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Chronic in vivo imaging of dendritic plasticity and functional remapping after cortical stroke

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Chronic in vivo imaging of dendritic plasticity and functional remapping after cortical stroke

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Neuroscience

By

David Johnston

2012

ABSTRACT OF THE DISSERTATION

Chronic in vivo imaging of dendritic plasticity and functional remapping after cortical stroke

By

David Johnston

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2012

Professor Carlos Portera-Cailliau, Chair

One of the most amazing aspects of stroke is how survivors often exhibit partial recovery from their deficits. This occurs presumably because of remapping of lost capabilities to functionally related brain areas in both hemispheres. Several functional imaging studies in humans and rats suggest that remapping in the contralateral (uninjured) cortex might represent a transient stage of compensatory plasticity, until more permanent forms of circuit rewiring in peri-infarct cortex can take over. Some postmortem studies in fixed tissue have suggested that cortical lesions (including stroke) trigger extensive dendritic plasticity in the contralateral hemisphere, but this remains controversial (as others could not replicate the results) and no mechanism has yet been established as to how this remapping might occur. I used longitudinal in vivo two-photon microscopy in the contralateral homotopic cortex to record changes in

dendritic spines in apical tufts of layer 5 (L5) pyramidal neurons in adult thy1 GFP-M mice before and after stroke. In addition, I used intrinsic optical signal (IOS) imaging to investigate whether sensory functions that were lost after stroke remapped to the contralateral homotopic cortex. Strokes were induced by either rose Bengal photothrombosis or unilateral middle cerebral artery occlusion, both of which produced cortical infarcts that spanned the forelimb region of primary somatosensory cortex. For spine dynamics, mice were imaged longitudinally every 4 days for a baseline period before the stroke, and for up to one month thereafter. For IOS imaging, I mapped the sensory responses to ipsi- and contralateral forelimb and hindlimb stimulation at baseline conditions and then again every few days at regular intervals for up to 28 days after stroke in both adult and juvenile mice. The peri-infarct region showed significant remapping after stimulation of a whisker bundle after stroke, and stimulation of the contralateral forepaw always produced a strong intrinsic signal, but stimulation of the ipsilateral forepaw never resulted in a detectable signal in the spared homotopic cortex opposite to the side of the stroke. Similarly, I could not detect de novo growth or branching of dendrites or any changes in the density or turnover of spines after stroke. Thus, the contralesional cortex does not show evidence of structural plasticity after stroke, at least at the level of apical dendritic tufts of L5 neurons, or of functional remapping as detectable with IOS. This suggests that, at least in mice, the peri-infarct cortex plays the dominant role in post-ischemic reparative plasticity.

The Dissertation of David Johnston is approved.

Felix Schweizer

Dean Buonomano

Tom Carmichael

Carlos Portera-Cailliau, Committee Chair

University of California, Los Angeles

2012

Dedicated to my parents, my sisters, and my friends without whom this would not have been possible.

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Acknowledgements:

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I must also recognize the many professors and colleagues for sharing their time and wisdom with me to complement and challenge my other training. Among the many people that I am deeply indebted to I would first like to thank my committee members Drs. Felix Schweizer, Dean Buonomano, and Tom Carmichael who helped guide me through my thesis project and instruct me in how to properly develop a scientific story.

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obstacle, technical advice for my experiments, or just a friendly outing to relax; without him, I would not be where I am today.

Finally, I would like to acknowledge the National Heart, Lung, and Blood Institute (NHLBI) for providing the images used in figure 1.

Vita (Publications):

1. Boehmerle W, Splittgerber U, Lazarus MB, McKenzie KM, Johnston DG, Austin DJ, Ehrlich BE. Paclitaxel induces calcium oscillations via an inositol 1,4,5-trisphosphate receptor and neuronal calcium sensor 1-dependent mechanism. Proc Natl Acad Sci U S A. 2006 Nov 28;103(48):18356-61.
2. Mostany R, Chowdhury TG, Johnston DG, Portonovo SA, Carmichael ST, Portera-Cailliau C. Local Hemodynamics Dictate Long-Term Dendritic Plasticity in Peri-Infarct Cortex. J Neurosci. 2010 Oct 20;30(42):14116-26..
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Chapter 1:

Background

Introduction

The high incidences of stroke make it one of the leading causes of death and the number one cause of adult onset disability in the United States (Lloyd-Jones D et al.). As a result, the financial costs of stroke are estimated at around $73 billion annually in the United States (Lloyd-Jones D *et al.*). The speech, sensory, and motor deficits after stroke are a direct result of neuronal death in areas of brain infarction. Amazingly, many patients recover some of these deficits over days to months (Skilbeck CE et al., 1983; Twitchell TE, 1951), and the potential for recovery appears to be far greater in children, perhaps because their developing brain has a greater potential for neuronal plasticity (Johnston MV, 2009; Kim CT et al., 2009).

Unfortunately, the physiological mechanisms that underlie the limited functional recovery or how to improve the endogenous forms of adaptive plasticity are currently not well understood. Functional recovery after stroke is presumably mediated by compensatory remapping of lost capabilities to functionally related brain regions in both hemispheres that were performing similar functions but were spared by the infarct (Cao Y. DOL, Vikingstad E.M., Levine S.R., Welch K.M.A., 1998; Chollet F et al., 1991; Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein S.P., 2001; Glees P et al., 1950; Schaechter J.D. PKL, 2008). The peri-infarct cortex, which suffers slight decreases in blood flow but little if any neuronal damage (Witte O.W. BHJ, Schiene K., Redecker C., Hagemann G., 2000), exhibits both functional and structural forms of plasticity (Mostany R et al., 2010; Murphy CR et al., 2009; Zhang Z et al., 2009). It is conceivable, however, that such remodeling in

peri-infarct cortex could be triggered by mild ischemia, inflammation, or pathological changes in the nearby infarct, and thus might represent a maladaptive form of plasticity that could eventually lead to epilepsy or other circuit dysfunction. For example, synaptic plasticity seen in this region (Brown C.E. LP, Boyd J.D., Delaney K., Murphy T.H., 2007; Ito U et al., 2006; Mostany R *et al.*, 2010) could simply be a manifestation of deafferentation. Moreover, in mice, changes in dendritic spines and functional remapping in peri-infarct cortex are somewhat delayed compared to the fast behavioral recovery (Mostany R *et al.*, 2010; Sigler A et al., 2009; Winship I.R. MTH, 2008) so it is difficult to attribute functional recovery solely to plasticity in this region.

Instead, the contralateral hemisphere might be better equipped to support structural and functional plasticity since it is unaffected by ischemia or inflammation after stroke. Functional brain imaging studies in humans have suggested the existence of two sequential phases of remapping (Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein S.P., 2001; Marshall RS et al., 2000). Lost functions would initially shift to the spared hemisphere and later, as inflammation subsides and blood flow improves, those functions are taken over by the peri-infarct cortex. However, long-term functional recovery is inversely correlated with how long the impaired function is remapped to the contralateral cortex (Rossini P.M. CC, Pauri F., Baron J.C., 2003), suggesting that the contralesional cortex may not be able to permanently maintain a compensatory role. In addition, a few postmortem studies in fixed tissue have also documented that cortical lesions (including stroke) trigger extensive dendritic growth in the contralateral hemisphere (Jones T.A. S, T., 1992; Jones TA and T Schallert, 1994), while others could not replicate those results

using analogous techniques (Biernaskie J et al., 2004; Forgie M.L. GR, Kolb B., 1996; Gibb RL et al.; Prusky G. WIQ, 1996). These discrepancies, which could arise from sampling biases from using the Golgi method, must be resolved in order for us to understand the role of dendritic plasticity after stroke.

While the mechanisms of the neuronal plasticity in the peri-infarct region have been studied in some detail, the mechanisms and exact role of the contralesional cortex in adaptive plasticity remain unknown. This led us to several unanswered questions regarding plasticity after stroke: What is the role of the contralateral cortex? What is the timing of functional remapping and dendritic plasticity? Is stroke plasticity developmentally regulated? I examined these issues longitudinally in vivo with chronic intrinsic optical signal (IOS) imaging and high-resolution two-photon imaging of dendritic spines of layer (L) 5 pyramidal neurons over 5 weeks before and after stroke. I found no evidence of dendritic plasticity or functional remapping in the contralesional cortex after stroke induced by Rose Bengal photo-thrombosis (RBPT) or permanent unilateral middle cerebral artery occlusion (MCAO). These findings shed new light on the mechanisms of post-ischemic adaptive plasticity and suggest a reevaluation of our current understanding of the contralesional hemisphere's role in recovery after stroke.

Background

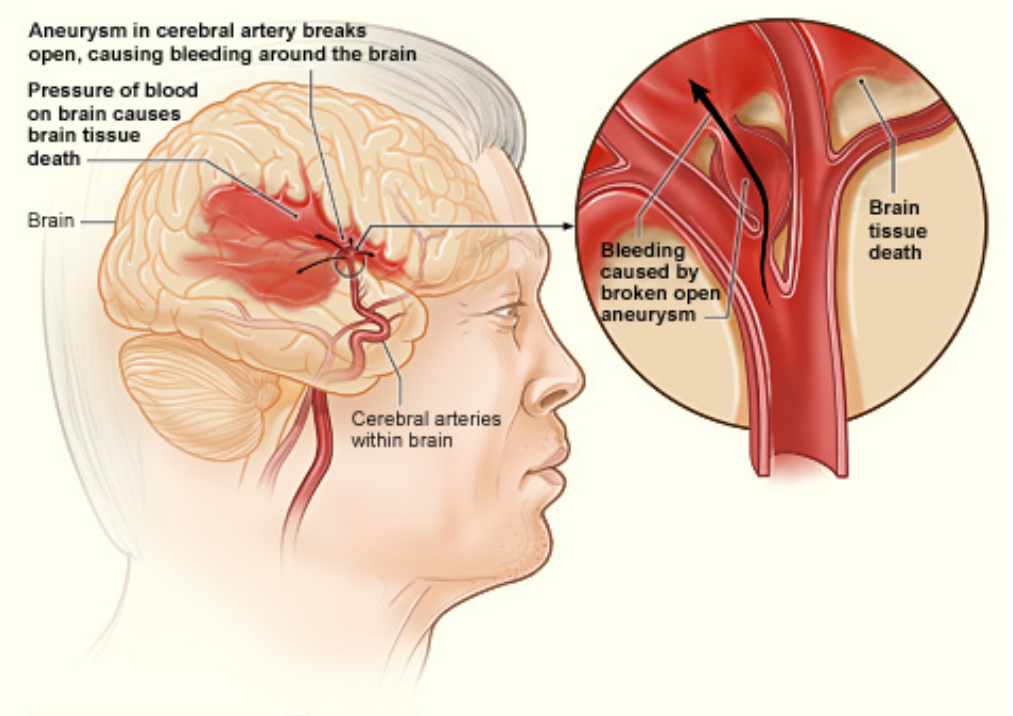
1.1 Ischemic and hemorrhagic strokes

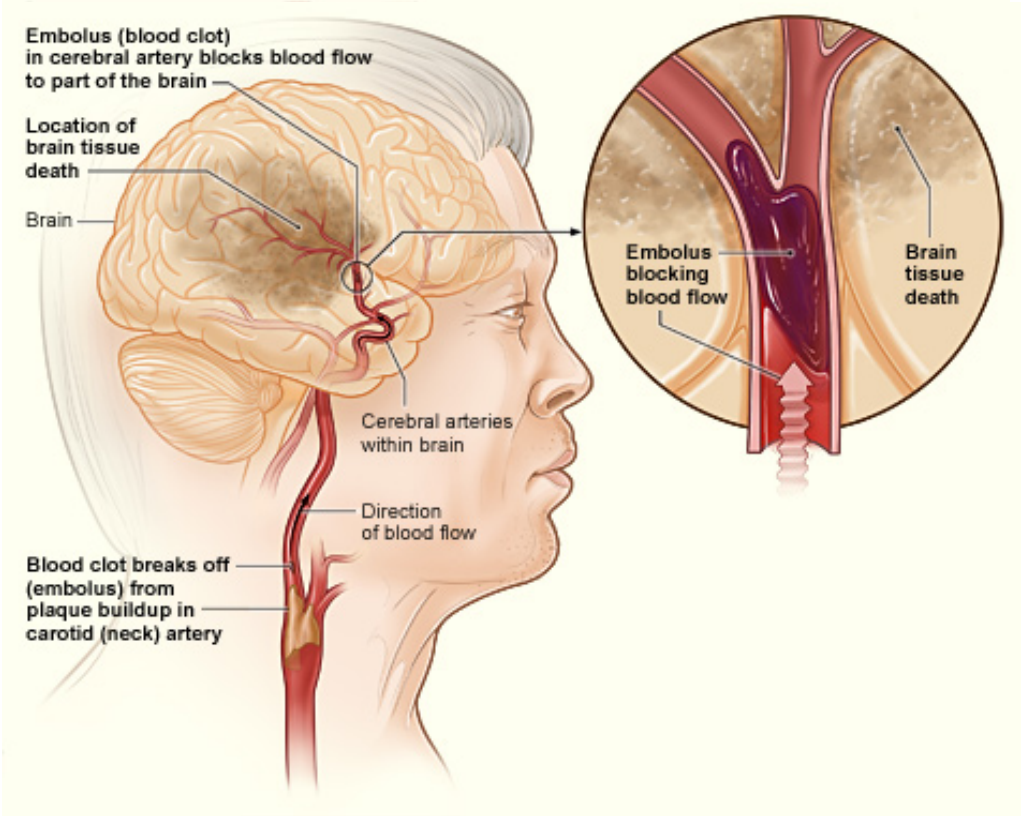
There are two main types of stroke: hemorrhagic and ischemic. They are caused by ruptured blood vessels and blocked blood vessels respectively (**Fig 1**). Approximately 87% of all strokes are ischemic and the majority of those (80 cases per 100,000 people) involve the middle cerebral artery (Lloyd-Jones D *et al.*). Ischemic stroke is caused by blockage of a blood vessel, usually from atherosclerotic or blood clots, preventing flow of oxygenated blood to an area of the brain.

Hemorrhagic strokes, while more rare, are more likely to lead to death or epilepsy (Burn J et al., 1997). Uncontrolled bleeding in the brain can cause increases in intracranial pressure (ICP) that can damage or distort the surrounding tissue or lead to ischemia. In addition, many of the components in blood, such as iron, are toxic and they can directly lead to cell death (Wu H et al.). Hemorrhaging in the brain is usually due to either hypertension that causes rupture of blood vessel walls or from bursting of aneurysms (Lloyd-Jones D *et al.*).

While hemorrhagic stroke remains a serious clinical problem, it may require unique mechanisms of recovery from ischemic stroke due to the added complications of ICP changes and toxicity (Wu H *et al.*). Because of this and the significantly higher incidence of ischemic stroke (Lloyd-Jones D *et al.*), I chose to focus my investigations into the role the contralesional hemisphere after ischemic stroke only. Nevertheless, investigating plasticity after ischemic stroke is likely to be relevant to

Figure 1: Cartoon diagrams depicting hemorrhagic (Reifenberger JG et al.) and ischemic (bottom) stroke. Image taken from the NHLBI.


Aneurysm in cerebral artery breaks open, causing bleeding around the brain
Pressure of blood on brain causes brain tissue death
Brain
Cerebral arteries within brain
Bleeding caused by broken open aneurysm
Brain tissue death


Embolus (blood clot) in cerebral artery blocks blood flow to part of the brain
Location of brain tissue death
Brain
Cerebral arteries within brain
Direction of blood flow
Blood clot breaks off (embolus) from plaque buildup in carotid (neck) artery
Embolus blocking blood flow
Brain tissue death

other forms of brain injury, including intracerebral hemorrhage and traumatic brain injury.

1.2. Clinical relevance and current treatments

Stroke is currently the fourth leading cause of death and the largest cause of adult onset disability in the United States (Murphy SL et al., 2012). The financial costs associated with stroke including the direct hospital costs and the indirect costs associated with loss of income and rehabilitation is estimated at 73.7 billion dollars annually (Lloyd-Jones D *et al.*). Furthermore, the majority of stroke victims survives the initial injury and suffers from a lifetime of debilitating symptoms such as aphasia, motor deficits, blindness, speech impediments, depression, and cognitive decline (Dobkin BH, 2005). The resulting drop in the quality of life for the patient creates an enormous financial and emotional burden on the family and caregivers of stroke survivors (Lloyd-Jones D *et al.*).

