UNIVERSITY OF CALIFORNIA,
IRVINE

A sensory stimulation-based collateral therapeutic for ischemic stroke: new insights and limitations

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Aneeka Mikael Hancock

Dissertation Committee:
Professor Ron Frostig, Chair
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2015

Dedication

To my mother Ariel Hancock,

my grandmother Alpha Loris Hancock,
1914-2008

and my father, Michael Odle,
1951-1990

in recognition of their love, support and wisdom

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CURRICULUM VITAE

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TEACHING AND RESEARCH EXPERIENCE

Department of Neurobiology and Behavior, **University of California, Irvine**

Graduate Student — Sept 2010–Dec 2015
Advisor: Prof. Ron Frostig

- Extensive utilization of techniques for *in vivo* imaging of neural activity and blood flow: intrinsic signal optical imaging, and laser speckle imaging. Conduct behavioral (Bederson scoring, cylinder task, and whisker-guided exploration) and histological assays to assess the effectiveness of stroke treatment
- Confirmed long-term protection from ischemic stroke in rats treated with a sensory stimulation-based treatment
- Elucidating the role of collateral vasculature in protected, treated subjects
- Introduced 2 new stroke models to the lab (mice and spontaneously hypertensive rats), and developed experimental protocols for their use
- Collaborate with various labs, and train colleagues and fellow graduate students

Undergraduate Mentor, Frostig Lab — June 2012–Dec 2015

- Trained three student volunteers in my lab and one Undergraduate Research Opportunities Program (UROP) Fellow
- Advising UROP fellow on the development and execution of a research project that will culminate in a poster presentation of their findings at the UCI Undergraduate Research Symposium
- Encourage students to become independent researchers and assist them with attaining their professional goals

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"Stroke and Stem Cells", lecture for N172: Regenerative Neurobiology and BIO SCI 44: Stem Cells and Brain Repair courses

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BIO SCI 36: Drugs and the Brain	Fall 2015
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BIO SCI 44: Stem Cells and Brain Repair	Winter 2015
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- Modified the lab manual and experimental designs to improve usability for better lab outcomes for students

Graduate Student Instructor, N113L Neurobiology Lab Spring 2011, Winter 2012

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- Designed and delivered weekly lectures, supervised students during each lab, designed and graded assignments and exams, and held office hours
- Created a classroom environment that fostered thoughtful student questions and enhanced interest in the course material

Department of Pathology, University of Washington School of Medicine

Research Scientist July 2008–Aug 2010

Supervisor: Prof. Jing Zhang

- Discovered novel Parkinson's disease biomarkers in human brain tissue and body fluids utilizing molecular and high-throughput proteomic techniques
- Spearheaded the development and management of a database to streamline the organization and usage records of thousands of samples, which additionally aided in charting the progress of the numerous projects in the lab
- Developed standard operating procedures for lab specific assays

- Collaborated with lab members, as well as with clinicians and researchers nationally and internationally, on experiments and for sample acquisition, and trained undergraduate student volunteers

Department of Psychology, University of North Carolina, Wilmington

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Supervisor: Prof. Julian Keith

- Researched the restoration of memory function after brain injury through the promotion of hippocampal neurogenesis in rats
- Discovered that BrdU does not have toxic effects on hippocampal cell proliferation or its population of immature neurons
- Implemented stereotactic neurosurgery, behavioral paradigms (Morris water maze), light and confocal microscopy, and immunohistochemistry
- Trained undergraduate student volunteers

PUBLICATIONS AND PRESENTATIONS

Publications:

9. **Hancock AM**, Lay CC, Davis MF and Frostig RD. (2013) *Sensory stimulation-based complete protection from ischemic stroke remains stable at 4 months post-occlusion of MCA.* J Neurol Disord. 1(4):135. doi:10.4172/2329-6895.1000135.

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1. Keith JR, Priester C, Ferguson M, Salling M and **Hancock A.** (2007) *Persistent increases in the pool of doublecortin-expressing neurons in the hippocampus following spatial navigation training.* Behav Brain Res. 188(2):391-7. doi: 10.1016/j.bbr.2007.11.026.

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Presentations:

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Hancock AM, Lay CC, Davis MF, Frostig RD. *Protection of Rodent Cortex from Ischemic Stroke by Mild Sensory Stimulation Remains Stable Over Time*. 42nd Annual Meeting of the Society for Neuroscience, New Orleans, LA; October 2012. Poster Presentation.

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Chancellor's Award (University of North Carolina, Wilmington)	2003

ABSTRACT OF THE DISSERTATION

A sensory stimulation-based collateral therapeutic for ischemic stroke: new insights and limitations

By

Aneeka Mikael Hancock

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2015

Professor Ron D Frostig, Chair

Stroke is currently the leading cause of long-term disability and the fifth leading cause of death in the United States, and prevalence is on the rise due to increasing aging populations. Nearly 90% of strokes are ischemic, where there is a blockage of blood flow to the brain. Despite many clinical and pre-clinical attempts to develop novel treatments, there remains only one FDA-approved treatment for stroke, rtPA, which breaks down the clot, however only about 5% of patients are eligible to receive it. Numerous stage III clinical trials have failed, partially due to not taking major stroke risk factors into account during pre-clinical research. Given the poor prognosis and the fact that 'time is brain' when it comes to stroke damage prevention, there is a clear need to develop a rapid and long-lasting treatment to protect from impending ischemic stroke damage. Harnessing the brains endogenous mechanisms for protection via collateral therapeutics has become an important area of research and a potentially viable solution to prevent ischemic stroke damage. Our lab has discovered that when using a rat model of ischemic stroke (permanent middle cerebral artery occlusion), intermittent sensory stimulation treatment, when delivered within two hours of ischemic onset, completely protects the cortex from

impending ischemic stroke damage. This protection is due in part to retrograde blood flow through collateral vessels and into the occluded middle cerebral artery. This collateral-based treatment is appealing as it is non-invasive and non-pharmacological, and has the potential to be delivered rapidly. This dissertation will present evidence of the translational capabilities of this treatment, and evidence demonstrating potential clinical limitations by testing it under conditions in which the brains vasculature has been altered, either due to anesthesia, stroke risk factors or the species used to model ischemic stroke. We found that the protective effects of this treatment are not dependent on the type of anesthesia utilized, and are long-lasting. Additionally, this treatment was tested in the presence of hypertension, the number one risk factor for ischemic stroke, and also in mice. The results suggest that this collateral-based sensory stimulation treatment does not prevent ischemic stroke damage in hypertensive conditions, in which the vasculature, including collateral vessels, are known to be impaired. Additionally, when a normotensive mouse model was tested, subjects were not protected. Although this strain reportedly has collaterals, they appear to be impaired under the ischemic conditions utilized in our lab. These mice may represent a human subpopulation that do not have fully developed or fully functional collateral vessels. If translational, this work serves to further characterize this treatment and potentially identifies groups of patients that may not exhibit complete protection from ischemic stroke, thus highlighting the importance of the collateral vasculature in the protection from ischemic stroke damage and emphasizing the need for further research on the collateral system to identify variables that can enhance its function.

