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by

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**Transcallosal Axonal Sprouting Patterns After Ischemic Motor Cortical  
Lesions and Varying Forelimb Experiences**

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Lesions and Varying Forelimb Experiences**

**by**

**Jessica Leighila Lubs, B.A.**

**Thesis**

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## **Abstract**

# **Transcallosal Axonal Sprouting Patterns After Ischemic Motor Cortical Lesions and Varying Forelimb Experiences**

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The University of Texas at Austin, 2014

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In rodent models of motor cortical stroke, skill learning with the non-paretic forelimb worsens rehabilitation outcomes of the paretic forelimb. The neural basis of this effect is not fully understood. A possible mechanism for this effect is activity-dependent synaptic competition between projections from remaining regions of the ipsi- and contralesional motor cortex, specifically from the ipsilesional rostral forelimb area (RFA) and the contralesional caudal forelimb area (CFA). Previous studies have found that this effect is negated by callosal transections or inhibition of the contralesional cortex, suggesting that the contralesional hemisphere plays a key role. The purpose of this study was to investigate the influence of differential forelimb experience on the cortical plasticity of callosal projections from the contralesional CFA, which is known to contribute to the reinnervation of peri-lesion cortex. Since axonal sprouting is activity

dependent, one would expect animals trained with the non-paretic forelimb to have an increase in axonal fibers and bouton densities from the contralesional CFA.

Adult male Long-Evans rats were trained to proficiency using their dominant (for reaching) forelimb on the single-pellet-retrieval skilled reaching task. Animals subsequently received unilateral cortical ischemic lesions in the CFA of the hemisphere contralateral to the trained forelimb. On post-infarct day five, rats began 15 days of reach training either with their non-paretic forelimb (NPT), their paretic forelimb (rehabilitation training, RT), or no-training control procedures (CTRL). On post-infarct day 23 all animals received an injection of biotinylated dextran amine (BDA) into the contralesional CFA to label callosal projections from the spared hemisphere into peri-infarct motor cortex.

Contrary to the hypothesis, results indicate no significant differences in axonal fiber or synaptic bouton densities across any of the groups within any of the examined regions of peri-lesion cortex. This suggests that the mechanism behind the detrimental effects of NPT on the paretic limb does not involve a net change in densities of neural connections from the contralesional CFA. Future research should explore possible changes in the structure of synapses or variations in relative densities of excitatory and inhibitory post-synaptic cells as possible contributors to the neural basis of the deleterious effect of NPT.

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## **List of Abbreviations**

The following is a list of abbreviations that will be used throughout this document:

AL	Anterolateral portion of the peri-lesion cortex
AM	Anteromedial portion of the peri-lesion cortex
BDA	Biotinylated Dextran Amine
CFA	Caudal Forelimb Area
CTRL	Control
ET-1	Endothelin-1
M1	Primary Motor Cortex
NPT	Non-Paretic Limb Training
PL	Posterolateral portion of the peri-lesion cortex
PM	Posteromedial portion of the peri-lesion cortex
RFA	Rostral Forelimb Area
RT	Rehabilitation Training

## **Introduction**

A new or recurrent stroke occurs every 40 seconds in the United States, which accumulates to about 795,000 people annually (Go et al., 2014). Although great strides have been made to reduce mortality among these individuals, many stroke survivors are left with persistent neurological and motor impairments, resulting in stroke being one of the leading causes of severe long-term disability (Centers for Disease Control and Prevention, 2009). Between 50% and 75% of stroke survivors experience persistent difficulty with upper-limb and hand control, including muscle fatigue as well as decreased coordination and precision (Kelly-Hayes et al., 2003; Lai, Studenski, Duncan, & Perera, 2002; Olsen, 1990; Parker, Wade, & Hewer, 1986). Because of the initial frustration encountered when first trying to use the paretic limb soon after stroke, a natural response in both animals and humans is to develop compensatory movement strategies with the non-paretic limb in order to maintain daily function (Jones & Schallert, 1992; Lang, Wagner, Edwards, & Dromerick, 2007). The increased reliance on the non-paretic limb can result in persistent disuse of the paretic limb (learned non-use; Taub et al., 1994), however recent animal models have shown that early learning with the non-paretic limb may actually go one step further and directly disrupt functional improvements in the paretic limb.

In rodent models of skilled reaching tasks after cortical ischemia, it has been found that non-paretic limb training (NPT) soon after a stroke reduces spontaneous recovery and impedes subsequent rehabilitation training (RT) of the paretic limb, as compared to those that received only control procedures prior to RT, without affecting

lesion size or cell loss (Allred, Cappellini, & Jones, 2010; Allred & Jones, 2008; Allred, Maldonado, Hsu, & Jones, 2005; Kerr, Wolke, Bell & Jones, 2013). This effect is seen in both rats that are naive to the skill (Allred et al., 2005) and those that had established it with the to-be paretic limb prior to the stroke (Allred & Jones, 2008), but the effect is not present in rats that were trained with *both* forelimbs after the lesion (Allred & Jones, 2008) or those with bilateral lesions (Allred et al., 2010). Furthermore, this effect is not present in intact rats (Allred & Jones, 2008) or those that undergo unskilled use of the non-paretic forelimb (Maldonado, Allred, Felthouser, & Jones, 2008). That is, in intact animals, learning the skill with the non-dominant forelimb does not interfere with the ability to re-learn the same skill task with the dominant forelimb, and the effect is not seen following undirected, non-task-specific experience with the non-paretic limb in lesioned animals. Rather, these findings suggest that the effect involves an interaction between activity-dependent motor-learning mechanisms and injury-induced neural modification.

The purpose of this project was to test a potential anatomical mechanism for the detrimental effects of prior NPT on future RT of the paretic limb. First, reviews of neural responses to learning a skilled motor movement and to injury will be presented separately, and then a possible mechanism for how they may interact will be offered.

### **MOTOR SKILL LEARNING IN INTACT ANIMALS**

In intact (uninjured) mammals, motor skill learning relies, in part, on activity-dependent neuronal and structural remodeling of the motor cortex (for review see Adkins, Boychuk, Remple, & Kleim, 2006). Briefly, within the cortex contralateral to the trained

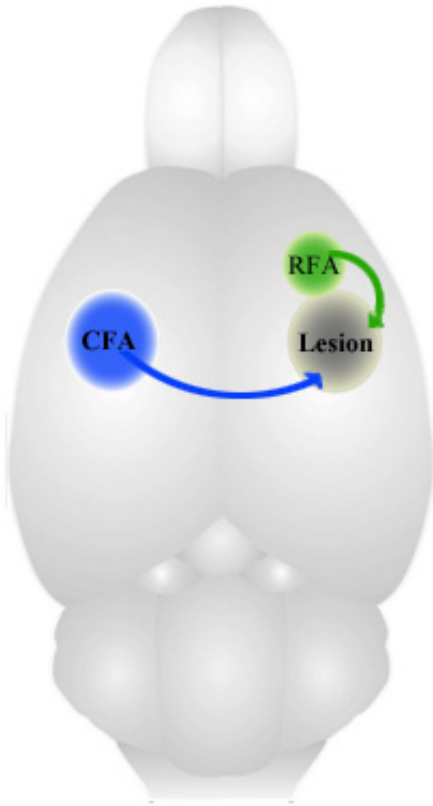
forelimb, motor skills training in the adult induces increases in dendritic arborization (Greenough, Larson & Withers, 1985) and synaptogenesis (Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996; Xu et al., 2009). Additionally, the functional topography of the motor cortex changes such that there is an expansion of motor cortical representations characteristic of the movements involved in the task (e.g., digit and wrist movements in reaching tasks; Kleim, Barbay, & Nudo, 1998; Nudo, Milliken, Jenkins & Merzenich, 1996). Yet these changes are not seen in association with unskilled motor (Kleim et al., 1998; Kleim et al., 2002) or strength training tasks (Remple, Bruneau, VadenBerg, Goertzen, & Kleim, 2001).

#### **NEURAL REORGANIZATION POST-INJURY**

Neural modification also occurs as a result of denervation and injury. Following cortical ischemic injury, there is an increase in growth-promoting proteins along with a decrease in normal growth-inhibiting proteins (Carmichael, 2006), ultimately causing axonal projections to sprout and dendrites to proliferate (Jones & Schallert, 1992; Nudo, 2007). These neuronal changes are also activity-dependent and sensitive to behavioral manipulations (Carmichael & Chesselet, 2002; Kerr, Cheng, & Jones, 2011; Overman et al., 2012). For example, while dendrites proliferate in response to motor skill learning, this growth is enhanced when the cortex has recently undergone denervation as a result of callosal transection (Bury et al., 2000) or a lesion in the homotopic cortex (Bury & Jones, 2002), suggesting an additive effect of denervation and learning induced plasticity.