Currently, there are limited approved recovery strategies to mitigate ischemic damage or improve recovery after the occurrence of a stroke (Lloyd-Jones D *et al.*). There are two main strategies when attempting to improve recovery after stroke. The first is to minimize the initial ischemic damage by increasing reperfusion to the ischemic area (Murphy TH and D Corbett, 2009). This can be accomplished in a number of different ways including cerebral angioplasty or thrombolysis. The second recovery strategy is rehabilitation of lost function by relearning.

Acute stroke treatments primarily attempt to improve reperfusion of blood to the ischemic area in order to minimize damage as cell death occurs rapidly after stroke (Small DL and AM Buchan, 2000). Cerebral angioplasty is a surgical procedure whereby a small balloon is inserted into the blood vessel with reduced blood flow. The balloon is then enlarged allowing greater blood flow to minimize the ischemic area. This procedure can be effective, but is not always recommended due to its invasive nature and the associated risks of blood vessel rupture and hemorrhage, or reperfusion injury (Higashida RT and PM Meyers, 2006).

Another surgical option is Merci clot retrieval to physically remove the clot from the affected vessel. This procedure involves threading a special nickel titanium wire into the affected vessel via a catheter system past the blockage. The wire forms a helical shape after the catheter is pulled back. The wire is then retracted pulling the blockage along the vessel until it can be removed at a more proximal location (Becker KJ and TG Brott, 2005). This procedure, while somewhat effective for a late start stroke treatment, is still only effective in the first hours after stroke onset.

One alternative that has shown significant benefit in improving clinical outcome is the administration of (intra-veinous or intra-arterial) tissue plasminogen activator (tPA) to break up the clot from the obstructed vessel (1995). tPA is a serine protease found endogenously in endothelial cells that when delivered intravenously within three hours after stroke onset can significantly improve reperfusion to the ischemic area (1995). Despite tPA's effectiveness in clinical studies and the fact that it is the only FDA approved acute treatment of ischemic stroke it is only used in a tiny percentage of stroke patients. In a multi-center study of the 29 hospitals in the Cleveland area it was

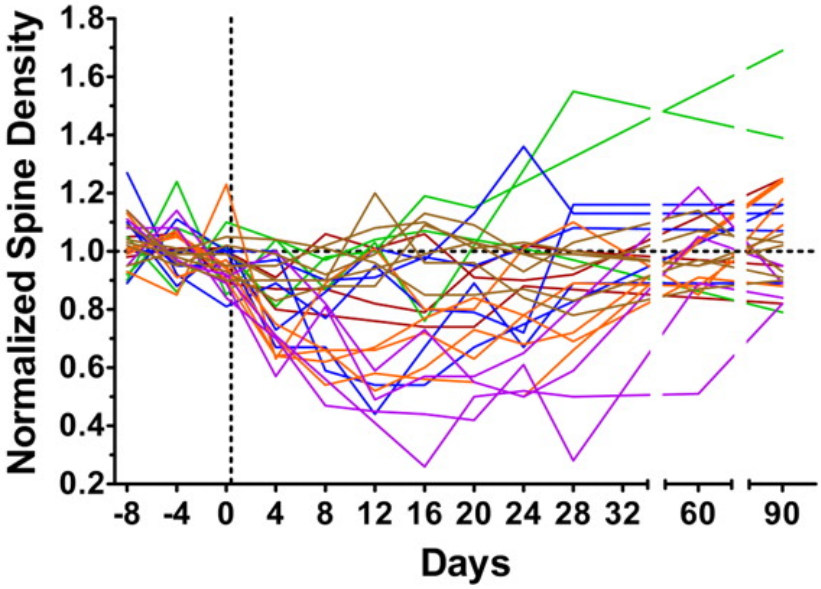
found that only 1.8% of stroke patients received tPA (Katzan IL et al., 2000). Without urgent administration shortly after symptom onset, thrombolytic treatments will fail to improve recovery because there is a short period of time before significant permanent neuronal damage occurs after stroke (Haddad GG and C Jiang, 1993). In fact, late delivery of thrombolytics can increase the chance for hemorrhagic transformation of the infarct (1995). Therefore, until changes in stroke care can be implemented to increase the percentage of patients that can receive tPA treatment or other intravascular interventions, it is necessary to investigate methods to improve recovery after the damage has occurred by improving adaptive plasticity after the acute period.

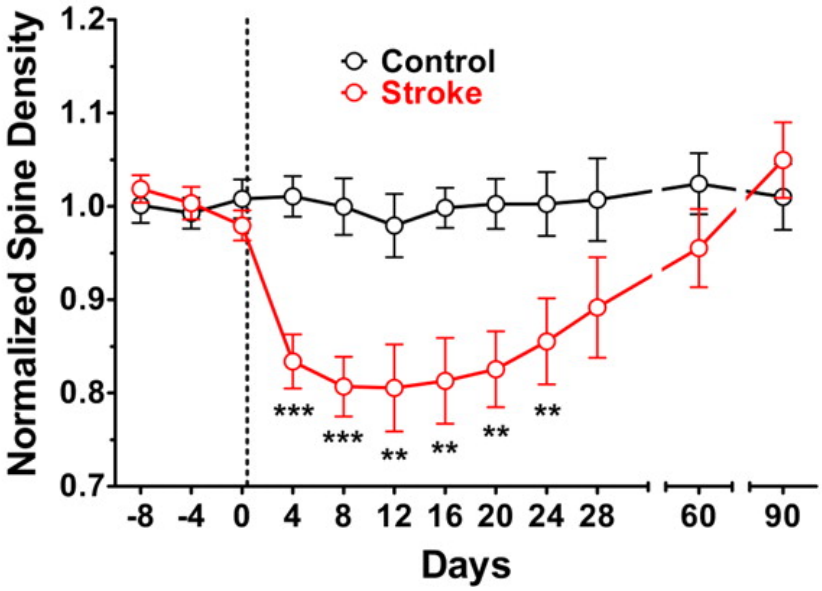
After the first few hours since stroke onset the treatment options for recovery become more limited (Dobkin BH, 2005). Multiple clinical trials were conducted for NMDA receptor antagonists to improve recovery after stroke by minimizing the excitotoxic effects of ischemia, but none of the trials were successful in improving recovery (Albers GW et al., 1999; Albers GW et al., 2001; Davis SM et al., 2000; Lees KR et al., 2000). Despite the well-known toxic effects of excessive glutamate, NMDA receptor antagonists failed to improve recovery after stroke or traumatic brain injury. One reason that these trials may have failed is that the window of NMDA receptor dysfunction is extremely small and that after this early destructive period NMDA receptors return to their normal physiological function (Ikonomidou C and L Turski, 2002).

Currently there are no pharmacologic therapies to enhance adaptive plasticity and improve functional recovery after stroke (Lloyd-Jones D *et al.*). Most treatment strategies involve occupational therapy and physical rehabilitation of lost function

through repetitive motor learning that ignores the cognitive deficits that stroke survivors often experience (Dobkin BH, 2005). Since current rehabilitative strategies are not driven by any knowledge of neuronal plasticity, we designed experiments to better characterize the endogenous plasticity mechanisms stroke.

Figure 2: (Reifenberger JG *et al.*) Normalized spine density over time for 26 L5 pyramidal neurons from 6 mice. (Bottom) Normalized spine density averaged across 26 neurons from 6 stroke mice (red) and 22 neurons from 5 control mice (Hilton E et al.). Data are shown as means ± s.e.m. (** $p < 0.01$; *** $p < 0.001$). The vertical dashed line in b and c indicates the time of MCAO.


Normalized Spine Density
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-8 -4 0 4 8 12 16 20 24 28 32 60 90
Days


Normalized Spine Density
1.2
1.1
1.0
0.9
0.8
0.7
Control
Stroke
***
***
**
**
**
**
-8 -4 0 4 8 12 16 20 24 28 60 90
Days

These experiments allowed us to better understand brain recovery and potentially highlight ways to enhance or accelerate the brain's endogenous plasticity after stroke.

1.3. The role of the peri-infarct region in post-ischemic plasticity.

Two areas of the brain are highlighted as the primary regions associated with adaptive plasticity after stroke to improve lost function: the peri-infarct, and homotopic contralesional hemisphere (Murphy TH and D Corbett, 2009). Due to the close proximity to the infarct, the peri-infarct cortex is already performing functionally similar roles as the affected area and would presumably require only limited structural remodeling to connect to the affected network. For these reasons the peri-infarct region is ideally prepared for post-ischemic plasticity (Murphy TH and D Corbett, 2009). Many previous studies have shown both structural (Brown C.E. LP, Boyd J.D., Delaney K., Murphy T.H., 2007; Brown C.E. WC, Murphy T.H., 2008; Brown CE et al.; Ito U *et al.*, 2006; Mostany R *et al.*, 2010) and functional remapping (Mostany R *et al.*, 2010; Winship I.R. MTH, 2008) in peri-infarct regions. Electron microscopic studies have shown a maximal decrease in synapse and spine number by four days after stroke that then recovered over 1 to 12 weeks later (Ito U *et al.*, 2006). In addition, in vivo longitudinal imaging using two-photon microscopy has shown similar results (Brown C.E. WC, Murphy T.H., 2008; Mostany R *et al.*, 2010). Studies from our lab have described an initial decrease in spine density in the peri-infarct region in the first three weeks after injury that recovers fours weeks after injury (**Fig. 2**). This suggests that connections that are pruned after stroke, presumably reflecting a removal of

inactive synapses due to loss of inputs from the core of the infarct, later reappear through cortical plasticity.

Despite the advantages the peri-infarct region has for adopting lost function after stroke due to its geographic proximity, there are also a number of challenges unique to this area. Because the peri-infarct region is by definition very close (within millimeters) of the infarct, it is also affected by mild ischemia as well as inflammation. Significant microglial activation can cause synaptic stripping (Nimmerjahn A. KF, Helmchen F.) that could inhibit new adaptive connections from forming. In addition, release of cytokines and other inflammation factors into the extracellular space can negatively impact normal cellular function. Electrophysiological studies in acute slices of striatal tissue after stroke have shown an ischemia-induced form of long-term potentiation (iLTP) that sets the synaptic weights to their maximum levels and prevents further learning (Calabresi P et al., 1999). Because the peri-infarct area is negatively effected by a number of factors in the first days to weeks after a stroke occurs, it is possible that other areas of the brain are better suited to compensate, at least temporarily, until the peri-infarct can recover from inflammation.

1.4. Ipsilateral cortical inputs.

One of the assumptions built into the theory that the spared hemisphere becomes involved in reparative plasticity after stroke is that there are direct anatomical connections from the affected limbs to the spared cortex. That is that there are ipsilateral cortical inputs that can become recruited or strengthened following stroke to

affect cortical plasticity. These ipsilateral connections, however, have not been well studied, at least as it is relevant to recovery after injury.

Anatomical studies in rats have characterized both the descending corticospinal tract (CST) and ascending pyramidal tract. Injections in the sensorimotor cortex of rats with tritiated proline revealed that the decending corticospinal tract has a small ipsilateral or uncrossed component that could be traced to the cervical level of the ventral spinal cord (Vahlsing HL and ER Feringa, 1980). Additional studies have characterized the ascending pyrmadal tract and shown that a small (~5%) portion remains uncrossed at the medulla oblongata and reaches ipsilateral sensorimotor cortex (Brosamle C and ME Schwab, 1997; Whishaw IQ and GA Metz, 2002). While most of these studies are purely anatomical characterizations, Whishaw and Metz investigated the functional role of the uncrossed component of the ascending pyramidal tract by performing a number of behavioral tests after a unilateral lesion of the CST at the medullary level rostral to the decussation. Using a variety of behavioral and motor tasks, Whishaw and Metz, were able to show clear impairment in contralateral limb use, while the ipsilateral limb functioned normally (Whishaw IQ and GA Metz, 2002). From this data it was concluded that although the uncrossed pyramidal tract had similar connection and branching patterns as the crossed pyramidal tract it is unlikely that the uncrossed path plays any role in skilled movement of the ipsilateral limb. Instead, it is suggested that the ipsilateral path plays a more modulatory role rather than direct motor control although they provided no direct evidence to support this hypothesis.

While it is clear from anatomical studies that an ipsilateral uncrossed tract does exist connecting the sensorimotor cortex to the periphery, it has not been elucidated as to what the function of this uncrossed path may be.

Multiple human studies have shown activation of the ipsilateral cortex during finger stimulation (Hlushchuk Y and R Hari, 2006; Sutherland MT, 2006) and median nerve stimulation (Kanno A et al., 2003; Korvenoja A et al., 1995). These studies find that in the majority of subjects there is evoked-activity in primary somatosensory cortex (Brodmann's areas 2 & 3b predominately) during ipsilateral stimulation. Most of the authors mentioned here, conclude however, that the ipsilateral activation (and deactivation in some cases (Hlushchuk, 2006 #1717)) is most likely due to transcallosal input from the contralateral hemisphere rather than a direct ascending pathway. This is partly attributed to an increased latency of the activation in the ipsilateral hemisphere as compared to the latency of activation in the contralateral hemisphere. Human studies are limited by methodology, and it is difficult to precisely pinpoint activation location and timing both with either fMRI or magnetoencephalography.

Animal studies have also investigated the origin of evoked ipsilateral activation during peripheral stimulation. Single and multi-unit recordings in macaque monkeys have shown neurons with bilateral and ipsilateral receptive fields during hand stimulation (Jones EG and SH Hendry, 1980; Taoka M et al., 2000). While callosal input from the contralateral cortex was still presumed to be behind the ipsilateral activation, the hand areas that were stimulated during these experiments was not found to be connected callosally.

Another strong piece of evidence suggesting that direct ascending ipsilateral inputs are important for sensory perception is from an IOS study in cats. IOS imaging showed ipsilateral activation in secondary somatosensory (SII) areas (Tommerdahl M et al., 2005). This study suggests that the primary importance of ipsilateral cortical activity is most likely modulatory rather than direct sensory perception.

At this point, the exact role of direct ascending cortical inputs from the periphery remains unclear. Evidence suggests that there is ipsilateral activation during sensory perception, however a large portion of this change in activity is likely due to interhemispheric connections through the corpus callosum. However, this does not seem to be the only source of ipsilateral input because activity persists during stimulation of areas not callosally connected (Jones EG and SH Hendry, 1980). These data suggest that the ipsilateral cortex may be involved in modulatory or higher order processing of peripheral stimuli projecting to the contralateral cortex. This is relevant to the stroke field because the lack of input to the ipsilateral hemisphere after ischemic injury could lead to unmasking of existing cortical networks. This seems a likely cause behind the multiple studies showing activation of the spared hemisphere after stroke.

1.5. Role of the contralesional cortex in post-ischemic plasticity and summary of relevant literature

The homotopic contralesional cortex has been shown to be an alternative brain region to compensate for lost function after stroke due to the fact that it is already

performing the same functions of the affected area but for the opposite side of the body. In addition inflammation, spreading depression, synaptic stripping, and iLTP are not negatively affecting it. Because the contralesional hemisphere is largely immune to most of the negative physiological changes in the peri-infarct zone, it may be better able to form new connections for increased adaptive plasticity.

The contralesional hemisphere is thought to be recruited after large cortical infarcts that damage multiple associated areas such as forelimb, hind limb and other somatosensory areas as well as damage to subcortical regions (Murphy TH and D Corbett, 2009). This severe cortical damage is thought to prompt the contralesional homotopic area through a currently unknown signal to begin compensating for any lost function. This compensatory role is thought to be short-lived, only lasting the first few weeks after the stroke until the peri-infarct region can adopt a more permanent fix (Murphy TH and D Corbett, 2009). In fact, studies have shown that prolonged contralesional activity after stroke is inversely correlated with neurological outcome (Rossini P.M. CC, Pauri F., Baron J.C., 2003).

Early studies investigating structural changes after electrolytic lesions in rats showed pronounced increases in dendritic arborization, specifically at higher order branches where there is a greater density of spines and synapses (Jones T.A. S, T.). Dendrites were counted and quantified using Golgi staining to show the increase in dendritic plasticity after injury. This showed that the contralateral hemisphere underwent significant structural changes after injury and suggested that it was involved in plasticity.

More recently, other labs have investigated the structural changes after stroke in more detail by using in vivo two-photon microscopy to count changes in spine density over 6 hour periods at different time-lengths after stroke in mice (Takatsuru Y et al., 2009). This study took the work of Jones & Schallert one step further by showing in vivo that spine turnover increased one week after stroke and then returned to normal levels afterwards.