CHAPTER 1: Introduction

Stroke has been afflicting humans for centuries and has been studied by many great historical figures throughout the years, such as Hippocrates, Galen, Rudolf Virchow, Thomas Willis and others (Pound et al., 1997). Originally referred to as 'apoplexy', used by the Greeks from the time of Hippocrates, it referred to the sudden loss of consciousness and the falling to the ground without sense or voluntary motion, often shortly before death, as if they had been struck by lightning (Cooke, 1820; Pound et al., 1997). Stemming from the work of Hippocrates and Galen, it was thought that apoplexy was caused by anything interfering with the flow of the 'vital spirit' to the brain (Thomas, 1907). There was a sense that the supernatural was at play, that "the stroke of God's hande" or "the stroke of justice" was responsible for this punishment for wrongdoing (Pound et al., 1997). This was a popular notion for centuries, until a shift began in the 17th century when Johann Jakob Wepfer dissected cadavers and by injecting dyes into cerebral vessels and ligating arteries, came to the conclusion in 1658 that either brain hemorrhage or an occlusion of a cerebral artery might be the origin of stroke symptoms (Karenberg, 2004). The perspective and definition of stroke began changing, however, its etiology remained unclear. Circulating theories ranged from the effect of weather to the possibility that things like overeating, restrictive clothing, alcohol, muscular exertion, and extremes of passion could be responsible, or even that particular postures in conjunction with excess body fat could compress the blood vessels and restrict flow (Pound et al., 1997; Daneski et al., 2011). Given this broad range of potential causes, the definition and diagnosis of stroke remained subjective. Treatments varied widely and included bloodletting, vomits and enemas, and it was thought that leading a balanced and moderate life could prevent stroke (Pound et al., 1997).

It wasn't until the late 19th century that the etiology of stroke became more grounded in anatomical and pathological findings from dissections, when WW Gull and HG Sutton described arteriosclerosis and suggested an association with hypertension, and research by Hammarsten on the structure of blood led to the understanding of clot formation (Osler, 1925; Pound et al., 1997). Additionally, with the development of Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) scans in the late 20th century, the diagnosis of stroke could now occur in the living patient.

Today, stroke is defined as an aberration in blood flow that results in damage to the brain or spinal cord. There are two types of stroke: hemorrhagic and ischemic. Hemorrhagic stroke, which is least common, results from the weakening of the blood vessel walls, such that blood leaks out into the surrounding parenchyma. Ischemic stroke, however, comprises nearly 90% of all strokes. This occurs when there is a blockage of a blood vessel by a clot, which reduces blood flow to the brain and results in a region of cell death called an infarct. Since this is the major form of stroke, ischemic stroke is the focus of the remainder of this dissertation.

Ischemic stroke can be the result of thrombosis or an embolism. Thrombosis, the most common cause of stroke, is a localized event due to plaque build-up or some other disturbance to a vessel wall, resulting in a blockage. On the other hand, an embolism occurs when a material from somewhere else in the body, often the heart, becomes lodged in a vessel and blocks blood flow to the brain. Both events reduce blood flow to brain regions downstream of the occlusion, starving the brain of oxygen and glucose, and it is this region that becomes the core of the ischemic tissue.

Ischemic stroke consists of a core and penumbral region, which are distinguished based on their perfusion thresholds. The core can experience <30% of baseline flow, while anywhere

between 30%-70% of baseline flow can be indicative of the penumbra (Astrup et al, 1981; Baron et al., 2001). Due to these varying perfusion thresholds, there are different levels of energy metabolism, ion pumping and neuronal electrical function in each region (Astrup et al., 1981). The perfusion threshold of the penumbra results in functional impairments, but a lack of morphological damage as exists in the core. This creates the potential for recovery if enough blood flow can be restored, and much research is currently focused on finding new therapeutic interventions to rescue the penumbra from evolving into the necrotic ischemic core.

The potential for penumbral tissue to be rescued from necrosis is due to the existence of collateral vessels. These collaterals are a subsidiary network of vascular channels that have been shown to play an important role in redirecting blood flow in ischemic regions, thereby increasing blood flow rates in the penumbral region. The result of this is a rescue of the still viable penumbral tissue from further damage due to the core of the ischemic insult expanding over days after the stroke. However, the extent of this prevention of damage relies not only on the type of stroke that occurs, but the amount of collateral vascularization as well.

Despite advances on this front and in medical imaging, there is still no medical cure for stroke. In fact, out of the numerous studies on therapeutic interventions or neuroprotective therapies, only one has resulted in improved outcome in phase III clinical trials in the past 25 years (Sahota and Savitz, 2011). During the 1990's, the first FDA approved drug treatment was developed, recombinant tissue plasminogen activator (rtPA), which acts by breaking down a clot in order to reduce impending damage. However, it is only approved for a small portion of stroke patients, and can sometimes even be harmful (Adams et al., 1996). Making the discovery of a viable treatment more difficult has been the identification of various risk factors (hypertension, diabetes, and smoking for example), and the resulting heterogeneity of stroke among patients.

Due to the lack of an immediate and long-lasting treatment to prevent stroke damage, clinically, the main focus has been on rehabilitation therapy in order to help stroke patients regain some lost functions after the damage has already occurred. However, even this is not completely successful for all stroke patients, as it can have varying results depending on the type, size and location of the stroke, as well as the length of rehabilitation. Furthermore, many stroke sufferers do not know, or do not immediately recognize the symptoms of stroke, thereby preventing rapid intervention by hospital staff. As a result, stroke is currently the fifth leading cause of death in the United States (the second leading cause of death worldwide), and remains a leading cause of serious, long-term disability (Kochanek et al., 2014; Mozaffarian et al., 2015). With nearly 800,000 people experiencing a stroke each year in the United States, and annual direct and indirect costs nearing 40 billion dollars (Mozaffarian et al., 2015), there is a clear need for a rapid and long-lasting treatment to protect from impending stroke damage.

As numerous phase III clinical trials testing the efficacy of pharmacological interventions on stroke outcomes have failed, it is worthwhile exploring the brains endogenous mechanisms for protection and noninvasive methods for harnessing them. One such mechanism is the collateral vasculature, which affords a high level of redundancy to allow the redirection and redistribution of blood flow in the event of an occlusion. An initial level of redundancy is supplied by the primary collaterals of the circle of Willis, which is formed by the merging of the vertebral, basilar and internal carotid arteries, while another major level is formed by the pial vasculature.

The term ‘collaterals’ was first defined by the 18th century British physician, John Hunter, who described them as native (preexisting) arteriole-to-arteriole anastomoses interconnecting arterial trees (Zhang et al., 2010). Hunter presumed these collaterals existed

within the human thigh and calf, but in 1874 Heubner described their presence in the brain, referring to them as leptomeningeal anastomoses (LMA) that connect some of the outer branches of the anterior (ACA), middle (MCA), and posterior cerebral artery (PCA) trees which supply the human cerebral cortex (Brozici et al., 2003, Zhang et al., 2010). Although LMA were first described by Thomas Willis and others in the 17th and 18th centuries, Heubner was the first to systematically study them, and demonstrated that the ACA, MCA and PCA (pial arteries) have different cortical territories, but that anastomoses between these vessels allowed blood to flow between them (Brozici et al., 2003). The importance of this cerebrovascular redundancy has not always been clear throughout the centuries, but today these collateral vessels are thought to help reduce ischemic damage by improving blood flow within the penumbral region. As such, it comes as no surprise that the salvaging of the ischemic penumbra is currently the prime goal of neuroprotection once a patient arrives at the hospital, and there are studies currently looking at ways to augment this collateral flow in hopes of identifying a new therapeutic target (Shuaib et al., 2011; Ramakrishnan et al., 2012).