Through use of a rodent model of ischemic infarct, one can examine how both learning and injury induced neural modification can interact to produce behavioral effects

of NPT on future RT of the paretic limb. The sensorimotor cortex of the rat contains two distinct areas that contribute to forelimb function: the caudal forelimb area (CFA) and the rostral forelimb area (RFA; Wise & Donoghue, 1986; see Figure 1). The CFA is part of the primary motor cortex (M1) while the RFA's functions have been compared to the supplementary motor cortex and the premotor cortex of apes (Rouiller, Moret, & Liang, 1993). Damage to the CFA of rats creates pronounced deficits in the limb contralateral to the lesion, which mimics those deficits seen in humans (Barth, Jones & Schallert, 1990). Functional recovery can be made in this limb, both spontaneously and through focused rehabilitation of the paretic limb that drives functional plasticity within the remaining cortex (Maldonado et al., 2008; Nudo, 2007). This remaining tissue adjacent to the lesion, also known as the peri-lesion cortex, shows considerable synaptic remodeling post-injury (Sigler & Murphy, 2010), and, depending on use of the paretic limb, can undergo extensive changes in motor map representations, such that it acquires functions lost within the damaged tissue (Castro-Alamancos & Borrel, 1995; Nudo, 2003). Additionally, functional improvements within the paretic limb are lost and deficits reinstated if the peri-lesion cortex is ablated (Castro-Alamancos & Borrel, 1995) or disrupted (Hsu et al., 2007). Furthermore, NPT decreases the activation of this area, as demonstrated by FosB expression (Allred & Jones, 2008). Therefore, the peri-lesion cortex is a site of functional recovery and is vulnerable to post-injury experience, suggesting that it is likely the location for the effects of NPT training on future RT of the paretic limb.



**Figure 1:** Diagram of rat motor areas and connections of interest. The rat motor cortex has two areas that control forelimb movement: the caudal forelimb area (CFA) and the rostral forelimb area (RFA). Each area is heavily connected to the other and those in the opposite hemisphere, such that, with a CFA lesion, the peri-lesioned cortex receives profuse projections from both the ipsilesional RFA and the contralesional CFA.

### **EXPERIENCE-DEPENDENT COMPETITION IN POST-INJURY NEURAL REMODELING**

Within the peri-lesion cortex it is possible that the interaction between lesion-induced plasticity and activity-dependent motor learning results in synaptic competition, reminiscent of processes that occur in development (such as the formation of ocular dominance columns within the striate cortex; see Greenough, Black, & Wallace, 1987 for review). Many patterns that shape the brain during development are also seen in the adult damaged brain, including dendritic overgrowth and subsequent pruning and spine remodeling (Brown & Murphy, 2008; Jones, 1999; Jones & Schallert 1992), reactive

axonal sprouting (Nafieralski, Butler, & Chesselet, 1996; Nudo, 2007), and rhythmic neuronal activity (Carmichael & Chesselet, 2002; for review see Jones & Jefferson, 2011). Many of these changes are also time-dependent (Carmichael, 2006; Liu, 2009). Thus, as with critical periods in development, behavioral patterns and subsequent cortical remodeling that are established early after injury may be relatively resistant to later change.

After CFA ischemic infarcts, there are two projection pathways that likely participate in synaptic competition over the peri-lesion cortex. The remaining CFA tissue receives profuse projections from the ipsilesional RFA and the contralesional CFA (Rouiller et al., 1993; Wise & Donoghue, 1986; see Figure 1 for diagram). The CFA is heavily connected to each of these areas, such that denervation caused by destruction of part of M1 would result in partial denervation to the other areas. Such denervation ultimately results in a cascade of molecular responses that may prompt these areas to grow and proliferate new neural connections (Carmichael, 2006). Moreover, since the ipsilesional RFA and contralesional CFA are located in opposing hemispheres, they will be activated differently by varying forelimb experiences. The ipsilesional RFA is presumably activated during paretic limb rehabilitation and the contralateral CFA during non-paretic limb training. Thus the peri-lesion cortex is a growth-promoting environment that receives connections from two areas that vary in activity level based on variations in forelimb training, making this area conducive to synaptic competition founded on forelimb training.



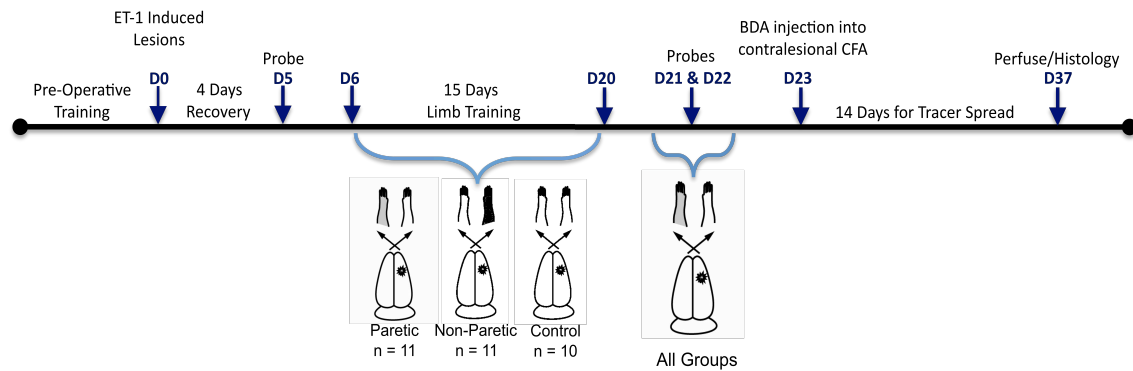
Current evidence is consistent with the idea of synaptic competition within the peri-lesion cortex after RT and NPT. Recently it was demonstrated that after a CFA infarct, NPT decreases axonal fiber density originating from the ipsilesional RFA within the peri-lesion cortex (Jefferson et al., 2012). However, while axonal projections from the contralesional hemisphere have been observed to proliferate into the peri-lesion cortex post ischemic CFA infarct (Carmichael & Chesselet, 2002), it is unknown how NPT may effect these proliferations. There is evidence, though, that supports that the contralesional hemisphere and its callosal projections are somehow involved in the deleterious effects of NPT. Inhibition of the contralesional cortex by Muscimol injections in rats (Mansoori et al., 2014) or by disruptive TMS in humans (Nowak, Grefkes, Ameli & Fink, 2009) can improve functional outcomes of the paretic limb. Additionally, rats with prior NPT can overcome the detrimental effects on their paretic limb if Muscimol is infused into the contralesional cortex while rats undergo subsequent RT with the paretic limb (unpublished data). Furthermore, callosal transections block the maladaptive effect of learning with the non-paretic forelimb on paretic forelimb function after stroke (Allred et al., 2010) but do not prevent the dendritic proliferation of layer V pyramidal neurons in the contralesional CFA when combined with behavioral learning (Bury et al., 2000). These data suggest that projections from the contralesional CFA likely play a role in the deleterious effects of NPT on subsequent RT of the paretic limb.

### **CURRENT DIRECTION**

Following ischemic cortical infarct, the peri-lesion cortex undergoes extensive activity-dependent remodeling. The increases in dendritic overgrowth, axonal

proliferations, and synaptogenesis within this area suggest that synaptic competition could play a role in the establishment of new connections within this area. In CFA cortical infarcts, the remaining surrounding tissue receives extensive connections from the ipsilesional RFA and the contralesional CFA, which may compete for the newly denervated tissue. Similar to developmental critical periods, if callosal projections from the contralesional hemisphere are stimulated to proliferate in response to NPT, the resulting synapses might be difficult to displace with connections from the ipsilateral RFA upon subsequent RT of the paretic limb. It is therefore conceivable that synaptic competition between ipsilateral RFA projections and contralesional CFA projections may be the underlying mechanism behind NPT's detrimental effects on subsequent RT of the paretic limb.

The purpose of the current experiment is to begin to test this mechanism by investigating whether contralesional projections to peri-lesion cortex are influenced by NPT. Briefly, rats will be trained on a skilled reaching task prior to being given CFA ischemic lesions. After a period of rest, they will then be given 15 days of training with their non-paretic limb (NPT), paretic limb (RT), or control procedures (CTRL), after which a tract tracer will be injected into the contralesional hemisphere and the resulting axonal fiber projections and bouton densities will be measured within the peri-lesion cortex (see Figure 2 for summary of experimental timeline). If there is synaptic competition, one would expect that training the non-paretic limb would increase axonal densities from the contralesional CFA in peri-lesion cortex, as compared to those who undergo paretic limb RT or control procedures.



**Figure 2:** Summary of experimental design. This experiment was designed to test the hypothesis that NPT would increase the axonal fiber and bouton densities in the peri-lesion cortex from the contralesional CFA. After pre-operative training on the single-pellet retrieval task, ischemic strokes were induced in the CFA opposite the dominant for reaching forelimb. Beginning on Day 6 (D6), rats underwent 15 days of either RT of the paretic forelimb, NPT, or control procedures. All animals were then given an injection of BDA into the contralesional CFA and allowed to rest for 14 day before they were perfused for histological examination.

