1986; Wise and Donoghue, 1986; see also Chu & Jones, 1999). Briefly, the AGm has a dense layer II and a pale staining layer III. The AGl has a homogenous appearance of superficial layers and a thick layer V with large pyramidal neurons. The FlOL zone and the more caudally and medially appearing HlOL zone have both large layer V pyramidal neurons and a dense granular layer IV, characteristic of both MI and SI.

Because the infarcts can distort the clarity of cytoarchitectural delineations, these subregions of the intact hemisphere were first identified in sections 350 μm apart, and then the identified boundaries were reversed and superimposed onto the lesion hemisphere to guide their delineation in the infarcted cortex. The volume of each subregion was estimated using the Cavalieri method and systematic point counting (Gundersen and Jensen, 1987). A grid of test points was superimposed onto the digital images and the number of points falling within each subregion was counted. Volume was calculated using the formula $\Sigma P \times a(p) \times T$, where ΣP is the total number of points counted summed across all sections, a(p) is the area per point (122500μm), and T is the

average distance between the sections used for measurement (350μm).

3.3.8.3 Analysis of Lesions. For cortical injuries, interhemispheric volume differences are often used as an indirect estimation of lesion size. However, morphological changes over the chronic period can be expected to confound this measure (e.g., cortical volume increases or atrophy would mask, at least partially, cortical volume loss resulting from the lesion). Thus, in addition to carefully matching groups based on initial behavioral impairment levels, we characterized the placement of the lesions by reconstructing them based on cytoarchitectural characteristics of remaining tissue (Chu and Jones, 2000). The lesion cavity was reconstructed onto schematic cortical coronal sections (adapted from Paxinos and Watson, 1998). For the sham-operated groups, any damage created by the electrode implantation procedure was also reconstructed.

The volume of the lesion cavity would be reduced if there were an expansion of surrounding cortex into the cavity. Furthermore, it would appear increased if there were a loss of surrounding tissue (e.g., as might occur if there is a gradual atrophy of this tissue over time after the lesions). In either event, if initial lesion volumes were similar between groups, then the net volume of the lesion cavity plus remaining sensorimotor cortex would be increased in animals showing either a subsequent increased volume or a reduced atrophy of remaining tissue relative to other groups. To verify this, we estimated the volume of the reconstructed lesion cavity and summed it with the volume of the remaining ipsilesional SMC region (inclusive of SI and ventrolateral cortex). The volume of the reconstructed lesion cavity was estimated using Image J (image processing program). Volume was calculated by finding the area of the lesion cavity per coronal

section and multiplying it by the average distance between sections, T, which was 500 μm. The volume of all remaining cortex within the SMC region between ~ +2.7 to -1.8 mm relative to bregma was estimated. Moving in a caudal direction, all cortex of the infarcted hemisphere appearing in coronal sections was outlined in a total of ten sections beginning at the appearance of the head of the caudate, as described previously (Jones et al., 1999). Neurolucida software was used to estimate the cortical areas and volume was calculated using the Cavalieri method (T = 350 μm).

3.3.9 Statistical Analyses

For behavioral results, planned comparisons were used for the primary analyses to address these questions: After the lesions does (1) CS improve function (CS versus NoCS), (2) forced use improve function (NoCS+Forced Use versus NoCS) and (3) forced

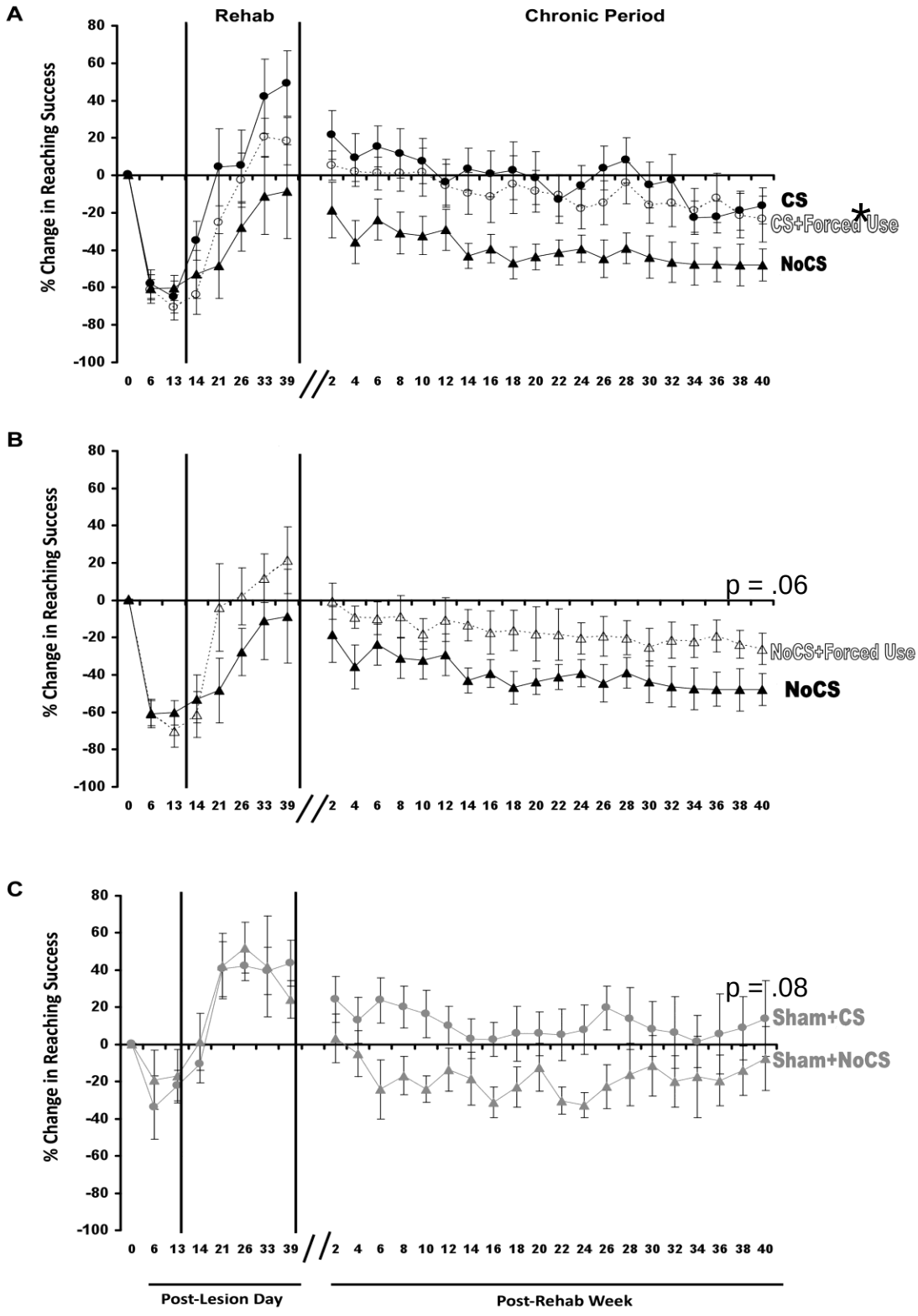

A
Rehab
Chronic Period
% Change in Reaching Success
CS
CS+Forced Use
*
NoCS
B
p = .06
NoCS+Forced Use
NoCS
C
p = .08
Sham+CS
Sham+NoCS
Post-Lesion Day
Post-Rehab Week

Figure 3.4: Reaching performance on the single pellet retrieval task. A: Animals receiving CS had persistent improvements in reaching performance compared to the NoCS group during the CS treatment and rehabilitative training (Rehab) period and over the 9-10 months after the end of treatment (Chronic Period). Forced use did not improve the effects of CS during the rehabilitative training period or over the following 9-10 months. * p = .01 vs NoCS in the chronic period. B: The effects of forced use in NoCS rats failed to reach significance. C: In intact animals, reaching performance during the Rehab period was similar between CS and NoCS treated rats. In the chronic period, CS effects in intact animals did not reach significance compared to NoCS. Data are means ± SEM change in reaching performance relative to pre-infarct performance.

use improve the effects of CS (CS+Forced Use versus CS)? Additionally (4) does CS influence function in sham-operates? Data from the rehabilitation period were analyzed separately from those from the chronic period to investigate the persistence of CS effects. Behavioral data were analyzed with repeated measures ANOVA using SPSS (SPSS Inc., Chicago, IL) to analyze effects of Day, Group and Group by Day interactions. When warranted, posthoc T tests were used with Bonferonni's corrections. There were no significant differences in any behavioral measures among the lesion and sham groups prior to the surgeries. For volume differences based on cytoarchitectonics, we used planned comparisons to test for effects of CS versus NoCS in both lesion and sham-operated groups. In addition we tested for CS or NoCS effects compared to the sham-operates.

3.4 Results

3.4.1 Skilled Reaching Performance

3.4.1.1 Performance During the Rehabilitative Training Period. As shown in Figure 3.4, lesion groups declined in reaching performance on the single pellet retrieval task as measured prior to the onset of rehabilitative training. All groups, including sham-operates, improved in performance during the rehabilitation period (during which time rats were being trained on a tray reaching task, as reported below). In the analysis of CS versus NoCS (Fig. 3.4A), there was a significant interaction effect of Group by Day of testing ($F(7, 140)=2.80$, $P=0.009$). In post-hoc analyses, there were no individual training days that reached significance, although the CS group tended to perform best later in the rehabilitative training period. The effects of forced use (Fig. 4B) did not reach significance (NoCS+Forced Use vs. NoCS, Group by Day: ($F(7,141)=1.43$, $P=0.18$). Furthermore, forced use did not further improve the effects of CS (CS vs. CS+Forced Use, Group by Day: $F(7,154)=0.87$, $P=0.54$). In intact animals (Fig. 4C), CS did not alter reaching performance during this period (Group by Day: $F(7,70)=0.51$, $P=0.82$). There were no significant main effects of Group in any of these comparisons (P's>0.05). All analyses indicated significant effects of Day (F's=12.49-31.50, P's<0.001), reflecting time-dependent improvements in reaching performance during this period.

3.4.1.2 Performance During the Chronic Period The primary question of the present study was whether CS combined with rehabilitative training improves function in the chronic period after SMC infarcts. As shown in Figure 3A, CS significantly improved skilled forelimb performance during the chronic period compared to rats receiving

rehabilitative training alone (Group: $F(1,20)=8.06$, $P=0.01$), as assessed during the 9-10 months following the rehabilitative training period. On average, CS groups performed better than NoCS groups throughout the chronic period and there was no interaction effect of Group by Day of testing ($P>0.05$). Forced use did not further improve the effects of CS (CS vs CS+Forced Use, Group: $F(1,22)=0296$, $P=0.59$). The effect of forced use alone (Figure 3B) missed significance in ANOVA (NoCS+Forced Use vs. NoCS, Group: ($F(1,21)=3.96$, $P=0.060$) although animals that received forced use performed better, on average, on every week of post-treatment testing. In intact rats, the effects of CS on reaching performance also failed to reach significance compared to Sham+NoCS (Fig. 3C, Group: $F(1,10)=3.84$, $P=0.079$). All analyses indicated significant effects of Day (F's=4.22-5.32, $P's<0.001$), reflecting gradual declines in reaching performance in the chronic period.

3.4.2 Performance on the Rehabilitative Task

Although the single pellet reaching task was the primary outcome measure, performance on the rehabilitative tray reaching task was also assayed by measuring the total amount of pellets retrieved (out of 100) per training session. Initially, SMC lesions reduced performance on the tray reaching task compared to sham-operates, but by the ninth day of training, all groups were retrieving most pellets (Figure 4). On average, the CS group performed best in the first week of training. In repeated measures ANOVA, there was a significant Group by Day effect ($F(18,360)=1.69$, $P=0.04$) between CS and NoCS, but no main effect of Group. There was also a significant Group by Day effect

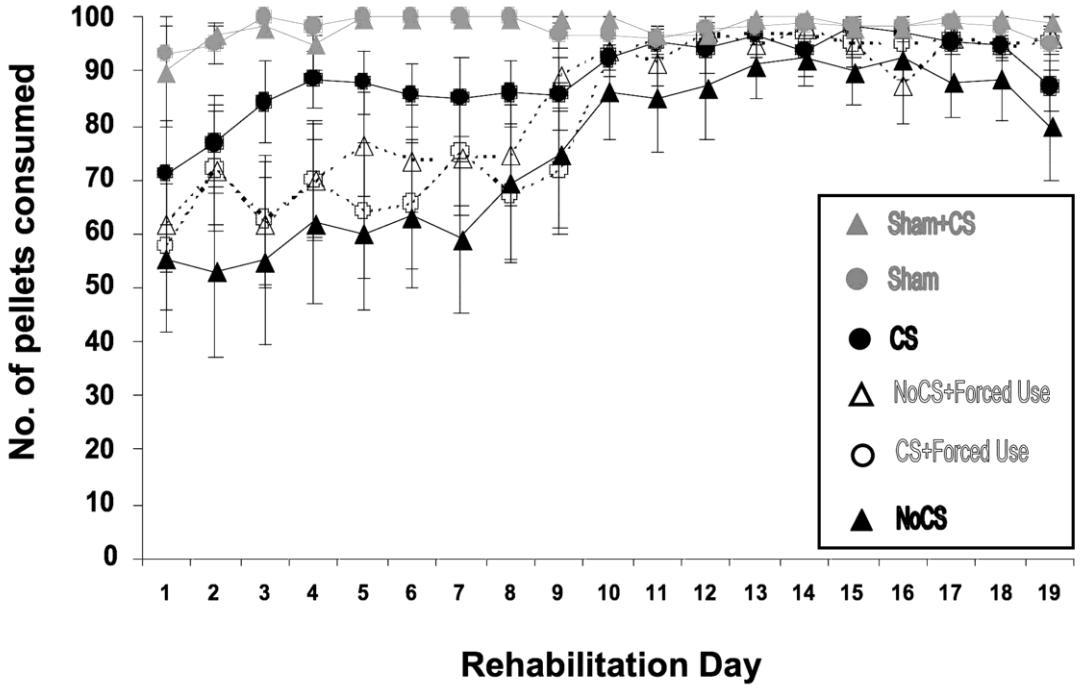


Figure 3.5: Performance on the rehabilitative tray reaching task. Rats receiving CS performed significantly better than NoCS depending upon day. The CS+Forced Use group had poorer performance compared with CS during early training. Data are means ± SEM.

between CS and CS+Forced Use ($F(18,396)=1.88$, $P=0.016$), reflecting the poorer performance of the latter group during the period of time in vests. In post-hoc analyses, however, there were no individual days that reached significance in CS versus NoCS. There was no significant difference between NoCS+Forced Use and NoCS (Group by Day: $F(18,378)=0.43$, $P=0.98$). There were also no significant differences in sham-operates as a result of CS. All analyses indicated significant effects of Day (F's=8.58-9.03, P's<0.001), reflecting improvements in reaching performance with ongoing training.

3.4.3 Forelimb-Use Asymmetry

As shown in Figure 3.6, lesions increased asymmetrical use of the forelimbs for postural support behaviors as measured in the Schallert cylinder test. Rats with lesions had an increased reliance on the less-affected ("unimpaired") forelimb ipsilateral to the lesion. The magnitude of this effect was similar between lesion groups as measured on post-lesion day 6, prior to the onset of CS treatment and reach training.

During the rehabilitation and chronic period, the CS group had a significant *decrease* in forelimb asymmetry compared to NoCS. That is, the CS group used the impaired limb proportionately more for postural support behavior than did the NoCS Group during both the rehabilitation period ($F(1,20)=5.30$, $P=0.032$) and the chronic period ($F(1,20)=4.55$, $P=0.045$).

As intended, forced use of the impaired limb effectively reduced the use of the less-affected (ipsilesional) forelimb, as measured on post lesion day 24. Excluding this time point in which vests were worn, there were no significant differences resulting from forced use. In NoCS+Forced Use, a return of asymmetry was seen after vest removal (Figure 3.6B).

Although CS did not significantly alter the asymmetry score in intact rats during the early time period ($P=0.10$), there was a significant Group effect ($F(1,10)=8.72$, $P=0.014$) between the Sham+CS and Sham+NoCS in the chronic period, reflecting an increased use of the reach-trained limb for postural support behaviors in Sham+CS.

3.4.4 Other Behavioral Measures

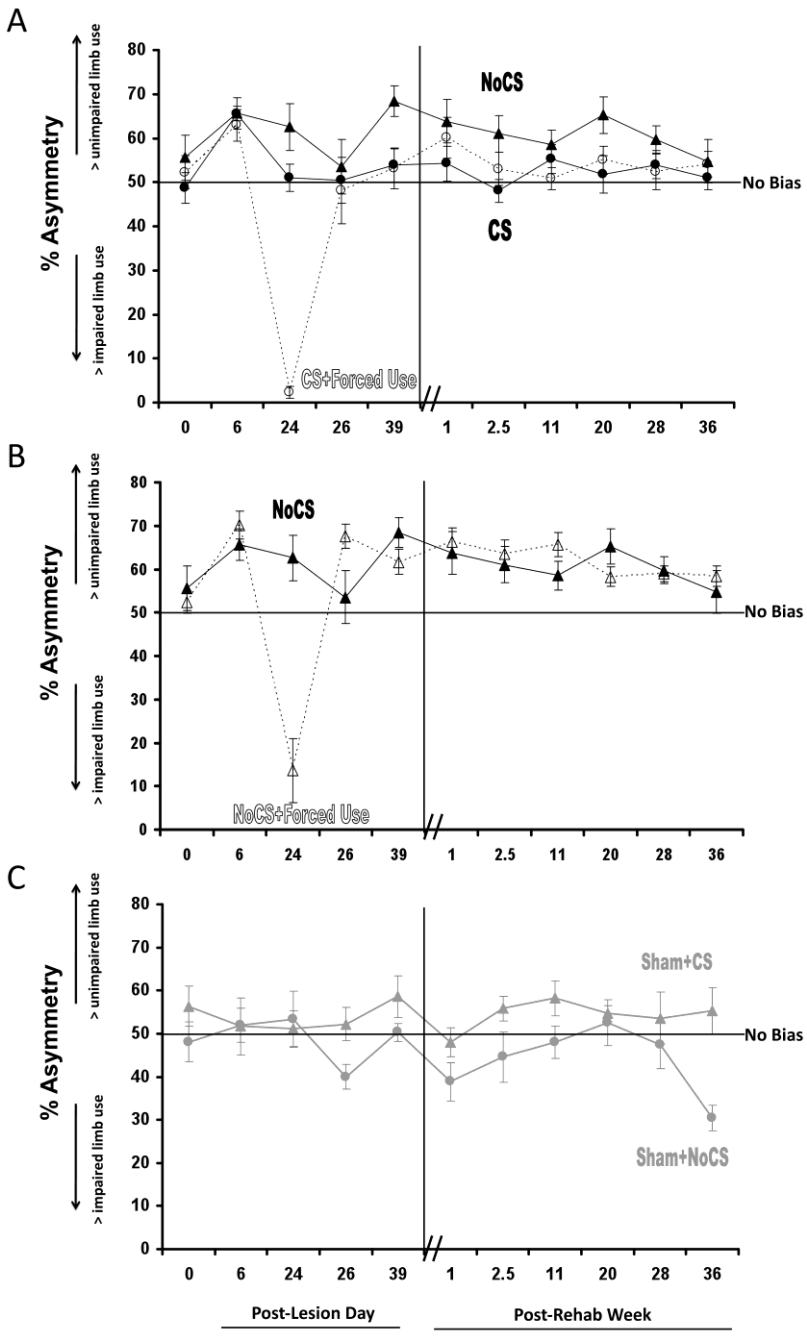


Figure 3.6. Forelimb use asymmetry as measured on the Schallert cylinder test. Larger asymmetry scores indicate greater disuse of the impaired limb. Lesions increased the use of the ipslesional ("unimpaired") limb. A: Early after the lesions (Day 6), CS and NoCS groups had similar forelimb asymmetries. During the chronic period, the CS group had a significant decrease in forelimb asymmetry compared to NoCS. B: There were no differences between the NoCS groups during the chronic period. As measured on Day 24, animals in the Forced-Use groups had an increased use of their impaired forelimb, supporting the effectiveness of this manipulation (A and B). However when the period of Forced-Use ended, they no longer relied largely on their impaired forelimb. Data are means ± SEM. * p = .014 vs Sham + NoCS in the chronic period.