Despite the promise of this collateral flow, studies have only shown a reduction of ischemic damage or behavioral improvements, not complete protection or recovery. These findings may be due to a lack of sufficient blood flow within these collateral vessels soon after a stroke occurs, as a patient may not immediately recognize the symptoms. This situation can delay arrival at a hospital, where the time it takes to image the patient's brain in order to determine the type and location of the stroke (and potentially commence treatment) can further compound the issue. If sufficient collateral flow commences too long after the stroke, the return of blood flow to the ischemic region could promote damage due to reperfusion injury, where

oxygenated blood can interact with reactive oxygen species in the ischemic region, resulting in inflammation, blood-brain barrier disruption and cell death.

Collateral vessels are formed during the prenatal period, but functional and structural changes can occur in adulthood after the onset of ischemia (Liebeskind, 2003), such as vasodilation and an increase in the length and tortuosity of anastomosing vessels due to growth stimulated by greater blood flow through them (Coyle and Heistad, 1991). Several factors are responsible for these changes. When an artery is occluded, blood pressure decreases in the distal branches of that arterial vessel, resulting in a pressure gradient between the territory of the occluded vessel and healthy arteries. This gradient results in autoregulation of the collateral vessels, where reflex vasodilation occurs in order to maintain normal blood flow in the affected region via retrograde flow in the occluded vessel (Derdeyn et al., 1998; Liebeskind, 2003).

Additionally, the resting luminal diameter of the major cerebral vessels and the collateral vessels is also an important factor in collateral flow. Luminal diameter ultimately determines the collateral ability of a vessel (Liebeskind, 2003; Qureshi et al., 2008; Tariq and Khatri, 2008), as blood flow is inversely related to resistance, so a reduction in luminal diameter increases resistance, thereby reducing blood flow. Immediately after ischemia, vasodilation of the collateral vessels is triggered, thereby reducing vascular resistance and increasing blood flow. This retrograde flow, although necessary for reduction of the penumbra, is not always sufficient to maintain normal blood flow after a middle cerebral artery occlusion in order to completely protect the affected tissue (Derdeyn et al. 1998). This situation may be due to variable vascular changes from common risk factors, like hypertension and diabetes that are known to reduce luminal diameters (Dirnagl, 2010), which could explain the variability in infarct size in humans, and could also be partly responsible for the discrepancy between the lack of complete ischemic

stroke protection in humans in studies augmenting collateral blood flow and our labs previous findings and those presented in this dissertation.

When developing animal models for neurological disorders and testing potential treatments, it is important to consider that many stroke patients present with at least one risk factor, such as hypertension. However, many studies do not take into account the heterogeneity of stroke and comorbidity when developing new treatments, and for good reason, as it can introduce multiple confounding factors, making results difficult to interpret. However, because the typical stroke patient is around 65 years old with one or more risk factors, it would be advantageous to test any potential therapeutics in the presence of comorbidities, as this is often a reason that clinical trials fail for otherwise promising treatments (Dirnagl et al., 1999). Out of numerous comorbidities and environmental and genetic factors, hypertension is the most important risk factor for ischemic stroke.

Additionally, experimental models of stroke always involve an anesthetized animal, which does not accurately represent the natural state of an individual when they experience stroke. Importantly, anesthesia can affect neurovascular regulation, which could potentially confound stroke research, particularly studies aimed at assessing collateral therapeutics.

We have previously demonstrated that a form of sensory stimulation, intermittent mechanical single-whisker stimulation, when delivered immediately (within 1 hour, and in most cases within 2 hours) after permanent middle cerebral artery occlusion (pMCAO), completely protects rodent cortex from impending functional and structural ischemic stroke damage (Lay et al., 2010; Frostig et al., 2013). We have confirmed that this mild stimulation results in the gradual recovery of cortical function and reperfusion of the MCA via collateral vessels during the treatment period itself (Lay et al., 2010; Lay et al., 2011). Additionally, not only has this

protection been observed in young adult rats (3–4 months of age), but it has also been confirmed in aged rats (21–24 months of age) (Lay et al., 2010; Lay et al., 2011; Lay et al., 2012).

Collateral vessels that anastomose (connect) the distal middle cerebral artery branches with the anterior and posterior cerebral arteries are critical to our sensory stimulation-based treatment. We have shown that occlusion of the distal branches of the MCA, in addition to occlusion of the M1 segment of the MCA, prevents complete protection from impending ischemic stroke damage (Lay et al., 2010). This is because blood originating from other cerebral arteries (collateral vessels) flows through these anastomoses back into the occluded MCA, in the reverse direction (retrograde blood flow). The recall of blood back into the MCA via these collaterals after occlusion is due to activation flow coupling, where the evoked neuronal activity from single whisker stimulation is linked to an increase in blood flow. In this case, the increase in blood flow to the ischemic region completely eliminates a necrotic core and penumbra from forming.

Thus, the focus of this dissertation is to further characterize the translational capabilities of this sensory stimulation-based treatment developed by our lab that is non-invasive, non-pharmacological, rapid, and has no side effects, particularly in cases where the vasculature has been altered, either due to anesthesia, stroke risk factors, or species used to model ischemic stroke.

Chapter 2: Chapter 2 will address whether this sensory stimulation-based treatment can completely prevent impending ischemic stroke damage independent of the type of anesthesia utilized during experimentation. In our previous studies, subjects were anesthetized with sodium pentobarbital, whereas human stroke patients are typically awake. To overcome this drawback, we tested the inhalational anesthetic isoflurane, which allows rats to rapidly recover from

pMCAO within minutes in order to test stimulation treatment in awake rats and to determine whether isoflurane has an effect upon the pMCAO stroke model. We found no difference in infarct volume between pMCAO in untreated controls under either sodium pentobarbital or isoflurane, and the primary finding was that rats that received treatment immediately post-pMCAO maintain cortical function and no stroke damage, whereas rats that received treatment 3 h post-pMCAO exhibited eliminated cortical activity and extensive stroke damage. The only difference between anesthetics was the broad extent of evoked cortical activity observed during both functional imaging and electrophysiological recording, suggesting that the extent of evoked activity evident under isoflurane anesthesia is supported by underlying neuronal activity. Given the high degree of similarity with previous data, we can conclude that the pMCAO stroke model is upheld with the use of isoflurane.

Chapter 3: Chapter 3 investigates the long-term stability of this protection. Instead of the typical assessment at 24 hours post-pMCAO, rats were assessed 4 months post-occlusion. Subjects that received treatment immediately after ischemic onset exhibit normal neural and vascular function, and they are behaviorally and histologically equivalent to healthy controls (surgical shams). Thus, the complete neuroprotection due to cortical activation via sensory stimulation remains stable with time, adding support to the translational potential of this treatment.

Chapter 4: Chapter 4 assesses this treatment's effectiveness in the presence of hypertension, the major risk factor for ischemic stroke. Spontaneously hypertensive rats were utilized which, similar to humans, are known to have impaired vasculature, including dysfunctional collateral blood vessels. These experiments demonstrate that spontaneously hypertensive rats are not protected from ischemic stroke damage at 24 hours post-pMCAO

despite receiving immediate treatment. This finding is critical because it informs us of an important limitation for this collateral-based therapeutic.

Chapter 5: The fifth chapter attempts to extend our findings to mice. Two commonly utilized mouse strains for stroke research, C57BL/6J and CD1 strains, were tested for protection from ischemic stroke. We found that neither strain exhibited protection despite receiving immediate treatment post-pMCAO. Since it is documented that collateral blood vessels exist in these strains, it is possible that these mice have different pathways involved in functional hyperemia compared to rats, resulting in a lack of protection when given this treatment. These mice may represent what could occur when humans with poorly developed collateral vasculature receive immediate intervention with this ischemic stroke treatment.