While these studies show significant structural changes after stroke they are somewhat limited by their experimental design. For example, the in vivo imaging study only looked at spine dynamics in different groups of animals for each time point and therefore was not a longitudinal study that compared changes in structural plasticity both before and after stroke in the same mice. Thus, neither study was able to describe changes in plasticity as they developed. For this reason, I designed a longitudinal in vivo study to examine structural plasticity after stroke over a 40-day period both before and after injury. This way I was able to describe changes in plasticity with temporal precision to know when they did or did not occur.

In addition to changes in structure after stroke, many studies have shown significant changes in functional activity in the contralesional cortex using functional magnetic resonance imaging (fMRI), voltage-sensitive dyes (VSD), and positron-emission tomography (PET). Multiple studies using fMRI have shown activation of the ipsilateral (contralesional) cortex during stimulation or movement of the affected forelimb after stroke in both human (Cao Y. DOL, Vikingstad E.M., Levine S.R., Welch K.M.A.) and animal (Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein S.P., 2001; Dijkhuizen R.M. SAB, Mandeville J.B., Wu O.,

Halpern E.F.. Finklestein S.P., Rosen B.R., Lo E.H., 2003) studies. Human studies using PET imaging have confirmed these results showing, with some variability between patients, that movement of the affected fingers caused a shift in activity to the spared hemisphere (Calautti C. LF, Guincestre J.Y., Marié R.M., Baron J.C.). Furthermore, more recent studies in mice using VSD imaging in vivo have shown a prolonged and exaggerated contralesional activity after stroke (Brown RF et al.). Taken together these experiments showed that after stroke the homotopic contralesional hemisphere expanded its receptive field to include that of the affected limb, which under normal circumstances would not elicit any activity on the ipsilateral side of the brain.

The evidence for functional plasticity after stroke, while compelling, is restricted to human and rat models. The evidence is all that more convincing because it persists across species using multiple imaging methods, but it is unclear, however, whether the same mechanisms of plasticity exist in the mouse. Mice are increasingly being used in clinical and basic scientific testing due to their increased facility in transgenic experimentation. In addition, the small number of data points collected limits the conclusions from previous functional imaging studies. It would be better to examine functional plasticity after stroke over multiple time points both before and after stroke to observe any changes that developed on a day-by-day basis. For this reason I chose to examine changes in evoked sensory maps every other day for the first week after stroke and then every week thereafter for a month to allow for a more detailed characterization of changes in functional maps.

The goals of the following experiments described herein were to answer several questions regarding recovery after stroke. First, what exactly is the role of the contralateral cortex after stroke? What is the timing of functional remapping and dendritic plasticity? Is stroke plasticity developmentally regulated? Previous studies have touched on some of these issues, but no one study to our knowledge has attempted to characterize in vivo both structural and functional plasticity in a mouse model of stroke longitudinally both before and after injury.

1.5 Experimental models of ischemic stroke.

Multiple experimental models of ischemia exist for studying the effects of stroke on the brain. There are two distinctions made in stroke models and each one offers unique advantages and disadvantages. First, stroke models can cause either permanent loss of cerebral blood flow (CBF) to the brain or can be temporary and allow reperfusion of CBF after a set period of time. Second, experimental strokes can either be global, restricting CBF to the entire brain, or focal, causing ischemia in a very distinct, specific area of the brain.

Global ischemia is used to model the brain's response to loss of blood flow to the entire brain simultaneously, as occurs after cardiac arrest. Permanent global ischemia would eventually lead to death, but temporary global ischemia differentially impairs certain neuronal populations that are especially vulnerable to ischemia such as the hippocampus (Schmidt-Kastner R and TF Freund, 1991), striatal aspiny interneurons

(Centonze D et al., 2001), and cortical pyramidal neurons (Haddad GG and C Jiang, 1993).

There are a number of experimental global ischemia models used in animals including neck cuff, cardiac arrest followed by cardiopulmonary resuscitation (CPR), 2-vessel occlusion (VO), and 4-VO (Traystman RJ, 2003). These models have been used in larger animals, like dogs, and smaller animals, like rats and mice, but it should be noted that mouse models of global ischemia have been difficult to use due to complication such as high mortality rates and seizures (Traystman RJ, 2003). While these models are useful for measuring cerebral energy metabolism and other cellular responses to a hypoxic environment, they are not useful for examining cortical reorganization as there is no focal injury (Traystman RJ, 2003). Since all cortical pyramidal neurons are similarly affected by global ischemia, presumably the mechanisms for recovery are different from when the injury is restricted to targeted area. I chose to focus on focal ischemia models rather than global ischemia models because I was more interested in understanding cortical rewiring rather than the mechanisms of energy metabolism and neuronal injury.

Focal ischemia models primarily target the middle cerebral artery (MCA) territory because this is the most common location for strokes in humans (Lloyd-Jones D *et al.*). MCA occlusion results in ischemia in the neocortex and striatum, but the degree of ischemia depend on the amount of collateral blood flow in the area from other sources. Focal stroke models, unlike global models, result in infarction of a specific location in the brain with a surrounding peri-infarct area experiencing minimal ischemia and increased plasticity. Because these models only damage a specific

area of the brain, they are useful for investigating how the brain reorganizes itself to compensate for the lost function in the infarct.

Temporary ischemia models allow a greater chance for reperfusion of the ischemic area because blood flow is not permanently blocked. Despite the fact that ischemia is temporary, these models still cause neuronal damage and death in a focal area, but allow for faster recovery of the surrounding tissue. The advantage of using temporary ischemia models is that the degree and duration of ischemia can be experimentally controlled and thus the direct effects of loss of CBF can be measured. One common temporary ischemia model in rodents is to use an intraluminal filament inserted proximally at the common carotid artery and advance it until it blocks the MCA. With this method, 80% of blood flow is reduced to the neocortex and caudoputamen area, but reperfusion easily occurs once the filament is removed (Memezawa H et al., 1992). In this way, the experimenter can control the magnitude of ischemia and therefore questions can be answered as to what plasticity occurs as a result of different levels of ischemia.

The advantage of temporary stroke models is the ability to more precisely control the level of ischemia than can normally be achieved with permanent ischemia models. However, contralateral involvement in recovery after stroke is thought only to occur after severe damage to the cortex and subcortical areas (Murphy TH and D Corbett, 2009) that is difficult to achieve with temporary ischemia models. Permanent stroke models are achieved by permanently blocking blood flow to the brain, usually by occluding the MCA. This can be accomplished through cauterization, embolization, or photothrombosis. Models that permanently occlude the MCA are one of the most

clinically relevant stroke models as almost all strokes in humans result from blockage in the MCA territory (Lloyd-Jones D *et al.*).

We utilized a permanent MCA occlusion (MCAO) model via cauterization of the MCA before its primary branching point and a Rose Bengal photothrobotic (RBPT) model. These two models create permanent loss of blood flow to a targeted area. The MCA provides blood to primary somatosensory and parts of the motor cortex. Depending on the amount of blood flow from nearby collateral arteries, the MCAO model causes infarction centered near the barrel field in somatosensory cortex and often affects the forelimb area as well. With this model, mice experience infarct sizes of 4.8 ± 1.4 mm^3 (**Fig. 7**) and experience sensorimotor deficits in the contralateral forelimb (**Fig. 6**). This model is a clinically relevant model that closely represents the majority of strokes in humans.

Photothrombotic stroke models, on the other hand, offer more precision in targeting strokes experimentally than occlusion of the MCA because photothrombosis is induced optically, either fiber optically or via a laser, to a very specific area after administering Rose Bengal (injected i.p.). Because occlusion is induced optically, it can be targeted to individual capillaries or arteries to investigate the effects of ischemia on a very precise targeted area. The MCA can be occluded photothrombotically and produce similar infarcts to cauterization, but it can also be targeted to smaller branches off the MCA to measure how local ischemia affects neighboring neurons. In addition to targeting single vessels, photothrombotis can be induced in entire areas of the brain. For example, after Rose Bengal is injected, the neocortex can be illuminated with green light fiber optically to target infarcts to specific

areas such the forelimb area of primary somatosensory cortex (S1FL). Because occlusion of the MCA can cause infarcts of varying size and location due to variability in collateral blood flow between animals, RBPT stroke models can better target infarcts to locations of interest because it cause occlusion of every illuminated vessel in a volume of tissue.

Chapter 2:

Materials and Methods

Methods

2.1 Materials:

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2 Animals:

We used adult (2-6 months of age) and juvenile (<3 weeks of age) male and female C57BL/6J mice. For in vivo imaging of dendritic structure, we used the GFP-M line of transgenic mice (GFP expression under the Thy-1 promoter; (Feng, 2000 #1727). The University of California Chancellor's Animal Research Committee approved all the procedures described in this study.

2.3 Cranial window surgery for in vivo two-photon imaging:

Chronic glass-covered cranial windows were implanted as previously described (Holtmaat A et al., 2009; Mostany R and C Portera-Cailliau, 2008). Mice were anesthetized with isoflurane (1.5% via nose cone) and placed on a stereotaxic frame over a warm water re-circulating blanket. Dexamethasone (0.2 mg/kg; Baxter Healthcare Corp., Deerfield, IL) and carprofen (5 mg/kg; Pfizer Inc., New York, NY) were administered subcutaneously to reduce brain edema and local tissue

inflammation. A 2.5 mm diameter craniotomy was performed under sterile conditions with a pneumatic dental drill, centered over the left hemisphere, 3 mm lateral to the midline and 1.95 mm caudal to Bregma (Figure 1*a*). A sterile 3 mm glass cover slip was gently laid over the dura mater (without using agarose) and glued to the skull with cyanoacrylate-based glue. Dental acrylic was then applied throughout the skull surface and the edges of the cover slip. A titanium bar (0.125 x 0.375 x 0.05 inch) was embedded in the dental acrylic to secure the mouse on to the stage of the microscope for imaging. Cranial windows were implanted at least three weeks prior to imaging dendritic spines because transient upregulation of astrocytic and microglial markers has ended by this time, and because we have shown that control mice have stable spine dynamics within a few days after window implantation {Holtmaat, 2009 #639}.

2.4 Distal MCAO stroke:

Unilateral distal MCAO was performed immediately after the third imaging time point (i.e., last time point for basal conditions; Fig. 1), as previously described (Tamura A et al., 1981) with some modifications (Mostany R *et al.*, 2010). Mice were anesthetized with isoflurane and placed on a stereotaxic frame over a water re-circulating blanket with the head tilted at a 45^{o} angle. The skin between the left eye and ear was incised and gently retracted, together with the temporalis muscle. The MCA was identified through the translucent squamosal bone (~0.8 mm rostral to the anterior junction of the zygomatic and squamosal bones) and a tiny (0.7 mm in diameter) craniotomy was performed using a dental drill, ~0.5 mm ventral to the

temporal ridge. Next, the MCA was cauterized with the tip of a small forgery iron and transected with a 21G syringe needle. The craniotomy was then covered with a small piece of Gelfoam (Pfizer Inc., New York, NY). The temporalis muscle and skin were then glued with Vetbond (3M Animal Care Products, St. Paul, MN) to the temporal ridge and the acrylic cement of the headset, respectively.

2.5 Rose Bengal Photo-Thrombosis stroke (RBPT):

Ischemic infarcts were targeted to the forelimb region of S1 (S1FL) using RBPT as previously described (Watson B.D. D, W.D, Busto R., Wachtel M.S., 1985; Winship I.R. MTH, 2008). Rather than using standard coordinates from a mouse brain Atlas, we used IOS (see below) to record the location of the FP sensory representation in order to target strokes to that area. Mice were anesthetized with isoflurane and placed on a stereotaxic frame. After a midline incision, the scalp over S1 was retracted and the periosteum gently scraped off with a scalpel blade and cleaned with sterile saline. A Rose Bengal solution (0.4 mL, 1% w/v, 133 mg/kg) was injected i.p., followed immediately by illumination. White light was delivered directly onto the skull surface for 18-19 min using a halogen light source (Schott North America, KL 1500 LED) coupled to a fiber-optic bundle (aperture 2.75 mm). We performed several calibration experiments to characterize and optimize the RBPT procedure and to produce consistent infarcts in every animal. After illumination, the scalp was sutured and the mouse transferred to a warm (37° F) recovery chamber before being returned to its home cage.

2.6 Behavioral test:

The limb-use asymmetry (cylinder) test (Schallert T FS, Leasure JL, Tillerson JL, Blnd ST, 2000) was used to examine the preference for using each forelimb during recovery from MCAO. Mice were tested 1 day before and 1, 5, 21 and 33 days after MCAO. Animals were placed in a transparent glass cylinder 10 cm in diameter and 16 cm high for 10 min per session. Activity of the animals was recorded from above with a digital camera. Forelimb use was measured during vertical exploration counting all forepaw contacts with the cylinder. The contralateral forelimb use was calculated [i.e. contralateral forelimb/(contralateral + ipsilateral)], where 0.5 represents perfect symmetry. To compensate for preference for one paw over the other during basal conditions, forelimb use scores for every post-stroke session were normalized to their respective basal value.

The adhesive removal test (Starkey ML et al., 2005; Tennant KA and TA Jones, 2009; Wells JE et al., 2005) was also used to characterize deficits in sensory and motor function in the forelimbs of mice after stroke. This test detects deficits in forelimb sensation by measuring the time it takes for the mouse to become aware of a piece of tape stuck to its paw. In addition, the adhesive-removal test detects motor impairment by recording the time it takes for the removal of the piece of tape after it has been discovered and comparing the time against control animals. To obtain a baseline, behavioral testing will be done three times prior to stroke induction, every day thereafter for the first week, every 4 days thereafter for another three weeks. In

control animals, sham RBPT surgeries will be performed whereby all details will be identical as stroke animals except that either no Rose Bengal will be injected or the skull will not be illuminated.

2.7 High-resolution two-photon imaging:

All imaging was done with a custom-built two-channel two-photon microscope, using a Ti:Sapphire laser (Chameleon Ultra II, Coherent Inc., Santa Clara, CA) tuned to 910 nm, a 40X 0.8 NA water immersion objective (Olympus, Tokyo, Japan), photomultiplier tubes (Hamamatsu, Japan) and ScanImage software (Pologruto TA et al., 2003) written in MATLAB (MathWorks, Natick, MA). Imaging of apical dendritic tufts of L5 pyramidal neurons (within 150 μm below the dura) expressing GFP was performed every four days (to match previous imaging protocols investigating spine plasticity after input deprivation and stroke; (Holtmaat, 2005 #243;Mostany, 2010 #342) for 40 days (from day -8 to day +32 after MCAO; **Fig. 1**). Low-magnification image stacks of sparsely labeled L5 neurons were collected (512 x 512 pixels, 0.72 μm/pixel, 5 μm *z* steps down to the soma) to confirm the identity and dendritic arborization pattern of the cells imaged. High magnification images (512 x 512 pixels, 0.152 μm/pixel, 1.5 μm apart) were obtained for the analysis of dendritic spines. Regions of interest (Nabekura T et al.) containing long dendritic segments (average length 53.6 μm) were selected at random for chronic imaging throughout the window. All dendritic protrusions were scored, including those projecting along the *z*-axis, as long as they protruded > 0.5 μm from the shaft. In total, 654 spines were counted over

696.8 μm of dendrite. The same dendritic ROIs were located every imaging session using the unique superficial vasculature pattern on the surface of the brain as a reference (Holtmaat A *et al.*, 2009). Turnover ratio was defined as (the fraction of spines gained + fraction of spines lost)/2*total spines for each time point.

2.8 Intrinsic optical signal (IOS) imaging:

IOS imaging was performed either through the skull covered with a thin layer of dental cement (**Fig. 4**) or through standard cranial windows (**Fig. 5**). For the former we performed a midline scalp incision to expose the skull over S1. The periosteum was gently scraped off and a thin transparent layer of dental acrylic was then applied to the exposed skull up to the wound margins. Mapping of the receptive field for ipsilateral and contralateral stimulation of the forepaw (FP) was done in the cortex contralateral to the infarct 1-3 days before (PRE) and at several time points after RBPT or unilateral permanent distal MCAO stroke (+1, +3, +5, +7, +14, +21, +28 d and +35 d for adult mice and +1, +3, +7, +14 for juvenile mice). For IOS imaging, mice were lightly anesthetized with 0.5-0.75% isoflurane and a single injection of chlorprothixene (6 mg/kg i.p.). In juvenile mice, we implanted cranial windows at P17-P20 and began baseline IOS imaging within 2 d, and at the same time points before and after MCAO (as above). As a control, to document that we can see remapping in peri-infarct cortex with IOS through the skull, we also performed IOS imaging after contralateral stimulation of a bundle of 4 whiskers (C2-C3 and D2-D3; WS), and the forepaw (FP) and hindpaw (HP) before and +5, +13, and +24-28d after unilateral permanent distal

MCAO (**Fig. 8**). The cortical surface was illuminated by green (535 nm) and red (630 nm) sets of light-emitting diodes (LEDs) mounted around a 'front-to-front' tandem arrangement of objective lenses (135 mm and 50 mm focal lengths). The green LEDs were used to visualize the superficial vasculature and the red LEDs were used for IOS imaging. The microscope was focused to 200-350 μm below the cortical surface, depending on the age of the mouse (deeper for adult mice). Imaging was performed at 10 frames per second using a fast camera (Pantera 1M60, Dalsa), frame grabber (64 Xcelera-CL PX4, Dalsa) and custom routines written in MATLAB. Each session consisted of 30 trials, taken 20 s apart, of mechanical stimulation for 1.5 s (at 10 Hz for WS and 100 Hz for FP and HP) using a glass microelectrode (blunt tip for HP and FP) coupled to a piezo bender actuator (Physik Instrumente). Frames 0.9 s before onset of stimulation (baseline) and 1.5 s after stimulation (response) were collected. Frames were binned 3 times temporally and 2 x 2 spatially. Stimulated cortical areas were identified by dividing the response signal by the averaged baseline signal (*DR/R*) for every trial and then summing all trials. Response maps were then thresholded at 62.5% of maximum response to get the responsive cortical areas for D2W, FP and HP.