In the other behavioral measures, although there were typical lesion-induced changes (Adkins et al., 2004), there were no significant effects of CS or forced use. In the footfault test and in the Whishaw movement analysis of reaching, lesion-induced deficits did not completely recover during the course of the experiment (and therefore there was room for further improvement). On the footfault test, pooling across lesion groups, means ± SEMs for % contralateral errors per step were 3.09 ± 0.42% pre-operatively, 15.58 ± 1.34% on day 7, 10.88 ± 0.89% one month post-rehabilitation and 10.43 ± 0.72% in the last month of testing. In the analysis of reaching movements using Whishaw's rating scale, lesions resulted in increases in abnormal movements from which there was little subsequent recovery. In lesion groups, means ± SEMs total number of abnormal movements per trial (including moderate and severe abnormalities) were 9.07 ± 0.40 preoperatively, 18.62 ± 0.69 post-operatively, 17.17 ± 0.84 on post-rehabilitation Day 40 and 14.08 ± 0.77 in month 12. In the vibrissae stimulated placing test, lesions resulted in more transient impairments (as expected for these lesions, Hsu and Jones, 2006) which recovered by post-rehabilitation week one.

3.4.5 Movement Thresholds

In all groups movement thresholds declined over time, consistent with previous findings (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003). There was a significant Group by Day effect between NoCS and NoCS+Forced Use ($F(3,63)=3.07$, $P=0.034$). On average, NoCS required less current to evoke movement during week 2 of rehabilitation compared to NoCS+Forced Use, but

this failed to achieve significance in post-hoc tests with Bonferonni's correction (*P*=0.024). There were no other significant group differences.

3.4.6 Volume of Remaining Cortex

As shown in Figure 3.7 CS groups had significantly greater volumes of some remaining motor cortical subregions compared to NoCS groups. Preliminary analyses indicated no significant differences resulting from the forced use condition in the CS or NoCS groups (F's(1,18)=.38-.97, F's(1,14)=.49-.95, respectively, *P*'s>.05) and these were combined for further statistical analyses. Using one-way ANOVAs, the CS lesion group had a significantly greater volume than the NoCS lesion group in the AGl (F(1,33)=9.79, *P*=.004), AGm (F(1,33)=13.28, *P*=.001) and FlOL (F(1,33)=13.86, *P*=.001). There were no differences between the CS and NoCS lesion groups in the volume of the HlOL (F(1,33)=1.17, *P*=.288), which is largely posterior to the lesion (and more remote from the electrode than the other areas). There were no significant differences in the volumes of motor cortical subregions between the two sham-operated groups (*P*'s>.05). The

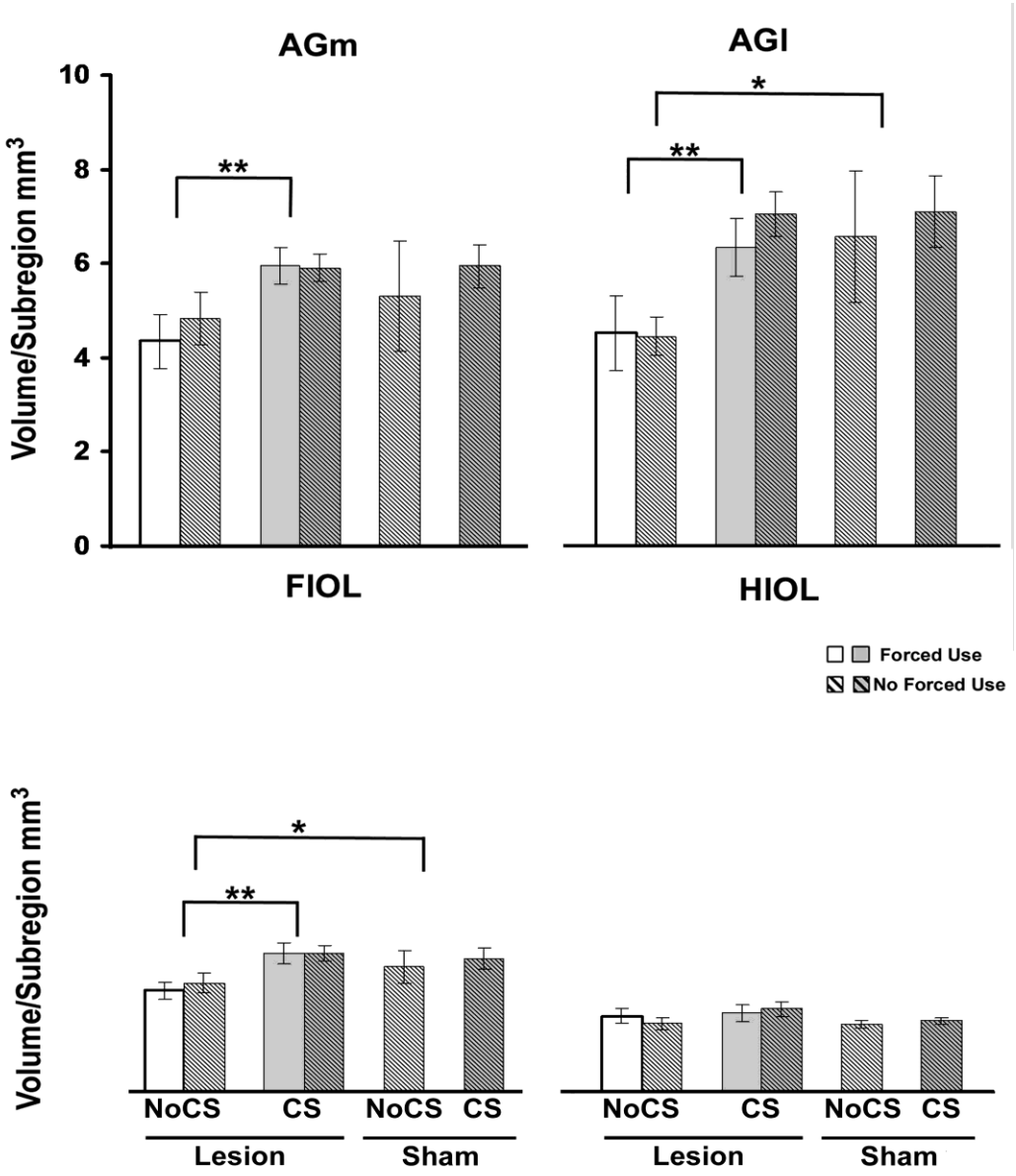


Figure 3.7. Volume of subregions of the SMC. The CS lesion groups had significantly greater volume of motor cortical subregions surrounding the infarct and near the site of stimulation compared to the NoCS lesion groups, i.e., the AGm, AGl and FlOL subregions. However, there were no significant differences in the volume of the HLOL subregion, which is posterior to the infarct and more remote from the focus of stimulation than the other regions. There were no significant differences in the volume of the motor cortical subregions in the CS lesion group compared to the sham-operated groups. The NoCS group had significantly less volume of the AGl and FlOL subregions compared to sham-operated groups. There were no significant differences between the two sham-operated groups (NoCS versus CS treated). AGm (Agranular Medial cortex), AGl (Agranular Lateral cortex), FlOL (forelimb overlap zone), HlOL (hindlimb overlap zone). Data are means ± SEM. * P<.030, ** P<.010.

NoCS lesion group had significantly reduced volume compared with sham operates in the AGl (F(1,24)=7.47, *P*=.011) and FlOL(F(1,24)=5.22, *P*=.032) but not the AGm or HlOL (F's(1,24)= 2.44 and .35, respectively, P's>.05). However, there were no significant differences between the CS lesion group and sham operates in any subregion (F's (1,28)=.024-2.30, *P*'s>.05).

As shown in Table 2, the CS groups also had significantly greater overall volumes of the ipsilesional remaining SMC. Using one-way ANOVAs, the CS lesion group had a significantly greater volume than the NoCS lesion group (F(1,33)=5.66, *P*=.023). There were no differences between the CS and sham-operated groups (F(1,33)=.52, *P*=.477) or the NoCS and sham-operated groups (F (1, 24) = 1.39, p = .250). There was a tendency for NoCS treated rats to have smaller lesion cavities than CS treated rats, though this failed to reach significance (Table 2, (F (1,33) = 3.68, p = .063, forced use conditions combined). There were no significant differences between forced use conditions. In sham-operates, damage resulting from the electrode was not significantly different between sham NoCS and CS subgroups (F (1,28) = 21, p = .649). Although there was a tendency for the NoCS lesion group to have significantly greater lesion cavity compare to sham-operates, the difference failed to reach significance (F (1,24) = 3.52, p = .073).

Table 2: Volume Differences of Remaining Motor Cortex

	Lesion Cavity	Ipsilesional	Lesion Cavity + Ipsilesional Cortex
NoCS	11.16 ± 1.71	107.75 ± 3.73 *	118.91 ± 3.33
CS	6.41 ± 1.73	119.24 ± 3.12	125.65 ± 2.95
Sham	4.88 ± 3.21	115.12 ± 5.23	120.01 ± 3.51

When the summed volume of the entire SMC region (lesion cavity and remaining cortex) was considered, there were no significant differences among the groups.

3.4.7 Infarct Characterization

In reconstructions of evident cortical damage relative to cytoarchitectural subregions (Figure 7), in all lesion groups, the lesion cavity included the AGm, AGl and FlOL subregions. The HlOL was relatively spared, which was expected because the lesion model does not target the HlOL. Most animals showed superficial damage to the underlying white matter. Eight animals (from NoCS, NoCS + Forced Use and CS + Forced Use groups), showed greater extension of damage into the corpus callosum, however, the damage did not penetrate deep into the corpus callosum and direct damage to the striatum was not evident in any of the animals. Lesion placement was similar

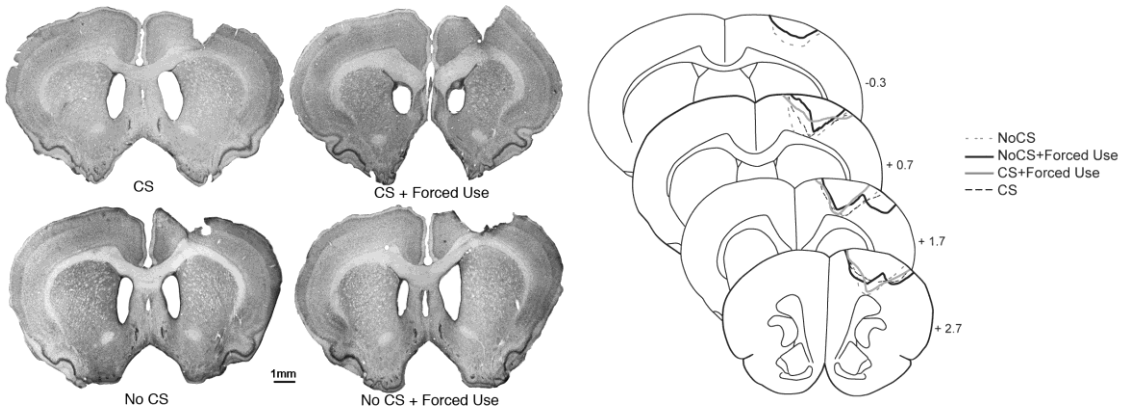


Figure 3.8. Representative lesions. A: Representative injury extent as viewed in Nissl stained coronal sections. B: A representative infarct reconstruction for each lesion group for each coronal plane. Lesion placement was similar among groups.

between the animals sacrificed in the chronic period and the small set of rats (n=4) sacrificed at the end of the rehabilitation period.

3.5 Discussion

In rats with unilateral sensorimotor cortical infarcts, CS combined with rehabilitative reach training significantly and persistently improved motor function in the impaired forelimb during the chronic recovery period compared to rats receiving training alone. This was, in part, a result of a greater maintenance of performance improvements gained during the rehabilitative training period. A two week period of forced use of the impaired forelimb did not further improve the effects of CS treatment. CS-treated rats also had greater volumes of motor cortical subregions, specifically the AGm, AGl and FlOL, compared to NoCS rats. CS combined with reach training has previously been found to change movement representations, neural activation and neuronal structure in the peri-infarct motor cortex and to improve behavioral performance of the impaired limb during and shortly after the training period (Adkins-Muir and Jones, 2003; Adkins et al., 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). The present findings support that CS combined with rehabilitative training can also be beneficial for inducing persistent functional improvements after stroke.

Although CS chronically improved skilled reaching performance, these improvements did not generalize to some other measures. Animals in this study receiving CS or forced use failed to completely recover on two of the measures--the footfault test, a test of coordinated foot placement during locomotion, and Whishaw's movement

analysis, which measures abnormalities in the reaching and grasping movements used to perform the single pellet retrieval task. It may be that greater normalization of reaching movements requires that CS be coupled with rehabilitative training on the more precise (single pellet) task. A surprising finding was that CS reduced forelimb asymmetries for postural support in the chronic period, as measured on the Schallert Cylinder Test. CS also reduced reliance on the untrained limb in sham-operates on this test. This was unexpected because previous research suggests that behavioral improvements resulting from CS are specific to the task with which it is coupled, at least as assessed during the treatment period (Adkins et al., 2006). Thus, it appears that CS has the ability to chronically influence postural support function in the impaired forelimb even when overt training in this function is not provided during CS administration.

As predicted, the CS-induced functional improvements were associated with greater volumes of motor cortical regions. It is possible that the greater volume of remaining cortex seen in CS-treated rats coincides with functional and structural changes that occur in the stimulated motor cortex, because, in intact animals, the addition of synapses, dendrites and glial processes is associated with increased cortical volume. For example, animals housed for a period of time in complex environments show an increase in the number of dendrites, synapses, brain weight, cortical thickness and volume (Black et al., 1997; Green et al., 1983; Grossman, Churchill, Bates, Kleim, & Greenough, 2002; Turner and Greenough, 1985). Motor skill training has also been shown to increase motor cortical volume, which is associated with increases in dendritic arborization (Greenough et al., 1985; Withers et al., 1989) and the number of synapses per neuron (Kleim et al.,

1996).

In the present study, the regions showing increased volume correspond to the regions showing CS-induced functional and structural changes in previous studies, though cortical volume changes have not previously been found. Previous studies have shown that CS increases dendritic (Adkins-Muir and Jones, 2003) and synaptic *density* (Adkins, Hsu and Jones, 2008). It also enhances motor cortical evoked potentials (Teskey et al., 2003) and increases in the size of forelimb movement representations (Kleim et al., 2003; Plautz et al., 2003). In these studies, lesion sizes and remaining cortical volume were consistently similar between CS and NoCS groups (Adkins-Muir et al., 2003; Adkins et al., 2006; Adkins, Hsu and Jones, 2008; Plautz et al., 2003; Teskey et al., 2003). The previous volumetric analyses did not distinguish between cortical subregions, so it is possible that there were subtle changes that were missed. However, the cortical measures were also made early after the CS treatment and, therefore, it is likely that the increased volume observed in the present study occurs subsequently, and possibly gradually, over time after the end of CS. If so, this would indicate that the previous findings of CS effects on neuronal structure and activity reflect an early stage of the changes instigated by CS. This calls for more detailed study of the chronic effects of CS on neural morphology and neural activity in this region.

Cortical lesions can result in atrophy of connected regions and this atrophy is often not evident until long after the lesions. For example, SMC lesions have been found to result in atrophy of the thalamus and striatum as measured three months post-lesion (Jones & Schallert, 1992). In addition, bilateral lesions to the visual cortex result in

atrophy of remote subcortical regions (Hovda et al., 1987). Excitotoxic striatal lesions result in long-term greater neuron loss and thickness of the whole cortex, and layers V and VI when assessed 12 months post-infarct (Munoz et al., 2001). Thus, it is possible that greater volume of peri-infarct motor cortical subregions in the CS-treated rats reflects, at least in part, a reduction in atrophy compared with CS. However, the pattern of volume differences is inconclusive, therefore, CS may result in a decrease of atrophy or an increase in volume. CS rats had greater volume of subregions and remaining cortex compared to NoCS, supporting an increase in volume influenced by CS. This data, along with the previous findings of increases in dendrites and synapses when assessed acutely after treatment suggests that CS results in increased volume when assessed chronically after treatment. However, although it is not significant, there is a tendency for CS rats to have decreased lesion cavity and lesion cavity summed with remaining cortex volume. In addition, the volume of the lesion cavity and the lesion cavity summed with remaining cortex is not significantly different in CS rats compared to sham-operates. This suggests that CS contributes to a decrease in atrophy. If CS facilitated increases in volume and not a decrease in atrophy, the NoCS lesion cavity volume should be similar to the sham-operates and the CS groups should have the least lesion cavity volume compared to NoCS and sham-operates. However, since the CS groups and the sham-operates are similar, this suggests that the CS is decreasing atrophy of the remaining cortex compared to the NoCS groups during the chronic period. Thus, CS may influence post-lesion atrophy, and the volume increase in the CS lesion group in the volume of the subregions is likely due to volume increases.