## **Method**

### **SUBJECTS**

Thirty-two well-handled 4-month old adult male Long-Evans hooded rats were housed in standard laboratory cages on a 12 hr:12hr light/dark cycle. Rats were housed in pairs when possible, and given standard supplementary housing materials (a polyvinyl chloride pipe, small wooden toys, and cardboard paper rolls). Before the start of the experiment, rats were placed on a food-restricted diet in order to increase motivation for the skilled-reaching task, with weight being maintained at or above 95% of their original weight. Animals were randomly assigned to participate in NPT (n = 11), RT (n = 11) or CTRL procedures (n = 10), with the exception of being matched as closely as possible for the severity of their post-operative deficits. All protocols pertaining to the health and well being of the rats were approved by The University of Texas Institutional Animal Care and Use Committee, an AAALAC accredited program.

### **BEHAVIORAL PROCEDURES**

**Single Pellet Retrieval Task.** Rats were trained on the single pellet retrieval task, adapted from Wishaw and Pellis (1990) as described previously (Allred & Jones, 2008; Bury & Jones, 2002), which was used for both training and assessment. Briefly, rats were placed in a Plexiglas reaching container and shaped to retrieve 45 mg banana flavored pellets (Bioserve, Inc.) from a shelf located outside of the reaching chamber. A Plexiglas wall was placed ipsilateral to the dominant for reaching forelimb and pellets were placed in a shallow well located diagonally from the reaching forelimb so that

successful reaches could only be achieved with the desired forelimb. For each trial, rats were given the opportunity to reach at most five times for the desired pellet. A trial concluded when the rat either grasped the pellet and transferred it to his mouth (success), grasped the pellet but dropped it on the floor of the chamber (drop), or knocked the pellet from its well or more made more than five reach attempts (fail). Performance was calculated by dividing the total number of successful reaches by the total number of reach attempts (successes + drops + fails). All rats were trained pre-operatively to a criterion of a 60% success rate or for a maximum of 30 days.

The experimental manipulation was post-operative reach training with either the paretic or non-paretic forelimb. To accomplish this, the wall was moved so that it was ipsilateral to the forelimb to be trained. Rats were then allowed to train for 60 trials per day for 15 days. Animals in the control condition were placed in the reaching chamber without an inner wall and given pellets on the floor of the chamber at a rate yoked to those in the manipulated conditions.

**Schallert Cylinder Test.** In order to measure stroke-induced forelimb asymmetries, use of each forelimb for postural- support during exploratory movements within a cylinder was recorded pre-operatively, post-operatively, and post training procedures. As described by Schallert, Kozlowski, Humm, and Cocke (1997), rats were filmed in a Plexiglass cylinder (19 cm diameter) and the first 30 instances of forelimb use (ipsilesional, contralesional, or bilateral) for upright support against the cylinder walls were noted during slow-motion playback of the videotapes by an investigator blind to the

experimental conditions. The asymmetry score was calculated as the percent use of the non-dominant forelimb:  $[(\text{non-dominant use} + \frac{1}{2} \text{ bilateral use}) / \text{total forelimb use}] \times 100$ .

## **SURGICAL PROCEDURES**

**Lesion Induction.** On day 0 all rats were given unilateral ischemic lesions with a topical application of the vasoconstricting peptide endothelin-1 (ET-1; American Peptide Co.) in the CFA contralateral to the dominant for reaching forelimb. Animals were anesthetized with an i.p. injection of a ketamine (100 mg/ml) and xylazine (10 mg/ml) cocktail, and were maintained on a surgical plane on anesthesia throughout the procedure with ketamine boosters and/or isoflurane gas (1-2%) when necessary. Rats were placed in a stereotaxic frame, an incision was made at the midline of the scalp, and a craniectomy was performed between 1.0 mm posterior and 2.0 mm anterior to Bregma and between 2.0 mm and 4.5 mm lateral to midline. The dura was then removed just prior to the application of 3  $\mu$ l of ET-1 to the surface of the brain. The rat was then left undisturbed for 10 minutes before a skull cap was placed using silicone sheeting (Invotec International) and dental cement (SDI Inc.) and the incision was sutured.

**BDA Pressure Injection.** After 15 days of limb training, rats were once again deeply anesthetized with a ketamine and xylazine i.p. injection and maintained on the surgical plane with ketamine boosters and isoflurane gas when necessary. Rats were in a stereotaxic frame and an incision was made up the midline. Two burr holes were then made in the contralateral skull at 0.5 mm and 0.9 mm anterior to Bregma and 2.75 mm lateral to midline. These burr holes were then connected and the underlying dura was gently removed as much as possible. A micropipette was then filled with 3  $\mu$ l of

biotinylated dextran amine (BDA; 10kDA; Invitrogen) and lowered to a depth of 1400  $\mu\text{m}$  and brought back up to a depth of 1250  $\mu\text{m}$  using a micropositioner. Three consecutive injections at a rate of 1  $\mu\text{l}/2$  min across three injection sites approximately 1 mm apart were given before the hole was filled with dental cement and the incision was sutured.

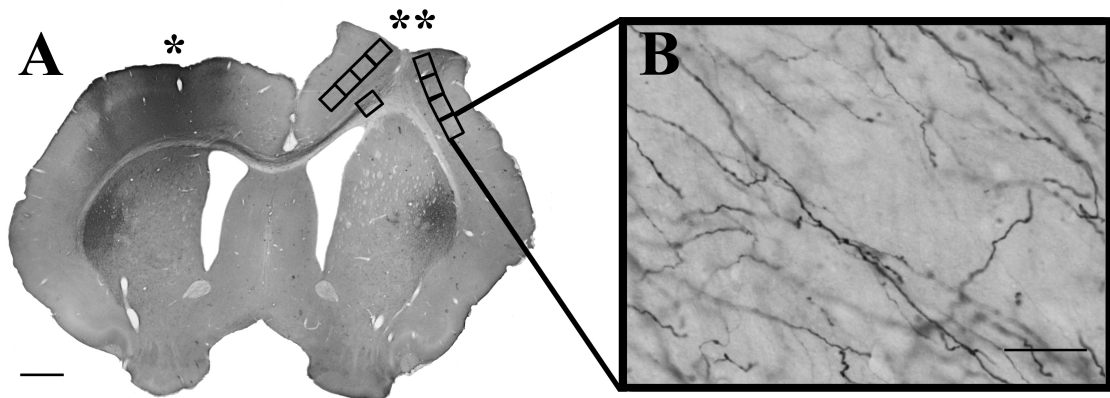
## **HISTOLOGY**

After 14 days of rest to allow for tracer transport, rats were anesthetized with a lethal dose of sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde in the same buffer. Brains were removed and post-fixed in 4% paraformaldehyde in .1 M PB for a minimum of one week. Six alternating sets of 50  $\mu\text{m}$  coronal sections were then collected using a Leica VT1200S vibratome and stored in cryoprotectant at 4  $^{\circ}\text{C}$ . One set was stained with toluidine blue, a Nissl stain, in order to reconstruct the lesion and to analyze the volume of the remaining cortex. Another set underwent a free-floating immunocytochemistry stain for visualization of fibers and injection placement. A standard immunocytochemistry procedure was used with peroxidase-linked avidin–biotin complex (ABC kit, Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) with nickel ammonium sulfate (NAS) intensification. All tissue was processed in one batch and included no-primary controls (tissue that was processed identically with the exception of exposure to the ABC) in order to verify the specificity of the antibody labeling. During data collection, the investigator was blind to the experimental condition of the tissue.

**Lesion and injection analysis.** To assess the extent of cortical damage across groups, the volume of the remaining ipsilesional CFA was measured using Neurolucida (Microbrightfield Inc.) perimeter tracing software. The ipsilesional cortical areas of seven 50  $\mu\text{m}$  Nissl stained sections (ranging from +2.7 mm to -0.80 mm relative to Bregma, with sections being 600  $\mu\text{m}$  apart) were traced at 17x magnification. The product of the summed cortical areas and the distance between sections was used as an estimate of remaining CFA cortical volume (Gundersen et al., 1988). Additionally, coronal schematic templates were used to estimate lesion location, lesion extent, injection location, injection depth, and tracer spread relative to macrostructural landmarks.