In summary, this dissertation aims to further characterize the mild sensory stimulation-based treatment developed by our lab, and determine some potential clinical limitations. Our findings highlight the importance of the collateral vasculature in the prevention of ischemic stroke damage. They suggest that despite being a fast-acting, noninvasive, long-term solution for preventing ischemic stroke damage, this treatment may only be completely protective in certain subgroups of patients that do not have impaired vasculature. Not only is it critical to determine the type of stroke that a patient presents with and what comorbidities may exist, care should also be taken to determine the amount of collateral vascularization of the patient before delivering this collateral-based therapeutic.

CHAPTER 2: Experiment I- Early Stimulation Treatment Provides Complete Sensory-Induced Protection from Ischemic Stroke Under Isoflurane Anesthesia

INTRODUCTION

Using a rodent model of ischemia [permanent middle cerebral artery occlusion (pMCAO)], previous studies have shown that single whisker stimulation treatment can completely protect the cortex from impending stroke damage when initiated within 2 h of pMCAO, but when initiated 3 h post-pMCAO, treatment resulted in significant stroke damage (for a recent review see Frostig et al., 2013) (Lay et al., 2010, 2011, 2012; Davis et al., 2011; Frostig et al., 2013). Although these findings demonstrate the translational potential of a drug-free, non-invasive, protective treatment from acute ischemic stroke, subjects in this study, similar to the vast majority of other stroke models, were anesthetized, whereas human stroke patients are typically awake. A better animal model of human stroke should therefore be based on studying the consequences of pMCAO in awake, behaving animals, following only brief anesthesia exposure necessary for the pMCAO procedure. However, the previously described studies were performed using sodium pentobarbital anesthesia, rendering animals unconscious for hours after anesthetic cessation. In this situation, awake, behaving studies, following even brief use of sodium pentobarbital anesthesia for the pMCAO procedure, would be impossible to conduct, as the great majority of experimental subjects would recover from even brief anesthesia beyond the 2 h 'window of opportunity' for sensory-induced protection. Employment of the volatile anesthetic isoflurane, from which rats recover within minutes of anesthetic cessation, seems to be the optimal solution for the shift toward studies of awake, behaving animals following pMCAO, which must be performed under anesthesia.

A potential problem, however, for the use of isoflurane for an animal model of ischemic stroke has emerged from investigations that have assessed the impact of isoflurane on ischemic outcome (Warner et al., 1986; Nehls et al., 1987; Baughman et al., 1988; Gelb et al., 1989; Ishikawa et al., 1989; Kawaguchi et al., 2000; Warner, 2000; Sakai et al., 2007; Makaryus et al., 2011). Results have been discrepant, with many conflicting reports regarding the protective ability of this anesthetic (Warner et al., 1986; Warner, 2000; Dirnagl, 2010). As a result, whether the use of isoflurane positively or negatively affects ischemic outcome remains an open question.

In the present study, we sought to determine if isoflurane can be used effectively within our ischemic stroke model, and whether isoflurane has positive or negative effects on outcome following pMCAO. Our strategy to assess this issue includes a two-pronged approach: (i) the comparison of infarct volume in rats that undergo pMCAO and never receive stimulation treatment under either type of anesthesia, and (ii) replication of our previous findings on the protective or harmful effects of sensory-based treatment obtained under sodium pentobarbital. Functional imaging was conducted on the first day of each experiment prior to pMCAO and stimulation treatment, and again the following day. At the conclusion of day 2 of experimentation, postmortem histology was then performed (Fig. 2.1). The rationale of these studies was that, if we found no difference between animals using both approaches, then the experimental ischemic stroke model would be upheld with the use of isoflurane.

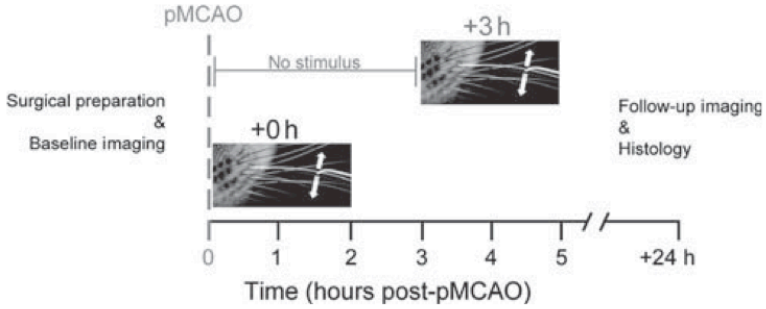

Figure 2.1. Schematic of the experimental design. For both experimental groups, vertical lines indicate the time of pMCAO, time of treatment, and the following 24 h reassessment.

MATERIALS AND METHODS

All procedures were in compliance with NIH guidelines and approved by UC Irvine Animal Care and Use Committee (protocol no. 1997-1608, assurance ID no. A3416.01).

Surgical preparation and anesthesia

Experimental subjects (295–400 g male Sprague Dawley rats) were individually housed in standard cages. All subjects were anesthetized using the inhalational anesthetic isoflurane (halogenated ether 2-chloro-2-difluoromethoxy-1,1,1-trifluoro-ethane), and maintained at 1.0–2.0% (E-Z Anesthesia Machine) isoflurane in 100% oxygen. The current experiments using isoflurane anesthesia were conducted alongside previously published experiments (Lay et al., 2010, 2011), which had used sodium pentobarbital anesthesia. An 'imaging' area (approximately 5 x 6 mm) of the skull over the left somatosensory cortex was thinned. Dextrose (5%; 3 mL) was administered initially and subsequently every 6 h during the experiment. Body temperature was maintained at 37 °C. In a subset of animals, arterial oxygen saturation (min–max, mean ± SE) (77–99, 92 ± 3% oxygen saturation), respiration (21–90, 63 ± 4 respirations per minute), pulse

distension (a proxy for blood pressure; 5– 64, 25 ± 8% vessel distention), and heart rate (275–413, 336 ± 15 beats per minute) were measured using pulse oximetry (Starr Life Sciences, Allison Park, PA, USA) to ensure that any observed changes following pMCAO were not due to variability in vital parameters. Previous studies in our laboratory have shown that pMCAO and stimulation treatment do not alter systemic vital parameters (Lay et al., 2010, 2011), and we again did not observe any significant alterations in vital parameters in this study.

Baseline data collection was followed by pMCAO [double ligature and transection of the stem (primary branch of the middle cerebral artery segment; just distal to the lenticulostriate branch) of the left proximal middle cerebral artery] (Lay et al., 2010; Frostig et al., 2013).

Histology (2, 3, 5-triphenyltetrazolium chloride staining for infarct)

At the conclusion of each experiment, rats were euthanized with sodium pentobarbital (2-3 mL, intraperitoneally), the brain was removed, sectioned into 2 mm coronal slices, and incubated in 2% 2,3,5-triphenyltetrazolium chloride at 37 °C for 20 min in the dark (Bederson et al., 1986). The infarct volume was determined by an observer blind to experimental condition. A small amount of damage occasionally produced at the surgical site was excluded from infarct analysis (Tamura et al., 1981). Infarct volume comparisons were performed by employing two-sample t-tests.

Stimulation treatment

As in previous studies (Lay et al., 2010, 2011, 2012; Davis et al., 2011), a total of 1280 whisker deflections, delivered in 256 events (five whisker deflections per event in a 5 Hz pattern) at varying intervals of 21 ± 5 s between onsets of consecutive events, was distributed over 120

min.