The pattern of results, combined with previous findings, also indicate that it is highly unlikely that CS altered the size of the ischemic injury. When assessed during earlier time points, there is no evidence that CS changes infarct size (Adkins et al., 2003; Adkins et al., 2006; Adkins, Hsu and Jones, 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). This is not surprising because the onset of CS treatment (days to weeks following ischemic induction) should minimize any influence on injury extent. In the current study, the volume of the lesion cavity plus the remaining cortex was increased in the CS group relative to the NoCS group, but similar between the NoCS and sham-operates. These results suggest that CS facilitated an increase in volume of the remaining cortex rather than altering the ischemic injury. In addition, there were no differences between the CS and NoCS lesion groups in behavioral performance on the single pellet retrieval task and the Schallert cylinder test as assessed early after the infarct and prior to the onset of treatment. Furthermore, animals in both treatment conditions remained similarly impaired on some behavioral measures over the 9-10 month period of chronic testing. Together, these findings suggest that any group difference in early injury severity is unlikely to be an important contributor to the present findings.

Although CS has been shown to be a beneficial adjunct to rehabilitative training in multiple animal models and in early phase human trials (Brown et al., 2006; Levy et al., 2008b), a recent large multicenter trial of epidural CS in humans showed no additional benefit of this treatment compared to physical therapy alone (Harvey and Winstein, 2009; Levy et al., 2008a). However, in a critical review, Plow et al. (2009) argue that the failure of this large clinical trial could have been due to differences in its

CS parameters compared with earlier trials. For example, in animal models and earlier clinical trials, CS was delivered at defined levels relative to movement thresholds. However, in the failed trial, movements could not be evoked within predefined CS intensity limits in the majority of patients (Plow et al., 2009). This suggests that CS intensity, and possibly localization, may be critical variables in its efficacy. It also raises the possibility that CS is particularly effective in a select population in which there remains sufficient corticospinal tract integrity. In rats, CS has been found to be less effective in improving forelimb function when initial impairments are severe compared with more moderate impairments (Adkins et al., 2008).

Noninvasive stimulation may also be a beneficial adjunct when paired with rehabilitative treatments. In humans, the combination of tDCS and occupational therapy (Nair et al., 2007), or rTMS and physical therapy (Khedr et al., 2005), has been shown to result in more enduring functional benefits than therapy alone. In addition, tDCS delivered to the motor cortex improved motor function of the paretic hand in tasks relevant to daily activities (Hummel et al., 2005). Recently, higher frequencies of rTMS applied to the ipsilesional motor cortex were found to be safe and resulted in modest improvements in behavioral function of the impaired hand (Yozbatiran et al., 2008). Furthermore, high frequency rTMS applied to the injured motor cortex, when combined with a complex, sequential motor task, was found to improve motor learning in human stroke survivors during the chronic period (Kim et al., 2006). This convergence of findings points to potential commonalities in the neural mechanisms of epidural CS and those of tDCS and rTMS (Hummel and Cohen, 2006).

Although forced use did not improve the effects of CS, it tended to improve reaching performance when administered without CS ($p = 0.06$), and it seems possible that a larger sample size would have revealed a significant effect. In humans, CIMT has been found to significantly improve arm motor function (Mark and Taub, 2004; Wolf et al., 2006) and, following striatal hemorrhagic injury in rats, CIMT-like treatment combined with rehabilitative training improved motor deficits and decreased tissue loss (DeBow et al., 2003). It should also be noted that the two weeks of forced use implemented in this study was on par in duration but does not closely resemble the effective CIMT used in humans (e.g. Wolf et al., 2006). For example, compared to the protocols for human rehabilitation, rats in the current study received less intense training each day. Therefore, it remains possible that an alternative strategy for combining CS and forced use, such as increasing the amount of time that rats in the forced use condition receive both CS and motor training or coupling CS with forced use outside of the reaching chamber, might more effectively influence forelimb function than either treatment alone. Furthermore, since CS is less effective in animals with severe initial impairments (Adkins et al., 2005), it may be that forced use (with or without CS) would be more beneficial in animals that are more severely impaired.

Another consideration is that forced use can increase injury severity in rats if initiated too early after lesions (Schallert et al., 2000); however, this seems unlikely to have occurred in this study because forced use was initiated at 9 days post-lesion and there were no differences in injury extent linked to forced use. The subgroup of rats receiving forced use with CS had a significant initial reduction in tray reaching

performance compared to control vests, an effect which was not found in rats receiving forced use without CS. This therefore raises the possibility that CS and forced use interfered with one another in their influence on reaching function.

In conclusion, the present findings support the efficacy of CS combined with rehabilitative training in chronically enhancing functional outcome following unilateral ischemic SMC lesions in rats. This improved function is linked with a greater volume of cortical subregions surrounding the infarct which, in combination with previous findings of increases in dendrites, synapses and movement representations, suggests that the behavioral improvements might be due, at least in part, to the greater functionality of residual motor cortex. Forced use of the impaired forelimb did not further improve the CS effects. However, given the benefits of CIMT identified in other studies, it remains possible that an alternative means of combining forced use with CS could further improve function. Further work is needed to understand the neural basis of these chronic behavioral changes. It remains important to further test how these persistent CS effects generalize to primates (Plautz et al., 2003) and to recovery from other types and loci of injury.

Chapter 4

Delayed Onset of Motor Rehabilitative Training, but not Cortical Stimulation, Improves Functional Deficits after Unilateral Cortical Infarcts in Rats.

4.1 Abstract

Unilateral ischemic sensorimotor cortex (SMC) lesions result in enduring impairments in contralesional forelimb function that can be partially improved by repeated practice on a skilled motor task as rehabilitative training (RT). In addition, in rats and monkeys, electrical stimulation of the peri-lesion cortex (cortical stimulation, CS) during practice on a skilled motor task improves impaired forelimb function compared to (RT) alone. However, these studies initiated CS and motor skill training within days to weeks after unilateral ischemic SMC lesions. The aim of this study was to determine whether a more delayed initiation of CS and RT improves forelimb function. Adult male rats were pre-operatively trained to criterion with their preferred forelimb on a unilateral reaching task, the single-pellet retrieval task. All rats then received an endothelin-1 induced ischemic SMC lesion contralateral to the trained forelimb. Three months later, all rats received a 24 day period of cortical stimulation concurrent with rehabilitative training (CS/RT), rehabilitative training alone (RT), cortical stimulation alone (CS) or neither cortical stimulation or rehabilitative training (noCS/noRT). CS consisted of 100 Hz cathodal stimulation given at 50% of the movement threshold. Rehabilitative training (both CS/RT and RT) resulted in major improvements in skilled

forelimb function compared to CS alone or neither manipulation. However, the addition of CS during RT did not further improve reaching performance compared to RT alone. Thus, motor skill training in the chronic period can greatly enhance function following ischemic damage to the SMC. However, the benefits of CS appear to be limited by time after lesion. It remains possible that an alternative strategy of delayed onset of CS administration would yield further improvements in forelimb function.

Key words: ischemia, recovery of function, motor learning, skilled reaching, epidural cortical stimulation

4.2 Introduction

In rats, initiation of rehabilitative motor skill training early after cortical ischemic injury results in functional improvements of the impaired forelimb (Biernaskie et al., 2004; Maldonado et al., 2008; Clark et al. 2009). The effect of motor skill training can be enhanced when combined with other therapies, such as an enriched environment (Biernaskie and Corbett, 2001; Knieling et al., 2009). In addition, the efficacy of rehabilitative training seems to be time-dependent. Early initiation of rehabilitative training results in greater performance on motor skill tasks and a decreased reliance on the less-affected forelimb. However, these improvements decline when rehabilitative training is initiated 14-days post-injury, and disappear completely when the onset of rehabilitative training is delayed by 1 month. In addition, enriched housing early after lesion induction results in greater performance on motor skill tasks, but enriched housing

administered during the chronic recovery period does not result in improvements in motor performance (Clarke et al., 2009).

Following unilateral infarcts of the sensorimotor cortex (SMC), cortical electrical current delivered to the peri-infarct motor cortex (cortical stimulation; CS) while animals undergo rehabilitative training (RT) improves recovery of impaired forelimb function compared to motor training alone (Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008; Brown et al., 2006; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003) and results in greater changes in structural plasticity. In the stimulated region of the motor cortex, the functional and structural plasticity includes increased dendritic (Adkins-Muir and Jones, 2003) and synaptic densities (Adkins, Hsu & Jones, 2008), larger forelimb movement representation areas (Kleim et al., 2003; Plautz et al., 2003) and enhanced motor cortical evoked potentials (Teskey et al., 2003).

All of these previous animal studies using CS in conjunction with RT initiated the therapy within a few days to weeks after unilateral ischemic SMC lesions. Therefore, it was unknown whether initiating CS and RT during a chronic phase of recovery (3-months post-infarct) would result in functional improvements of the impaired forelimb. The purpose of this study was to determine whether a delayed initiation of CS after cortical ischemia would be similarly effective in facilitating the effects of motor rehabilitative training.

4.3 Materials and Methods

4.3.1 Experimental Design Overview

Preoperatively, animals received 30 trials/day or 10 min (whichever came first) skilled reach training on the single pellet retrieval task until they reached a criterion of 45% successful retrievals/attempt (~9 days). This criterion was chosen based on asymptotic performance using these task parameters in this substrain of rats (O'Bryant et

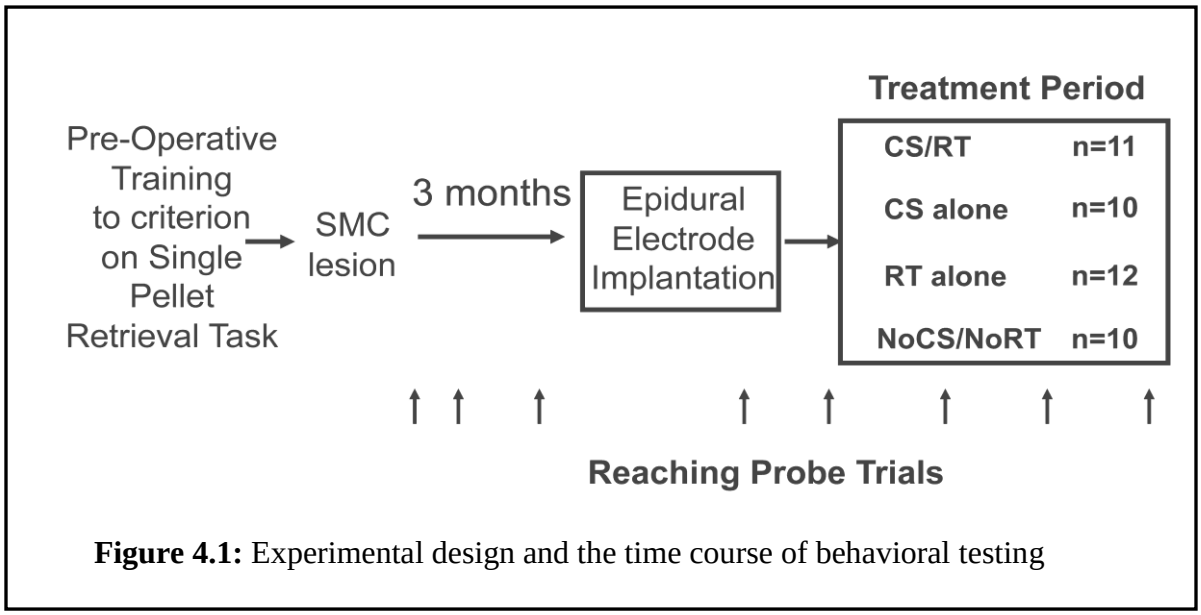


Figure 4.1: Experimental design and the time course of behavioral testing

al., 2010). Post-operative performance was tested on day 5 (30 trials) and day 24 (15 trials) and prior to electrode implantation (days 90 and 91, data pooled). Performance was calculated as the percentage of successful retrievals (success + drops) per reach attempt. Following electrode implantation, animals received a 15 trial test on days 99 and 100. During the rehabilitation period, all groups were tested for 14 trials once weekly on the single pellet retrieval task on days 106, 113, 120 and once at the termination of rehabilitation training, day 127. In addition, on day 134, thirty minutes prior to

perfusion, all groups were tested on the single pellet retrieval task (14 trials) to investigate the expression of c-fos, an immediate early gene. Post-implantation performance was also calculated as the percentage of successful retrievals (success + drop) per reach attempt. Initial lesion-induced impairment in reaching success and post-implant reaching performance was matched across groups.

4.3.2 Animals

Forty-three male Long-Evans rats were obtained from Charles Rivers Laboratories (Wilmington, MA) at one month of age and housed in polycarbonate cages in pairs or triplets on a 12:12 h light:dark cycle with water ad libitum. Housing included standardized cage supplements (a PVC tube, cardboard rolls, small wooden objects to gnaw and manipulate, and 6g of a complex food mixture, given twice weekly, that consisted of in-shell nuts, in-hull seeds, macaroni, and dried fruit pieces). Rats were tamed by weekly gentle handling and placed on scheduled feeding (15g chow/day, gradually increased to permit age-related weight gain). All rats began behavioral training at 3 months of age and received lesions at 4 months of age. The protocol for all procedures was approved by the University of Texas Animal Care and Use Committee.

4.3.3 Testing and Training in Skilled Reaching

The single pellet retrieval task has been described in detail previously (Bury and Jones, 2002; O'Bryant et al., 2007). Briefly, animals were placed in a plexiglas chamber and learned to reach through a tall narrow window to retrieve a banana flavored pellet

(45mg, Bio-Serve, Frenchtown, NJ) placed in a well 1 cm from the opening (Figure 2.1). After shaping to determine endogenous limb preference (Whishaw, 1992), a removable wall was inserted ipsilateral to the preferred limb and pellets were placed, one at a time, in a well opposite this limb. Rats were permitted to make up to five reach attempts to retrieve each pellet. Performance was measured as the number of successful retrievals (grasping, retrieving and eating the food pellet)/reach attempt.

An adaptation of the Whishaw qualitative rating scale (Gonzalez et al., 2004; Whishaw et al., 1993; Whishaw and Metz, 2002) was used to analyze ten specific reaching movements in frame-by-frame video playback, as previously described (O'Bryant et al., 2007). Each of the 10 movements was classified as being normal (0), mildly abnormal (0.5) or severely abnormal (1). The numbers of mildly and severely abnormal movements per 5 successful trials were analyzed. Animals were tested pre-operatively, on post-operative day 8, prior and following electrode implantation, and again at the end of rehabilitation training.

4.3.4 Surgical Procedures

4.3.4.1 Cortical Infarcts. In all rats, ischemic damage (Fig. 3.2, Ch.3) was induced via a topical application of endothelin-1 (ET-1), a vasoconstricting peptide, to the SMC (Adkins-Muir and Jones, 2003; Fuxe et al., 1997). Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the skull was removed between 0.5 mm posterior, 2.5 mm anterior, and 3.0-5.0 mm lateral to Bregma. The dura was removed and 2.2 µl (200 pmol) of ET-1 (Peninsula Laboratories Inc.) was administered to the pial

surface. After ten minutes, Gelfoam (Pfizer, New York, NY) was applied on top of dura, and the craniectomy was covered using UV-cured dental cement (Wave A2; Southern Dental Industries, Victoria, Australia).

4.3.4.2 Electrode Implantation. Three months after lesion induction, the craniectomy was enlarged ~1.0 mm both rostrally and medially to expose perilesion areas of motor cortex. The electrode and its configurationwas previously described in detail (Fig. 3.3, Ch.3).

4.3.5 Movement Threshold Testing. During the rehabilitation period, all animals were tested weekly for movement thresholds. A movement threshold was defined as the minimum current needed to evoke an observable forelimb, head/neck, or shoulder movement. Rats were placed in a Plexiglas cylinder and observed during delivery of cathodal stimulation that consisted of 100 μs pulses delivered at 100 Hz frequency in 3 s trains of increasing amplitude. Stimulation was delivered using a Sunrise PSS2 stimulator (Northstar Neuroscience, Inc., Seattle).

4.3.6 Rehabilitation Training and Cortical Stimulation (CS) Procedures

Rats received RT on the tray reaching task (Figure 4.2) 6 days a week for 24 days, with probe trials on the single pellet retrieval task given every 7th day. During rehabilitative training sessions, rats were placed into a reaching chamber equipped with a 7.5 cm long × 5.2 cm high × 6 cm wide inclined (25°) metal tray positioned outside the reaching chamber in front of the reaching window. The rats were then permitted to reach

for a total of 100 banana flavored food pellets through the reaching window for 10 minutes. Limb use was restricted to the impaired limb through the use of in-chamber walls, as described above. Animals received 50 pellets during the first five minutes, followed by the remaining 50 pellets during the last five minutes of training. For all groups not receiving RT, 100 banana pellets were placed on the floor of the chamber. Performance was measured as the number of pellets consumed out of 100.

Animals receiving CS received continuous 100 Hz cathodal stimulation delivered 50 % of the MT for the entire 10 minutes of the rehabilitation session. All other animals were connected to the stimulation cable but received no stimulation. These parameters were chosen because they have previously been found to be particularly effective with epi- or subdural electrodes in acutely improving function (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003). On probe trials of performance on the single pellet retrieval task during the rehabilitation period, the CS

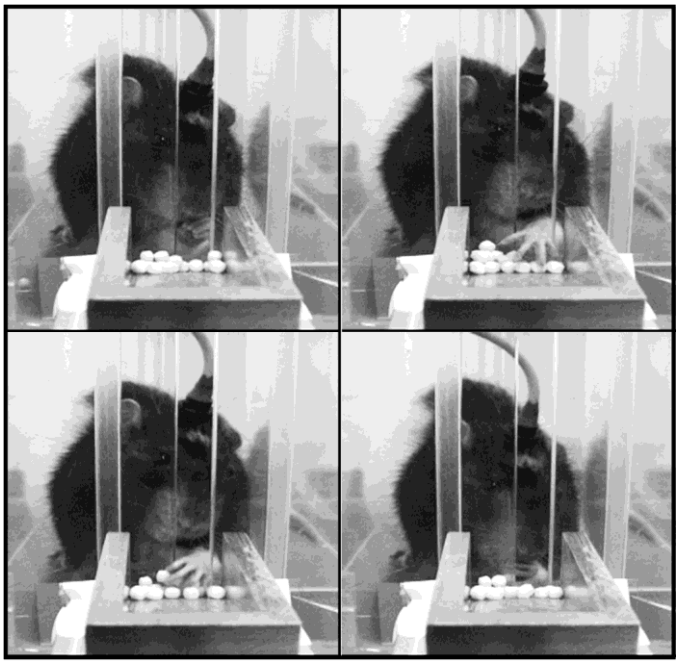


Figure 4.2: Motor Rehabilitative Tray Reaching Task. Rats received daily training on the tray reaching task for a total of 24 days. Rats reached for 100 food pellets for 10 minutes, with our without continuous stimulation.

groups received stimulation for half of the trials, with the order alternated weekly. This was done to assess whether reaching performance altered in the presence of CS. NoCS rats were connected to stimulator cables but received no stimulation during these training and testing sessions.