**Axonal Density Analysis.** The surface densities of BDA-labeled axonal fibers in the peri-lesion area were determined using a cycloid grid method (Baddeley, Gundersen, & Cruz-Orivet, 1986). Four sections per brain were selected for analysis: two representing the anterior portions of the lesion (approximately 1.8 mm and 1.2 mm anterior to Bregma) and two representing the posterior portions of the lesion (approximately 0.2 mm anterior and 0.3 mm posterior to Bregma). Sample frames were obtained on a Nikon Optiphot-2 light microscope under a 50x oil-immersion Nikon objective. Each sample was taken from a plane near the top on the section and was captured using a SPOT Idea 3.0 megapixel color digital microscope camera (Diagnostic Instruments, Inc.). Within each section, nine samples were collected (see Figure 3). First, a sample from the dorsal portion of the splenium of the ipsilesional corpus callosum was taken in order to determine the extent of transcallosal BDA staining. The image was rotated so that fibers ran horizontally through the sample. These samples were used as a





**Figure 3:** Representative lesion, BDA infusion, and fibers. A) Photomontage of BDA labeled coronal section depicting labeled fibers and locations of samples taken. Eight peri-lesion samples were taken per section, four medial and four lateral to the lesion. One additional sample was taken of the splenium of the corpus callosum as a control for tracer uptake. \* = Infusion site, \*\* = CFA ischemic lesion. Scale bar = 1 mm. B) Representative axonal fiber sample frame. Scale bar = 30  $\mu$ m.

control for labeling efficiency since it is not expected that axons will sprout long distances in response to injury (Cheatwood, Emerick & Kartje, 2008). Additional samples were then collected from layer V of the cortex immediately adjacent to the lesion, and images were rotated so that the vertical axis was parallel to the orientation of cortical columns. The first image was placed as close to the medial border of the lesion as possible, excluding any necrotic tissue. Three subsequent samples were then taken by moving one frame over from the adjoining image so that there was no overlap between samples. The process was then repeated in the cortex lateral to the lesion so that a total of eight cortical images per section were captured: four medial and four lateral to the

lesion. Thus, 36 images were collected in total per brain. This sampling strategy, although not systematically random, was chosen in order to obtain data from areas most proximal to the infarct region, an area that undergoes the most change in response to experiential manipulations post neuronal damage (Kerr, Cheng, and Jones, 2011).

Images were then imported into ImageJ software and analyzed on a 29.46 cm LED-backlit computer monitor with 1366 x 768 pixel resolution, yielding a final magnification of 1100x. To quantify axon density, a set of rolling cycloid arcs was superimposed onto the image and the number of intersections between the cycloid and in-focus BDA-labeled axonal fibers were counted. Adjusted for magnification, the individual arc length was 0.02 mm, and a grid containing 72 arcs was placed across the 1.31 mm<sup>2</sup> sample frame. This method yielded an average of 199 cortical intersections per brain. The surface density (Sv) of each sample was calculated using the formula:  $Sv = 2(I/L)$ , where I is the total number of intersections counted and L is the total cycloid arc length per sample (1.44 mm).

The Sv of splenial fibers in the anterior and posterior portions of the CFA were then found by averaging the Sv of splenial fibers from the two sections within in each respective area. The peri-lesion cortex was then divided into four areas relative to the lesion location: anteromedial, anterolateral, posteromedial, and posterolateral. The number of intersections for each of these regions was then averaged together. That is, for each region a total of eight samples were averaged: two sections within the anterior/posterior portion each with four samples ranging from proximal to distal relative to the lesion (see Figure 3). This averaged value was then divided by the corresponding

splenium average to obtain the final relative surface density of axons within each perilesion area.

**Bouton Density Analysis.** The optical disector method (Harding, Halliday, & Cullen, 1994) was used in order to quantify the density of boutons on axonal fibers. The same sample sites were used for bouton and axonal densities measures. Per each site, after capturing images for axonal measures, a smaller unbiased sample frame was superimposed over the live image and the number of boutons contained within the frame were counted. This method was conducted on a 75.44 cm LCD display with 2560 x 1600 pixel resolution with a final magnification of 2400x. Boutons that were in focus in the top plane were excluded from the count, while all other boutons were counted. In addition to the boutons that were contained within the frame, boutons that touched the top and right border of the frame were counted, while those that touched the bottom and left border were excluded.

Bouton counts were averaged within the four regions (anterolateral, anteromedial, posterolateral, and posteromedial), and divided by their respective axonal intersection counts from the cycloid grid method. Each number was then multiplied by a constant correction factor (14,284.83) to yield boutons per mm<sup>2</sup> fiber surface area, which was calculated using the formula  $\Sigma Q / ((a(\text{frame}) * T) / (2(I/L)))$ ; where  $\Sigma Q$  equals the number of boutons counted;  $a(\text{frame})$  equals the area of the bouton sample frame (0.001008 mm<sup>2</sup>);  $T$  equals the average depth of the tissue, which was assumed to be equal to the average section thickness (50  $\mu\text{m}$ ) based on the work of Harding et al. (1994);  $I$  equals

the average number of intersections on the cycloid grid; and L equals the total length of the cycloid per sample (1.44 mm).

### **INFERENCEAL ANALYSIS**

All data were analyzed using a prior planned comparisons: 1) NPT versus CTRL, 2) RT versus CTRL, and 3) RT versus NPT. Of most concern was to test NPT versus CTRL, in order to determine the detrimental anatomical properties of NPT. Additionally, RT will be compared to CTRL in order to test the effects of rehabilitation, and NPT versus RT will be compared in order to compare variations in limb use. Behavioral reaching success data was analyzed using repeated measure analysis of variance (ANOVA) in order to examine the effects of Group, Day, and Group by Day interaction. The post-training day data point represents the average of the two probes taken on days 21 and 22. Ipsilesional cortical volumes were compared using one-way ANOVAs for each planned comparison. Fiber and bouton densities were analyzed with one-way ANOVAs for each planned comparison within each of the four designated anatomical areas (anteromedial, anterolateral, posteromedial, and posterolateral). All data was analyzed using SPSS statistical software (SPSS Inc.) and used two-tailed tests with a set alpha level of .05. Descriptive statistics are reported as mean  $\pm$  SEM.

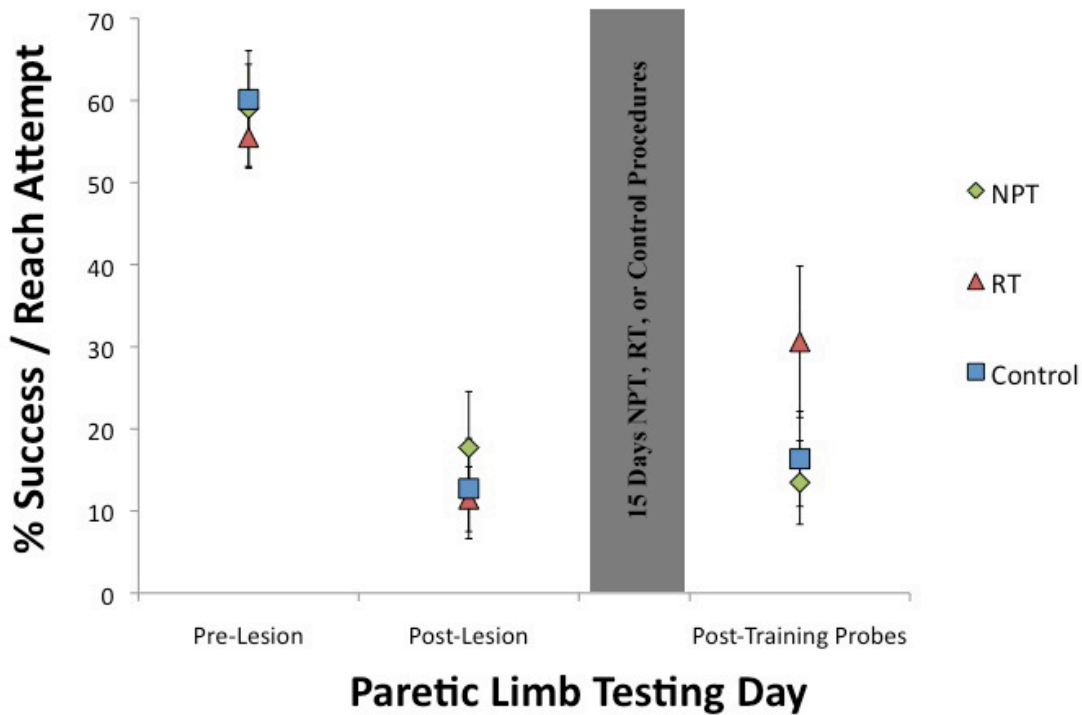
## Results

### BEHAVIORAL DATA

The percentage of successful reaches of the paretic forelimb can be seen in Figure 4. Several rats were excluded from the analysis due to lesion size and insufficient labeling, as described below, yielding final sample sizes of  $n = 7$  for NPT,  $n = 11$  for RT, and  $n = 8$  for CTRL. In the planned comparisons repeated-measures ANOVAs, all comparisons showed a significant effect of Day [ $F_s(2, 26 - 34) = 54.76 - 64.75, p_s < .01$ ] reflecting that all groups demonstrated a significant impairment of the contralesional forelimb after unilateral ischemic lesions (“Post-Operation”) compared to pre-operation performance levels. There was also a significant Group by Day interaction between NPT and RT rats [ $F(2,32) = 4.32, p = .02$ ]. Post-hoc analysis by way of independent  $t$  tests found that there was a significant difference between NPT and RT at the Post-Training time point [ $t(16) = -3.32, p < .01$ ] such that RT rats had significantly higher success rates ( $30.61 \pm 9.23\%$ ) than NPT rats ( $13.46 \pm 5.09\%$ ). No significant group main effects were found within the NPT versus CTRL analysis. Behavioral results on postural support were not informative (see Appendix A for additional information).