2. 9 Statistics:

To avoid having to make assumptions required by traditional parametric statistical methods (e.g., normally distributed data), we used resampling methods, including bootstrap versions of Student's t test, one-way ANOVA and two-way ANOVA

with repeated measures, and linear regression. To perform a one-way ANOVA with repeated measures, we used the ratio of between-group over within-group variability as the test statistic, not as sums of squares as in a traditional F-test, but as sums of absolute values of the distances from the grand/group means. The ANOVA ratio was calculated as ($F=\Sigma_{j=1\ldots m}|(\text{mean } X_j - \text{grand mean})|/\Sigma_{i=1\ldots n;j=1\ldots m}|Xij - \text{mean } Xj|$) where m = number of imaging time points, n = number of cells, i = the cell number, and j = the column number. For two-way ANOVA with repeated measures, we performed three analyses for the factors (time, experimental treatment, and interaction between factors) as described above. Individual test statistics were calculated for each analysis: time, experimental group, and interaction effect.

To determine statistical significance, data sets were compared against the null hypothesis of no difference between groups. Data sets were resampled with replacement to create new data sets. We then calculated a new test statistic for the resampled data set. This process was repeated 10,000 times to create a distribution of possible test statistics. The actual test statistic was then compared against this distribution to determine if it was outside of the 95% confidence interval. A two-tailed method was used unless otherwise stated, meaning that statistical significance was determined if the actual test statistic was found in the bottom 2.5 or above the 97.5 percentile of the test statistics from the 10,000 resampled distributions.

For IOS experiments, we used a modified form of linear regression. For this "intercept test" we calculated a distribution of y-intercepts under the null hypothesis to determine if two groups had significantly different distributions of y-intercepts. The mean was calculated for each x value and then plotted and a regression line was then

generated using the formula: $y = mx + b$, where m equals the slope of the line and 'b' equals the intercept on the y-axis. We chose to use y-intercepts as our test statistic because we found that all of our groups of IOS data had similar slopes and therefore differences might be masked if we compared the entire regression, since 'b' was the only factor that differed between groups. The data was then bootstrapped 10,000 times to generate a distribution of resampled y-intercepts. Two groups were said to be significantly different if their 95% confidence intervals did not overlap. Significance was set at $p < 0.05$. Error bars in figures are the standard error of the mean.

For peri-infarct IOS experiments a two-group comparison was performed. The difference of the means was used as the test statistic and to determine significance the two groups were resampled randomly creating two new resampled data sets from which a new test-statistic could be calculated. This process was recorded and then repeated 10,000 times to generate a distribution of resampled test-statistics. If the real test statistic was found in the outside 5% of this distribution it was said to be significant $p < 0.05$.

Chapter 3:

Results

Results

3.1. Absence of dendritic plasticity in the contralateral cortex after stroke

Others and we have used chronic high-resolution in vivo two-photon microscopy to test whether dendritic spine remodeling in peri-infarct cortex contributes to brain repair and functional recovery after stroke (Brown, 2009 #858;Brown, 2007 #860;Mostany, 2010 #851). To investigate whether dendritic spine plasticity also occurred in the contralateral homotopic cortex after stroke, we used the same approach to image GFP-expressing pyramidal neurons through a cranial window before and after permanent unilateral distal MCAO. We chose the same MCAO model of stroke (Tamura, 1981 #907;Roof, 2001 #933) as before (Mostany, 2010 #851), in order to compare both studies and also because the mechanism of neuronal injury (ischemia from arterial occlusion) is the same as the most common stroke pattern in humans (Lloyd-Jones, 2009 #913). We focused our studies in S1 because it is commonly affected by stroke in humans and many studies

Figure 3: Experimental design and typical MCA stroke.

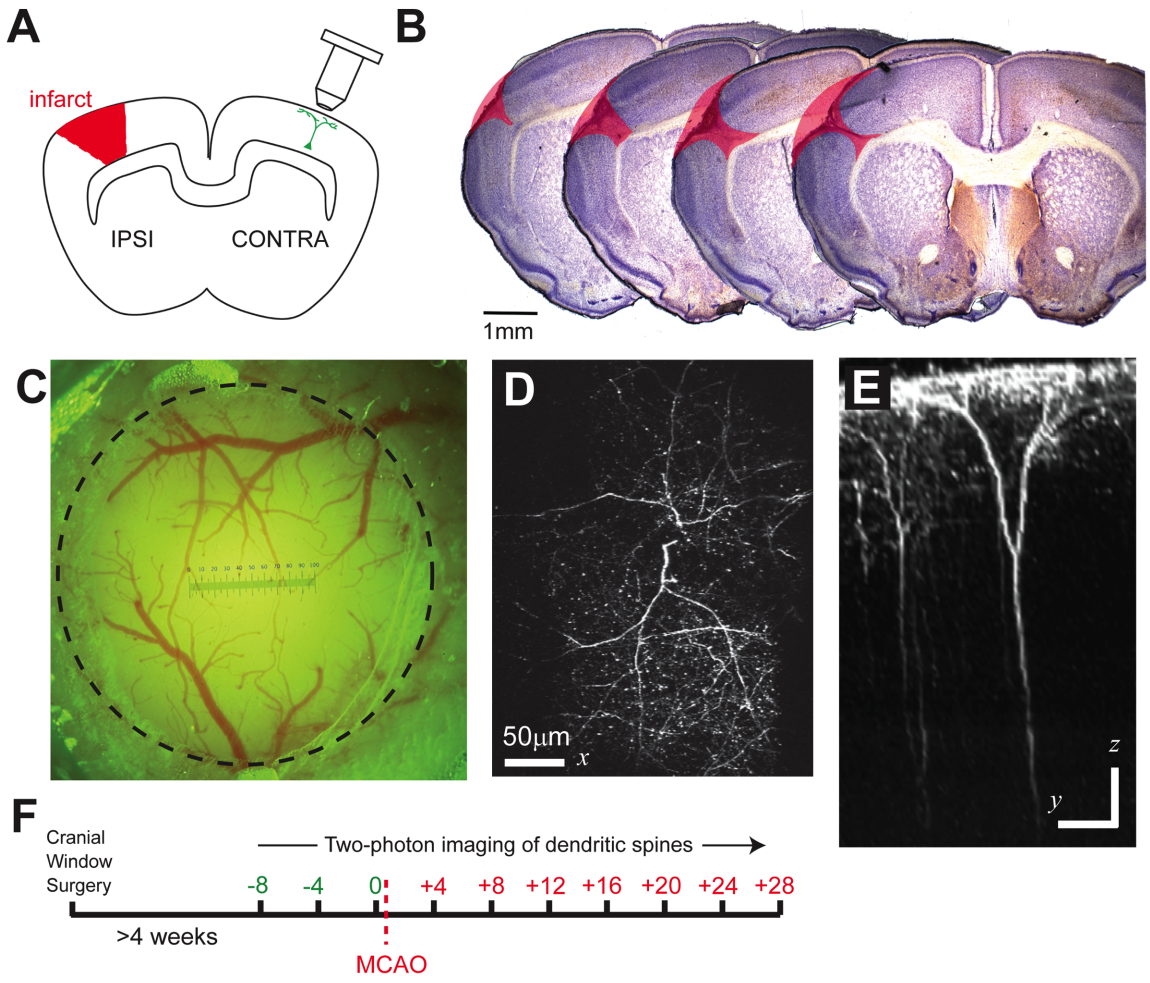
(A) Location of cranial window for in vivo imaging area (green neuron) in relation to the location of the infarct (red dashed lines).

(B) Four coronal sections stained with cresyl violet from a mouse in this study, showing the location and size of a representative infarct after MCAO (the red shaded area represents the portions of cortex that was missing when the mouse was perfused at +32 d).

(C) Sample optical window used for in vivo 2-photon imaging. The black dashed circle outlines the window perimeter.

(D, E) Representative top view (D) and side view (E) of a L5 pyramidal neuron expressing GFP in the thy1 GFP-M mouse line at a baseline imaging session before the MCAO. Maximum intensity projection of a stack of 115 images, 1.5 μm apart (scale bar = 100 μm).

(F) Experimental timeline for in vivo two-photon imaging experiments.


A
infarct
IPSI
CONTRA
B
1mm
C
D
50μm
x
E
z
y
F
Cranial
Window
Surgery
Two-photon imaging of dendritic spines
-8
-4
0
+4
+8
+12
+16
+20
+24
+28
>4 weeks
MCAO

addressing functional and structural plasticity in mice, including those investigating experience-dependent spine plasticity with in vivo two-photon microscopy, have focused on S1 (Holtmaat, 2009 #686).

Cranial windows for imaging spines in vivo were deliberately implanted in the contralesional hemisphere (**Fig. 3A**) so we could image neurons in homotopic areas of cortex corresponding to those that were destroyed by the infarct. MCAO produced moderate size infarcts (**Fig. 3B**), which spared the hindpaw representation but included the lateral barrel field, rostral vibrissae, lower lip, and the forelimb portions of the primary somatosensory cortex (S1), as well as a small part of the primary motor cortex (Mostany et al., 2010). We selected the thy1 GFP-M line of transgenic mice because they express GFP sparsely in L5 pyramidal neurons, which makes it easier to image unobscured dendrites (**Fig. 3D-E**).

The apical tuft of layer 5 pyramidal cortical neuron dendrites were examined over weeks before and after MCAO stroke to determine the level of plasticity at the level of individual spines as well as growth and retraction of dendrite tips. We chose L5 pyramidal neurons for two main reasons. The first is that most previous two-photon studies of spine dynamics in vivo have been of L5 pyramidal neuron dendrites and therefore their baseline dynamics have already been carefully characterized (Holtmaat A. WL, Knott G.W., Welker E., Svoboda K., 2006; Holtmaat AJ et al., 2005; Trachtenberg J.T. CBE, Knott G.W., Feng G., Sanes J.R., Welker E., Svoboda K., 2002). Because of the wealth of data describing spine dynamics, both at baseline and under other conditions such as experience-dependent plasticity, it allowed us to compare our own results both at baseline and after ischemic stroke to a well-

characterized body of data. With most of the basic characterization of spine dynamics already described, it allowed us to focus our results on whether or not ischemia induces structural changes in the fine architecture of the cortex.

The second and more important reason we chose to examine L5 of pyramidal neurons in S1 is that L5 acts as the major output layer of the cortex. Most signals leaving the cortex do so through L5 and therefore we assumed that changes in the circuitry of cortical networks due to plasticity after stroke would be evident in L5. Layer 5 pyramidal neurons receive inputs from L2/3 and L4 on their apical dendrites. Therefore L5 integrates information from multiple sources and should remodel its inputs in the event of network rewiring in response to ischemic damage.

In humans, the majority of clinical improvement occurs in the first 3 months after stroke (Skilbeck, 1983 #879). In rodents, the recovery is even faster, particularly when rehabilitative strategies are initiated within 2 weeks after stroke (Biernaskie, 2004 #864). We have previously shown that mice subjected to distal MCAO exhibit signs of functional impairment, as manifested by reduced use of the contralateral forelimb in the cylinder test at +1 d and +5 d after stroke but significant functional recovery was observed in affected mice by +21 d post stroke (Mostany et al., 2010). Thus, if the contralateral cortex plays a significant role in post-stroke recovery, it must do so within a few weeks after the initial insult. For this reason, we focused our imaging experiments on the first month after stroke and mice were imaged every four days for ~40 days, including three baseline imaging sessions before the MCAO (**Fig. 3F**).

Figure 4: Chronic imaging of dendritic spines in contralateral cortex before and after MCAO shows no evidence of structural plasticity

(A) High-resolution two-photon images acquired in vivo of a typical apical dendritic segment from a L5 pyramidal neuron in the contralateral cortex before and after MCAO. All are maximum intensity projections (4-12 slices, 1.5 μm apart). A few examples of always present spines (yellow arrows), gained spines (green

arrowheads), and lost spines (open red arrowheads) are shown. The day of imaging is shown in the upper left hand corner (scale bar = 5 μm).

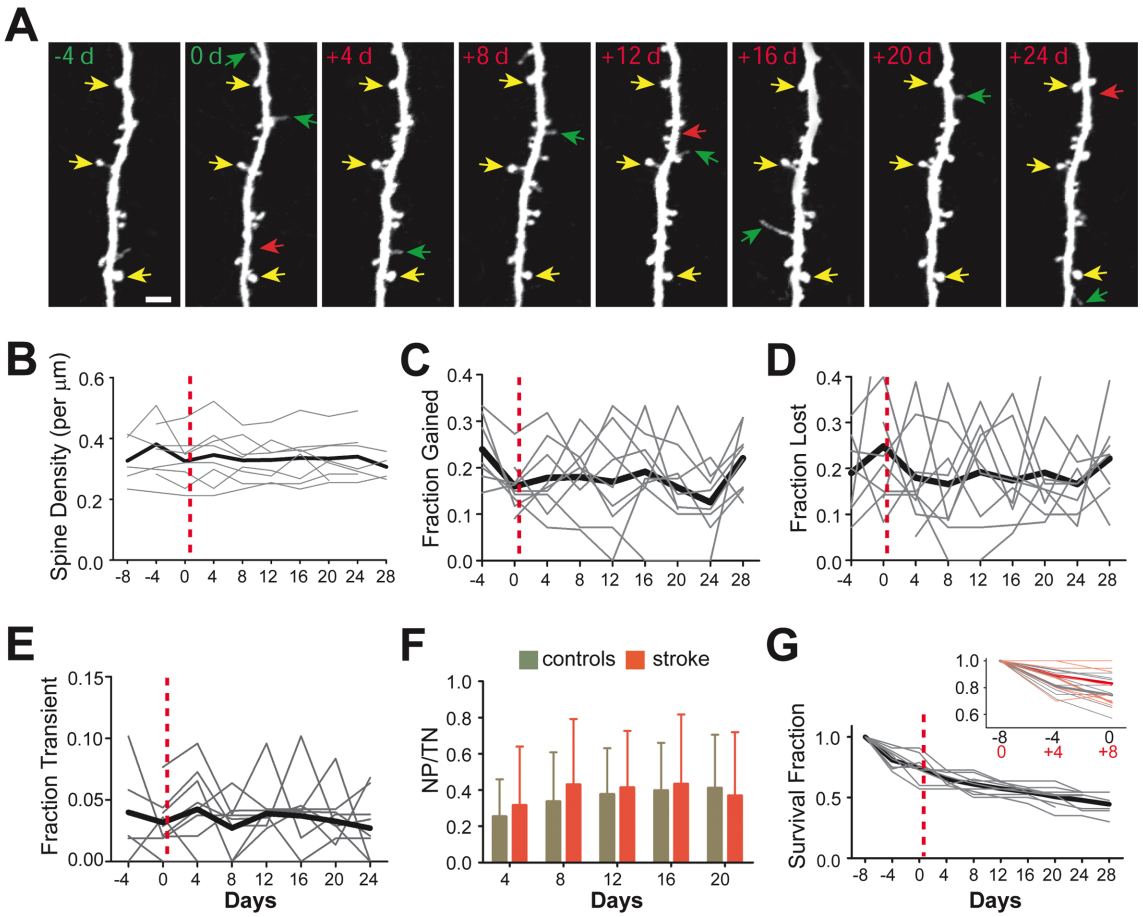
(B) Normalized spine density over time for 9 L5 pyramidal neurons from 4 mice. Gray lines represent individual cells and the thick black line is the average in panels D-F. The vertical dashed line in panels B-G indicates the time of the MCAO. No significant change in spine density was found (one way ANOVA with repeated measures $p = 0.125$)

(C, D) Fraction of spines gained (D) and lost (E) over the total number of spines, as a function of time before and after MCAO. No statistical significance was found for either parameter before vs. after MCAO (one way ANOVA with repeated measures $p = 0.192$ and $p = 0.46$ respectively).