4.3.7 Other Behavioral Measures

Animals were tested on a sensorimotor test battery once pre-operatively, on post-operative day 8, prior and following electrode implantation, and again at the end of rehabilitation training.

4.3.7.1 The Vermicelli Handling Test. This test measures dexterous forepaw function in rodents (Whishaw and Coles, 1996; Allred et al., 2008; Tennant et al., 2010). Rats were given 7 cm lengths of uncooked vermicelli pasta (1.5 mm diameter; 0.15 g/piece; Skinner brand, distributed by New World Pasta Co., Harrisburg, PA) marked at 1.75 cm intervals with an ultrafine tip marker. In order to control for variations in texture and diameter, a single source of Italian style vermicelli was used. All rats were given vermicelli pieces in their homecages prior to behavioral testing so they could become accustomed to pasta handling. A test consisted of 4 trials with of one pasta piece per trial. During testing, rats were placed into a clean, clear Plexiglas chamber, isolated from their cagemates. All trials were videotaped and replayed in slow motion. Data were collected without knowledge of experimental condition and/or injury side.

4.3.7.2 The Schallert Cylinder Test. The Schallert cylinder test (Schallert et al., 2000) was used to measure asymmetrical forelimb use for postural support and increased

reliance on the ipsilesional forelimb. Animals were placed in a Plexiglas cylinder (19 cm diameter) and video-recorded until 30 paw placements occurred. The cylinder encourages animals to vertically explore the environment while supporting themselves against the cylinder wall using their forepaws. This sensitively reveals asymmetries in the use of the forepaws for postural support. In slow-motion video playback, each instance of forelimb use was recorded. The asymmetry score was calculated by the following formula: (ipsilateral + 1/2 simultaneous bilateral use) / total limb use × 100.

4.3.7.3 The FootFault Test. This test was used to measure coordinated forelimb placement during locomotion as rats traversed an elevated grid. Animals were placed on the elevated grid platform (33 cm × 30 cm; grid openings: 8.4 and 6.25 cm^2) and allowed to move across the platform, placing their paws on the rungs of the grid openings. The experimenter counted the number of slips with impaired forelimb through the grid openings. Performance was calculated as the number of errors ("foot faults") that occurred in ratio to the total number of total steps.

4.3.8 Neuroanatomical Methods

4.3.8.1 Histology. At the end of the rehabilitation period, rats received an overdose of sodium pentobarbital and were ranscardially perfused with 0.1 M phosphate buffer and 4% paraformaldehyde solutions. Coronal sections (50μm) were taken throughout the cerebrum using a vibrotome (Leica Microsystems, Wetzlar, Germany) and Nissl stained with toluidine blue. All anatomical samples were coded to blind for

experimental condition and collected from digital images captured using an Olympus BX61 microscope.

4.3.8.2 Volume Measures and Lesion Analysis. For cortical injuries, interhemispheric volume differences are often used as an indirect estimation of lesion size by subtracting the remaining tissue on the injured hemisphere from that of the intact hemisphere. For each rat, the area of cortex remaining within brain sections that included the SMC region was determined using Neurolucida perimeter tracing software (Microbrightfield, Inc.) at a final magnification of X 17. A total of seven sections, 400 μm apart, moving in a caudal direction, were measured beginning with the appearance of the head of the caudate, as described previously (Jones et al, 1999). The Cavalieri method (Gundersen and Jensen, 1987) was used to calculate volume as the product of the summed areas and the distance between section planes. The extent and placement of each lesion was also reconstructed onto schematic cortical coronal sections adapted from Paxinos and Watson (1986).

4.3.9 Immunohistochemical Procedure

Immunostaining was performed on 50 μm thick sections, using the avidin-biotin peroxidase (ABC) method (ABC kit, PK-6101, Vectastain, Vector). Sections were rinsed with PBS and then incubated for 30 min with 0.3% hydrogen peroxide to extinguish any endogenous peroxidase activity. The sections were then incubated in a block solution for 2 h to prevent non-specific background staining. Immediately after, sections were incubated with the primary antibody for 48 h to determine the expression levels of c-fos

or MAP-2. The primary antibodies used were anti-cfos (X source info) and anti-MAP2A+B (1 : 500, clone AP20, Sigma Chemicals, St. Louis, MO, USA). After incubation in appropriate secondary antibodies, sections were incubated in avidin-biotin-horseradish peroxidase complex, which reacted with the enzyme substrate, DAB (diaminobenzidine tetrahydrochloride in PBS), until desired staining intensity appeared. For each batch of immunocytochemical processing, specificity of antibody binding was verified using tissue sections processed without the primary antibody.

4.3.10 Statistical Analyses

To analyze reaching and sensorimotor behavioral data, the SPSS (SPSS, Inc.) program for general linear models for repeated measures analysis of variance (ANOVA) was used to determine the effects of Day, Group, and Group by Day interactions. For behavioral results, planned comparisons were used for the primary analyses to address these questions: After the lesions does (1) RT improve performance (RT groups vs. NoRT groups), (2) CS further improve function compared to RT (CS/RT versus RT), (3) does CS alone improve function (CS versus NoCS). Post hoc T tests were used to further analyze reaching data. The a priori α level for all comparisons was 0.05. To analyze the reaching movement analysis data, SPSS general linear model multivariate analysis was used.

4.4 Results

4.4.1 Skilled Reaching Performance on the Single Pellet Retrieval Task.

As shown in Figure 4.3, following lesions and prior to the onset of rehabilitative training, all animals declined in reaching performance on the single pellet retrieval task. There was minor spontaneous recovery, as evident by a partial improvement in performance between days 6 and 99, but analysis revealed significant impairments compared to pre-injury performance.

RT alone or in conjunction with CS, but not CS alone, improved reaching

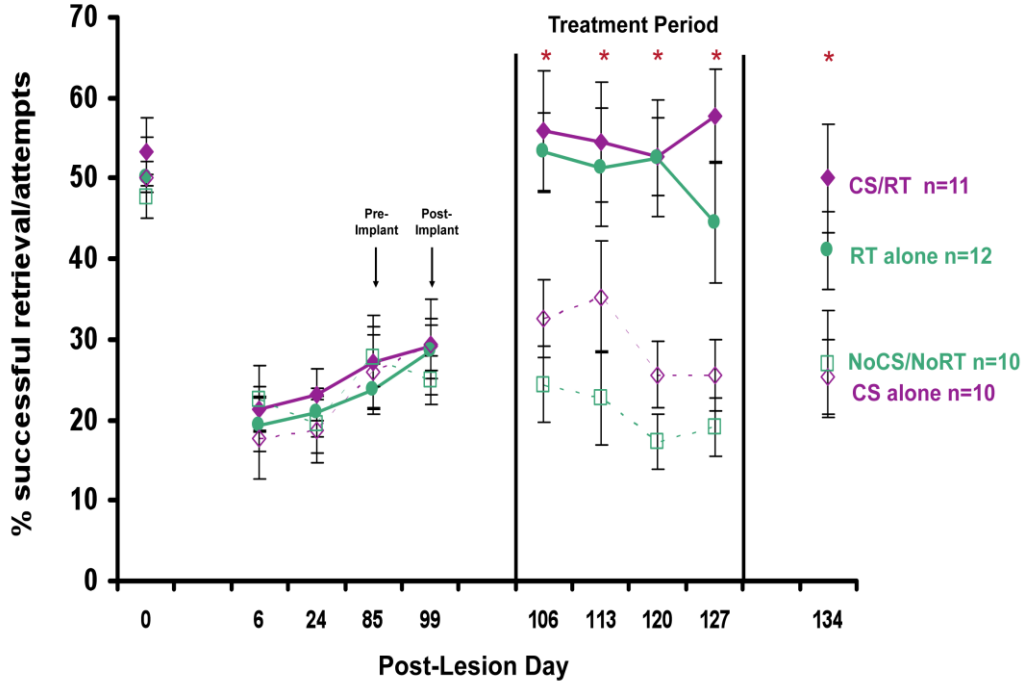


Figure 4.4. Performance on the Rehabilitative Tray Reaching Task. Initially, the no rehabilitative training groups (CS alone and NoCS/NoRT) consumed more banana flavored food pellets. In addition, delayed onset CS tended to improve function weeks after rehabilitative training begins. Data are means ± S.E.M.

performance when initiated during the chronic phase of recovery. Using a repeated measures ANOVA, RT animals had greater reaching performance compared to NoCS/NoRT. Results indicate there was a significant Group by Day ($F(9,180) = 6.79$, $p < .001$), Group ($F(1,20) = 10.84$, $p = .004$) and Day effect ($F(9,180) = 9.40$, $p < .001$). Post-hoc analyses revealed significant differences in reaching performance on the single pellet retrieval task during all days assessed during the rehabilitative training period. There were no significant differences in reaching performance between the CS/RT and RT alone groups, or the CS alone and NoCS/NoRT groups (p's > .05)

4.4.2 Performance on the Rehabilitation Task.

Although the single pellet reaching task was the primary outcome measure, performance

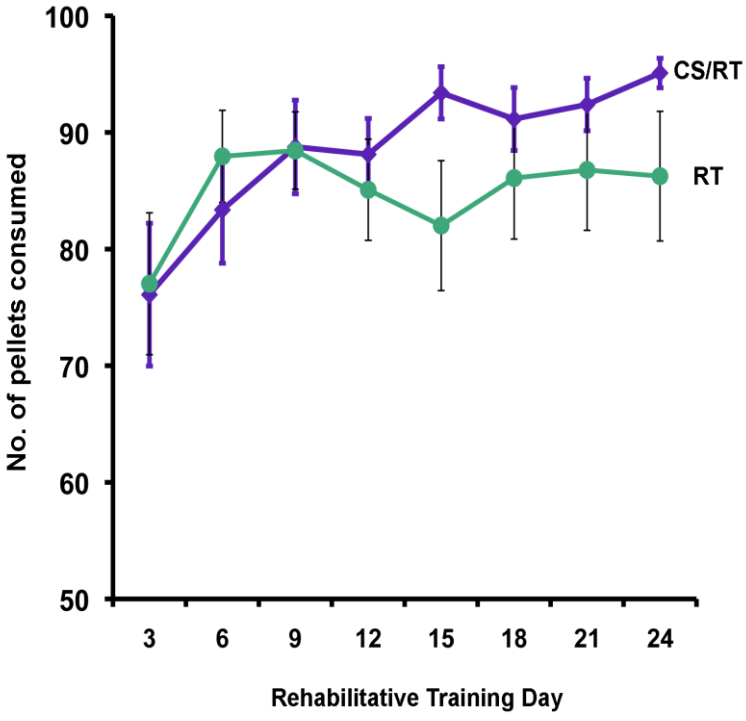
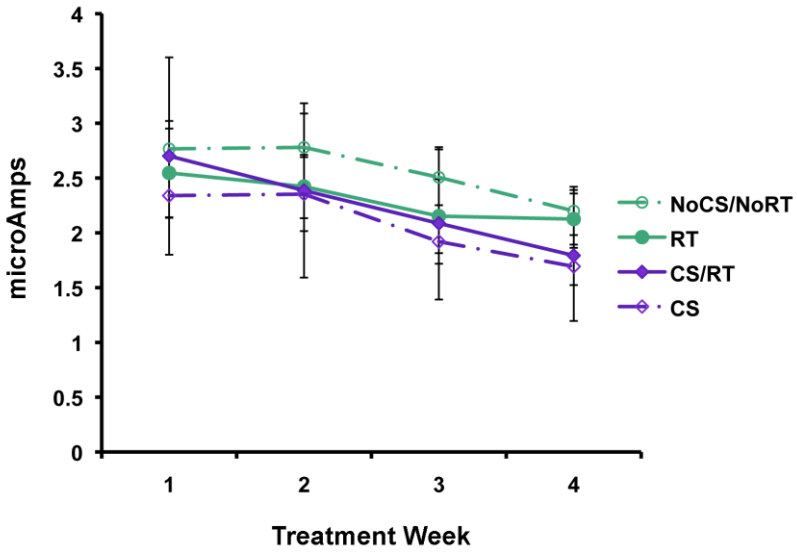


Figure 4.3. Performance on the single pellet retrieval task. Rats receiving motor rehabilitative training had greater performance during the treatment period compared to the no rehabilitative training groups. CS did not result in additional reaching improvements when paired with rehabilitative training. Data are means ± S.E.M. (* $P < .05$, RT groups vs. noRT groups.

on the rehabilitative tray reaching task was also assayed by measuring the total number of pellets retrieved (out of 100) per training session. Rats receiving rehabilitative training (CS/RT and RT) had improvements over days of training, (as measured by the number of pellets consumed) on the tray reaching task compared to rats not receiving rehabilitative training (CS alone, NoCS/NoRT). However, the group receiving CS with motor training (CS/RT) had greater performance on the tray reaching task compared to motor training alone (RT) (Group by Day ($F(7,147) = 3.55$, $p = .001$). Posthoc analyses revealed no significant differences in tray reaching performance, although there was a tendency for the CS/RT group to perform better on days 13-15 ($p = .08$). There was no significant difference between the CS alone and NoCS/NoRT groups in the number of pellets eaten from the floor of the chamber.

4.4.3 Movement Thresholds



As seen in Figure 4.5, movement thresholds for all groups declined over time, consistent with previous findings

ds, the minimum
kly on all animals.

(Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003). There were no significant differences among the groups in weekly movement threshold levels.

4.4.4 Other Behavioral Measures

In both Qualitative Movement Analysis and the FootFault test, although there were typical lesion-induced changes (Adkins et al., 2004), there were no significant differences in performance among the groups during both the pre-CS/RT period and after the treatment period. In the FootFault test, animals did not fully recover from lesion-induced deficits during the course of the experiment (which indicates there was room for further improvement). Pooling across lesion groups, means ± SEMs for % contralateral errors per step were 5.16 ± 0.63 % pre-operatively, 31.50 ± 1.27 % on day 7, 23.26 ± 1.31 % pre-implantation, 21.81 ± 0.94 % post-implantation, and 19.91 ± 1.20 % post-rehabilitative training. In the analysis of reaching movements using Whishaw's rating scale for Qualitative Movement Analysis, lesions resulted in increases in abnormal movements from which there was little subsequent recovery. The means ± SEMs for total number of abnormal movements per trial (including moderate and severe abnormalities) were 0.56 ± 0.04 pre-operatively, 1.67 ± 0.08 post-operatively, 1.53 ± 0.09 pre-implantation, 1.49 ± 0.06 post-implantation, and 1.33 ± 0.09 post-rehabilitative training.

On the Schallert Cylinder test, there was a significant lesion-induced change between pre-operative and post-operative performance ($p < .001$). Pooling across all groups, means ± SEMs for % asymmetry were 49.81 ± 1.76% pre-operatively, 57.52 ±

1.92% on day 7, 56.17 ± 1.99% pre-implantation, 54.12 ± 2.53% post-implantation and 51.40 ± 2.21% post-rehabilitative training. However, animals failed to recover on this measure both pre-treatment or during treatment.

4.4.5 Training-induced changes in MAP2-labeled dendritic processes and cFos-IR cells in peri-infarct motor cortex

As shown in Figure 4.6, RT animals with unilateral ischemic SMC lesions had significantly greater surface density of MAP2-IR dendritic processes in layer V of the peri-infarct motor cortex compared with NoCS/NoRT (F (1,21) = 6.74, $p < 0.017$) and CS/RT (F (2,31) = 3.35, $p < 0.049$). There were no significant increases in MAP2-IR between the CS alone versus NoCSNoRT ($p > .05$). The surface density of MAP2-IR

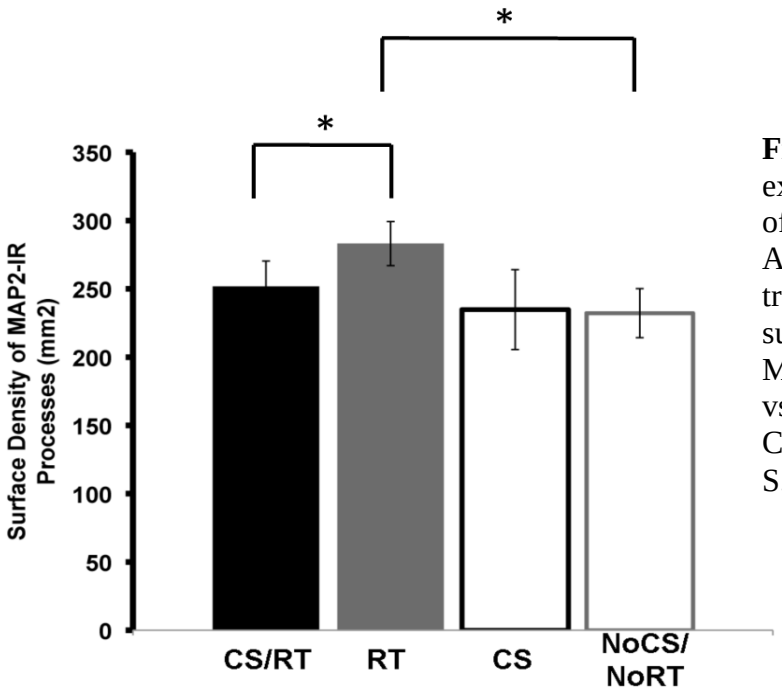


Figure 4.6. MAP2-IR expression in layer V of peri-infarct cortex. Animals receiving RT training had greater surface density of MAP2-IR compared vs NoCS/NoRT and CS/RT. Data are +/- S.E.M. * $p < .05$

processes for all groups were 251.78 ± 18.55 (CS/RT), 283.16 ± 29.27 (RT), 234.78 ± 29.27 (CS) and 232.19 ± 17.94 (NoCS/NoRT). There were no significant differences in the cFos-IR expression in peri-infarct motor cortex (*p*'s > .05).