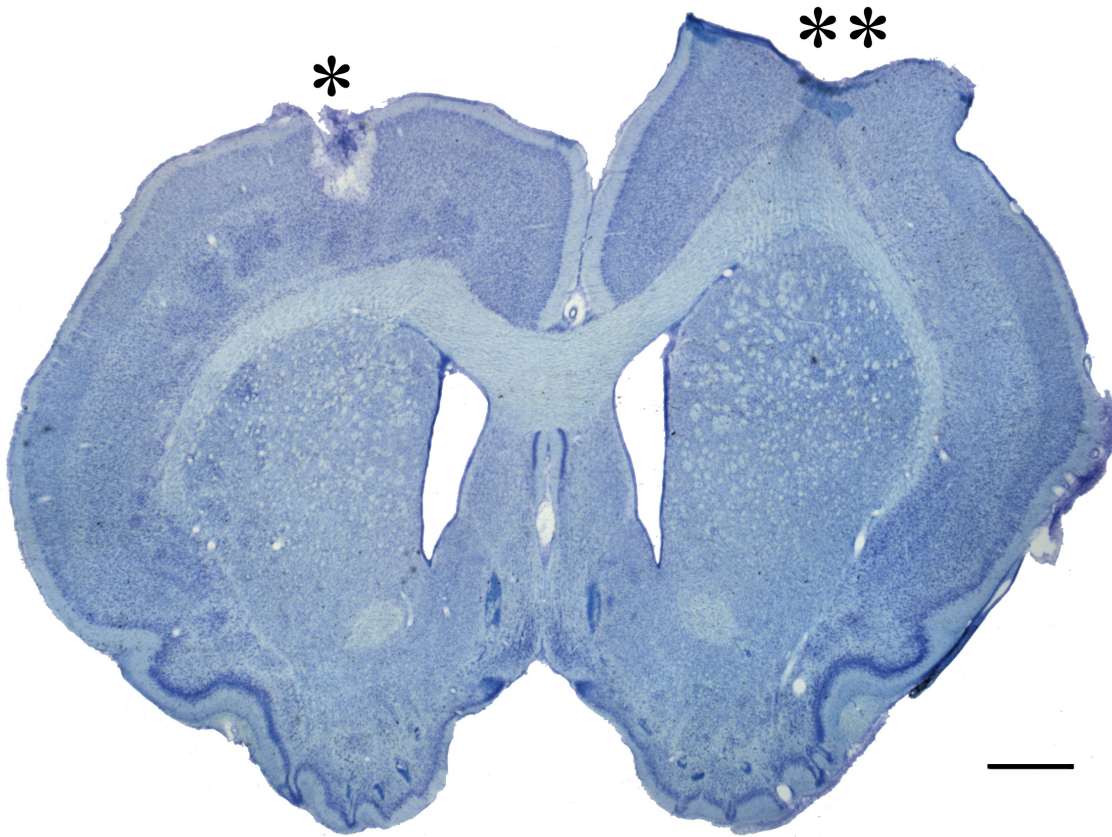
### LESION EXTENT AND INJECTION SITE

All animals sustained cortical injury within the CFA region, between approximately 3.5 mm rostral and 0.4 mm caudal to Bregma, that extended to layer V (see Figure 5 for representative Nissl stained coronal section). Upon examination of the Nissl stained tissue, two rats from the NPT group were omitted from the analysis due to



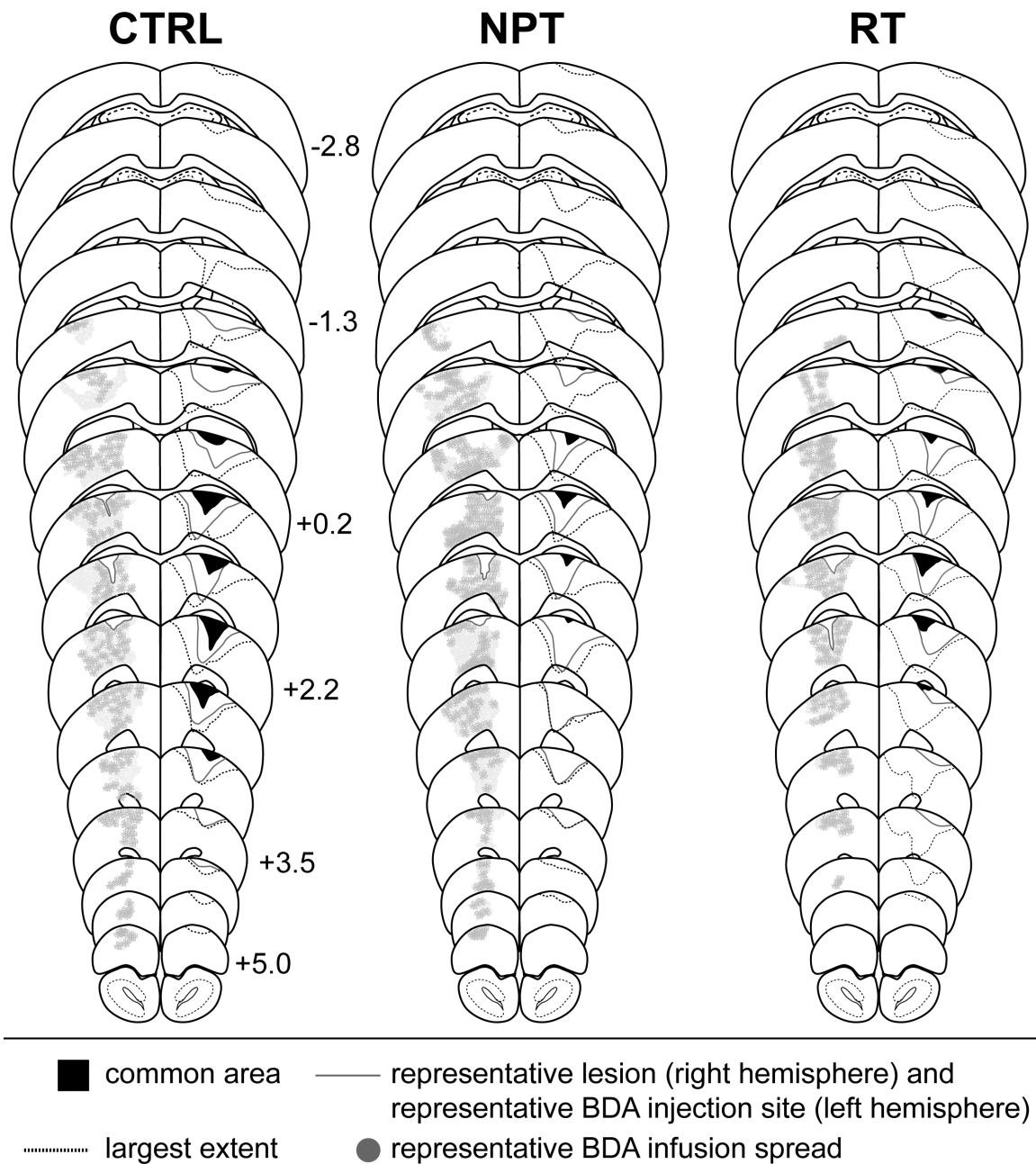
**Figure 4:** Behavioral reaching data. All animals, NPT ( $n = 7$ ), RT ( $n = 11$ ), and control procedures ( $n = 8$ ), had a significant deficit following ischemic lesion induction. Following the 15 days of training, RT rats had a significantly higher success rate than the NPT rats ( $p < .05$ ). Although there is no significant difference between NPT and CTRL rats, this is consistent with previous studies for this time point (Allred et al., 2010). The post-training day data point represents the average of the two probes taken on days 21 and 22. Error bar =  $\pm$ SEM.

extensive lesions that eliminated all tissue medial to the lesion as well as severed the corpus callosum such that no callosal fibers could cross into the remaining lateral tissue. Additionally, one rat from the NPT group sustained a deep cortical infarct that severed



**Figure 5:** Photomontage of Nissl stained coronal section depicting a representative lesion. \* = Tracer injection site; \*\* = CFA ischemic lesion. Scale bar = 1 mm.

the corpus callosum but spared medial tissue, and thus only data from the medial tissue was used in subsequent analysis. After the omission of these animals, there were no obvious observable differences between placement or extent of lesions between groups (see Figure 6). One-way ANOVA analysis indicates that there was a non-significant tendency [ $F(1,16) = 3.16, p = 0.09$ ] for RT animals to have slightly smaller ipsilesional cortical volumes ( $64.19 \pm 3.01 \text{ mm}^3$ ) than NPT animals ( $72.04 \pm 2.83 \text{ mm}^3$ ), suggesting a tendency towards larger lesions in the RT animals. While this may affect the



**Figure 6:** Schematic of coronal sections reconstructing lesion size and extent (right hemisphere) as well as a representative BDA injection site and spread (left hemisphere) for CTRL, NPT, and RT animals. Numbers represent distance from Bregma in mm.



comparisons between RT and NPT anatomical measurements of axonal fiber and bouton densities, it does not contribute to our primary comparison of interest: that of NPT versus CTRL. No significant group differences in ipsilesional cortical volumes existed between NPT versus CTRL ( $66.21 \pm 2.99 \text{ mm}^3$ ;  $p = .18$ ) or between CTRL versus RT ( $p = .65$ ).