Intrinsic signal optical imaging and analysis

We used intrinsic signal optical imaging (ISOI) to assess the evoked functional response to single whisker stimulation (whisker functional representation). For a recent review of ISOI see Frostig & Chen-Bee (2009). A detailed description of ISOI (Grinvald et al., 1986; Frostig et al., 1990; Ts'o et al., 1990) data acquisition and analysis can be found elsewhere (Chen-Bee et al., 2000, 2007). Briefly, a charge-coupled device camera was used for imaging with red light illumination. Post-stimulus ratio images were created by calculating the fractional change (FC) values relative to activity collected immediately before stimulus onset. The first two phases of evoked functional representation, the 'initial dip' and 'overshoot', were analyzed. The ratio image containing the maximum areal extent was quantified at a threshold level of 5.0×10^{-4} away from zero. Although previous work has utilized 2.5×10^{-4} FC, the higher 5.0×10^{-4} FC threshold was chosen here to achieve areal extent values that were comparable to the previous studies. Peak amplitude was quantified in FC units from the pixel with peak activity within the maximum areal extent for each of the two phases.

As there were no responses to quantify in animals that received whisker stimulation 3 h after ischemic onset (+ 3 h), the post-pMCAO imaging evoked area and amplitude were converted to difference score values (post-occlusion - baseline), with values away from 0 signifying a change from baseline. A constant was added to allow for ANOVA, scores were transformed with a natural log function to better satisfy the assumptions of an ANOVA, and inferential statistics were performed on the transformed data. The alpha level was set to 0.05 and Bonferroni adjustments were applied to account for multiple contrasts. Separate ANOVAs followed

by respective contrasts were performed for the two phases of the whisker functional representation. The alpha level was set to 0.05 and a Bonferroni adjustment was applied to accommodate the two contrasts (P = 0.05/2 = 0.025).

Electrophysiology – extracellular recording and analysis

We used extracellular recording to confirm that the extent of activity observed during functional imaging was reflective of whisker-evoked subthreshold/suprathreshold cortical activity. ISOI was performed to identify the location of peak optical activity evoked by whisker stimulation in order to guide the proper placement of electrodes for subsequent neuronal recording (Masino et al., 1993; Brett-Green et al., 2001; Frostig et al., 2008). Neural activity was filtered and amplified simultaneously from an in-line straight array of seven low-impedance (1–2 mΩ) tungsten microelectrodes (Microprobe) using a multi-channel acquisition system (Alpha Omega). The electrode array was positioned with the second electrode placed at the peak of ISOI activity. Suprathreshold [multi-unit activity (MUA)] and subthreshold [local field potential (LFP)] evoked neuronal activity was obtained from a depth of approximately 300–400 μm (supragranular layer) below the cortical surface. Recorded signals were amplified and bandpass (1–3000 Hz) filtered to allow the simultaneous capture of MUA and LFP from the same electrode, and then digitized at a rate of 24 kHz. Recording sessions consisted of the same whisker stimulation parameters as used during ISOI. SPIKE2 software was used for the off-line extraction of MUA and LFP by refiltering the collected data in either the 1–300 Hz range (LFP) or 300–3000 Hz range (MUA) for the subsequent analysis. A single average LFP waveform or MUA response in 1 ms bins was generated from 64 stimulation trials. LFP and MUA magnitudes were then calculated based on the first peak 'on' response minus the mean obtained from a 1 s duration of pre-stimulus data.

RESULTS

Isoflurane does not alter infarct volume post-permanent middle cerebral artery occlusion in untreated rats

In order to establish whether isoflurane by itself has an effect upon the damage sustained following pMCAO, our first goal was to determine whether rats anesthetized with isoflurane (Fig. 2.2 A) sustain a similar degree of infarct volume compared with those anesthetized with sodium pentobarbital. Untreated controls (rats underwent pMCAO but never received stimulation treatment; n = 8) were compared with an identical untreated control group anesthetized with sodium pentobarbital (n = 10, previously published data) (Lay et al., 2010, 2011). We found that untreated controls anesthetized with isoflurane sustained the same degree of infarct [range, 19.4–45.9 mm^3, 30.6 ± 3.2 mm^3 (mean ± SEM)] as those anesthetized with sodium pentobarbital [previously published data; range, 13.0–35.0 mm^3, 23.2 ± 2.3 mm^3 (mean ± SEM); $t_{16} = -1.94$, $P = 0.07$; Fig. 2.2 B, left] (Lay et al., 2010). Under these conditions, isoflurane did not influence infarct volume following pMCAO, and is therefore not protective in this experimental model.

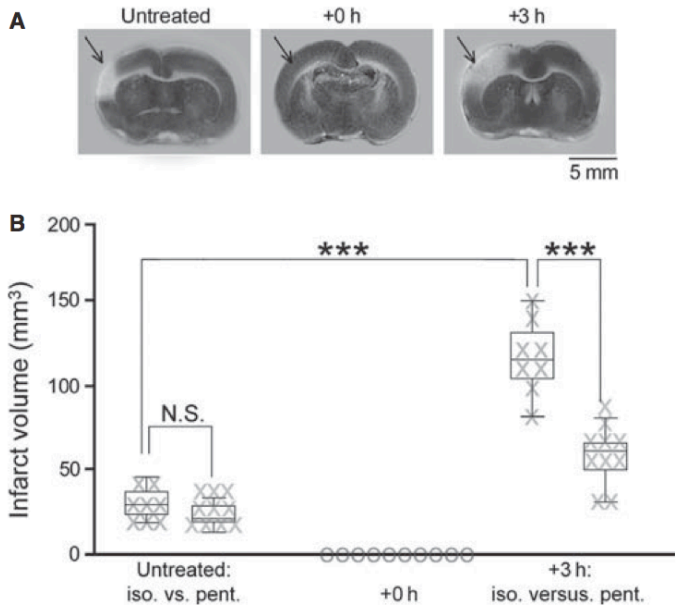

Figure 2.2. Box-and-whisker plots, with individual data plotted, of the volume of infarct sustained by animals that underwent – pMCAO (Untreated controls), pMCAO and single whisker stimulation immediately (+ 0 h), or 3 h (+ 3 h) post-occlusion as assessed via 2,3,5-triphenyltetrazolium chloride assay for infarct. No-stimulation controls never received whisker stimulation, yet underwent anesthetic and surgical procedures (including pMCAO) that were identical to those of the experimental groups. Significant difference in infarct volume between groups (***$P < 0.0001$).