4.4.6 Volume of Remaining Cortex

All lesions appeared to result in major damage in the forelimb area of the SMC. It appeared that no animals had any direct damage to the striatum. Volume measurements of the remaining SMC revealed no differences in infarct size among the groups (Figure 4.X). Using a one-way ANOVA, results indicate similar lesion size among groups ($F(3,37) = 0.73$, $p = .542$). Pooling across lesion groups, the mean ± SEM of remaining

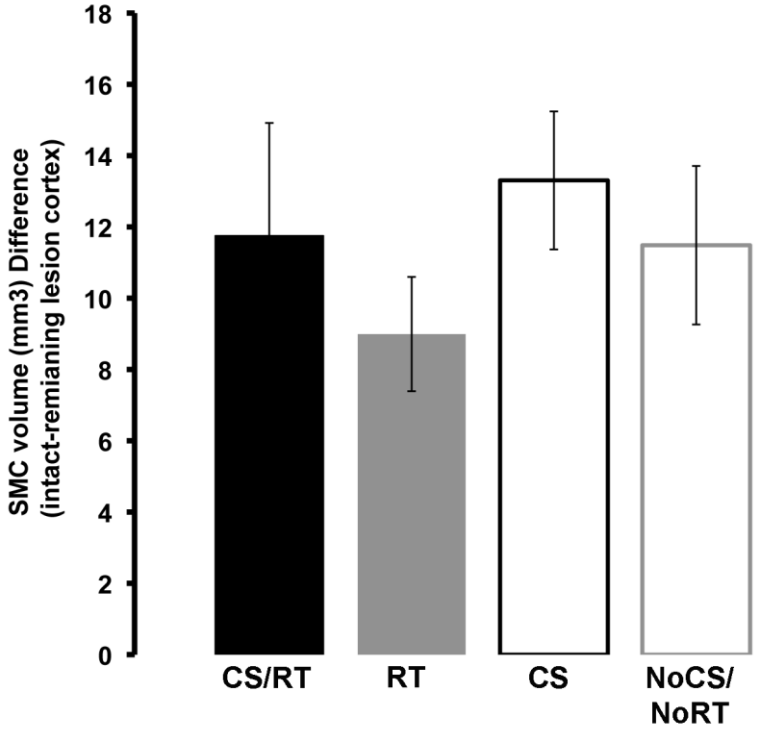


Figure 4.7 Interhemishpheric Volume Differnce. There were no differences in the volume of remaining motor cortex among the groups. Data are +/- S.E.M.

SMC region volume in mm^3 was 70.33 ± 1.22 in the damaged hemisphere, and the interhemispheric difference in volume (contra - ipsi) in the SMC region was 11.26 ± 0.97 (Figure 4.7).

4.5 Discussion

Rehabilitative reach training significantly improves motor function of the impaired limb and resulted in greater surface density of MAP2-IR in layer V of remaining motor cortex, when initiated during the chronic phase of recovery, in rats with unilateral sensorimotor cortical infarcts. However, the addition of CS during rehabilitative training did not result in greater improvements in impaired forelimb function compared to rehabilitative training alone. As expected, rats in the groups not receiving rehabilitative training (CS alone and NoCS/NoRT) did not have improvements in impaired forelimb function. Thus, rehabilitative reach training in the chronic period can greatly enhance function following ischemic damage to the SMC. However, the benefits of CS appear to be limited by time after lesion. It remains possible that an alternative strategy of delayed onset of CS administration would yield further improvements on forelimb function.

Previous research has shown that rehabilitative training results in neuroplastic changes in the cortex and contributes to behavioral improvements. In the unilateral middle cerebral artery occlusion (MCAo) and more focused stroke models, complex rehabilitative training has been found to enhance synaptic and dendritic plasticity in the

intact cortex (Jones et al.,1999; Biernaskie and Corbett, 2001) and decrease the loss of remaining tissue in peri-lesion cortex (Chu and Jones, 2000). Focused training of the impaired forelimb after small ischemic lesions promotes neuronal reorganization within adjacent cortical tissue and is associated with motor recovery (Nudo et al., 1996). Previous studies have demonstrated that, following ischemic injury to the motor cortex, early initiation of motor skill rehabilitative training (i.e. 5-15 days post-injury) results in significant improvements of the impaired forelimb (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Maldonado et al., 2008). However, previous research suggests there is a time-sensitive window for the initiation of rehabilitative training. A study by Biernaskie et al. (2004) demonstrated that animals receiving early (beginning post ischemic injury day 5) rehabilitative training combined with enriched environment have greater improvements in behavioral assays compared to animals that receive it at a later time point (post-ischemia day 30). Nudo et al. (1996) found that rehabilitative training prevented further loss of hand territory in the undamaged cortical tissue adjacent to the infarct suggesting that rehabilitation training may contribute to subsequent reorganization of this tissue and to motor recovery. A recent study assessing the motor performance of rats receiving early rehabilitative training paired with an enriched environment following ischemic damage to the motor cortex and dorsolateral striatum suggested that motor performance greatly improves in rats receiving motor rehabilitative training and an enriched environment compared to rats receiving no motor training and housing in standard cages (Clark et al., 2009). In addition, post-rehabilitative “tune-ups”, which consisted of intense sensorimotor/cognitive activities, did not result in additional benefits

on recovery. These data suggest that the most sensitive time to initiate rehabilitative training for optimal motor recovery is early following ischemic injury. However, adult squirrel monkeys trained on a motor skill task, followed by ischemic injury to the motor cortex, showed significant improvements in motor recovery when rehabilitative training was initiated 30 days post-infarct (Barbay et al., 2006). Delayed rehabilitative training and the subsequent recovery of the motor impairments were similar to previous findings that initiated rehabilitative training early after infarct. However, unlike early initiation of rehabilitative training, this study failed to find sparring of hand representations in the motor cortex (Barbay et al., 2006). This suggests that although delayed initiation of motor rehabilitative training results in motor recovery, alterations in the movement representations may not be similar. The current study found that initiation of rehabilitative training during a chronic recovery period (i.e. 3-months post-infarct) results in significant improvements in reaching performance on a motor skill task compared to rats not receiving motor rehabilitation. Thus, the use of adjunct therapies may be particularly important for rehabilitative training efforts that are delayed in onset.

It should be noted that, unlike previous studies, the current study did not have a direct comparison with a group that received an earlier onset of rehabilitative training (e.g. Biernaskie et al., 2004; Barbay et al., 2006). However, a previous study in our lab investigated early initiation of rehabilitative training and the potential CS-induced behavioral improvements. The results indicate that early onset rehabilitative treatment results in a greater magnitude of behavioral improvements (O'Bryant et al., in progress). Although in the current study RT and CS/RT animals were able to return to pre-operative

reaching performance, early onset rehabilitative treatment CS/RT resulted in reaching performance that surpassed the pre-operative reaching success. This suggests that, although delayed onset rehabilitative training does result in a return to pre-operative reaching performance, early onset results in the greatest magnitude of improvement.

An additional question of the current study was to investigate the potential effects of CS on rehabilitative training initiated during a chronic recovery period. Although research suggests that CS coupled with rehabilitative training early after cortical injury improves performance and increases plasticity, little is known about its effects when initiated during a chronic recovery period. Compared to the rehabilitative training groups, CS did not result in additional improvements. Thus, while motor skill training in the chronic period can greatly enhance function following ischemic damage to the SMC, the benefits of CS appear to be time-dependent. However, it should be noted that CS/RT rats began differentiating from RT and showing improved performance on the daily tray reaching rehabilitative task in the later stages of the treatment period. This suggests delayed onset of CS, when paired with motor rehabilitative training, could result in greater improvements, compared to RT alone, if the length of the treatment period was extended.

CS enhances both the cortical and behavioral effects of rehabilitative training when initiated early post-infarct. Motor cortical CS combined with rehabilitative training changes functional activity patterns and neuronal structure in the peri-infarct area and improves behavioral performance of the impaired limb (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003). It also increases in the size of forelimb motor map

representations in peri-infarct motor cortex (Kleim et al., 2003; Plautz et al., 2003), increases in synaptic density (Adkins, Hsu and Jones, 2008) and increases in dendritic processes in the peri-infarct area (Adkins-Muir and Jones, 2003). CS concurrent with rehabilitative training also results in a decrease in movement thresholds (the amount of electrical current needed to elicit a movement) and enhances motor cortical evoked potentials (Teskey et al., 2003). Previous human clinical studies have found that CS/RT enhanced functional recovery of the impaired arm compared to rehabilitative training alone (Brown et al., 2006; 2008; Huang et al., 2008; Levy et al., 2008). The results of the currents study suggest that CS does not further improve reaching performance compared to RT alone. However, the CS group appeared to improve on reaching performance in the single pellet retrieval task and motor rehabilitative tray reaching task late into training. Therefore, it is likely that a longer period of training would result in greater improvements. In addition, it may be that CS does not further improve forelimb function compared to RT when initiated too late following stroke-like injury. It remains possible that an alternative strategy of delayed onset CS would yield further improvements in forelimb function.

Although rehabilitative training improved skilled reaching performance when initiated during the chronic recovery period, these improvements did not generalize to other behavioral measures. Animals in this study receiving RT failed to completely recover on two of the measures--the FootFault test, a test of coordinated foot placement during locomotion, and Whishaw's movement analysis, which measures abnormalities in the reaching and grasping movements used to perform the single pellet retrieval task. It

may be that greater normalization of reaching movements will occur if a more precise rehabilitative motor task (i.e., the single pellet task) is used.

In conclusion, the present findings support the efficacy of delayed initiation of RT in enhancing functional outcome following unilateral ischemic SMC lesions in rats. This improved function is linked with a greater surface density of MAP2-IR cells in layer V of peri-infarct motor cortex. These two findings suggest that functional recovery of the impaired forelimb is, at least in part, due to greater functionality and plasticity in remaining motor cortex. CS did not further improve reaching performance when combined with RT. Further work is needed to understand the neural basis of the motor rehabilitative changes. It remains important to further test the beneficial CS effects at different timepoints during recovery.

Chapter 5

Effects of transcranial cortical stimulation and motor rehabilitative training on functional recovery following unilateral cortical infarcts in rats.

5.1 Abstract

In rats and monkeys, forelimb function can be improved with rehabilitative training following unilateral ischemic sensorimotor cortex (SMC) lesions. Greater improvements in forelimb function are found when practice on a motor skill task is paired with electrical stimulation of the peri-lesion cortex (cortical stimulation, CS) compared to rehabilitative training (RT) alone. However, these studies have administered CS at 50% of movement thresholds using electrodes implanted just above dura or with dura removed. Transcranial stimulation is less invasive and, in human stroke survivors, it can result in improved motor function. The aim of this study was to determine whether the epidural CS parameters effective for improving post-stroke forelimb function on a skilled motor task would be similarly effective if administered transcranially. A second aim was to determine if forelimb improvements were specific to stimulation of peri-lesion cortex, or if transcranial stimulation of a non-motor cortical region (i.e., occipital cortex) would result in similar improvements. In addition, ICMS was used to investigate if transcranial stimulation would potentially change forelimb motor map representations in the remaining motor cortex.

Adult male rats were pre-operatively trained to criterion with their preferred (for reaching) forelimb on a unilateral reaching task, the Single-Pellet Retrieval Task. All rats then received a unilateral endothelin-1 induced ischemic SMC lesion, followed by the placement of the electrode transcranially over peri-lesion motor cortex. Following

recovery, all rats received one of five treatments: (1) a 24 day period of transcranial cortical stimulation concurrent with rehabilitative training on a tray reaching task (tCS/RT), (2) rehabilitative training alone (RT), (3) cortical stimulation alone (CS), (4) no cortical stimulation or rehabilitative training (noCS/noRT) and (5) occipital cortex cortical stimulation and rehabilitative training (OC/RT). A subset of rats received epidural CS (eCS/RT) with RT or RT alone in order to compare their behavioral performance to those receiving transcranial CS. CS consisted of 100 Hz cathodal stimulation given at 50% of the movement threshold. In contrast to the effects of epidural CS, transcranial CS administration had minimal effects on forelimb function compared to RT alone. In addition, there was a tendency for eCS/RT, but not tCS/RT, to increase motor map representations of the forelimb compared to RT alone. Thus, the benefits of CS as applied in this study appear to be limited by method of delivery. It remains possible that an alternative strategy of transcranial CS administration would yield further improvements in forelimb function.

Key words: ischemia, recovery of function, motor learning, skilled reaching, cortical stimulation

5.2 Introduction

In rats and squirrel monkeys with unilateral infarcts of the sensorimotor cortex (SMC), delivery of epidural electrical current to the surface of the peri-infarct motor cortex (cortical stimulation; CS) in concert with daily practice in skilled forelimb

reaching improves recovery of forelimb function compared to motor training alone (Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003; see also Brown et al., 2006). The resultant behavioral improvements coincide with functional and structural plasticity in the stimulated region of motor cortex, including increased dendritic arborization (Adkins-Muir and Jones, 2003) and synaptic density (Adkins, Hsu & Jones, 2008), larger forelimb movement representation areas (Kleim et al., 2003; Plautz et al., 2003) and enhanced motor cortical evoked potentials (Teskey et al., 2003). Human clinical trials have found that epidural cortical stimulation combined with motor training is safe and, at least sometimes, efficacious in improving motor function after stroke (Brown et al., 2006; Harvey and Winstein, 2009; Huang et al., 2008; Levy et al., 2008).

Noninvasive transcranial stimulation may also be a beneficial adjunct when paired with motor rehabilitative treatments. In humans, the combination of transcranial direct current stimulation (tDCS) and occupational therapy (Nair et al., 2007) or repetitive transcranial magnetic stimulation (rTMS) and physical therapy (Khedr et al., 2005) have been shown to result in more enduring functional benefits than therapy alone. In addition, tDCS delivered to the motor cortex improved motor function of the paretic hand in tasks relevant to daily activities (Hummel et al., 2005). Recently, higher frequencies of rTMS applied to the ipsilesional motor cortex were found to be safe and resulted in modest improvements in behavioral function of the impaired hand (Yozbatiran et al., 2009). Furthermore, high frequency rTMS applied to the injured motor cortex, when combined with a complex, sequential motor task, was found to improve motor learning in

human stroke survivors during the chronic period (Kim et al., 2006). A recent study found that administration of bilateral repetitive transcranial magnetic stimulation, administered in a manner which inhibits the unaffected cortex and stimulates the lesioned cortex, resulted in greater performance of the paretic hand following motor training (Takeuchi et al., 2009). This convergence of findings points to potential commonalities in the neural mechanisms of epidural CS and those of tDCS and rTMS (Hummel and Cohen, 2006).

Following middle cerebral artery occlusion in rats, TMS applied to the infracted or injured hemisphere resulted in a greater recovery of neurological function and an increase in an immediate early gene and neurotrophic factor (Zhang et al., 2007) as well as greater glucose metabolism and a decrease in apoptosis (Gao et al., 2010). In addition, rTMS has recently been used in a rat model of Parkinson's disease. Following 6-hydroxydopamine (6-OHDA)-induced lesions, rats treated with rTMS had a reduction in neurobehavioral deficits and a greater preservation of dopamine neurons in the substantia nigra (Yang et al., 2010).

Taken together, the benefits of tDCS and rTMS, in both rats and humans, suggest that transcranial stimulation which is less invasive than epidural CS, results in improvements in brain and motor function. Therefore, the purpose of this study was to determine whether the epidural CS parameters effective for improving post-stroke forelimb function on a skilled motor task would be similarly effective if administered transcranially. An additional aim was to determine if forelimb improvements were specific to stimulation of peri-lesion cortex, or if stimulation to a non-motor cortical

region (i.e. occipital cortex) would result in similar improvements. Futhermore, we included a subset of rats that received the standard, epidural CS in order to compare their behavioral performance to those receiving transcranial CS. Reorganization of motor map representations occurs following CS/RT (Kleim et al., 2003; Teskey et al., 2003). Therefore, intracortical microstimulation (ICMS) was used to investigate potential changes in the forelimb representation area of peri-lesion motor cortex.

5.3 Materials and Methods:

5.3.1 Animals

Fifty-five male Long-Evans hooded rats were obtained from Charles Rivers Laboratories at one month of age. Forty-three were used for the transcranial stimulation study and fourteen for the epidural stimulation study. All rats were housed in polycarbonate cages in pairs or triplets on a 12:12 h light:dark cycle with water *ad libitum*. Housing included standardized cage supplements (a PVC tube, small wooden objects to gnaw and manipulate, and 6g of a complex food mixture, given twice weekly, that consisted of in-shell nuts, in-hull seeds, macaroni, and dried fruit pieces). Rats were tamed by weekly gentle handling and placed on scheduled feeding (15g chow/day, gradually increased to permit age-related weight gain). All rats began behavioral training at 2.5 months of age and received lesions at 3.5 months of age.

Rats receiving transcranial stimulation were divided into the following groups: (1) tCS/RT: transcranial cortical stimulation concurrent with rehabilitative training (n = 10), (2) RT: rehabilitative training alone (n = 9), (3) CS: cortical stimulation alone (n = 7), (4)

NoCS/NoRT: no cortical stimulation or rehabilitative training (n = 8) and (5) OC/RT: occipital cortex cortical stimulation and rehabilitative training (n = 9). For the epidural electrode implantation, rats were divided into two groups: (1) eCS/RT (n = 6) and (2) RT (n = 5). The protocol for all procedures was approved by the University of Texas Animal Care and Use Committee.

5.3.2 Testing and Training in Skilled Reaching

5.3.2.1 Single Pellet Retrieval Task. The single pellet retrieval task (Fig.1, Ch.2) has been described in detail previously (Bury and Jones, 2002; O'Bryant et al., 2007). Animals were placed in a plexiglas chamber and learned to reach through a tall narrow window to retrieve a banana flavored pellet (45mg, Bio-Serve, Frenchtown, NJ) placed in a well 1 cm from the opening. After shaping to determine their limb preference for this task (Whishaw, 1992), a removable wall was inserted ipsilateral to the preferred limb and

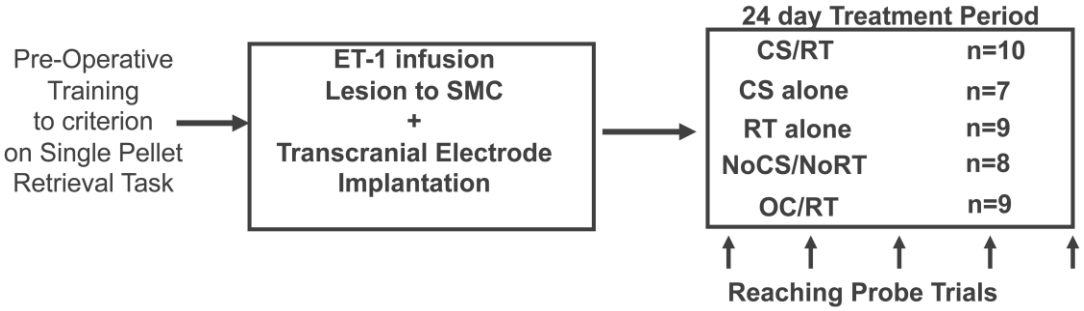


Figure 5.1: Experimental design and time course of behavioral training.

pellets were placed, one at a time, in a well opposite this limb to limit training to the preferred reaching limb. Rats were permitted to make up to five attempts to retrieve each pellet. Performance was measured as the number of successes (grasping, retrieving and eating the food pellet)/reach attempt. Preoperatively, animals received 30 trials/day or 10 min of training (whichever came first) until they reached a criterion of 45% success/attempt (~9 days).