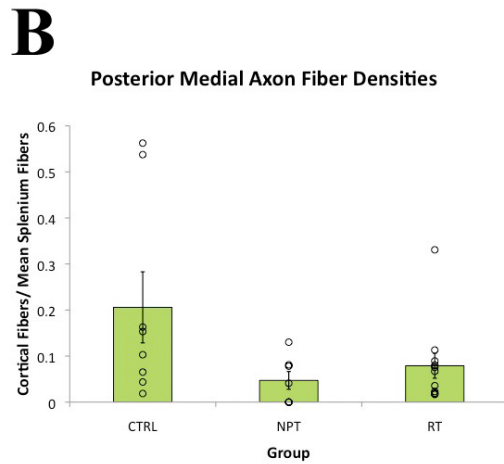
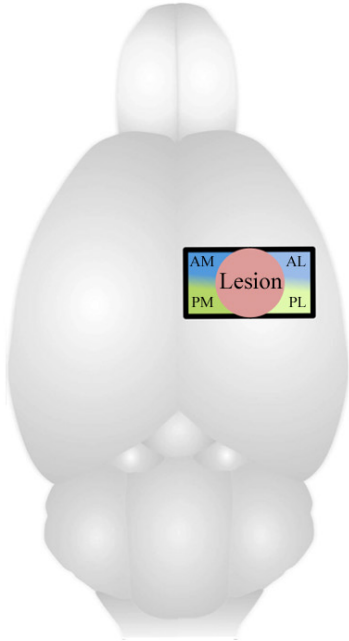
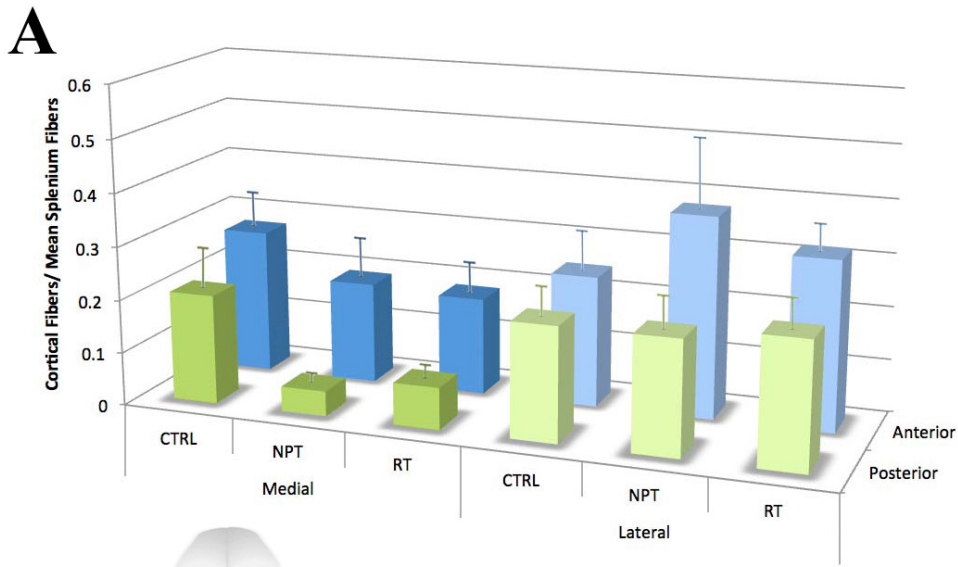
BDA injection sites were located in the same anterior to posterior region as CFA ischemic infarcts, typically between 2.2- 1.2 mm anterior to Bregma, and did not appear to spread into the underlying white matter (see Figure 6). The BDA tracer typically spread throughout all layers of the cortex and throughout the entirety of the CFA (see Figure 3 for representative BDA stained coronal section). To account for possible variability in infusions and tracer uptake between animals, a sample of axonal fibers from the ipsilesional splenium of the corpus callosum was quantified within each section, and final values were reported as a ratio between cortical and splenial fiber densities. After initial splenial analysis, four rats (2 NPT and 2 CTRL) demonstrated insufficient labeling within the corpus callosum and were omitted from subsequent analysis.

#### **AXONAL FIBERS AND BOUTON DENSITIES**

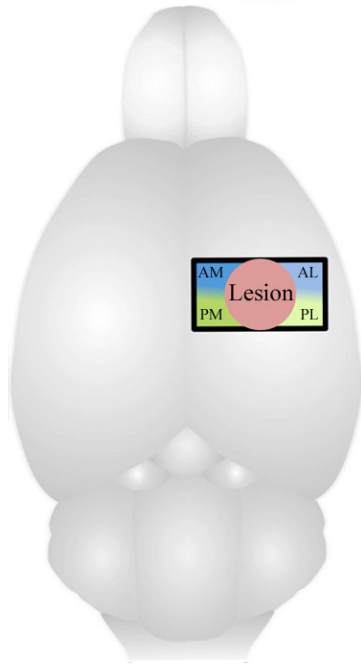
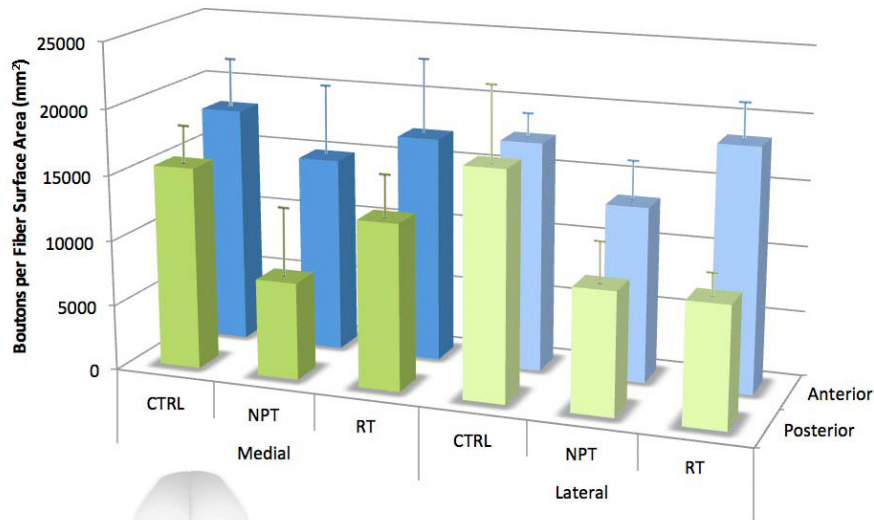
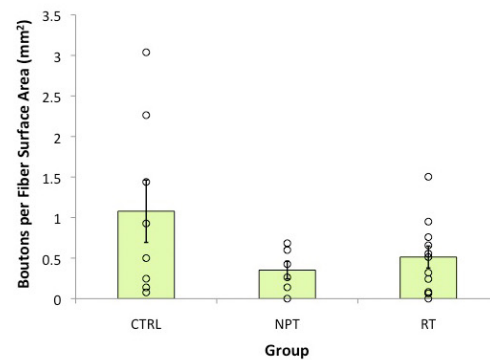
As seen in Figure 7, no significant differences between groups were found in any of the examined regions of the peri-lesion cortex. The data indicate a tendency, within the posteromedial portion of the peri-lesion cortex, for the control group to have greater axonal densities [CTRL versus NPT:  $F(1,13) = 3.50$ ,  $p = .08$ ; CTRL versus RT:  $F(1,17) = 3.06$ ,  $p = .10$ ] than either training manipulation, which did not differ [NPT versus RT:  $F(1,16) = 0.72$ ,  $p = .41$ ]. However, upon further examination it became clear that this tendency was highly influenced by only two data points, and thus was not likely a true

trend. No significant differences in axonal densities were found for any of the other planned comparisons within anteromedial, anterolateral or posterolateral portions of the peri-lesion cortex [ $F_s(1,13 - 17) = 0.00-0.80, p_s = .39 - .95$ ], indicating that differential forelimb training did not produce a change in axonal fiber densities from the contralesional hemisphere within the peri-lesion cortex (mean  $CE = 0.22$ ).