Cortical function is completely protected as a result of immediate stimulation treatment

After confirming that isoflurane does not alter the ischemic challenge placed upon the cortex by pMCAO, we proceeded by studying the effects of stimulation treatment. All subjects underwent pMCAO, and were randomly assigned to one of two experimental groups (n = 8 per group). Following pMCAO, whisker stimulation treatment (designed to mimic the rodent's natural whisker use) was delivered either immediately (+ 0 h group), or 3 h (+ 3 h group) after ischemic onset (Fig. 2.1). The ISOI revealed that rats that received treatment immediately after pMCAO (+ 0 h) maintained whisker functional representation 24 h post-pMCAO compared with baseline, and did not sustain stroke damage (Fig. 2.3A, bottom row). In contrast, treatment

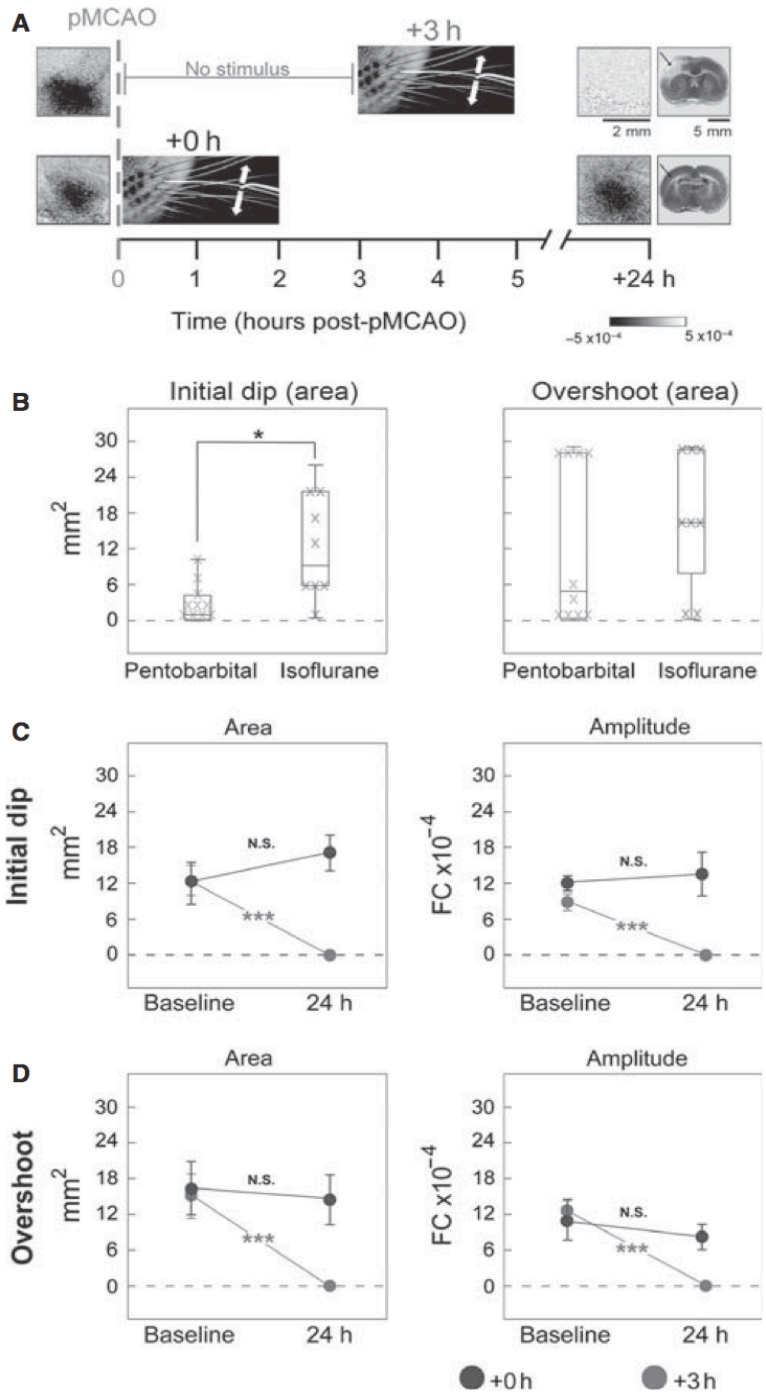

Figure 2.3. Isoflurane is an effective alternative to sodium pentobarbital for use in sensory-induced neuroprotective studies. (A) Representative data from ISOI of the initial dip for + 0 h and + 3 h groups, and from 2,3,5-triphenyltetrazolium chloride (TTC) staining of + 0 h and + 3 h groups for comparison. All + 0 h subjects regained whisker functional representation and did not sustain infarct, whereas + 3 h rats never demonstrated any post-pMCAO cortical activity and sustained infarct larger than that of untreated controls (staining indicates healthy tissue, lack of staining would indicate ischemic infarct).

(Figure 2.3 cont.) Linear grayscale bar indicates intrinsic signal strength x 10^{-4}. Scale bar below imaging data indicates 2 mm, the bar below TTC indicates 5 mm, and arrows indicate approximate region vulnerable to pMCAO infarct. (B) Box-and-whisker plots, with individual data plotted, for quantitative comparison of whisker functional representation observed at baseline in + 0 h animals anesthetized with isoflurane (black) or sodium pentobarbital (gray). Both the area of the initial dip (left) and overshoot (right) are included for comparison. Significant difference between the area of the initial dip evoked while under sodium pentobarbital vs. isoflurane anesthesia (*P < 0.05). Quantitative analysis of whisker functional representation in terms of area (left) and amplitude (right) of the initial dip (C) and overshoot (D). Group baseline and 24 h post-pMCAO data are plotted in each graph. A value of zero indicates no response. Means and SEs are provided for the area and amplitude of the whisker functional representation. Significant differences between group baseline and 24 h values (***P < 0.001).

delivered 3 h post-pMCAO (+ 3 h) failed to restore whisker functional representation, and resulted in a substantial infarct (Fig. 2.3A, top row). Importantly, we also observed a large increase in the initial dip (t_{16} = -2.27, P = 0.04) at baseline for all rats anesthetized with isoflurane compared with previous studies in which rats were anesthetized with sodium pentobarbital (Fig. 2.3 B, left). In addition, the overshoot phase frequently extended beyond the region of the cortex being monitored (maximum area of cortex monitored was 28.04 mm^2), artificially limiting the full areal extent of overshoot activity. As a result, there was also a trend for the overshoot to increase compared with rats anesthetized with sodium pentobarbital (Fig. 2.3 B, right), although the difference was not significant.

Given the increase in activity, we chose to quantify all ISOI data (Fig. 2.3 C and D) at a higher threshold of analysis to achieve areal extent values that were comparable to the previous studies (Fig. 2.4). The area and amplitude of evoked activity were quantified for the first two phases (initial dip and overshoot) of the whisker functional representation. Between-subject ANOVAs of imaging data were conducted between + 0 h and + 3 h rats at baseline, and at 24 h following treatment. At baseline, there were no between-group differences in the initial dip (area: $F_{1,14}$ = 0.01, P = 0.91; amplitude: $F_{1,14}$ = 3.40, P = 0.09; ANOVA), or overshoot phases (area: $F_{1,14}$ =

0.07, P = 0.80; amplitude: $F_{1,14}$ = 0.07, P = 0.79; ANOVA).

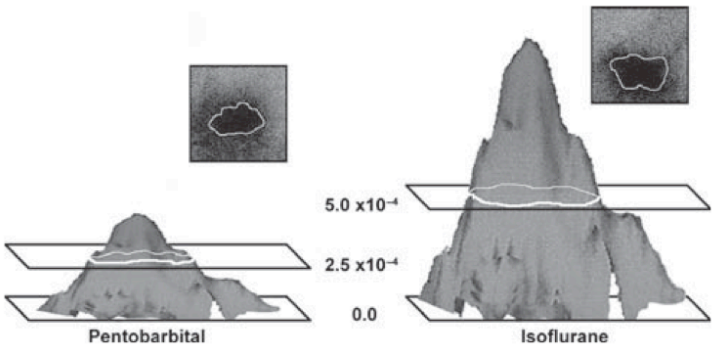

Figure 2.4. The whisker functional representation is quantified at a higher threshold of analysis under isoflurane anesthesia. Each phase of the whisker functional representation may be rendered three-dimensionally by plotting FC along the z-axis and its two-dimensional areal extent may be visualized and quantified at incrementally higher thresholds. The threshold used in this study is 5.0 x 10^{-4} FC (used to quantify the area of activity). Although pre- vious work has utilised 2.5 x 10^{-4} FC, the higher 5.0 9 10^{-4} FC threshold was chosen here to achieve areal extent values that were comparable to the previous studies. Outlined in white, a + 0 h subject's initial dip is visualised at 2.5 x 10^{-4} FC under sodium pentobarbital anesthesia (left), and a + 0 h subject's initial dip is visualised at 5.0 x 10^{-4} FC under isoflurane (right).