Post-operatively and prior to the onset of the rehabilitation period, animals' performance was tested on day 5 in a 15 trial session. Performance was calculated as the percentage of successes per reach attempt. During the rehabilitation period all groups were tested once weekly for 14 trials on the single pellet retrieval task on days 9, 16, 23, 30 and once at the termination of rehabilitation training, day 37. Performance on the probe trials during rehabilitative training was also calculated as the percentage of success per reach attempt. Pre-operative and initial lesion-induced losses in reaching success and post-implant reaching performances were matched as closely as possible among all groups.

An adaptation of the Whishaw rating scale (Gonzalez et al., 2004; Whishaw et al., 1993; Whishaw and Metz, 2002) was also used to qualitatively analyze nine specific reaching movements **i**n frame-by-frame video playback, as previously described (O'Bryant et al., 2007). Each of the 9 movements was classified as being normal (0), mildly abnormal (0.5) or severely abnormal (1). The numbers of mildly and severely abnormal movements per 5 successful trials were analyzed. Animals were tested pre-operatively, on post-operative day 8, and again at the end of rehabilitation training.

5.3.3. Surgical Procedures

5.3.3.1 Cortical Infarcts. In both topical and infusion applications, ET-1 produces focal lesions followed by reliable behavioral impairments (Adkins-Muir and Jones, 2003; Fuxe et al., 1992; 1997; Hughes et al., 2003). For the transcranial

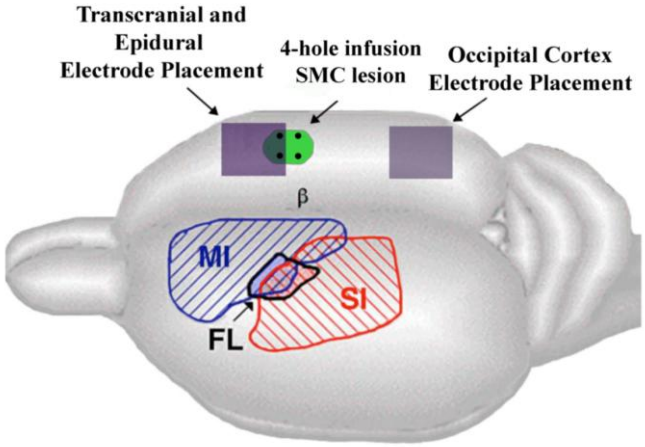


Figure 5.2. Focal unilateral ischemic lesions were made in the SMC using ET-1. Transcranial electrodes were implanted over remaining motor or occipital cortex.

stimulation, we used an infusion method to minimize the perturbations of the skull prior to electrode implantation. In rats receiving epidural electrodes, we used the infusion method for lesions as well in order to decrease the likelihood of lesion size confounds due to differing methods of inducing the lesion. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). As shown in Figure 1, rats receiving transcranial electrode implants first received ischemic damage via four holes in the skull placed according to coordinates of the forelimb overlap zone of the SMC. The coordinates of the holes were 0.2 mm posterior, 1.7 mm anterior, 3.5 mm medial and 4.6 mm lateral to

Bregma. At each hole, a micropipette was lowered into cortex to an initial depth of 1500 μm (depth of layer V of the motor cortex), then retracted to 800 μm. An infusion of 0.75 μl of endothelin-1 (ET-1), a vasoconstricting peptide, was administered into the cortex at each site and 1.5 minutes of rest was allowed before retraction of the micropipette. Five minutes after the final ET-1 injection, the holes were covered with bone wax.

In order to verify our ability to reproduce the CS effects previously reported (Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003; Chapters 3 & 4), two groups of rats received the infusion application of ET-1 to the SMC. As mentioned, the infusion method was used in order to minimize possible lesion size differences between the topical application previously used (Chapters 3 & 4) and the infusion method.

5.3.3.2 Electrode Implantation. Five minutes after the last injection of ET-1, all rats in the tCS/RT, RT, CS alone and NoCS/NoRT groups had an electrode placed trancranially, ~1.0 mm both rostral and medial to Bregma. For rats in the OC/RT group, the electrode contact was placed slightly anterior to lambda, targeting the occipital cortex. The electrode (Northstar Neuroscience Inc., Seattle, WA) consisted of parallel platinum wire contacts (0.4 X 2.0 mm) mounted on a 3.0 X 3.0 mm supporting plate that extended from a connector pedestal (Plastics One Inc., Roanoke, VA). For all groups the contacts were positioned parallel to midline above the skull, which placed the electrode over the remaining motor cortex, including the remaining forelimb representation area and surrounding areas. This position was chosen because it has previously been found to reliably elicit movements of the contralateral forelimb, shoulder, and/or face when

current is applied (Adkins-Muir and Jones, 2003; Adkins et al., 2006). For the OC/RT group, the electrode was placed over occipital cortex, parallel to midline above the skull. A metal disk inserted subcutaneously near Lambda served as a ground for all animals.

For the two groups receiving the infusion ET-1 application followed by the standard electrode implant, the craniectomy was enlarged ~1.0 mm both rostrally and medially to expose perilesion motor cortex. The electrode (Fig. 3.3, Ch.3) was composed of two parts. First, the Ghinder hub, supporting plate and two parallel platinum wire contacts (0.4 X 2.0 mm) were made by Bill Rowe (previously of Northstar Neuroscience Inc., Seattle, WA). Second, the metal disk that serves as a ground was modeled off of the previous design and made in our lab. Briefly, a local jewelry store made the platinum metal disc by cutting 4.0 X 4.0 mm squares, and by pressing the center, raised the contact to the exact size of the previous grounds. Next, we soldered the two platinum wires to the concave side of the metal disc. The outer cover of the disc was made using UV-cured dental cement (Wave A2; Southern Dental Industries, Victoria, Australia).

5.3.4 Rehabilitation Training and Cortical Stimulation (CS) Procedures

5.3.4.1 Rehabilitation Training Procedures. For the transcranial and epidural electrode implants, rats received RT on the Tray Reaching Task (Fig. 4.4, Ch.4) for 6 days a week for 24 days, with probe trials on the Single Pellet Retrieval Task given every 7th day. In this task, rats were placed in a reaching chamber, and 100 pellets were placed on a metal tray, 7.5 cm long × 5.2 cm high × 6 cm wide and inclined at 25º, that was

positioned outside the reaching chamber in front of the reaching window. The rats were then permitted to reach for pellets for 10 minutes. Animals received 50 banana flavored food pellets during the first five minutes, followed by the remaining 50 pellets for the last five minutes of training. Removable partial walls were placed in the reaching chamber to force the use of their preferred/impaired forelimbs for reaching. For all groups not receiving RT, all banana pellets were placed on the floor of the chamber. Performance was measured as the number of pellets consumed out of 100. Animals receiving CS received continuous cathodal stimulation delivered for the entire 10 min of the rehabilitation session. All other animals were connected to the stimulation cable but received no stimulation.

5.3.4.2 Movement Threshold Testing. During the rehabilitation period, all animals were tested weekly for their movement threshold, defined as the minimum current needed to evoke an observable forelimb, head/neck, or shoulder movement. These movements were consistently evoked in both the transcranial and epidural stimulated groups. For the OC/RT rats, the typical movements observed were head/neck and shoulder. Rats were placed in a Plexiglas cylinder and observed during delivery of cathodal stimulation that consisted of 100 μs pulses delivered at 100 Hz frequency in 3 s trains of increasing amplitude. Stimulation was delivered using a Sunrise PSS2 stimulator (Northstar Neuroscience, Inc., Seattle).

5.3.4.3 CS during Rehabilitative Training. In both the transcranial and epidural CS treated rats, 100 Hz cathodal current was delivered at 50% of movement threshold for the entire 10 minutes of each day's training on the tray reaching task. These parameters

were chosen because they have been found in previous studies to be particularly effective in improving function with epi- or subdural electrodes (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003). On probe trials of performance on the single pellet retrieval task during the rehabilitation period, the CS groups received stimulation for half of the trials, with the order alternated weekly. NoCS rats were connected to stimulator cables but received no stimulation during these training and testing sessions.

5.3.5 Intracortical microstimulation mapping

The intracortical microstimulation procedure in rats is described in detail in Kleim et al. (1998). At the end of the rehabilitative treatment period, all animals were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (8 mg/kg). Supplemental ketamine (10 mg per injection) and low levels of isoflurane (2-3% in oxygen) were given when necessary to maintain plane of anesthesia and prevent wakefulness. A unilateral craniectomy was made over the motor cortex and a small puncture of the cisterna magna was made to reduce cortical swelling. Warm silicone oil (37 °C) was then applied to the exposed cortex to prevent drying. A digital image of the exposed cortical surface was taken using a computer-interfaced stereomicroscope and a grid (500 μm), used to serve as a guide for sites of stimulation, was superimposed over the image using Canvas software (ACD Systems International, Inc.). A pulled glass pipette and platinum wire stimulating electrode (filled with 3M NaCl and controlled by a

hydraulic microdrive) was used to make systematic penetrations into the exposed cortex. The tip of the electrode was lowered to approximately 1550 µm. Stimulation consisted of a 40 ms train of 13, 200 µs, monophasic cathodal pulses delivered at 350 Hz from an electrically isolated constant current stimulator (BAK Electronics, Mount Airy, MD). At each stimulation site, current was increased to a maximum of 60 µA. The forelimb was supported throughout the observations to facilitate viewing of movements. If a visible movement was evoked, the current was decreased until the movement was no longer visible and the movement threshold, the minimum current needed to elicit a response, was recorded. A non-response site was recorded if no movement was observed with 60 µA of stimulation. Systematic penetrations were made across the cortex until the entire extent of the caudal (CFA) and rostral (RFA) forelimb areas were resolved. Jaw, neck, whisker, trunk, and hindlimb movements bordered the forelimb area. In cases where movements of multiple body parts were evoked simultaneously, the site was considered responsive to the body part with the lowest movement threshold. Forelimb movements were classified as distal (wrist and digits) and proximal (elbow and shoulder), and the areal extent of each was calculated.

5.3.7 Neuroanatomical Methods

5.3.7.1 Histology. At the end of the ICMS, when rats were 5-5.5 months old, they were overdosed with sodium pentobarbital (100 mg/kg) or pentobarb with phenytoin (100 mg/kg) ("Euthasol") and transcardially perfused with buffer and fixative solutions. Coronal sections (50 µm) were taken throughout the cerebrum and Nissl stained with

toluidine blue. All anatomical data were collected from digital images captured or visualized in real time using an Olympus BX61 microscope. All data were collected by an experimenter blinded to experimental condition.

5.3.7.2 Analysis of Lesions. For cortical injuries, interhemispheric volume differences are often used as an indirect estimation of lesion size. For each rat, the area of cortex remaining within brain sections that included the SMC region was determined using Neurolucida perimeter tracing software (Microbrightfield, Inc.) at a final magnification of 17 X. A total of six sections, 400 μm apart, moving in a caudal direction, were measured beginning with the appearance of the head of the caudate, as described previously (Jones et al, 1999). The Cavalieri method (Gundersen and Jensen, 1987) was used to calculate volume as the product of the summed areas and the distance between section planes (400 μm). The extent and placement of each lesion was also reconstructed onto schematic cortical coronal sections adapted from Paxinos and Watson, 1986. Due to poor tissue quality, one animal from the transcranial and three from the epidural study were removed from final lesion analysis.

5.3.8 Statistical Analyses

To analyze data from the reaching test and all other sensorimotor behavioral tests, SPSS (SPSS, Inc.) program for general linear models for repeated measures analysis of variance (ANOVA) was used to determine the effects of Day, Group, and Group by Day interactions. For behavioral results, planned comparisons were used for the primary

analyses to address these questions: After the lesions does (1) RT improve performance (RT groups vs. NoRT groups), (2) CS further improve function compared to RT (CS/RT versus RT), and (3) does CS alone improve function (CS versus NoCS). Additionally, (4) is there any dependence on stimulation site (tCS/RT versus OC/RT). For the ICMS data, One-way analyses of variance (ANOVAs) were conducted to compare areal extent of movements. Post hoc T tests were used to further analyze reaching data. The *a priori* α level for all comparisons was 0.05. To analyze the reaching movement analysis data, SPSS general linear model multivariate analysis was used.

5.4 Results

5.4.1 Skilled Reaching Performance on the Single Pellet Retrieval Task.

As shown in Figure 5.3A and B, following the infusion or topically induced ET-1 lesions, all animals declined in reaching performance on the single pellet retrieval task as measured prior to the onset of rehabilitative training. Using a repeated measures ANOVA, RT alone did not improve reaching performance compared to NoCS/NoRT ($p > .05$). Transcranial stimulation of remaining motor cortex was not effective in improving reaching success compared to RT alone or OC/RT. Using a repeated measures ANOVA, there were no significant group by day or group effects. In addition, there were no differences between the CS or NoCS/NoRT animals (p's > .05)

Further analysis indicated that there were animals in each transcranial implantation group showing a notable degree of post-operative spontaneous recovery

prior to the onset of the rehabilitative training period, as evident by performance improvements on day9 versus day5. Averaged over groups, the % success/attempt on day for the rats showing spontaneous recovery on day 9 was 23.95 ± 4.29 versus 9.84 ± 1.69 in the rats not showing spontaneous recovery. The number of spontaneous recovery rats in each group was as follows: tCS/RT (n = 3), RT (n = 2), CS (n = 2), NoCS/NoRT (n = 1) and OC/RT (n = 1). However, if these animals were removed from the analyses, there continued to be no effect of TCS/RT. Using a repeated measures ANOVA, there were no significant Group by Day or Group effects in any of the planned comparisons. (*p*'s > .05). For animals showing spontaneous post-operative recovery, results from a repeated measures ANOVA also indicated no significant differences in the planned comparison of the groups, including performance during the treatment period (*p*'s > .05)

Figure 5.3B shows animals receiving epidural cortical stimulation compared to RT alone. During the rehabilitative treatment period, eCS/RT rats had greater reaching performance on the single pellet retrieval task compared to RT alone. Repeated measures ANOVA indicated a significant Group effect ($F(1,9) = 5.65$, $p = .041$) but no Group by Day effect ($F(3,27 = 0.79$, $p = .51$) between eCS/RT and RT alone groups during the treatment period.

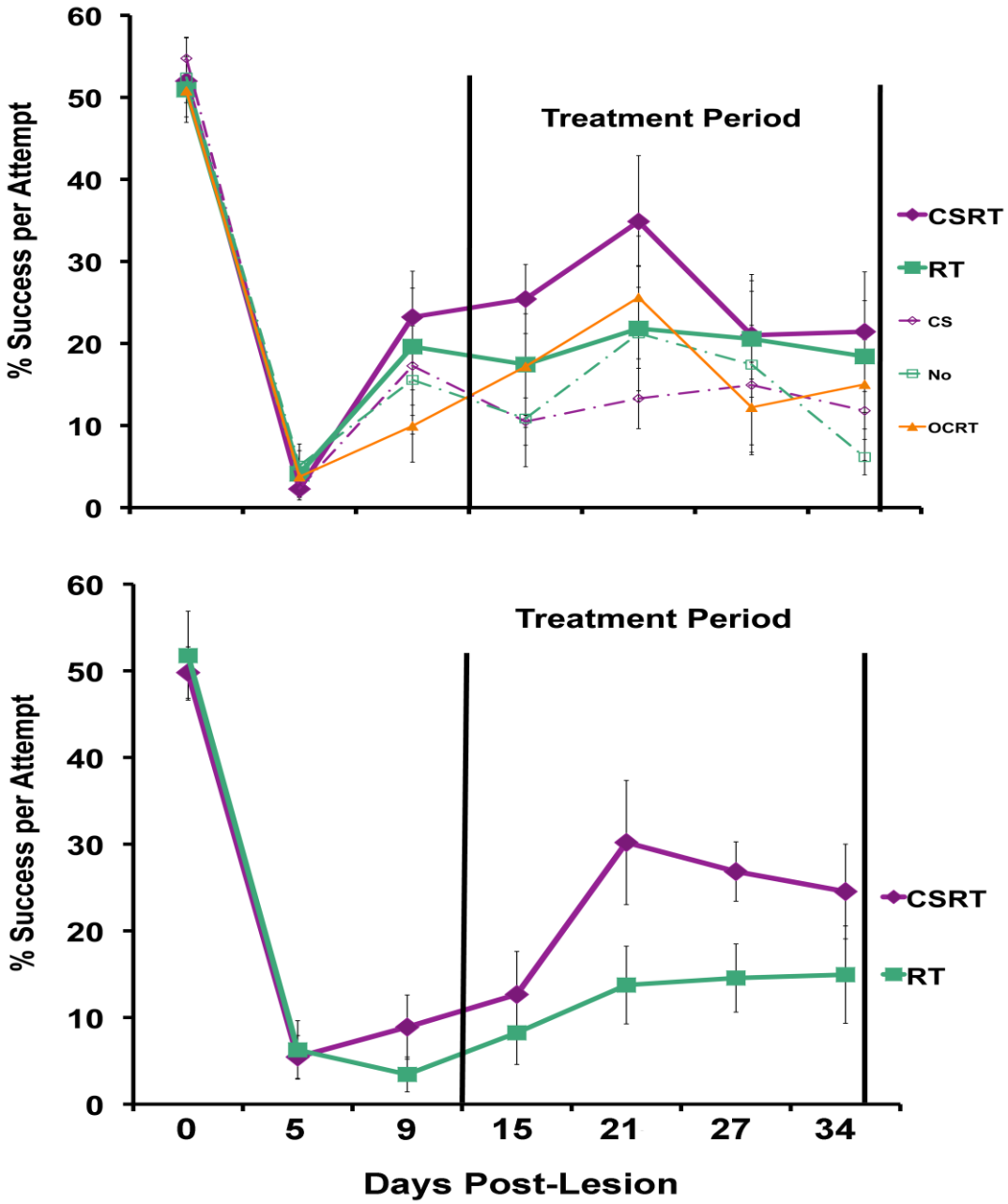


Figure 5.3: (A) Transcranial CS administration did not further improve forelimb function compared to RT alone. *p*'s > .05. (B) There was a main effect of Group between eCS/RT and RT during the treatment period ($p < .05$)