(E) Fraction of transient spines (i.e., new spines that appeared on only one imaging time point and then disappeared; one way ANOVA with repeated measures $p = 0.28$)

(F) Probability of new spine stabilization (a.k.a., spine stabilization ratio). The stabilization ratio was calculated as the number of new persistent spines (spines surviving 3 or more time points) divided by the total number of spines. No significant difference was found between the stabilization ratio of stroke group and a control group ($p = 0.197$).

(G) Survival fraction of dendritic spines over time. The inset compares the survival fraction over the baseline time period and over the first 8 d after stroke. No significant change in survival fraction was found ($p = 0.101$).

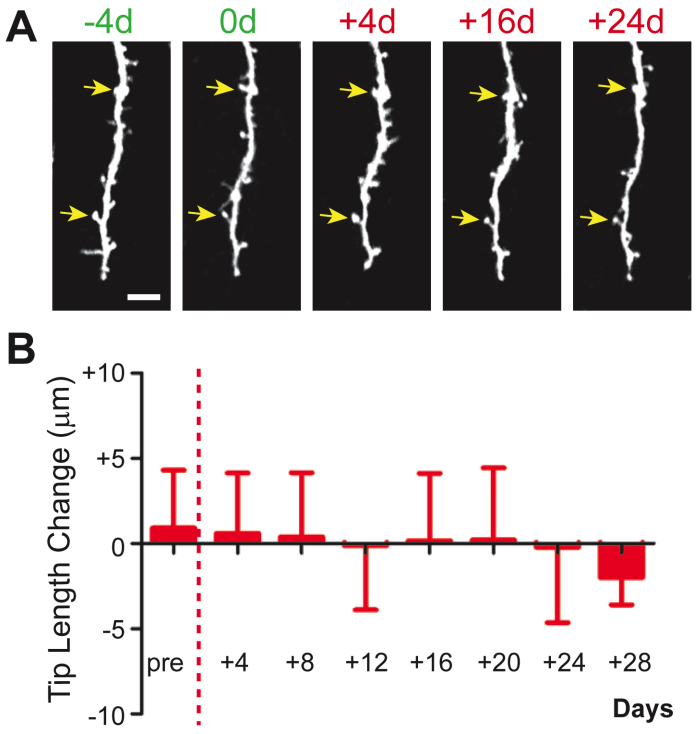

A
-4 d
0 d
+4 d
+8 d
+12 d
+16 d
+20 d
+24 d
B
Spine Density (per µm)
C
Fraction Gained
D
Fraction Lost
E
Fraction Transient
Days
F
controls
stroke
NP/TN
Days
G
Survival Fraction
Days

High-resolution two-photon microscopy allowed us to follow individual dendritic segments longitudinally before and after MCAO. We could not detect differences in the normalized spine density at any point after MCAO compared to the basal conditions (0.36 ± 0.09 spines/μm before vs. 0.34 ± 0.08 spines/μm after stroke; one way ANOVA with repeated measures p = 0.125; **Fig. 4B;** 13 dendritic segments from 9 different L5 neurons in 4 mice). Structural rewiring may involve changes in spine dynamics without a change in spine density. For example, experience-dependent plasticity is associated with changes in spine turnover even though spine density is unaffected (Hofer, 2009 #683;Holtmaat, 2006 #535). Therefore, we first quantified the fraction of spines gained and lost between imaging sessions, as well as the density of transient spines (those appearing in only 1 imaging session), but found no differences in any of these parameters after stroke (one way ANOVA with repeated measures p= 0.192, p= 0.46, p= 0.277, respectively**; Fig. 4C-E**). Next, we measured the probability that new spines would be stabilized, because this parameter has been found to increase after whisker deprivation (Wilbrecht, 2010 #826), which would suggest that new spines are permanently altering circuits after stroke. However, the spine stabilization probability (# new spines that persist for 3-5 imaging time points / # total new spines) was not significantly different in stroke vs. control animals at any point after stroke (on average 0.36 ± 0.26 in stroke animals and 0.38 ± 0.34 in control animals; +4 to +20 d after MCAO; two way ANOVA with repeated measures p > 0.197; **Fig. 4F**; control data comes from 31 dendritic segments from 14 different L5 neurons in 8 control mice). Finally, we found that the survival function of spines did not change abruptly after stroke (**Fig. 4G**), and was similar to that of normal adult wild

type mice imaged in vivo (Mostany, 2010 #851;Holtmaat, 2009 #639;Holtmaat, 2006 #535;Wilbrecht, 2010 #826). Also, there was no significant difference in the survival fraction of spines between the baseline period and the first eight days after stroke (74.7 ± 9.9 % for 8 d baseline vs. 83.3 ± 13.6 % after 8 d post-stroke; p = 0.101; **Fig. 4G, inset**). Thus, L5 neurons in the contralateral cortex neither add nor retain spines after stroke to compensate for the massive loss of synapses in the lesioned hemisphere.

Figure 5: Absence of large-scale plasticity in contralateral cortex after MCAO

(A) Two-photon images of representative tips of apical dendrites of L5 pyramidal neurons (best projections of image stacks consisting of 5-10 slices, 1.5 μm apart).
(B) Changes in dendritic tip length over time. Length was measured from fiducial points that could be identified at every time point before and after MCAO (stable spines; yellow arrows). No significant growth or retraction of tips was seen after the stroke (one way ANOVA with repeated measures $p = 0.159$).

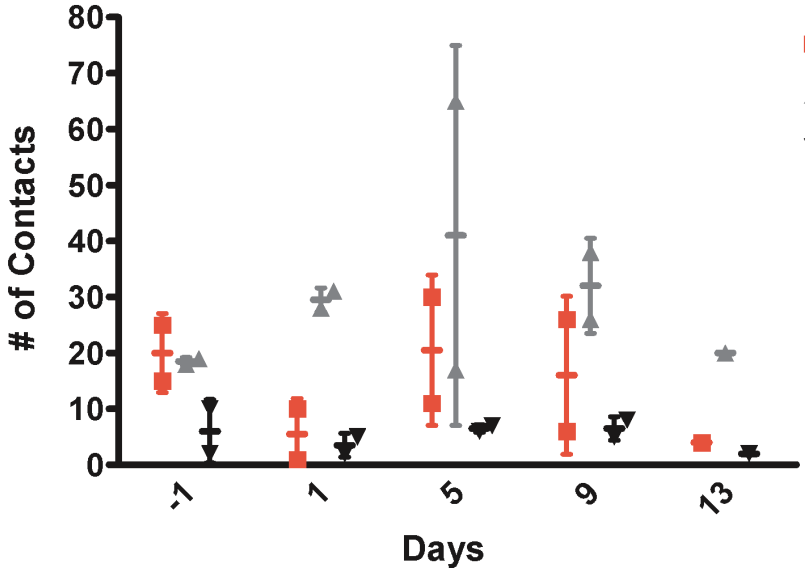

A
-4d
0d
+4d
+16d
+24d
B
Tip Length Change (μm)
+10
+5
0
-5
-10
pre
+4
+8
+12
+16
+20
+24
+28
Days

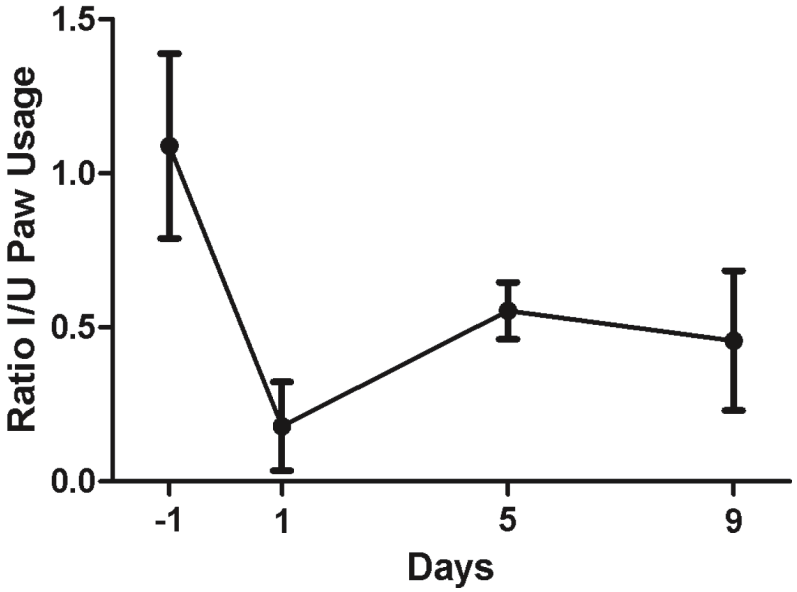
We also wondered whether, even in the absence of synaptic plasticity at the level of spines, stroke might have triggered large-scale remodeling of dendrites in the contralateral cortex to allow for more new connections to be made. Previous Golgi studies have shown that after cortical lesions dendritic complexity of L5 pyramidal neurons increases in the spared hemisphere (Jones, 1992 #863), particularly after rehabilitation (Jones, 1994 #567;Biernaskie, 2004 #864). However, others did not find any large-scale dendritic growth after cortical lesions (Forgie, 1996 #566;Prusky, 1996 #565). The idea that pyramidal dendrites grow after brain injury is not supported by more recent in vivo imaging studies. For example, dendritic arbors of L5 pyramidal neurons appear to be stable in adult mice under normal conditions (Lee, 2006 #862;Trachtenberg, 2002 #237), suggesting that dendrites may not have the ability to grow at all in mature animals. In agreement with this notion, our previous longitudinal in vivo imaging study showed that dendrites of L5 pyramidal neurons in peri-infarct cortex did not grow or add new branches after MCAO stroke (Mostany, 2011 #865). We measured the changes in length of 11 dendritic tips from 7 neurons of 4 mice (average tip segment length was 24 μm) after stroke and found no evidence for dendrite remodeling, as the tip length did not change significantly before or after stroke (on average -1.2 ± 1.5 μm after stroke vs. -0.4 ± 0.8 μm at baseline, one way ANOVA with repeated measures, $p = 0.159$; **Fig. 5)**.

Figure 6: Evidence of behavioral deficit in the affected forelimb after stroke.

(A) Absolute number of contacts made with the impaired paw (pink) drops while the number of contacts with the unimpaired paw (gray) increases after stroke while the number of contacts made with both paws (Hilton E *et al.*) remains constant before and after stroke.

(B) Ratio of impaired paw/unimpaired paw plotted to highlight the animals favoring the paw unaffected by stroke.


# of Contacts
80
70
60
50
40
30
20
10
0
-1
1
5
9
13
Days


Ratio I/U Paw Usage
1.5
1.0
0.5
0.0
-1
1
5
9
Days

3.2 Behavioral Testing

In the present study, we wanted to characterize the timeline of functional recovery of the affected limb after stroke so that we could see if functional recovery was correlated with the timeline of functional remapping to the spared hemisphere after stroke. To that end, we utilized two behavioral tests: the cylinder test and the adhesive-removal test (ART) to detect asymmetries in forelimb use. The cylinder test detects asymmetries in paw use by measuring the number of paw contacts on the wall of the glass cylinder while the mouse rears up during spontaneous exploration. The second test, the adhesive-removal test, detects deficits in forelimb sensation by measuring the time it takes for the mouse to become aware of a piece of tape stuck to its paw. In addition, the ART detects motor impairment by recording the time it takes for the removal of the piece of adhesive after it has been discovered and comparing the time against control animals.

To obtain a baseline, behavioral testing was performed once prior to stroke induction for the cylinder test and three times for the adhesive removal test. After baseline, mice were imaged every four days starting one day after stroke for the cylinder test and every day after stroke for the ART. In control animals, sham RBPT surgeries were performed whereby all details were identical as stroke animals, except that either no Rose Bengal was injected or the skull was not illuminated. Sham animals showed no signs of cell death when examined histologically (Nissl stain with Cresyl Violet) indicating that Rose Bengal alone was not neurotoxic.

The adhesive-removal test was used to detect deficits in both forelimb sensation and motor function by measuring a sense score (time until adhesive is noticed) and a motor score (time to remove adhesive once it has been noticed). However, preliminary findings proved inconclusive as this test was extremely biased by the mouse's activity level. Many of the mice tested were not motivated to remove the adhesive and therefore received a maximum score. The animal's activity level was not significantly different after stroke as many mice during the baseline period chose not to remove the adhesive even after it was noticed. Due to the subjectivity inherent in the design of the behavioral test the ART was abandoned for a more objective behavioral measure.

The cylinder test was chosen as a more unbiased measure of behavioral function as the activity level of the mouse did not skew the results to the same degree as in the ART. Mice subjected to distal MCAO exhibited signs of functional impairment, as manifested by reduced use of the contralateral forelimb during rearing in the cylinder test at +1, +5, and +9d after stroke. These preliminary data showed a marked imbalance in paw usage after stroke as evidenced by the drop in absolute number of contacts made with the impaired forelimb and an increase with the unimpaired forelimb (**Fig. 6A**).

After stroke, mice were sometimes lethargic during behavioral testing and did not complete as many rears as during the baseline period. To better quantify asymmetry in forelimb and avoiding bias from fewer absolute numbers of paw contacts a ratio of the impaired/unimpaired forelimb use was calculated. This ratio produced a single quantitative value describing changes in animal behavior after stroke. We found that

this ratio decreased significantly after stroke and remained impaired throughout the testing timeframe up to 13 d after stroke (**Fig. 6B**).

While we did not pursue behavioral testing to produce a high powered analysis of changes in forelimb function after stroke, the experiments described above as well as those performed by others (Mostany R *et al.*, 2010; Tennant KA and TA Jones, 2009) gave us a better understanding of how an ischemic insult impaired behavior so that we could better understand neural plasticity during functional imaging in a well characterized behavioral framework.

3.3 No functional remapping in contralateral cortex of adult mice

It is possible that the contralateral cortex might exhibit functional remapping in the absence of structural rewiring at the level of pyramidal dendrites. Thus, we next explored a functional readout of cortical plasticity and investigated the potential remapping of sensory-evoked cortical activity to the contralateral hemisphere after stroke. In addition, we wanted to test the biphasic theory of remapping, which suggests that remapping first involves the spared hemisphere (in the first few days after stroke) until the peri-infarct cortex can be recruited later after the initial ischemic insult subsides (Dijkhuizen, 2001 #871;Dijkhuizen, 2003 #924;Marshall, 2000 #870). That pioneering work was done in humans and rats using functional magnetic resonance imaging (fMRI), but suffers from limited temporal and spatial resolution. In most cases,

Figure 7: IOS experiments in adult mice show no evidence of functional remapping in the contralateral cortex after MCAO or RBPT stroke.

(A) Location of the cranial window in relationship to functional brain regions (adapted from (Diamond, 1999 #934;Grove, 2003 #935;Van der Gucht, 2007 #936)). Green shading is for primary somatosensory cortex (BF: barrel field; FL: forelimb; HL: hindlimb; LL: lower lip; No: nostril; Rv: rostral vibrissae; Tr: trunk; UL: upper lip), blue shading is for visual cortex and red shading for motor cortex.

(B) Mid coronal section (at the level of Bregma) stained with cresyl violet from an adult mouse in this study, showing the location and size of a representative infarct induced by focal RBPT.