At 24 h after pMCAO, differences were found between groups for both the initial dip and overshoot (initial dip area: $F_{1,14}$ = 16.37, P = 0.001; amplitude: $F_{1,14}$ = 22.58, P = 0.0003; overshoot amplitude: $F_{1,14}$ = 5.73, P = 0.03; ANOVA). In the + 0 h group, every subject's entire whisker functional representation remained at baseline levels, and the entire whisker functional representation for every subject in the + 3 h group had been reduced to zero (initial dip area: $F_{1,14}$ = 21.69, P = 0.0004; amplitude: $F_{1,14}$ = 33.62, P = 0.00005; overshoot amplitude: $F_{1,14}$ = 18.58, P = 0.0007; Fig. 2.3 C and D).

Although the overshoot area shared the same trends as the initial dip (+ 0 h maintain the whisker functional representation, whereas the whisker functional representation is reduced to 0 in the + 3 h group, for subjects anesthetized with isoflurane or pentobarbital), a between-group

ANOVA performed on the overshoot area did not find a significant difference ($F_{1,14} = 3.54$, $P = 0.08$; ANOVA). Given the strong qualitative difference observed between rats that maintained the overshoot area (+ 0 h) and rats that lost the overshoot area (+ 3 h), however, we sought to statistically evaluate this observation with two paired t-tests, performed separately on each group. The change in overshoot area after pMCAO was significant in the + 3 h group ($t_7 = 4.34$, $P = 0.003$), but remained unchanged in the + 0 h group ($t_7 = 0.35$, $P = 0.74$).

In summary, when stimulation treatment was delivered immediately (+ 0 h) after pMCAO, whisker functional representation was re-established to levels equivalent to baseline and there was no infarct 24 h post-pMCAO. Treatment delivered 3 h post-pMCAO (+ 3 h) failed to restore whisker functional representation and resulted in substantial infarct (Fig. 2.3 A, C and D).

Stimulation treatment results in an increase in cortical damage when delivered 3 h post-permanent middle cerebral artery occlusion

An additional critical factor of stimulation treatment 3 h following pMCAO (+ 3 h) was that treatment at this time increased the degree of infarct sustained compared with controls (Lay et al., 2010; Davis et al., 2011). In order to determine whether the same increase in stroke damage occurred under isoflurane, we compared the infarct volume sustained by + 3 h subjects with untreated controls. We found that + 3 h animals anesthetized with isoflurane sustained infarct volumes averaging 119.3 ± 8.1 mm^3 (range, 83.1–153.7 mm^3; Fig. 2.2 B, right). This value was significantly greater than that of untreated controls also anesthetized with isoflurane ($t_{14} = 10.20$, $P = 7.3 \times 10^{-8}$; Fig. 2.2 B). This result is in agreement with previous findings, which have shown that sensory stimulation delivered 3 h post-pMCAO exacerbates ischemic damage

(Lay et al., 2010; Davis et al., 2011).

Interestingly, differences in infarct volume were also observed between + 3 h subjects anesthetized with isoflurane and + 3 h subjects anesthetized with sodium pentobarbital (Fig. 2.2 B, right). We found that + 3 h subjects anesthetized with isoflurane sustained larger infarcts than those of + 3 h subjects anesthetized with sodium pentobarbital [previously published data; range, 30.4–82.6 mm^3; mean, 59.2 ± 4.4 mm^3 (mean ± SEM); t_{16} = _6.90, P = 3.6 x 10^{-6}] (Lay et al., 2010, 2011).

Neural recordings support the extent of activity observed during functional imaging

What might explain the increase in the volume of infarct sustained by + 3 h subjects anesthetized with isoflurane vs. those anesthetized with sodium pentobarbital? Previously, we have argued that cortical activation may play a key role in determining the extent of infarct sustained by + 3 h subjects (Lay et al., 2010; Davis et al., 2011; Frostig et al., 2013). Given that + 3 h subjects under isoflurane demonstrate a greater extent of cortical activation (measured here as whisker functional representation) in response to single whisker stimulation when compared with those under sodium pentobarbital, we wished to confirm that the increase in whisker functional representation was truly reflective of evoked cortical subthreshold and suprathreshold activity. We therefore corroborated our ISOI data by directly measuring neuronal activity.

The LFP (subthreshold activity) and MUA (suprathreshold activity) were recorded from control rats (n = 5, Fig. 2.5) using a whisker stimulation paradigm that was identical to that used during imaging. Two primary observations were made. First, unlike the neuronal response to whisker stimulation under sodium pentobarbital, LFP and MUA activity undergo a 'fast-

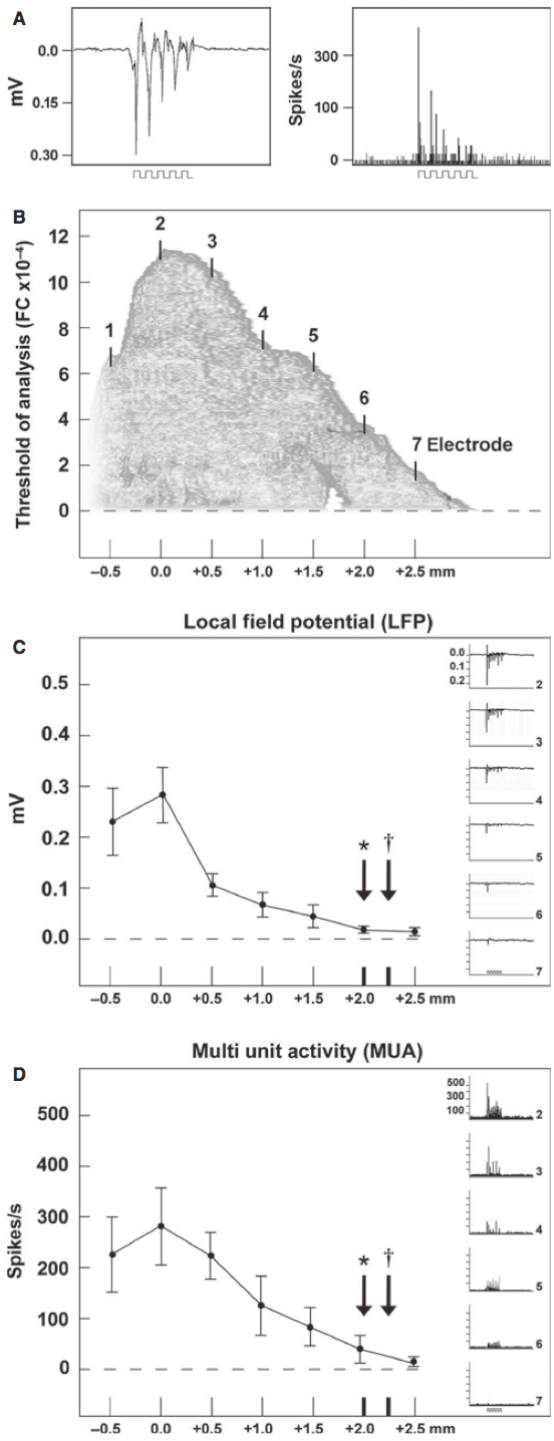

Figure 2.5. Evoked neuronal activity underlies the whisker functional representation. (A) Representative LFP (measured in mV) and MUA (measured in spikes/s) responses recorded from a control rat anesthetized with isoflurane. Stepping function indicates stimulus delivery. Note the fast adaptation of both the LFP and MUA response to whisker stimulation. (B) Approximate placement of the seven-electrode array superimposed upon the initial dip. The X-axis represents the distance from the peak of the