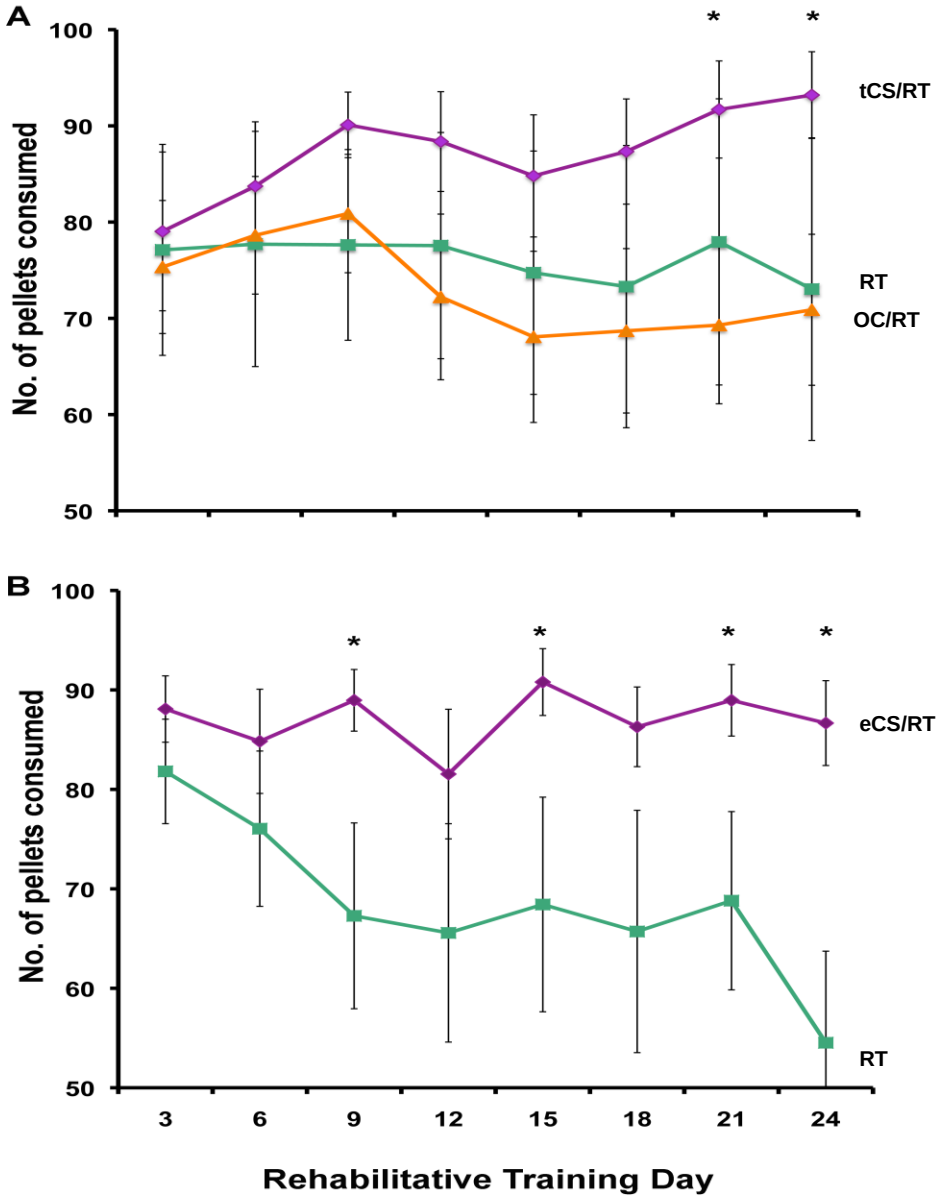
5.4.2 Performance on the Rehabilitative Task.

Although the single pellet reaching task was the primary outcome measure, performance on the rehabilitative tray reaching task was also assayed by measuring the

total amount of pellets retrieved (out of 100) per training session. For a more concise presention, the 24 data points were pooled into 3-day blocks. As shown in Figure 5.4A, did not improve function on the tray reaching task compared to RT alone. Using a repeated measures ANOVA,there was no significant Group

By Day (F (7,119) = 1.07, p = .39) or Group effect (F (1,119) = 1.93, p = .18) between the tCSRT group compared to RT alone. However, there was a significant Group By Day effect between the tCS/RT and OC/RT rats (F(7,91) = 2.94, p = .007). Posthoc analysis indicate the tCS/RT rats had better performance on pooled days 19-21 and 22-24.

Figure 5.4. Motor Rehabilitative Tray Reaching Task. Transcranial CS tended to improve function weeks after training compared to RT alone. There was a significant Group By Day effect between the CSRT group compared to RT alone *'s p< .05 versus RT; ** *p*'s < .05 versus OC/RT.



As shown in Figure 5.4B, eCS/RT rats had improved performance on the motor rehabilitative task compared to RT alone. Using a repeated measures ANOVA, there was a significant Group by Day ($F(7,70) = 2.53$, $p = 0.022$) and Group ($F(1,10) = 7.10$, $p = 0.024$) effect. Posthoc analysis indicates eCS/RT rats had better performance on pooled days 7-9, 13-15, 19-21 and 22-24.

5.4.3 Other Behavioral Measures

In the Qualitative Movement Analysis of performance on the Single Pellet Retrieval Task although there were typical lesion-induced changes (Adkins et al., 2004), there were no significant differences in performance among the groups. Results indicate that there were no differences between RT vs NoCS/NoRT, tCS/RT vs RT, CS vs NoCS/NoRT or tCS/RT vs OC/RT (p's > .05). In the analysis of reaching movements

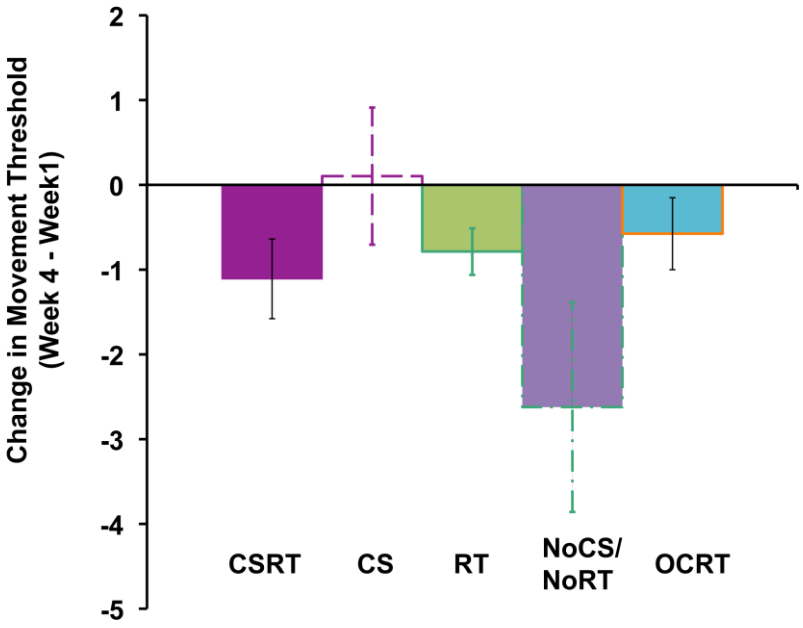


Figure 5.5: Weekly Movement Threshold Testing. Most groups declined in the amount of current needed to elicit a movement.

using Whishaw's rating scale (Qualitative Movement Analysis), lesions resulted in an increase in abnormal movements, from which there was little subsequent recovery. The means ± SEMs for total number of abnormal movements per trial (including moderate and severe abnormalities) were 1.05 ± 0.12 pre-operatively, 2.38 ± 0.29 post-operatively, and 1.66 ± 0.17 post-rehabilitative training.

5.4.4 Movement Thresholds

As seen in Figure 5.5, in the transcranial groups, with the exception of the CS group, all movement thresholds tended to decline over time. This is contrary to previous findings (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Kleim et al., 2003; Teskey et al., 2003; O'Bryant et al., 2010). Using a One-way ANOVA, there CS rats had less change in their weekly testing compared to NoCS/NoRT ($F(1,14) = 4.83$, $p = .047$) There were no significant differences on the weekly movement threshold testing between RT versus NoCS/NoRT, tCS/RT vs RT or tCS/RT vs OC/RT (p's > .05).

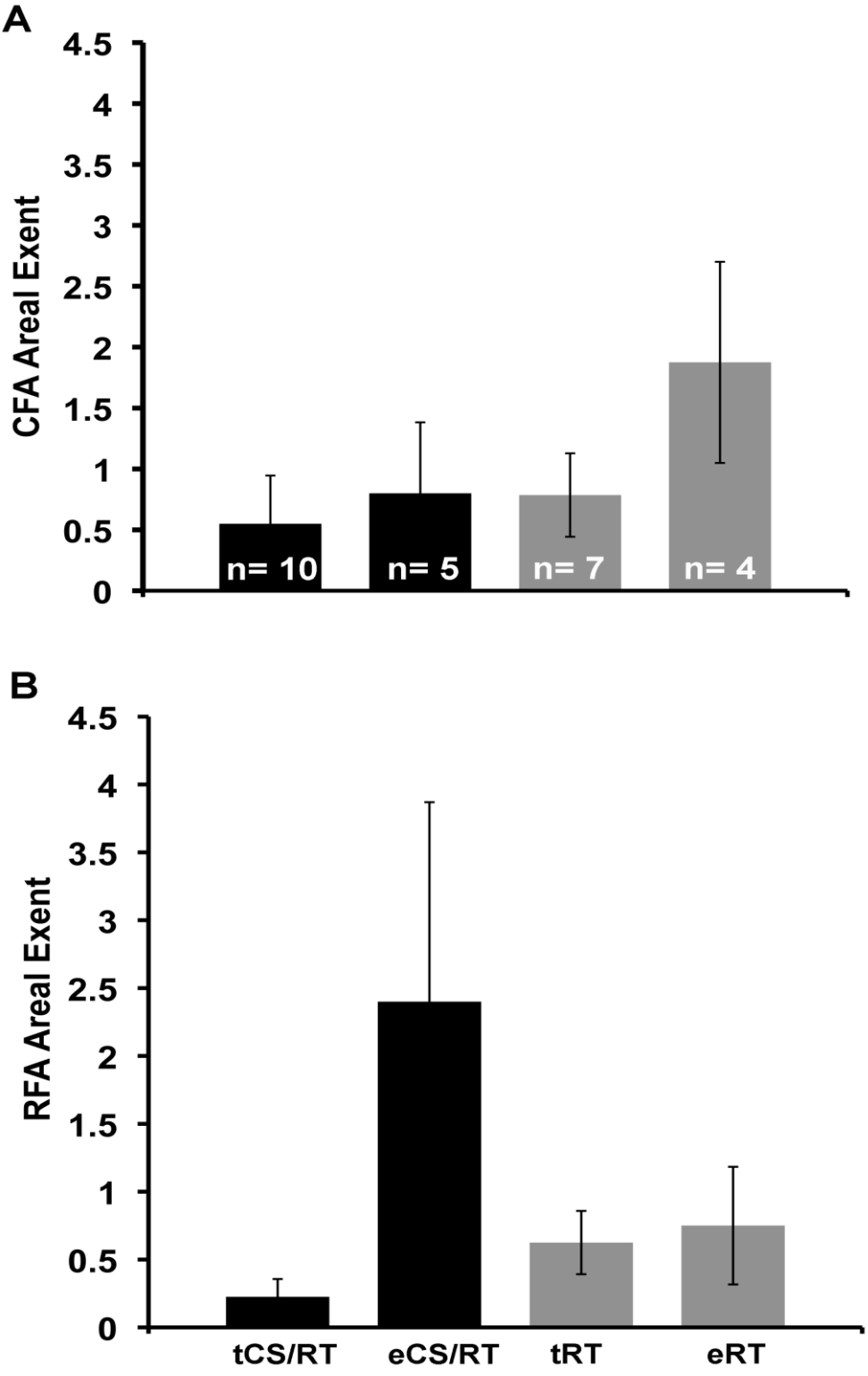


Figure 5.6: Motor Map Representataton. (A) CFA areal extent. (B) RFA areal extent. eCS/RT tended to increase the areal extent of distal (digits/wrist) and proximal (elbow and shoulder) in the RFA compared to tCS/RT.

5.4.5. Organization of movement representations Forelimb movement representations were organized into a caudal forelimb area (CFA) and included the distal (digits and wrist) and proximal (elbow and shoulder) movements. As shown in Figure 5.6, eCS/RT teneded to have greater motor map representation in RFA compared to tCS/RT. In the RFA, there were no significant differences in the planned comparisons. eCS/RT tended to have more forelimb response sites in RFA than did tCS/RT ($F(1,15) = 2.89$, $p = .11$). However there were no differences in the number of forelimb response sites between eCS/RT and RT ($F(1,10) = .95$, $p = .36$). tCS/RT tended to have a fewer number of sites that evoked a forelimb response in RFA than were found in the RT group ($F(1,16) = 2.58$, $p = .13$). This tendency was not found in the other transcranial groups. In contrast, in CFA, the mean number of forelimb response sites in the eCS/RT group was less than that of RT, but not significantly so ($F(1,X) = X$, $p = X$). Further more, there was little difference between eCS/RT and tCS/RT (P > .05)between between tCS/RT vs RT alone, eCS/RT vs RT alone, or tCS/RT and eCS/RT rats ($F(3,27) = 0.75$, $p = .53$)., $p = .355$). In addition, jaw representations were not different between the groups (p's > .05).

5.4.6 Volume of Remaining Cortex

All lesions in the transcranial and epidural groups appeared to result in major damage in the forelimb area of the SMC (Figure XA and B). It appeared that no animals had any direct damage to the striatum. One-way ANOVA results from the planned comparison analyses indicate that volume measurements of the contralesional, ipsilesional and interhemispheric difference in volume revealed no differences in infarct

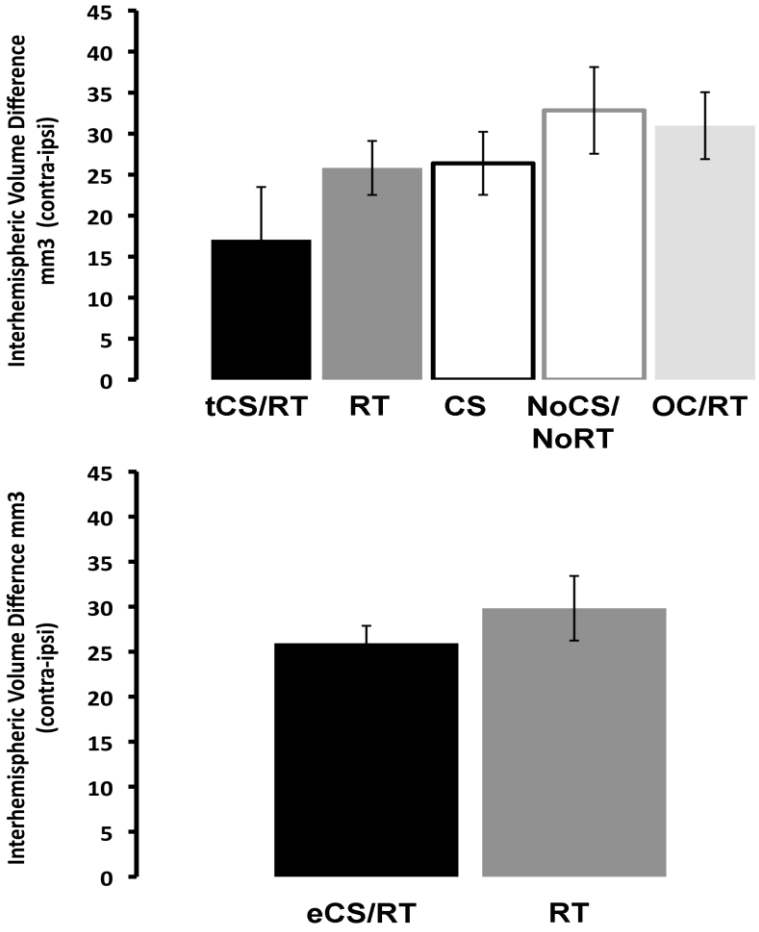


Figure 5.7: Interhemispheric Volume Difference. There were no significant differences in lesion size among the groups. Data are mean ± S.E.M.

size among the groups (p's > .05). Pooling across the transcranial lesion groups, the mean ± SEM of remaining SMC region volume was 51.73 ± 1.65 mm^3 in the damaged hemisphere, and the interhemispheric difference in volume (contra - ipsi) in the SMC region was 27.13 ± 1.55 mm^3. For the epidural groups, the mean ± SEM of remaining SMC region volume was 56.56 ± 3.05 mm^3 in the damaged hemisphere, and the interhemispheric difference in volume (contra - ipsi) in the SMC region was 23.90 ± 2.03 mm^3.

5.5 Discussion

The purpose of this study was to determine if cortical electrical stimulation delivered in a less invasive transcranial method would result in functional improvements of the impaired forelimb compared to motor rehabilitative training alone. Our method of delivering transcranial CS over remaining motor cortex did not further improve forelimb function compared to rehabilitative training alone However, epidural CS concurrent with rehabilitative training did result in greater improvements in impaired forelimb function compared to rehabilitative training alone. As expected, rats in the groups not receiving rehabilitative training (CS alone and NoCS/NoRT) did not have improvements in impaired forelimb function. In addition, transcranial stimulation to the occipital cortex did not result in reaching improvements of the impaired forelimb. Thus, at least with the CS parameters used in our previous studies (Adkins-Muir et al., 2003; Adkins et al., 2006; 2008, Chapters 3 & 4) and others (Boychuck et al., 2010; Kleim et al., 2003; Plautz et al., 2003;Teskey et al., 2003) it is more efficacious when delivered epidurally than when delivered transcranially. It remains possible that an alternative strategy of transcranial CS administration would yield further improvements in forelimb function.

An additional question of the current study was whether forelimb improvements were specific to stimulation of peri-lesion cortex, or if stimulation to a non-motor cortical region (i.e. occipital cortex) would result in similar improvements. In humans, non-specific stimulation using TMS resulted in a decrease in reaction time, potentially due to stimulus-dependent intersensory facilitation (Sawaki et al., 1999). However, the current

study did not show that stimulation to a non-motor cortical region, the occipital cortex, results in forelimb improvements compared to rehabilitative training alone. In addition, there were no significant differences in reaching performance on the single pellet retrieval task between rats receiving CS over remaining motor cortex compared to CS over occipital cortex. However, on the tray reaching task, tCS/RT rats did have greater performance compared to OC/RT over days of training. It remains possible that an alternative strategy of transcranial CS administration may result in differences between the CS stimulation over motor cortex compared to CS over non-motor areas.