Similarly, there were no significant effects of variable forelimb training on bouton densities found for any of the planned comparisons within any of the peri-lesion areas [ $F_s(1, 13 - 17) = 0.00 - 1.88, p_s = .15 - .97$ ; mean  $CE = 0.35$ ]. As seen in Figure 8, CTRL, NPT, and RT rats had a very similar number of boutons per fiber surface area within the anteromedial, anterolateral, posteromedial, and posterolateral portion of the peri-lesion cortex. Thus variable forelimb training did not produce a net change in bouton densities per fiber surface area. Differences in reach performance cannot explain the lack of differences in the anatomy. Exploratory Bivariate correlations assessing the relationship between post-training reaching success and axonal fiber and bouton densities revealed no significant correlations across any of the four peri-lesion areas (see Appendix B for more information).



**Figure 7:** Quantitative results of axonal fiber densities in peri-lesion cortex. No regions contained a significant difference between groups in axonal fiber densities (A). Although there was a tendency in the PM area for the control to have higher densities of axonal fibers, when individual data points are overlaid (B) it becomes clear that this tendency was highly influenced by a small number of animals. Error bars =  $\pm$ SEM.

**A****B****Posterior Lateral Bouton Densities**

**Figure 8:** Quantitative results of boutons per fiber surface area in peri-lesion cortex. Bouton densities did not vary by group in any area (A). Within the PL area, there was a tendency for CTRL animals to have higher densities, however, when examined with individual data points (B) it becomes clear that this effect is highly influenced by only a few rats. Error bars =  $\pm$ SEM.

## Discussion

Consistent with previous findings, animals that received rehabilitation training (RT) of the paretic limb improved the functional outcome of the paretic limb for the skilled reaching task (Adkins, Hsu & Jones, 2008; Maldonado et al., 2008), an effect that was not seen in rats trained with their non-paretic forelimb. Also consistent with previous findings, rats receiving control procedures experienced a moderate, though non-significant, amount of spontaneous recovery of paretic forelimb function during the post-training probe trials (Allred et al., 2010). Although there was no significant differences between post-training success rates of NPT and CTRL rats, this is consistent with previous studies in the literature that indicate no significant differences between these groups at the first time point after training (Allred et al., 2010), rather these groups may differentiate over time with subsequent rehabilitation training. However, subsequent RT was not done in this experiment because the purpose of the study was to investigate the effects of NPT and not NPT followed by RT on callosal proliferations.

The results presented in this study revealed that, contrary to the initial hypothesis, variations in forelimb training after an ischemic unilateral lesion of the CFA did not produce significant differences in transcallosal sprouting patterns in the peri-lesion cortex. Rats that received NPT, RT, and control procedures had comparable densities of axonal fibers from the contralesional CFA in the anteromedial, anterolateral, posteromedial, and posterolateral portions of the peri-lesion area. Similar results were seen for boutons per fiber surface area.

It is possible that the design of this study missed the changes occurring within the peri-lesion cortex that is responsible for the effect of NPT on paretic limb function. For instance, previous studies finding anatomical differences within the peri-lesion cortex examined the brains after CTRL and NPT animals had undergone *subsequent* RT of the paretic forelimb. One example of this is that neuronal activation, measured by presence of FosB/deltaFosB, increases with RT, an effect that is blocked by previous training with the non-paretic forelimb (Allred & Jones, 2008). We chose to investigate neuronal changes directly after NPT procedure because our developmental model of synaptic competition indicated that changes might occur at this time point that would make neuronal connections resistant to future change. However, it is possible that the time point chosen for this study missed important changes that may occur in response to subsequent RT.

Additionally, while there is no net change in bouton density, this method did not account for synaptic structural changes. In 2011, Kim, Allred, Adkins, Donlan and Jones conducted an electron microscopy study that revealed an increase in multiple synaptic boutons, but not perforated synapses, in the peri-lesion cortex when animals underwent NPT followed by subsequent RT of the paretic limb, as compared to those that received control procedures followed by RT. While perforated synapses have been associated with increased efficacy/ maturity of a synapse (Greenough, West, & DeVoogd, 1978), multiple-synaptic boutons are unstable and have been associated with the process of learning (Federmeier, Kleim & Greenough, 2002). This shows that it is possible for variations in forelimb training to cause differences in synaptic structure that may have a

direct impact on behavior. Again this was found at the conclusion of RT and does not rule out the possibility that axonal changes are occurring in the pathway from contra to ipsilesional cortex, which are detectable only with the RT paradigm. While this study did not account for the origin of synapses, it is feasible that there could be an unequal distribution of synapse type originating from ipsilesional RFA and contralesional CFA, that does not result in a net change in boutons but does ultimately lead to the detrimental effects of NPT on paretic limb function.

It is also feasible that the detrimental effects of NPT on subsequent RT are a result of changes to select pathways within the callosal network. The corpus callosum is made up of projections from neocortical pyramidal cells, which form excitatory asymmetrical synapses in the contralateral cortex (Cipolloni & Peters, 1983). While the vast majority of these cells synapse onto dendritic spines of excitatory pyramidal cells in the contralateral hemisphere, approximately 13% synapse onto  $\gamma$ -aminobutyric acidergic (GABAergic) inhibitory interneurons (Karayannis, Huerta-Ocampo & Copogna, 2007). The methods of the current study did not account for the types of post-synaptic neurons the callosal fibers projected onto. It is possible that the net excitatory and net inhibitory networks are differentially effected by NPT in such a way that does not result in a change in axonal fiber density and yet still has a profound effect on later behavioral recovery.

These results do not preclude the possibility that the effect resides primarily within the RFA rather than the CFA. As mentioned previously, has already been demonstrated that NPT causes a reduction in axonal fiber proliferations from the ipsilesional RFA (Jefferson et al., 2012), and it may be that these projections are the

primary mediator to the effect. If this is the case, then projections from the contralesional RFA to the ipsilesional RFA could be the callosal fibers that are involved and are affected by callosal transections (Allred et al., 2010). An experiment that may provide further insight into this possibility would be to selectively sever the corpus callosum such that only fibers from either the contralesional RFA or CFA are transected. If both or either outcomes are still able to block the detrimental effect of non-paretic forelimb training on subsequent RT it will determine which contralesional area contributes to the effect.

#### **METHODOLOGICAL CONSIDERATIONS**

The issue of controlling for variability in infusions and tracer uptake between animals poses a considerable problem for all tract tracer studies. This study used samples from the ipsilesional splenium of the corpus callosum as a control for this. However it is not clear whether this is the best area to sample as a control. Fibers may exit the corpus callosum into the tissue at any point after the midline and may project both anterior and posterior from its exit point. Thus the density of fibers within the splenium may not be an accurate representation of the expected density in the cortex within the same plane of section. In an attempt to compensate for this discrepancy, this study used an average of splenial densities from two section planes. However, future studies may wish to consider using other fiber tracts, such as striate fiber bundles or descending corticofugal tracts as controls.

Additionally, BDA has been known to occasionally participate in retrograde transport, causing a few parent cell bodies to be labeled in the peri-lesion cortex. While



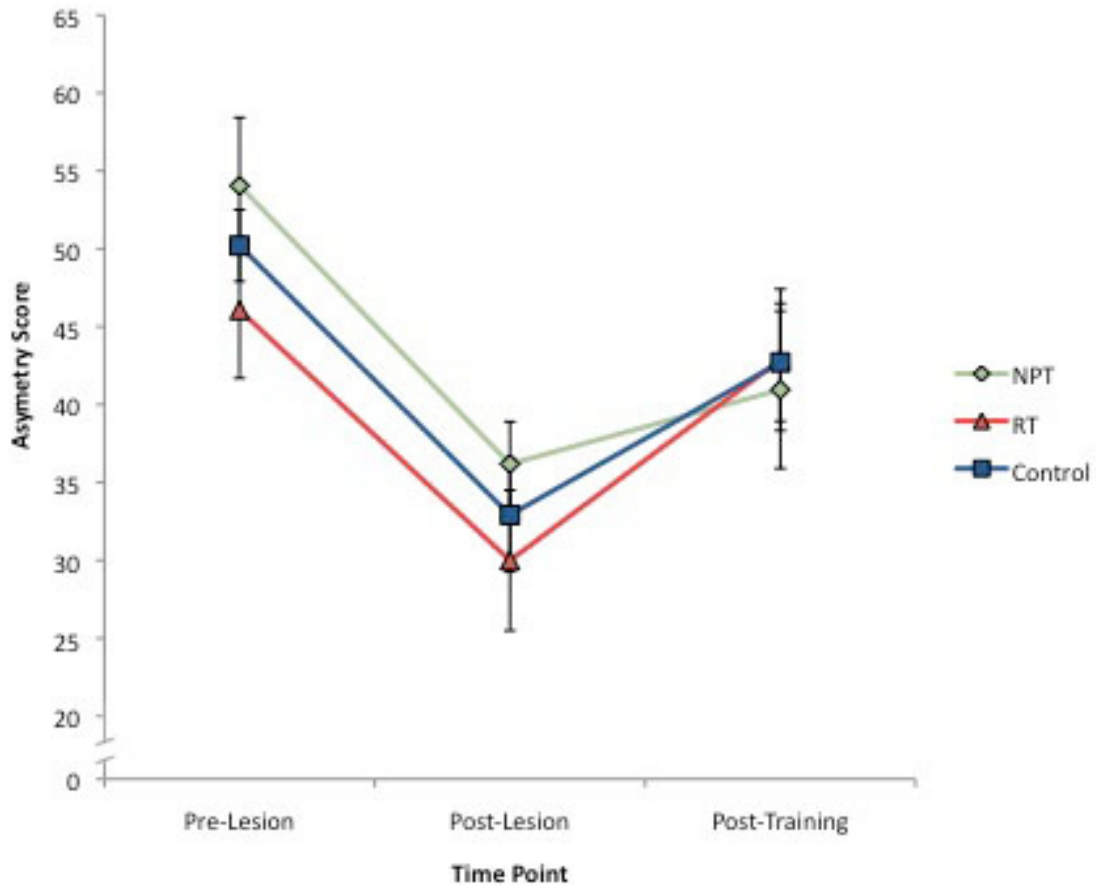
precautions were taken to minimize the number of dendritic fibers that were included in the analysis by adjusting the sample frame such that no cell bodies were included, this does not rule out the potential for some of the fibers analyzed to have been dendritic processes that originated in other planes of section. Nevertheless, this potential is minimized by the clear morphological differences between axons and dendrites.