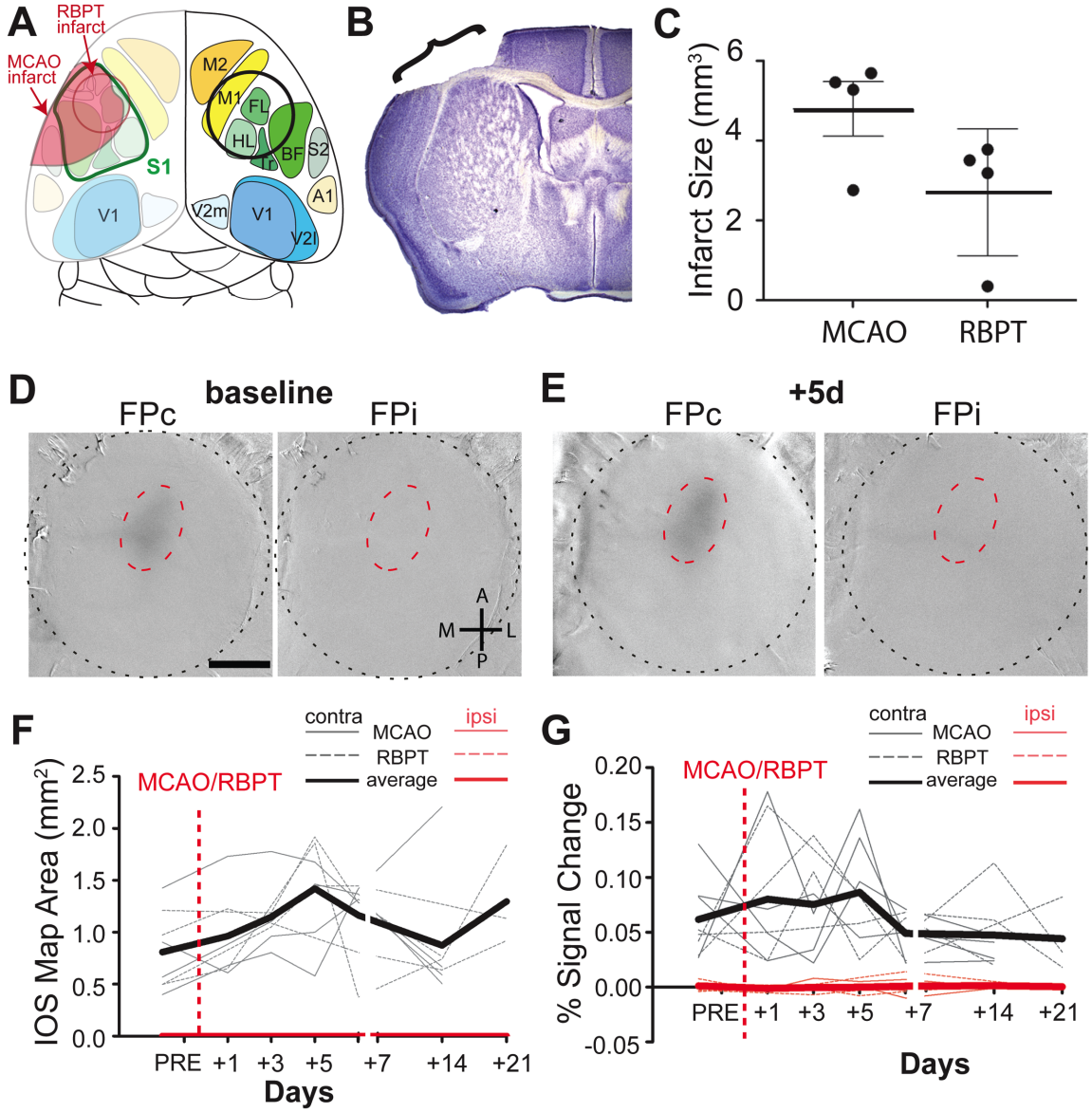
(C) Volume of infarcts for animals used for IOS experiments.

(D) Baseline IOS images after stimulation of the contralateral (FPc, left) and ipsilateral (FPi, right) in a representative mouse. A: anterior; P: posterior; L: lateral; M: medial. The dashed black circle outlines the window area and the dashed red oval outlines the actual FPc map area in S1FL. No map was detected during stimulation of the FPi. Scale bar (1 mm) is the same for panel F.

(E) IOS images for the same animal 5 days after stroke corresponding to stimulation of the FPc (left) and FPi (right).

(F) IOS map size (area in mm^2) corresponding to a 62.5% threshold of the maps resulting from stimulation of the FPc (solid grey line is after MCAO and dashed gray line is after RBPT) and FPi (red) over time before and after stroke. For analysis, data for MCAO and RBPT infarcts were pooled together and the average is shown in black. Ipsiliateral stimulation resulted in a map area that was not significantly different from zero, but the difference between ipsilateral and contralateral stimulation was significant at all time points before and after stroke (intercept test $p > 0.05$ and $p < 0.01$ respectively).

(G) Percent change in signal intensity for IOS maps corresponding to stimulation of the FPi (pink lines; average shown in red) and FPc (grey lines; average shown in black) over time before and after stroke. The magnitude of the IOS signal was quantified by taking the ratio of the average pixel value within the expected FPc map area (dashed red oval in E, F) divided by the average pixel value of an equal sized area just outside the FPc map area. This provided a measure of the strength of the IOS signal against the background level of noise. There was no significant IOS signal after FPi stimulation (intercept test $p > 0.05$).


A
RBPT infarct
MCAO infarct
M2
M1
FL
HL
BF
S2
Tr
S1
V1
V2m
V1
A1
V2l
B
C
Infarct Size (mm3)
6
4
2
0
MCAO
RBPT
D
baseline
FPc
FPi
A
M
L
P
E
+5d
FPc
FPi
F
contra
MCAO
RBPT
average
ipsi
MCAO/RBPT
IOS Map Area (mm2)
2.5
2.0
1.5
1.0
0.5
0.0
PRE +1 +3 +5 +7 +14 +21
Days
G
contra
MCAO
RBPT
average
ipsi
MCAO/RBPT
% Signal Change
0.20
0.15
0.10
0.05
0.00
-0.05
PRE +1 +3 +5 +7 +14 +21
Days

these were not longitudinal studies and subjects after stroke recovery were compared to normal controls. Therefore, we used longitudinal IOS imaging to record the cortical regions that respond to sensory stimulation of the contralateral and ipsilateral (affected) forepaw (FP) before and after stroke.

Due to variations in the intracerebral arterial anatomy from mouse to mouse, the exact infarct size and location after MCAO is somewhat variable, and this could confound the results of IOS mapping. For these reasons, we also used the rose Bengal photothrombotic (RBPT) stroke model so we could specifically produce an infarct of consistent size that was targeted to the FP region and then image the exact homotopic cortical area in the contralateral spared hemisphere. The main criticism of the RBPT model has been that both veins and arteries are occluded and there is virtually no reperfusion from collaterals. This was not a problem for our experiments because we investigated the mechanisms of plasticity in the homotopic cortex, the drive for which is presumably triggered by ischemic cortical injury in the opposite hemisphere, regardless of mechanism. A halogen lamp coupled to a fiber optic guide probe was used to focus white light on a 2.75 mm diameter area over S1FL (**Fig. 7A**) to create a focal infarct spanning all layers of the neocortex, but sparing subcortical regions (**Fig. 7B**).

After the last imaging session all mice were perfusion-fixed with 4% paraformaldehyde and brains were stained with cresyl violet to confirm the location and size of the infarct. An advantage of investigating functional remapping with two different stroke models was that we could test the theory that small strokes trigger remapping in peri-infarct cortex whereas large strokes are more likely to recruit the

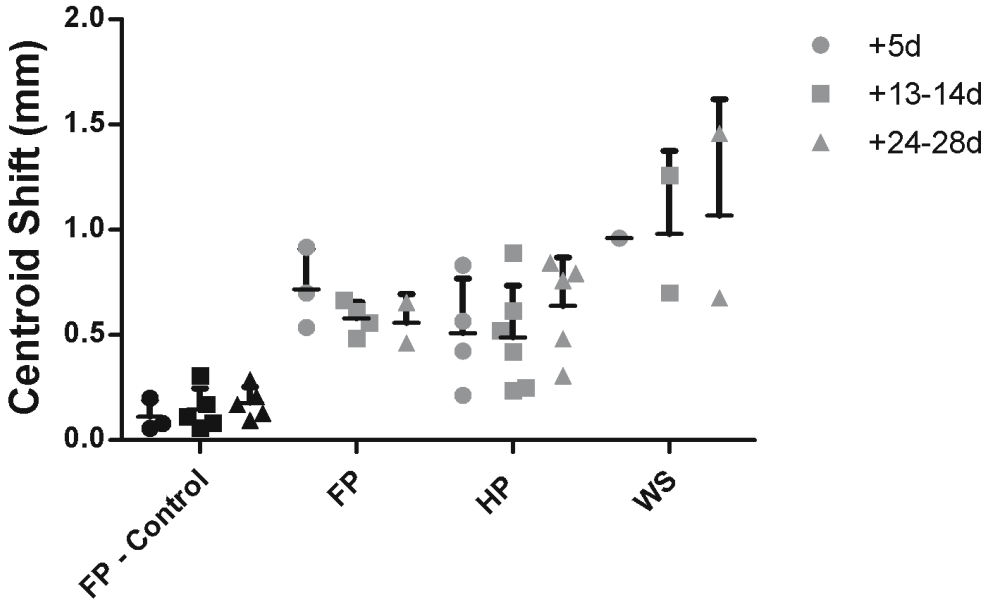
contralateral hemisphere (Abo, 2001 #925;Calautti, 2001 #897;Biernaskie, 2004 #864;Cramer, 2006 #887). However, although the volume of RBPT infarcts was smaller than that of MCAO infarcts (2.7 ± 1.6 mm^3 for RBPT vs. 4.8 ± 1.4 mm^3 for MCAO; **Fig. 7C**), they were directly targeted to the FL representation.

Mice were imaged at a baseline before stroke and then again at +1, +3, +5, +7, +14, +21, and +28 d after stroke. For every imaging session we acquired IOS signals during stimulation of each FP individually. There was no difference in IOS map size or signal intensity change between MCAO and RBPT stroke models (two way ANOVA with repeated measures; $p = 0.091$ and $p = 0.354$, respectively; n= 4 each), so both sets of data were pooled for analysis. Stimulation of the FP contralateral to the imaging window reproducibly evoked a detectable IOS signal in the expected location in S1FL (Diamond, 1999 #934;Grove, 2003 #935;Winship, 2008 #874), and this map did not change in location after MCAO or RBPT stroke (**Fig. 7D, E**, images on the left). As expected, in the baseline imaging session, we could not detect an IOS map by stimulating the affected FP ipsilateral to the imaging window (**Fig. 7D**, image on the right). Even after stroke, however, IOS imaging did not reveal an activity map after stimulation of the ipsilateral (affected) FP in the spared cortex (**Fig. 7D**, images on the right).

Quantitative analysis revealed that the area of IOS maps elicited by contralateral stimulation was significantly different from that elicited by ispilateral stimulation at every time point before and after stroke (intercept test, $p < 0.01$; **Fig. 7F**, n= 8 mice).

Figure 8: Shift in sensory-evoked IOS maps in the peri-infarct region after stroke in adult mice

Displacement of sensory-evoked activity maps after IOS imaging show a significant shift in map of the whisker bundle (C2-C3 and D2-D3; WS; $p < 0.01$ two-group comparison) when compared to the shift in forelimb maps during contralateral stimulation (Hilton E *et al.*).


Centroid Shift (mm)
2.0
1.5
1.0
0.5
0.0
FP - Control
FP
HP
WS
+5d
+13-14d
+24-28d

Thus, although contralateral FP stimulation consistently evoked activity maps of 0.5-2 mm^2 in the spared S1FL, the map area after ipsiliateral stimulation was not significantly different from zero at any time point (intercept test, $p > 0.05$). We also quantified the % signal intensity change over background before and after stroke in IOS maps elicited by contralateral and ipsilateral FP stimulation (**Fig. 7G**). To avoid any bias in selecting IOS maps, we calculated a signal-over-background ratio by dividing the average pixel intensity of the IOS images in the presumed region of the FP map (based on the contralateral stimulation map) by the average pixel intensity of a region of interest of equal size located outside of the FP map (see methods). The % signal change for the FP map evoked by contralateral stimulation varied slightly between sessions from 0.02 to 0.17% (due to inherent variability in chronic IOS measurements), and was significantly higher than the % signal change for the FP map after ipsilateral stimulation (intercept test, $p < 0.01$; **Fig. 7G)**. The signal change after ipsilateral stimulation was always less than 0.01% and did not change significantly after stroke ($p > 0.05$). We conclude that sensory stimulation of ipsilateral FP does not elicit an evoked response in S1FL that can be detected with IOS imaging up to 1 month after stroke.

It is possible that the sensitivity of IOS imaging through the skull was not sufficient to detect very small or weak signals from ipsilateral FL stimulation in the contralateral hemisphere. As a control, we used IOS to record the whisker representations in peri-infarct cortex using the same approach. At baseline, we were able to detect IOS signals resulting from contralateral stimulation of just 4 whiskers

(**Fig. 8**). The WS map disappeared acutely after stroke, but then reappeared by +13 d in a new location.

Figure 9: **IOS experiments in juvenile mice show no evidence of functional remapping in the contralateral cortex after MCAO stroke**

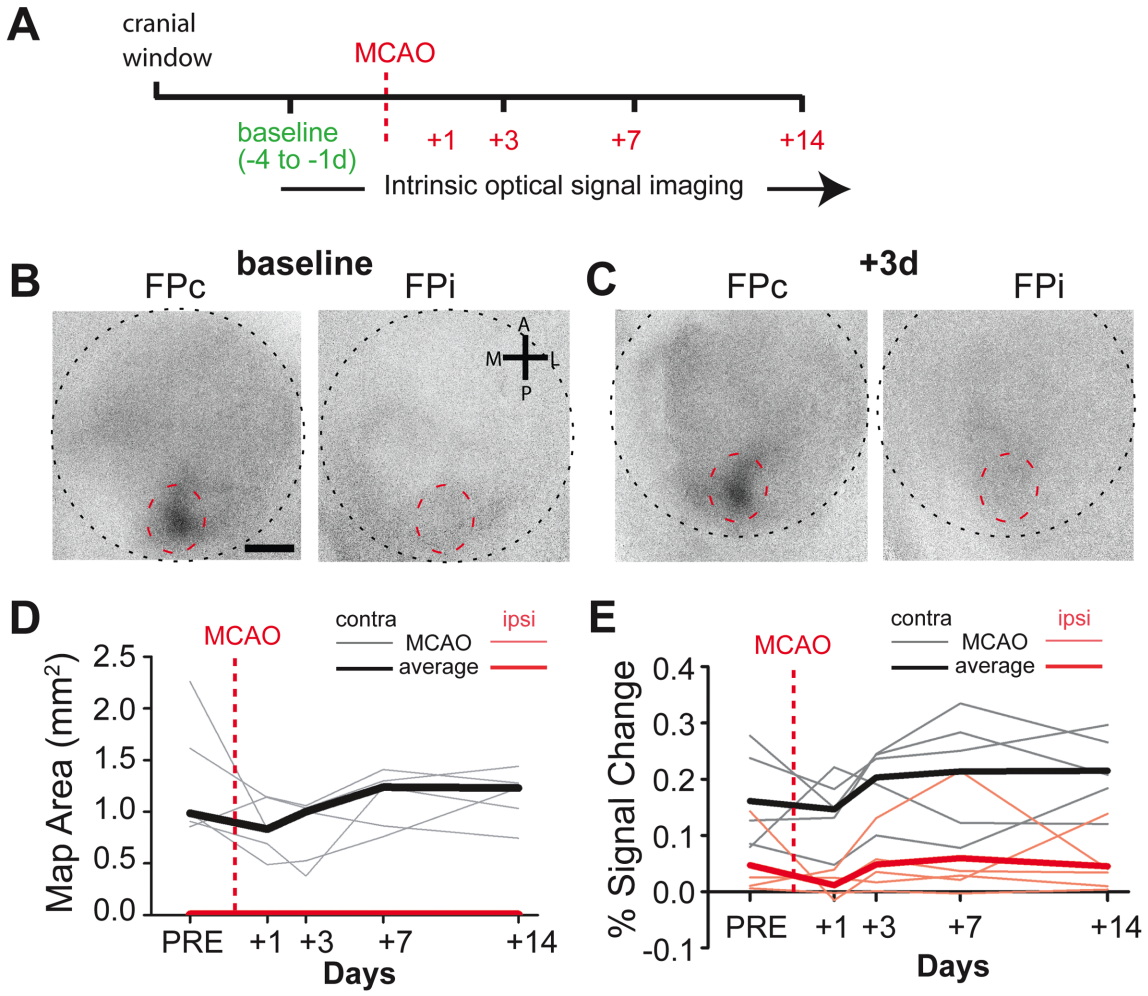
(A) Timeline of IOS imaging experiments in juvenile mice with respect to the time of stroke (dashed red line). Baseline images were taken at P18 and the infarct was induced at P19-P21.

(B) Baseline IOS images after stimulation of the contralateral (FPc, left) and ipsilateral (FPi, right) in a representative mouse. A: anterior; P: posterior; L: lateral; M: medial. The dashed black circle outlines the window area and the dashed red oval outlines the actual FPc map area in S1FL. No map was detected during stimulation of the FPi. Scale bar (1 mm) is the same for panel C.