As expected, epidural administration of CS resulted in greater reaching performance compared to rehabilitative training alone, consistent with previous findings. Motor cortical CS combined with rehabilitative training changes functional activity patterns and neuronal structure in the peri-infarct area and improves behavioral performance of the impaired limb (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003). CS results in increases in the size of forelimb motor map representations in peri-infarct motor cortex (Kleim et al., 2003; Plautz et al., 2003), increases in synaptic density (Adkins, Hsu and Jones, 2006) and increases in dendritic processes in the peri-infarct area (Adkins-Muir and Jones, 2003). CS concurrent with rehabilitative training also results in a decrease in movement thresholds (the amount of electrical current needed to elicit a movement) and enhances motor cortical evoked potentials (Teskey et al., 2003). In human clinical studies CS/RT enhanced functional recovery of the impaired arm compared to rehabilitative training alone in preliminary

motor assessments (Huang et al., 2008;Levy et al., 2008). Therefore, it was expected that epidural CS would yield greater improvements in reaching performance.

Although our method of epidural, but not transcranial, administration improved skilled reaching performance, these improvements did not generalize to other behavioral measures. Animals in this study receiving CS/RT failed to completely recover on Whishaw's movement analysis, which measures abnormalities in the reaching and grasping movements used to perform the Single Pellet Retrieval Task. It may be that greater normalization of reaching movements will occur if a more precise rehabilitative motor task (i.e. the single pellet task) is used.

In addition, there were no differences in the volume of remaining motor cortical regions or measures of interhemispheric differences among any of the groups. When assessed during a chronic period of recovery, epidural CS-induced functional improvements have been associated with greater remaining volume of motor cortical regions (O'Bryant et al., in progress; Chapter 3). However, in previous studies, lesion sizes and remaining cortical volume were consistently similar between CS and NoCS groups (Adkins-Muir et al., 2003; Adkins et al., 2006; Adkins, Hsu and Jones, 2008; Plautz et al., 2003; Teskey et al., 2003). Although epidural CS resulted in functional improvement of the impaired forelimb compared to RT alone, the magnitude of improvement was diminished compared to previous studies (Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008; O'Bryant et al., in progress, Chapter 3 & 4). In the current study, the transcranial and epidural groups do not show clear differences in post-operative D5 behavioral impairment levels or lesion size. In the transcranial study, rats

show less average impairment level on post-operative D9 compared to epidural rats. When animals showing greater than average spontaneous recovery were removed and analyzed, results did not change. Following ischemic injury, epidural CS enhances reaching performance in animal with moderate, but not severe impairments (Adkins et al., 2008). In addition, studies on traumatic brain injury (TBI) indicate that the effectiveness of rehabilitative training or additional adjunct therapies are not as effective due to increased severity of cortical injury. These findings suggest that CS effects vary with impairment level and could contribute to a muting of the effect in the CS study, given that their impairments were more severe.

There was a tendency for eCS/RT rats to have greater motor map representation in remaining motor cortex compared to tCS/RT. In rats and non-human primates, epidural CS/RT results in increases in motor map representations (Kleim et al., 2003; Plautz et al., 2003). Results from the current study suggest that the failure to find a significant difference between the eCS/RT and tCS/RT groups is possibly due to an inadequate number of rats in the eCS/RT group (n = 5) compared to tCS/RT (n = 10). Based on previous findings, more animals in the eCS/RT group are needed for an accurate comparison.

In conclusion, the present findings indicate that our method of transcranial CS administration did not result in functional improvements of the impaired forelimb compared to rehabilitative training alone. However, we were able to verify that epidural administration of CS does result in greater reaching performance compared to RT alone.

Non-specific stimulation of the occipital cortex was not effective in improving motor performance. However, given benefits of non-invasive transcranial stimulation in humans, it remains possible that an alternative means of administering transcranial CS would result in functional improvements of the impaired forelimb.

Chapter 6 General Discussion

6.1 Summary

These dissertation studies provide substantial support for the benefits, as well as the limitations, of CS for improving forelimb function after cortical stroke. Epidural CS combined with motor rehabilitative training resulted in functional recovery of the impaired forelimb when initiated early after ischemic injury to the SMC. Compared to RT alone, epidural CS combined with RT resulted in long-lasting (9-10 months post-RT) improvements in reaching performance and increased the volume of motor cortical subregions. However, the beneficial effects of epidural CS appear to be time-and method-dependent. Initiation of CS combined with RT during a chronic phase of recovery (3 months post-infarct) did not result in greater improvements in reaching performance compared to RT alone. Furthermore, our method of transcranial CS administration did not have a major effect on forelimb function compared to RT alone. Transcranial stimulation of a non-motor area, the occipital cortex, also failed to result in improved reaching performance compared to RT alone.

6.2 CS combined with motor rehabilitation: benefits of early initiation

CS combined with rehabilitative reach training significantly and persistently improved motor function in the impaired forelimb during the chronic recovery period compared to rats receiving training alone (Chapter 4). This was, in part, a result of a greater maintenance of performance improvements gained during the rehabilitative

training period in that CS-treated animals had greater reaching improvements during this period compared to those receiving motor training alone. The improvements during the treatment period are consistent with previous findings (Adkins-Muir and Jones, 2003; Adkins et al., 2006; Adkins et al., 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). The present study (Chapter 3) expands these findings by showing that the benefits of CS are not limited to the treatment period. CS-treated rats continued to have greater reaching performance compared to motor training alone during the chronic recovery period (9-10 months post-infarct).

Although the specific mechanisms of how CS improves functional recovery of the impaired forelimb are still unknown, the mechanisms may be similar to the less-invasive forms of administering CS, transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS). TMS results in enhanced cortical excitability in rats (Luft et al., 2001) and humans (Pascual-Leone et al., 1994; Gilio et al., 2009) as measured by the motor evoked potentials (MEPs). In addition, in human and rat studies, tDCS over the motor cortex results in enhanced cortical excitability (Nitsche and Paulus, 2000; Fregni et al.,2007). Therefore, CS appears to work by altering the excitability of neural networks in the peri-infarct tissue. Learning or re-learning of a motor task in conjunction with rTMS (Kim et al., 2005; Khedr et al., 2006), tDCS (Hummel et al., 2005; Fregni et al., 2007) and epidural CS (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Teskey et al., 2003) appears to strengthen existing neural networks by changing cortical excitability and contributes to enhanced performance on the motor task. RT does result in improvements, its effects on the strengthening of peri-

infarct neural networks are limited. However, CS, when paired with RT, increases the likelihood that neurons will reach threshold, which results in neuronal firing and strengthening. Previous research suggests that CS also contributes to changes in structural plasticity in remaining motor cortex by altering movement representations, neural activation and neuronal structure in the peri-infarct motor cortex thus improving behavioral performance of the impaired limb during and shortly after the training period (Adkins-Muir and Jones, 2003; Adkins et al., 2008; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). The current findings (Chapter 4) indicate that the functional and structural changes found shortly after the training period may continue to contribute to the greater maintenance of reaching performance in CS-treated rats. CS-treated rats also had greater cortical volumes in motor cortical subregions, specifically the AGm, AGl and FlOL, compared to NoCS rats. It is possible that the greater volume of remaining cortex seen in CS-treated rats coincides with functional and structural changes that occur in the stimulated motor cortex, because, in intact animals, the addition of synapses, dendrites and glial processes is associated with increased cortical volume (Black et al., 1997; Green et al., 1983; Grossman, Churchill, Bates, Kleim, & Greenough, 2002; Turner and Greenough, 1985). Taken together, previous and current findings suggest that CS-induced changes in structural plasticity result in improvements in reaching performance shortly after treatment and during the chronic recovery phase.

CS may contribute to improved behavioral performance and structural plasticity by strengthening remaining neural circuits that mediate fine motor movements in remaining cortex. In intact and ischemic-injury rats, motor skill training results in

structural changes in motor cortex such as increases in synapse number (Rioult-Pedotti et al., 2000) and synaptic efficacy (Monfils and Teskey, 2004), changes in evoked potentials (Monfils and Teskey, 2004) and structural changes and plasticity of motor maps (Kleim et al., 2003). These studies suggest that motor skill learning results in behaviorally-induced structural changes and motor cortical reorganization by LTP-like mechanisms. Similarly, when paired with motor training, CS may further facilitate adaptive structural plasticity. CS results in greater evoked potentials and a reduction in the amount of current required to elicit a movement (Teskey et al., 2003). In addition, low-intensity electrical stimulation of cortical and hippocampal neurons resulted in a decrease in the neural firing thresholds and an increase in the spontaneous depolarizations (Bikson et al., 2004; Nowak and Bullier, 1998). It is possible that motor rehabilitative training also induces neural activation, but the neural networks remain below threshold for sufficient depolarization. CS may contribute to cortical excitability and further depolarize neural networks when paired with specific motor training. The result is greater activation of existing neural networks in the peri-infarct area, and changes in functional and structural plasticity that improve reaching performance on of the impaired forelimb.

6.3 CS-induced behavioral improvements are time-dependent

Rehabilitative reach training significantly improves motor function of the impaired limb when initiated during the chronic phase of recovery in rats with unilateral sensorimotor cortical infarcts. However, the addition of CS during rehabilitative training

did not result in greater improvements in impaired forelimb function compared to rehabilitative training alone. Therefore, the benefits of CS appear to be limited by time after lesion induction. Following ischemic injury, early initiation (~5 days post-lesion) of motor skill rehabilitative training results in significant improvements of the impaired forelimb (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Maldonado et al., 2008). However, there is a time-sensitive window for the initiation of rehabilitative training. Animals receiving early motor rehabilitative training after cortical infarcts show improved behavioral performance (Biernaskie et al., 2004; Clark et al., 2009) and subsequent reorganization of cortical tissue adjacent to the infarct (Nudo et al., 1996). These data suggest that the most sensitive time to initiate rehabilitative training for optimal motor recovery is early following ischemic injury. However, adult squirrel monkeys trained on a motor skill task followed by ischemic injury to the motor cortex showed significant improvements in motor recovery when rehabilitative training was initiated 30 days post-infarct (Barbay et al., 2006). However, unlike early initiation of rehabilitative training, this study failed to find sparring of hand representations in the motor cortex (Barbay et al., 2006), suggesting that behaviorally-induced changes in movement representations during late initiation may not be similar to early initiation. Although research suggests that CS coupled with rehabilitative training early after cortical injury improves performance and increases plasticity, little is known about its effects when initiated during a chronic recovery period. The findings of the current study suggest that CS did not result in additional improvement to motor rehabilitative training when initiated during the chronic recovery period (Chapter 5). Therefore, the potential

mechanisms in which CS contributes to improvements appear to be time-dependent. Following ischemic injury to the motor cortex there is an increase in the expression of immediate early genes (IEG) in the ischemic core that appear to be neuroprotective and important for recovery. Acutely after stroke, in the peri-infarct area, axons sprout and form new connections. In addition, there is a migration of new neurons. Both of these phenomenon occur as a result of an increase in growth-promoting proteins and a decrease in growth-inhibiting proteins in the peri-infarct area and contributes to neuronal regeneration (reviewed in Carmichael, 2006) The functional improvements following CS/RT may, at least partly, depend on the time of CS initiation in conjunction with the post-infarct alterations in IEG activity in the ischemic core. Thus, while motor skill training in the chronic period can greatly enhance function following ischemic damage to the SMC, the benefits of CS appear to be limited by time after lesion.

Although CS did not further improve reaching performance compared to motor rehabilitative training alone, a longer duration of treatment may be more effective. Motor rehabilitative training alone increased MAP2-IR surface density in layer V of remaining motor cortex compared to NoCS/NoRT and CS/RT. When treatment occurs early after ischemic injury, CS combined with motor rehabilitative training increases the density of MAP2-IR in peri-infarct cortex compared to motor rehabilitative training alone (Adkins-Muir and Jones, 2003). Failure to find increases in MAP2-IR in CS-treated rats when treatment was initiated during the chronic period of recovery could suggest that CS was unable to facilitate behaviorally-induced neural plasticity in remaining motor cortex. Taken together, these data suggest that initiation of motor rehabilitative training, but not

necessarily CS, during the chronic period of recovery improves reaching performance and increases dendritic plasticity in remaining motor cortex.

6.4 Transcranial administration of CS and implications of lesion severity

The current method of delivering transcranial CS over remaining motor cortex did not further improve forelimb function compared to rehabilitative training alone (Chapter 6). This was surprising, given that transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS) over the motor cortex in humans is a beneficial adjunct when paired with motor rehabilitative treatments. In humans, the combination of tDCS and occupational therapy (Nair et al., 2007) or rTMS and physical therapy (Khedr et al., 2005) have been shown to result in more enduring functional benefits than therapy alone. In addition, tDCS delivered to the motor cortex improved motor function of the paretic hand in tasks relevant to daily activities (Hummel et al., 2005). Recently, higher frequencies of rTMS applied to the ipsilesional motor cortex were found to be safe and resulted in modest improvements in behavioral function of the impaired hand (Yozbatiran et al., 2009), and when combined with a complex, sequential motor task, were found to improve motor learning in human stroke survivors during the chronic period (Kim et al., 2006). Following middle cerebral artery occlusion in rats, TMS applied to the lesioned hemisphere resulted in a greater recovery of neurological function and an increase in cFos and brain derived neurotrophic factor (BDNF) (Zhang et al., 2007). In addition, rTMS has recently been used in a 6-hydroxydopamine (6-OHDA)-induced rat model of Parkinson's disease, resulting in a reduction in neurobehavioral

deficits and a greater preservation of dopamine neurons in the substantia nigra (Yang et al., 2010). Taken together, the benefits of tDCS and rTMS, in both rats and humans, suggest that transcranial stimulation can result in improvements in brain and motor function. Although the current study (Chapter 5) failed to find that transcranial CS administration improved forelimb function compared to motor rehabilitative training alone, it remains possible that an alternative strategy of transcranial CS would yield further improvements in forelimb function. The parameters of CS administration were designed for subdural or epidural administration and both anodal and cathodal currents were optimal (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). As previously mentioned, although the specific mechanisms for how CS works are unknown, previous research on epidural CS administration and tDCS/rTMS suggests that the functional improvements are mode and site-dependent. Epidural CS improves forelimb function when anodal or cathodal current is administered over remaining motor cortex (Adkins-Muir and Jones, 2003). However, tDCS works best when the stimulating current is anodal (Fregni et al., 2007). Anodal current results in an increase in cortical excitability which appears to result in faster learning and improvements in functional motor performance compared to cathodal current (Nitsche & Paulus, 2000; Kim et al., 2010; Stagg et al., 2011). In addition, epidural CS results in greater evoked potentials and a reduction in the amount of current required to elicit a movement (Teskey et al., 2003). CS may facilitate adaptive structural plasticity by increasing the likelihood that neural networks in the peri-infarct area are strengthened.

Therefore it remains possible that alterations to the CS parameters may result in more effective transcranial administration of CS.

The severity of motor impairments in the transcranial and epidural CS study (Chapter 6) appeared to mute the effectiveness of CS combined with RT. Following ischemic or traumatic brain injury, the effectiveness of the treatment (CS and/or rehabilitation training) appears to be dependent on the level of behavioral impairments (Adkins et al., 2008; Walker and Pickett, 2007). Although epidural CS and motor rehabilitative training in the current study (Chapter 6) improved impaired forelimb function compared to motor training alone, the magnitude of improvement was diminished compared to previous studies (Chapter 3 & 4; Adkins-Muir and Jones, 2003; Adkins et al., 2006; 2008). These findings suggest that the impairment level significantly contributes to the effectiveness of the rehabilitative treatment. This provides important insight into the potential limitations on behavioral recovery. Knowing which CS and RT parameters work for less severe impairments is important. However, it is equally important to know which parameters are less effective with more severe impairments because this range of impairment level is translatable to human stroke levels.

In addition to motor rehabilitative training, adjunct therapies such as rTMS and CIMT, have resulted in improvements of the paretic hand (Nowak et al., 2008; Ro et al., 2006; Takeuchi et al., 2009; Taub et al., 1993; Wolf et al., 2006) and a reorganization of motor movements in ipsilesional motor cortex (Ro et al., 2006). In the current study, anesthetization of the intact forelimb and restricted use of the intact forelimb using CIMT did not further improve reaching performance of the impaired forelimb (Chapter 3 and 4).

Anesthetization the ipsilesional forelimb did not interfere with the reaching performance of the contralesional forelimb. This suggests that, at least at later stages of recovery after relatively small cortical infarcts, it is possible for reaching movements in the contralesional forelimb to recover in this task without ongoing compensatory reliance on the ipsilesional forelimb. However, anesthetization of the intact forelimb partially normalized skilled reaching movements which may indicate interhemispheric and bimanual effects of peripheral anesthetization reported in humans most likely extend to rat reaching function. This seems important in light of recent findings indicating competitive interactions between the hemispheres that become unbalanced by unilateral brain damage and which might be targeted for therapeutic interventions (Rushmore et al., 2006; Takeuchi et al., 2005; Voller et al., 2006; Ward and Cohen, 2004). It should be noted that the two weeks of forced use (CIMT) implemented in this study (Chapter 4) was on par in duration but does not closely resemble the effective CIMT used in humans (e.g. Wolf et al., 2006). For example, compared to the protocols for human rehabilitation, rats in the current study received less intense training each day. Therefore, it remains possible that an alternative strategy for CIMT, such as increasing the amount of time rats in the forced use condition receive treatment, might more effectively influence forelimb function than either treatment alone.

6.5 Future directions and overall conclusion

There remain many unanswered questions and possibilities for future studies. One question is, since the benefits of CS appear to be task-specific, would CS administration

during other behavioral tasks result in functional improvements on that task? In animals with delayed onset of rehabilitative training (Chapter 5), would a longer duration of treatment result in CS improvements to motor rehabilitative training and changes in structural plasticity compared to RT alone? Would an alternative strategy for transcranial CS administration (i.e. changes in the parameters of CS) result in improvements in motor function compared to RT alone? In addition, little is known about how CS works. Although many studies have shown structural changes and greater performance with the impaired forelimb following CS, the mechanisms by which CS works is not well understood. Specifically, how do these mechanisms relate to time of CS administration, mode of CS delivery and CS site dependencies.

In conclusion, these dissertation studies have provided substantial evidence for the role and potential limitations of CS. The beneficial effects of CS persist long after treatment has ended. However, the efficacy of CS appears to be time-sensitive in terms of time of initiation following injury. In addition, the current method of transcranial CS administration does not result in additional functional improvements over RT alone, but may be restricted by lesion severity. There is great promise for the clinical relevance and application of CS combined with motor rehabilitative training, and further investigation is required to assess potential limitations and the mechanisms by which CS induces behavioral changes.

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