It is also important to note, that ideals of stereological procedures were not rigorously held to in this project. Most notably, samples used for cycloid grid analysis were not taken in a random orientation around the vertical axis. However, axons tend to proliferate in a relatively radial fashion in this dimension as they follow the basilar dendrites of pyramidal neurons in the cortical layers. Thus, these fibers should be largely isotropic, but it is possible that some anisotropy may still exist and could limit the findings of this experiment. However, the samples from the splenium of the corpus callosum clearly run parallel to one another and do not form a radial distribution, and thus will be greatly affected by the anisotropy bias. Since the results from the cycloid grid analysis were subsequently used in our analysis of bouton densities, it is possible that anisotropy may also have an effect on the bouton density estimates as well. An additional departure from ideal stereological procedures was that the sample frames were not chosen through systematic random sampling. This is because the question required samples from regions located as close to the lesion as possible (the peri-lesion area). Thus, it is believed that departures made from the ideal stereological procedures here are justified.

## CONCLUSIONS

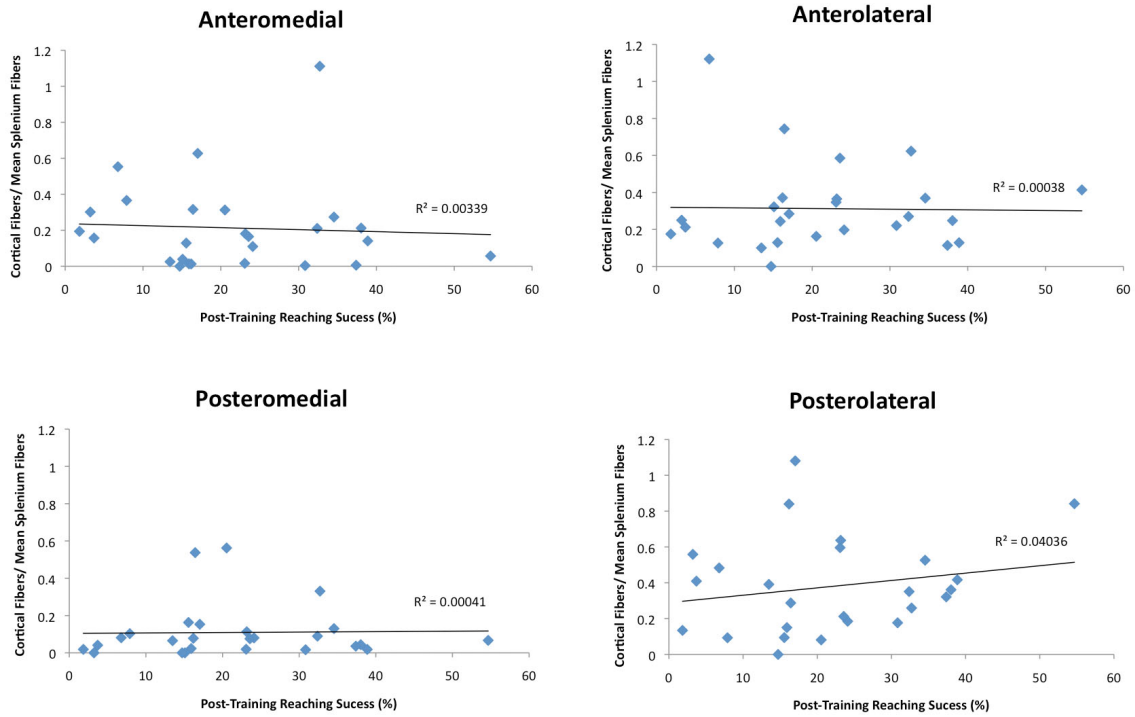
The present results indicate that variations in forelimb training do not cause a net change in axonal fiber or bouton densities from the contralesional cortex in the peri-lesion cortex, at least at the time point examined. This suggests that the peri-lesion cortex is not undergoing traditional synaptic competition between projections from ipsilesional RFA and contralesional CFA during the non-paretic forelimb training period, as previously thought. Further examination of variations in structural synapses and post-synaptic cell types would provide more information to the pursuit of the molecular mechanism for the maladaptive effects of prior non-paretic limb training. Determining the mechanism of this effect could lead to better treatment plans for human stroke survivors that enhance neural plasticity in such a way that allows for optimal use of both upper-extremities.

## Appendix A: Postural Support

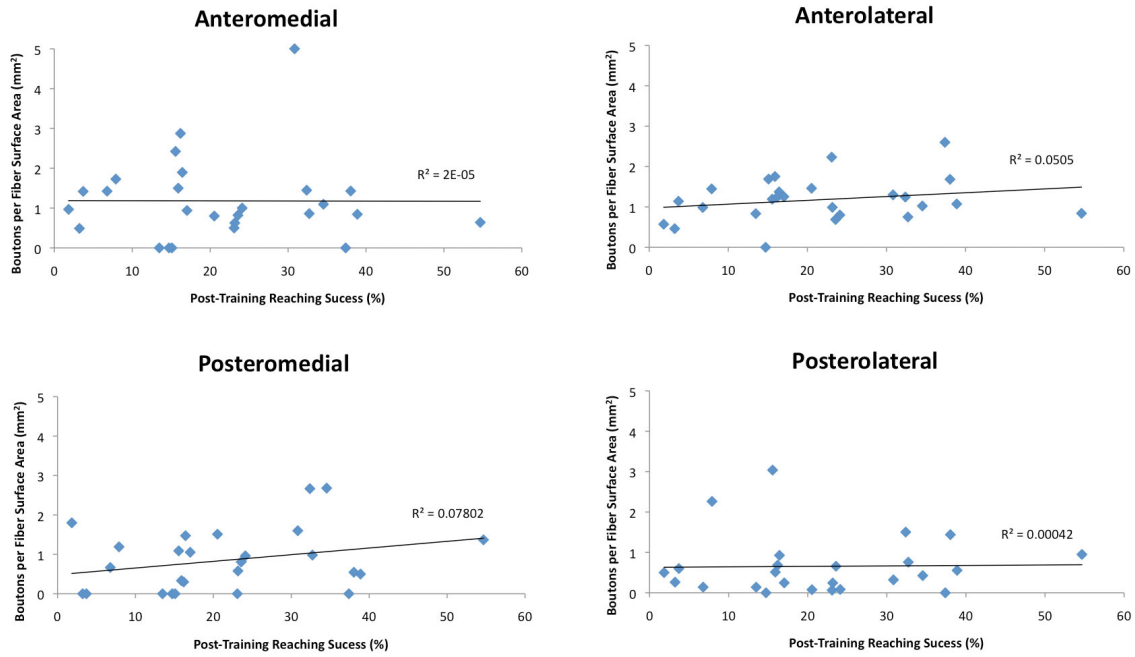


**Figure A.** Forelimb asymmetries in postural support. For all groups, CFA ischemic infarct resulted in increased use of the non-paretic forelimb for postural support in the Schallert cylinder test. All groups saw decreased asymmetries after the 15 days of training. Error bars =  $\pm$ SEM.

## Appendix B: Behavior X Anatomy Correlations



**Figure B1.** Post-training reaching performance (% successful reaches) is not correlated with axonal fiber densities in the peri-lesion cortex from the contralesional CFA.



**Figure B2.** Boutons per fiber surface area in the peri-lesion cortex are not correlated with post-training reaching performance (% successful reaches).

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