(C) IOS images for the same animal 7 days after stroke corresponding to stimulation of the FPc (left) and FPi (right).

(D) IOS map size (area in mm^2) corresponding to a 62.5% threshold of the maps resulting from stimulation of the FPc (grey) and FPi (red) over time before and after MCAO stroke. The bold black/red lines are the average of 5 mice. Note that the FPi map size was never significantly different from zero after ipsilateral stimulation (intercept test $p > 0.05$).

(E) Percent change in signal intensity for IOS maps corresponding to stimulation of the FPi (pink lines; average in red) and FPc (grey lines; average in black) over time before and after MCAO. The difference in signal change was always significant between ipsilateral and contralateral stimulation (intercept test $p < 0.01$).


A
cranial
window
MCAO
baseline
(-4 to -1d)
+1
+3
+7
+14
Intrinsic optical signal imaging
B
baseline
FPc
FPi
A
M
L
P
C
+3d
FPc
FPi
D
MCAO
contra
ipsi
MCAO
average
Map Area (mm²)
2.5
2.0
1.5
1.0
0.5
0.0
PRE
+1
+3
+7
+14
Days
E
MCAO
contra
ipsi
MCAO
average
% Signal Change
0.4
0.3
0.2
0.1
0.0
-0.1
PRE
+1
+3
+7
+14
Days

The medial-caudal displacement of the WS map after stroke was significantly greater than the average movement of the map with repeated measurements during the baseline period before the stroke (1.01 ± 0.34 mm after stroke vs. 0.15 ± 0.08 mm at baseline; $p < 0.01$; n= 2 mice). The remapped representation was of equivalent signal intensity to the original map in barrel cortex.

3.4 Lack of functional remapping in contralateral cortex of juvenile mice

Children recover from brain lesions, including stroke, more fully than adults (Kim, 2009 #922;Ganesan, 2000 #1038;deVeber, 2000 #1037). Therefore, we hypothesized that young mice might exhibit cortical remapping after MCAO stroke in the contralateral hemisphere. To test this we implanted cranial windows in juvenile mice and induced infarcts at postnatal day (P) 19-P24 and then carried out IOS imaging before and at +1, +3, +7, and +14 d after an MCAO stroke. In juvenile mice, the unilateral distal MCAO also produced infarcts that included the FP representation in S1FL, but their size was more variable than in adult mice (not shown). The first baseline IOS imaging session was usually done 1-2 days after implanting the cranial window and this was followed 2-3 days later by MCAO surgery after the stroke. In Juvenile mice, distinct maps could also be evoked by stimulation of the contralateral FP before and after stroke (**Fig. 9B, D**), and the size of thresholded maps was similar to that of maps in adults (on average 0.98 ± 0.31 mm^2 in juvenile vs. 1.13 ± 0.47 mm^2 in adults; **Fig. 7F and 9D**). Just as with adult animals, using IOS imaging we could not detect activity maps that were significantly different from zero after ipsilateral FP

stimulation at any point before or after stroke in juvenile mice (intercept test, $p > 0.05$; **Fig. 9C-D**). In juvenile mice we again found that the magnitude of IOS signal change in the spared hemisphere was significantly greater after contralateral stimulation than after ipsilateral stimulation in juvenile animals (on average 0.16 ± 0.09% change for contralateral vs. 0.05 ± 0.06% change for ispilateral; intercept test $p > 0.05$; **Fig. 9E**). Furthermore, the signal change after ipsilateral stimulation did not change significantly after stroke (0.047 ± 0.056 % for the -1 d baseline vs. 0.041 ± 0.047 % for the +1 d to +14 d post- stroke average; $p > 0.05$).

Chapter 4:

Discussion

Discussion

From examining the degree of cortical plasticity in the contralesional hemisphere, I could find no evidence for remapping of activity or alteration in the fine structure of neurons to suggest any involvement of the spared hemisphere in recovery after stroke. Utilizing a longitudinal characterization of both the structure and function of the spared cortex in living animals, there was no evidence for adaptive plasticity. The goal of this project was to answer three basic questions. First, what is the role of the contralateral cortex in recovery after stroke? Second, what is the timing of functional remapping and dendritic plasticity after stroke? Finally, is stroke plasticity developmentally regulated?

My experiments were designed to describe the degree of adaptive plasticity in the spared hemisphere after stroke in a novel manner and in greater detail then had been done previously. Despite the goal of characterizing the role of the contralateral hemisphere in recovery after stroke, however, the data did not support the findings of most previous studies. The results instead suggested that the contralateral hemisphere plays no role in recovery after stroke, at least at the level of changes in spine dynamics, growth of dendrite tips, or remapping of cortical activity from the infarcted area to the homotopic cortex. By extension, the question of what is the timing of functional remapping after stroke was answered as well. That is, there was no detectable change in functional remapping at any time point after stroke.

The potential for adaptive plasticity at younger ages was also measured and compared to that in adults. This question was examined by measuring changes in

sensory-evoked cortical maps in the S1FL area in juvenile animals (~P20) using IOS. However, despite the fact that the brain is thought to be inherently more plastic at younger ages (Johnston MV, 2009; Kim CT *et al.*, 2009), this was not manifested as an increased potential for cortical remapping after ischemic stroke, at least in the spared hemisphere.

4.1 Summary of Results

One initial goal of these experiments was to investigate the structural basis of cortical plasticity after stroke. We wished to take a step further in our investigations than had been done previously by utilizing an in vivo longitudinal approach to discover the degree of structural plasticity at the level of individual spines as well as growth of dendrite tips in the apical tuft of L5 pyramidal neurons in primary somatosensory cortex, both before and after stroke. This experimental design had never been used previously and provided the most detailed method to learn how spine dynamics might change in order to repair lost function after ischemic injury in the brain.

Using in vivo two-photon microscopy we imaged 13 individual dendrite segments from 8 L5 pyramidal neurons in 4 mice over a period of 40 days. Because the first 3 imaging sessions were performed before the stroke was induced, we were able to characterize the baseline spine dynamics as well as any changes that might occur after stroke. In this manner each cell would act as its own control, and we would be able to track structural changes that might occur over time in response to an ischemic insult. From these imaging sessions, we showed that there was no change in spine

density, survival fraction, fraction gained, fraction lost, or changes in spine stability as measured by the ratio of new persistent spines over total new spines (**Fig. 4F**). In addition, we found no gross dendritic remodeling as measured by growth or retraction of apical dendrite tips. Although not specifically investigated, we also did not observe any formation of new dendrite branches in the areas imaged for spine analysis and dendrite growth.

These findings suggest a profound reexamination of the role of the contralateral hemisphere in reparative plasticity after stroke. Multiple previous studies have shown that the contralateral hemisphere exhibits significant changes in structural plasticity (Jones T.A. S, T., 1992; Jones TA and T Schallert, 1994) after stroke, but provides no mechanism for how these changes might influence recovery. The idea that pyramidal dendrites can grow in mature animals is also not supported by more recent in vivo imaging studies. For example, dendritic arbors of L5 pyramidal neurons appear to be stable in adult mice under normal conditions (Lee WC et al., 2006; Trachtenberg J.T. CBE, Knott G.W., Feng G., Sanes J.R., Welker E., Svoboda K., 2002) suggesting that dendrites may not have the ability to grow in mature animals. In agreement with this notion, our previous longitudinal in vivo imaging study showed that dendrites of L5 pyramidal neurons in peri-infarct cortex did not grow or add new branches after MCAO stroke (Mostany R and C Portera-Cailliau). We measured the changes in length of 11 dendritic tips in 7 neurons of 4 mice after stroke and found no evidence for dendritic remodeling in the contralateral hemisphere, as the average tip length did not change more than -1.2 ± 1.5 μm after stroke. In fact, we found dendrite tip growth to not be significantly different from zero ($p > 0.5$; intercept test) nor change significantly over

time ($p = 0.159$ one-way ANOVA with repeated measures; **Fig. 5**) and therefore it is not likely to be functionally significant as this provided no new dendritic space for new spines. Using what we believe to be a more sophisticated in vivo longitudinal approach, our results do not support the hypothesis that there is a change in structural plasticity after stroke in L5 pyramidal neurons in primary somatosensory cortex and we therefore conclude that important reparative plasticity is occurring at a different locus in the brain.

Despite the fact that we could find no evidence for the contralateral hemisphere's role in structural plasticity after stroke, we went one step further and examined functional plasticity to test whether there might be remodeling that only occurred at the level of network activity in the absence of growth or loss of spines. To investigate functional activity we used an in vivo longitudinal IOS imaging approach aimed to overcome the limitations of previous work by following the same mice before and after stroke, and investigating the time course of ipsi- and contralateral remapping. Further, because IOS imaging has better spatial resolution than fMRI or positron emission tomography, we could reliably identify response maps as small as 0.5 mm^2 in size. Presumably, this would position us to detect even the slightest signs of contralesional activation. Surprisingly, we could not find any evidence of functional remapping to the spared hemisphere after stroke in adult mice over a period of 4 weeks after stroke (**Fig. 7**). Whether some remapping occurs there at later time points remains to be seen, but if that were the case, such delayed contralateral plasticity would not easily explain the functional recovery. Indeed, even though the majority of recovery in human stroke victims occurs within the first 3 months (Skilbeck, 1983 #879), recovery

in rodents is much faster. In addition, because children can recover more quickly and to a greater extent after cortical injury (Kim, 2009 #922;Johnston, 2009 #923), we also examined juvenile mice to test whether they might be better equipped than adults to manifest functional remapping in the contralateral cortex. However, stimulation of the affected forepaw did not elicit any detectable IOS map in the spared hemisphere even in 3-week old mice (**Fig. 9**). In summary, we conclude that, at least in mice, the spared cortex does not play a significant role in functional remapping in the first month after stroke.

4.2 Caveats

The goal of this project was to investigate the role of the contralateral hemisphere in reparative plasticity after stroke. To our knowledge, this is the first longitudinal, in vivo experiment in mice to determine the role of the spared hemisphere in recovery after stroke using both structural and functional imaging.

In the present work we failed to find evidence for any involvement of the contralateral hemisphere in recovery after stroke as measured by structural plasticity at the level of L5 pyramidal apical dendrites (**Fig 4-5**) or functional remapping of activity to the spared hemisphere (**Fig 7, 9**). While there are some limitations to the techniques used in this study we feel that they offer the most detailed characterization of cortical plasticity after stroke to-date. No other studies have looked at both structural and functional evidence for remapping of activity. In addition, we are the first to implement a longitudinal in vivo study where we can measure plasticity levels in

the same animal both before and after stroke. We believe that the previous studies, in which completely different groups of animals were compared, fail to provide a direct link between changes in cortical plasticity and ischemia.

Despite the power of our experimental tools, there are a few caveats that may help explain the difference in results seen here compared to previous work. First, there could be a species difference in the brain's response to ischemic injury. The majority of previous research, which has shown changes in plasticity in the contralateral hemisphere after stroke, has been conducted in rats (Carmichael S.T. CMF, 2002; Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein S.P., 2001; Dijkhuizen R.M. SAB, Mandeville J.B., Wu O., Halpern E.F.. Finklestein S.P., Rosen B.R., Lo E.H., 2003; Jones T.A. S, T., 1992) and humans (Calautti C. LF, Guincestre J.Y., Marié R.M., Baron J.C., 2001; Cao Y. DOL, Vikingstad E.M., Levine S.R., Welch K.M.A., 1998) and not mice. It is therefore possible that the same mechanisms of recovery that exist in rats and humans are not present in mice. This seems unlikely, however, if similar mechanisms exist across species as evolutionarily distant as rats and humans, but not between species as similar as rats and mice. Furthermore, more recent studies have shown changes in plasticity in the contralateral hemisphere after stroke in mice (Nabekura T *et al.*). This suggests that there are other explanations for the discrepancy between these two results and that it is not simply due to the use of different species.

One explanation might be that the strokes used in the present study were too small to require the involvement of the contralateral hemisphere in compensating for lost function. This seems unlikely, however, because the strokes induced for these

experiments generally encompassed the majority of primary somatosensory cortex as well as parts of motor cortex and other associated areas. Unfortunately, our attempts to make larger strokes, either through temporary bilateral carotid artery occlusion after MCAO or larger aperture light sources for RBPT increased significantly the mortality rates (25-50%) of our animals. In a recent review of reparative plasticity after stroke (Murphy TH and D Corbett), the authors explain that contralateral recruitment in recovery after stroke most likely occurs when neighboring, functionally similar areas are also affected by the infarct. That is, if the infarct is centered on the barrel field of S1 and the neighboring somatosensory and motor areas including forepaw are also affected, then this would require compensatory help from the spared hemisphere. This was often the case for the strokes induced for these experiments because they generally encompassed the majority of S1 as well as parts of motor cortex. This model proposed by Murphy and Corbett, however, has not been proven experimentally as the signal that might induce this remapping has yet to be identified. While our strokes affected large parts of somatosensory and motor cortex, they never penetrated deeper into subcortical areas. It is possible that subcortical infarction is necessary for contralateral remapping, although other groups have shown contralateral remapping without subcortical infarction (Brown CE *et al.*; Carmichael S.T. CMF, 2002; Jones T.A. S, T., 1992) suggesting that damage to the striatum or thalamus is not necessary for the spared hemisphere to become involved after ischemia. Because no mechanisms have been identified for remapping of activity to the spared hemisphere after stroke, we would argue that if contralateral plasticity truly

existed we would have detected it using our techniques and with infarct sizes comparable to the ones used in other studies.

Another possibility is that another cortical area besides L5 apical dendrites is the primary locus of plasticity after stroke. For example L4, L2/3, interneurons, and axons could instead be the primary loci of compensatory plasticity. However, because L5 receives inputs from all these areas we thought it reasonable that changes in upstream circuits would result in some manifestation of plasticity in L5 as well. Because we could detect no changes in the structure of apical dendrites in L5 pyramidal neurons, we infer that L5 did not experience any ischemia-induced plasticity. While it is possible that some other area upstream of L5 does experience plasticity, we believe this is unlikely given that we saw neither changes in L5 apical dendrites nor in functional activity. Presumably, if there had been evidence for remapping of activity to the spared hemisphere from our IOS experiments, then this might indicate that another part of the cortex besides L5 apical dendrites was undergoing some type of structural plasticity.

With intrinsic optical signal imaging (IOS) we hoped to characterize the functional remapping of the contralateral cortex. Specifically, the aim was to test whether the spared hemisphere expanded its receptive field to include the function of the infarcted area. IOS, like fMRI is based on measuring changes in blood flow that presumably reflect changes in neural activity. Currently, however, it is not fully understood what type of neural activity induces changes in blood flow detected by IOS and fMRI. It is possible that there were changes in neural activity in the spared hemisphere that did

not elicit a corresponding change in blood flow and therefore was not detectable with IOS.

While limitations of our methodology do exist, they are not unique to our study. Other groups have used similar methodology (Brown C.E. WC, Murphy T.H., 2008; Takatsuru Y *et al.*, 2009) and therefore suffer the same limitations. In future experiments it would be interesting to directly measure neural activity in the contralateral cortex using in vivo recordings of pyramidal neurons to test whether other methods might be able to detect new activity.

4.3 Limitations of previous work

The accumulated data concerning cortical recovery after stroke suggests that there are two major loci of adaptive plasticity contributing to functional recovery after stroke: the peri-infarct area and the homotopic contralateral hemisphere. The aim of this study was to investigate the role of the contralateral hemisphere in recovery with the hypothesis that there would be both structural evidence at the level of individual spines and dendritic branches as well as evidence for new activation in the spared hemisphere after stimulus of affected areas after stroke. This hypothesis was initially based on experiments performed in golgi-stained fixed tissue from rats (Jones T.A. S, T., 1992) showing that there was significant outgrowth of basal dendrite branches after injury. In addition, there is a large body of evidence from various functional imaging studies (Cao Y. DOL, Vikingstad E.M., Levine S.R., Welch K.M.A., 1998; Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein

S.P., 2001; Dijkhuizen R.M. SAB, Mandeville J.B., Wu O., Halpern E.F.. Finklestein S.P., Rosen B.R., Lo E.H., 2003; Schaechter J.D. PKL, 2008) that also contributed to our interest in exploring, in more detail, the exact role of the spared hemisphere in stroke recovery. However, since the experiments described here failed to reproduce the results of these other labs, we became more interested in what controversy existed in this topic.

We examined multiple measures of spine dynamics including spine density, turnover-ratio, and stabilization ratio that all failed to show a difference between pre and post-ischemia, or between stroke and control animals based on the spine stabilization ratio (**Fig. 4**). This finding contradicts other data that shows that dendritic plasticity occurs after stroke. Other investigators have investigated spine and dendrite plasticity after stroke, but none have utilized a longitudinal design detailing plasticity before and after stroke. One early study showed an increase in arborization of basal dendrites of L5 pyramidal neurons in rats after electrolytic lesions, but this study was performed in fixed tissue using Golgi staining (Jones T.A. S, T., 1992). Because each neuron reconstructed was imaged at only one time point it is impossible to know how that neuron changed due to stroke. Reconstructed neurons for each time point were compared to different neurons from different animals to try and show that there were differences before and after stroke. Since Golgi staining is done only in fixed tissues the same animal could not be investigated before and after stroke. This is an important distinction because it is possible that there is some sampling bias in the neurons stained by the Golgi method after stroke as it is currently unknown why some neurons are labeled with Golgi and some are not. This staining bias could lead to a

disproportionate number of complex cells being labeled after stroke through random sampling or some unknown bias in the Golgi method itself after stroke. Because this problem cannot be resolved, it is reasonable to question the conclusion that dendrites become more arborized after stroke.

The data presented here, however, is not the first to contradict some of these earlier findings. Multiple groups have sought to examine the role of dendritic and spine plasticity after stroke in L5 pyramidal cortical neurons and could not find evidence that such structural plasticity exists. One study sought to refine these earlier observations by examining corticospinal L5 pyramidal neurons in motor cortex by retrogradely labeling with the tracer DiO (Prusky G. WIQ, 1996). This revealed neurons in motor cortex that were involved in movement, which was the presumed basis for the plasticity seen in Jones & Schallert earlier work (Jones T.A. S, T., 1992). However, Scholl analysis failed to show any changes in dendritic branching of basal dendrites as had been described by Jones & Schallert.

Forgie et al (Forgie M.L. GR, Kolb B., 1996) also failed to identify structural changes after stroke. This study was undertaken to characterize sex differences in neuronal arborization after stroke. However, Forgie et al found no sex differences in dendrite growth after stroke, and furthermore found no evidence that the spared hemisphere displayed any increase in dendritic outgrowth at all. This study initially chose to use an aspiration lesion rather than an electrolytic lesion like that used by Jones & Schallert, but after failing to find changes in dendritic structures they replicated their experiments using an electrolytic lesion and could still find no changes in dendrite structure in the spared hemisphere. These two examples (Forgie M.L. GR,

Kolb B., 1996; Prusky G. WIQ, 1996) provide early clues that the role of the contralateral hemisphere in recovery related plasticity after stroke might not be as clear-cut as was previously assumed.

A more recent attempt to characterize plasticity in spine dynamics after photothrombotic stroke in mice showed that there was an increase in spine turnover one week after stroke when compared to baseline pre-stroke turnover rates (Takatsuru Y *et al.*, 2009). They showed that these changes in plasticity were restricted to the area of the cortex homotopic to the infarcted area. While this study makes some advances from earlier work in fixed tissue by using in vivo two-photon imaging to examine individual dendrites to make measurements of spine dynamics, it still suffers from the fact that at each time point a different set of dendrites was imaged. Mice were imaged three days, one, two, and four weeks after stroke and then imaged six hours later to determine turnover rates for each day. However, different animals were imaged for each time point so that changes in spine density could only be measured over a period of six hours. Therefore, just as in the work of Jones and Schallert, this study could not determine how the dendrites changed before and after stroke. For this reason, it is difficult to determine if any sampling bias might have occurred when picking new neurons to image each day. In addition, it is unclear whether spine turnover over a six-hour period is functionally meaningful in terms of recovery after stroke. Most other studies investigating spine plasticity use a similar protocol imaging dendrites every 3-4 days as it takes many hours to days for new spines to form functional synapses (Bonhoeffer T and R Yuste, 2002; Holtmaat AJ *et al.*, 2005; Trachtenberg J.T. CBE, Knott G.W., Feng G., Sanes J.R., Welker E.,

Svoboda K., 2002). It is unclear if Taketsura and colleagues study of the new spines detected one week after stroke stabilized and integrated into any cortical network involved in recovery after stroke over the six hour period monitored in their study. In addition, there is no mechanism proposed as to how these new spines seen only at the one-week post-stroke time and not three days or two weeks post-stroke could influence cortical repair in any meaningful way. Therefore, despite the technical advances of using in vivo imaging, this study provides no better proof that structural plasticity is occurring after stroke, and moreover how these structural changes could have any effect on reparative plasticity.

In addition to inconsistencies seen by multiple investigators in structural plasticity after stroke in the spared hemisphere, functional imaging studies have also failed to consistently show that the spared hemisphere is indeed activated by stimulation of the effected limbs after stroke. While the majority of functional imaging studies show contralateral remapping after stroke (Abo M. CZ, Lai L.J., Reese T., Bjelke B., 2001; Calautti C. LF, Guincestre J.Y., Marié R.M., Baron J.C., 2001; Cao Y. DOL, Vikingstad E.M., Levine S.R., Welch K.M.A., 1998; Chollet F *et al.*, 1991; Dijkhuizen R.M. RJ, Mandevill J.B., Wu O., Ozdag F., Moskowitz M., Rosen B.R., Finklestein S.P., 2001; Dijkhuizen R.M. SAB, Mandeville J.B., Wu O., Halpern E.F.. Finklestein S.P., Rosen B.R., Lo E.H., 2003; Marshall RS *et al.*, 2000; Rossini P.M. CC, Pauri F., Baron J.C., 2003; Schaechter J.D. PKL, 2008; Seitz RJ et al., 1998; Ward NS, 2004; Weiller C et al., 1992) there are still some studies that fail to find any evidence of contralateral involvement (Tuor UI et al., 2007; Weber R. R-CP, Justicia C., Widermann D., Strecker C., Sprenger C., Hoehn M., 2008). This contradictory evidence becomes

more interesting when combined with the fact that we could also find no evidence, functional or structural, that the contralateral hemisphere plays any role in recovery after stroke. Furthermore, previous functional imaging studies did not implement a truly longitudinal experimental design as different groups of animals were used for different time points. When the previous literature is reexamined, we found that the only two truly longitudinal functional imaging studies could not detect any remapping the in spared hemisphere (ours and Weber R., 2008). Weber and colleagues used a novel anesthesia protocol for fMRI that enabled them to image the same animals multiple times and is the only other study to use a longitudinal experimental design. They were the first to examine functional activity after stroke in the same animals over time and were unable to find any evidence of contralateral remapping after stroke (Weber R. R-CP, Justicia C., Widermann D., Strecker C., Sprenger C., Hoehn M., 2008). Similarly, when we attempted to uncover the role of the contralateral hemisphere after stroke using a longitudinal in vivo experimental design, we were also unable to find any evidence to support the theory that the contralateral hemisphere is instrumental in stroke recovery. While further study is required to fully address this issue, we feel that there is enough contradictory evidence from both this study and the general literature to redefine the role of the spared hemisphere in recovery after stroke.

4.4 Unmasking and homeostatic plasticity

One possibility that has not been investigated in relationship to stroke recovery, is the idea that the contralateral activity seen is due to either an unmasking of latent cortical circuits, or the nonspecific strengthening of inputs after the denervation of the spared hemisphere due to the neuronal cell death in the ischemic infarct. While, not studied in the context of ischemic injury, unmasking and homeostatic plasticity provides two possible explanations for nonspecific and potentially maladaptive forms of increased activity in the spared hemisphere after stroke. Multiple studies have shown that after the sudden loss of input, either through amputation, peripheral denervation, or neuronal injury, previously silent circuits are allowed to operate.

One model for cortical reorganization suggests that normally intracortical connections form a substrate for reorganizing cortical representations that is normally suppressed by critically placed inhibitory neurons (Harauzov A et al.; Jacobs KM and JP Donoghue, 1991). This suggests that loss of inhibitory control could induce reorganization of cortical representations of motor (Jacobs KM and JP Donoghue, 1991) or sensory (Harauzov A *et al.*) areas. It is known that ischemia can damage inhibitory interneurons, although excitatory neurons are usually most vulnerable (Centonze D *et al.*, 2001; Schmidt-Kastner R and TF Freund, 1991). While there is some loss of inhibitory neurons after stroke, the majority of cell death is of excitatory neurons (Haddad GG and C Jiang, 1993). However, there is significant inhibitory dysfunction after stroke (Clarkson AN et al.) in the form of increased tonic inhibition due to impairment of the GABA transporters GAT-3/GAT-4. If reorganization of cortical representation areas is truly mediated by normally suppressed intracortical excitatory connections, then it is hard to reconcile how this could occur after stroke

when there is an increase in tonic inhibition that would further suppress these excitatory connections. In addition, even without the increase in tonic inhibition, as excitatory neurons are more vulnerable to ischemia, these latent circuits presumably would be disrupted by ischemia-induced neuronal cell death.

Another interpretation derived from evidence supporting contralesional activation during stimulation of the affected limb is that homeostatic mechanisms are increasing synaptic strength non-specifically to all remaining inputs after the loss of inputs from the infarcted area. Unlike unmasking that is just uncovering already existing cortical circuits, homeostatic plasticity would entail strengthening of existing inputs non-specifically to compensate for the loss of signal after cell death in the infarcted area. Evidence for this type of homeostatic plasticity has been seen elsewhere in the sensory system. For example, after monocular deprivation there is a loss of responsiveness in the visual cortex during stimulation of the previously deprived eye (Gordon JA and MP Stryker, 1996; Hubel DH and TN Wiesel, 1970; Ranson A et al.). This suggests that cortical circuits can be strengthened or weakened in response to loss of input rather than a Hebbian like model of associated activity. After stroke, the spared hemisphere loses inputs from the infarcted area as these neurons die. In response to this loss of input, it is possible that to compensate the spared hemisphere strengthens its remaining inputs, although no direct evidence of this has yet been shown. One prediction from this model would be that if it were possible to provide exogenous input to mimic the connections lost after stroke to the spared hemisphere to prevent homeostatic plasticity from occurring then no remapping of activity should be observed.

4.5 A New Model for Post-Ischemic Plasticity and Future Directions

The goal of these experiments was to characterize cortical plasticity after stroke in the spared hemisphere. After examining structural changes in L5 pyramidal neurons in somatosensory cortex, we found no evidence to suggest that the contralateral hemisphere is involved in stroke related recovery. In addition, functional imaging of sensory-evoked activity also failed to show any evidence of increased plasticity after stroke in the spared hemisphere.

Based on these data, I propose a new model for understanding reparative plasticity after stroke. In this model, the peri-infarct region plays the primary role of compensating for lost function in the infarcted area while the spared hemisphere adopts no new function and experiences no adaptive plasticity as a result of an ischemic injury. Since we could find no evidence that the spared hemisphere undergoes any plasticity, either structural or functional, we cannot support the current model for post-stroke recovery that relies on homotopic brain regions in the neocortex to temporarily adopt the function lost in the infarcted area. The magnitude of computational and structural effort that would be necessary to innervate an entire hemisphere just does not seem feasible in the short time period (3 days post-stroke) suggested by previous studies. Furthermore, the energy required to create these supposed new networks does not seem realistic given that they will be completely abandoned 1-2 weeks later. As no mechanism has been established, the speculation that some unknown signal that rapidly induces the creation of new cortical networks to survive for no more than 2 weeks no longer seems as unassailable. For this reason, I

propose that the peri-infarct region is the primary area involved in reparative plasticity after stroke given that there is a large body of evidence without contradictions showing plasticity in the peri-infarct area after stroke (Brown C.E. LP, Boyd J.D., Delaney K., Murphy T.H., 2007; Ito U *et al.*, 2006; Mostany R *et al.*, 2010). In addition, it seems more likely that homeostatic or active forms of plasticity could occur in the close proximity of the injury rather than in the opposite hemisphere where more large-scale structural plasticity would be necessary.

Another explanation for the activity seen in the spared hemisphere after stroke that has not been discussed is the fact that there is behavioral plasticity to overcome the sensory and motor deficits that result in the recruitment of novel body parts in neighboring cortical areas to the forelimb. For example, after stroke rats' performance on skilled reaching tasks is severely reduced, but by about one month their success rates return to near baseline levels (Tennant KA and TA Jones, 2009). However, what is not obvious from these data is that although rodents reach baseline levels by around one month after, the manner in which they achieve the reaching task is profoundly different. In a normal healthy rat, the animal reaches one arm through a slot in a wall, grabs a food pellet, rotates it's arm, brings the pellet to it's face, and eats the food. Therefore, a healthy rat primarily uses the muscles in its forelimb to achieve this goal. After stroke, however, the animal no longer has the dexterity to successfully reach and grab the food pellet without using other body parts not recruited before stroke. The animal now has to shift onto its unaffected paw to support more of its weight recruiting postural muscles bilaterally. Then the mouse reaches and grabs the pellet, but now has to turn its head to eat the pellet whereas before stroke it had the

manual dexterity to rotate its arm to eat. Therefore, the recruitment of these new muscle groups to achieve the same task as before stroke would lead to increased activation of both sensory and motor areas neighboring the areas of the directly affected limbs. While we are not suggesting that all previous contralateral activity shown is due to recruitment of novel body parts, it is important to note that the interpretation of this previous remapping data may not be as clear as has been suggested previously.

While I argue that the contralateral hemisphere is an unlikely source of plasticity after stroke, there are a number of interesting experiments left undone to challenge or support the established model. For example, because it is thought that the homotopic spared hemisphere expands its receptive field to that of the infarcted area, then one ought to be able to directly stimulate the spared cortex and elicit movement in the affected limb. If movement was elicited by stimulation of the spared hemisphere, then this would strongly support the established model that it is indeed adopting the functional roles of the infarcted area. However, if no movement occurred after stimulation, this would correlate with our data suggesting instead that there is no role for the contralateral hemisphere in recovery after stroke.

While I do not expect other cortical layers in the contralateral hemisphere to exhibit structural plasticity after stroke, it might still be possible that other areas are involved in recovery and result in no downstream changes in L5 apical pyramidal dendrites. For example, it would be interesting to repeat our two-photon imaging experiments looking at basal dendrites instead of apical dendrites. Since some of the previous research showing plasticity after stroke was done in the basal and not apical

dendrites, it would be interesting to see if the basal dendrites showed the same forms of plasticity in vivo as they have previously in fixed tissue (Jones T.A. S, T., 1992). Unfortunately, it is not currently possible to resolve individual spines on the basal dendrites of L5 using two-photon microscopy. In the future it might be possible to improve the resolution to accomplish theses experiments.

Even though it is not currently possible to image basal L5 dendrites with two-photon imaging, it might be possible to track spine dynamics in L4, L2/3, or interneuron dendrites or alternatively track axonal plasticity. It would be interesting to characterize the structural plasticity in these other cortical areas to fully address the controversy of the role of the spared hemisphere in adaptive plasticity after stroke. To go one step further, it would be important to link any possible structural changes to changes in activity or behavior. For example, if future studies provided evidence that there is structural plasticity in inhibitory interneurons in the spared hemisphere after stroke it would be interesting to couple these imaging experiments with in vivo electrophysiology to show accompanying changes in the level of inhibition with the change in structure.

Another interesting future experiment would be to investigate the role of homeostatic plasticity in remapping after stroke. As described earlier, there is potentially a latent network of excitatory connections that are normally suppressed by inhibitory inputs. It would interesting to reduce the levels of inhibition pharmacologically in the affected hemisphere to see if this would encourage recovery through more rapid shifting of functional representations. In fact, there remains a

great deal of work left to be done in the basic characterization of shifting balance in excitation and inhibition in different cortical areas after stroke.

Chapter 5:

Summary and Significance

Summaries and Significance

1. Longitudinal in vivo two-photon imaging could not reveal any evidence for structural plasticity at the level of individual spines; no change in spine density, fraction gained, fraction lost, transient spines, spine stabilization ratio, and survival fraction was detected in the apical tuft of L5 pyramidal neurons in primary somatosensory cortex.
2. No large-scale plasticity of dendrites was detected in either the growth, retraction, or branching of dendrite tips in the apical tuft of L5 pyramidal neurons.
3. In vivo intrinsic optical signal imaging in adult mice could not detect any new activation in the contralateral hemisphere during stimulation of the affected forelimb. This suggests that there is no remapping of activity from the infarcted area to the homotopic contralateral cortex.
4. In juvenile mice there was also no evidence for remapping of activity to the spared hemisphere after stroke during stimulation of the affected forelimb. Even in the young brain where there is presumably a greater potential for plasticity there was no evidence for a role of the contralateral hemisphere in recovery after stroke.

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