(Figure 2.5 cont.) initial dip. The Y-axis represents the amplitude of whisker-evoked initial dip activity at a given distance from the peak. In this example, the initial dip is an average of the five rats used for extracellular recording. Evoked LFP (C) and MUA (D) activity plotted along the electrode array. Mean and SE are plotted for each recording site. The X-axis represents the distance from the peak of whisker-evoked activity, and arrows along the X-axis represent the average radius of the initial dip (arrows with asterisks) and overshoot (arrows with daggers) observed in recording subjects during ISOI mapping. Insets – representative LFP and MUA data from recording electrodes 2 (peak activity) to 7. Stepping function indicates stimulus delivery.

adapting' response to whisker stimulation such that evoked activity rapidly decreases across each of the five whisker deflections during stimulation (Fig. 2.5 A, left and right). Second, in order to compare the extent of LFP, MUA, and whisker functional representation, the seven-electrode array was positioned so that electrode 2 was located within the peak of the initial dip (Fig. 2.5 B). Both LFP (Fig. 2.5 C) and MUA (Fig. 2.5 D) met and surpassed the average radius of the initial dip and overshoot phases of the whisker functional representation, which were collected for each rat prior to neural recording and analyzed at the 5.0×10^{-4} (FC) threshold of analysis used in this study (Fig. 2.5 C and D). These results served as confirmation that our imaging data reflected the extent of whisker-evoked activity. Moreover, it should be noted that the whisker functional representation is most typically quantified at 2.5×10^{-4} (FC), half that of the threshold of analysis employed here. Even the seven-electrode array, which covered a line of 2.5 mm between the electrode located at the peak of the initial dip (electrode 2) and the furthest electrode (electrode 7), does not record the full extent of evoked activity, and therefore under-represents the striking degree of cortical activation that occurs as a result of whisker stimulation in rats anesthetized with isoflurane.

Furthermore, the observed increase in whisker functional representation, LFP, and MUA all coincided with an increase in infarct sustained in + 3 h subjects. This result further strengthens our previous suggestion that the extent of evoked cortical activity plays a critical role

in determining the extent of ischemic infarct. The finding that activity plays a central role in the evolution of infarct is perhaps surprising given that, currently in both humans and rats, dogma states that the area of the brain that sustains infarct is dictated solely by the spatial architecture of the vasculature that it is supplied by (Coyle, 1986, 1987; Wei et al., 1995, 1998; Dirnagl et al., 1999; Caplan, 2009). In contrast, however, we have suggested that cortical activity may play a central role in determining the extent of protection conferred during early treatment, and the extent of infarct sustained during late treatment (3 h) following pMCAO (Lay et al., 2010, 2011; Davis et al., 2011; Frostig et al., 2013).

DISCUSSION

This study characterized the impact of isoflurane use upon our laboratory's experimental model of stroke (pMCAO), and employed a two-pronged approach to determine whether isoflurane has positive or negative effects following pMCAO. First, we found that isoflurane does not affect the volume of ischemic damage sustained by adult rats that undergo pMCAO vs. those anesthetized with sodium pentobarbital. We then characterized the effects of single whisker stimulation treatment (stimulation was intermittently delivered 256 times, with random intervals averaging 21 s, totaling 4.27 min of stimulation, over the course of 2 h), and found that the adult rat cortex responds to protective (+ 0 h) treatment, and harmful (+ 3 h) treatment in a manner similar to previously published research utilizing sodium pentobarbital as the anesthetic (Lay et al., 2010, 2012; Davis et al., 2011; Frostig et al., 2013). Finally, we found that there was an increase in the extent of the cortex activated by single whisker stimulation, and report a strong correspondence between functional imaging (ISOI) and electrophysiological recordings taken under isoflurane administration. Together, these results demonstrate that sensory-induced

protection from ischemic stroke is not affected by the use of isoflurane, and allow for highly detailed investigation of potential novel treatments for ischemic stroke using awake, behaving animals.

In addition to the data presented here, a large amount of pre-clinical study has gone into the investigation of isoflurane as a neuroprotective agent. It has been reported that rats having undergone hemispheric ischemia had smaller infarct volumes after being exposed to isoflurane vs. nitrous oxide sedation (Baughman et al., 1988). However, in a study by Warner et al. (1986), rats showed no benefit from isoflurane pre-treatment vs. untreated controls vs. transient carotid artery occlusion, although this discrepancy could be due to the location or method of infarct. Baboons under isoflurane had a worse outcome vs. thiopental or fentanyl groups following a transient MCAO (Nehls et al., 1987), and macaques received no benefit from isoflurane vs. halothane (Gelb et al., 1989). In a human study, subjects maintained neuronal electrical activity at significantly lower levels of cerebral blood flow during isoflurane anesthesia vs. halothane controls (Michenfelder et al., 1987). When considering this body of work in totality, the two major challenges to making effective between-group comparisons are – the use of many forms of anesthesia between studies, and the lack of standardization of isoflurane delivery (isoflurane concentration, oxygen, oxygen/nitrogen mixes). Whether (and in what cases) isoflurane is capable of effective neuroprotection remains unclear. In our hands, pMCAO results in a consistent infarct size, and stimulation treatment remains effective in completely protecting both the function and structure of the cortex under isoflurane administration when delivered in 100% oxygen, compared with sodium pentobarbital-anesthetized rats.

With respect to our laboratory's utilization of isoflurane vs. sodium pentobarbital, two critical differences readily became apparent. (i) Even relatively low concentrations (1–1.5%) of

isoflurane can impact neuroimaging (Berger et al., 2007; Alkire, 2008; Alkire et al., 2008; Schummers et al., 2008). Whisker stimulation resulted in a large area of cortical activation under isoflurane, and necessitated the use of a higher threshold of analysis, double our typical threshold used for quantifying the whisker functional representation of animals anesthetized with sodium pentobarbital. (ii) Whereas untreated controls sustained equal infarct volumes when anesthetized with either anesthesia, rats that received stimulation treatment 3 h post-pMCAO (+ 3 h) sustained greater infarct volumes when anesthetized with isoflurane than those anesthetized with sodium pentobarbital.

Is there a relationship between these two key differences? Using ISOI to assess evoked cortical function, we found a significant increase in the extent of the initial dip under isoflurane when compared with functional imaging conducted using sodium pentobarbital. Using extracellular recording, we found that both subthreshold and suprathreshold neural activity underlie the large extent of evoked activity observed. Previous work in our laboratory has demonstrated that an increase in the extent of cortical activation 3 h post-stroke onset results in an increase in infarct volume sustained (Davis et al., 2011). In this case, an increase in cortical activation extent was achieved by stimulating the entire whisker array rather than a single C2 whisker. We therefore hypothesized that an isoflurane-induced increase in cortical activation would similarly result in an increase in infarct volume amongst + 3 h rats compared with + 3 h rats anesthetized with sodium pentobarbital. This hypothesis was supported by the finding that + 3 h rats anesthetized with isoflurane sustained a significantly greater infarct volume (119.3 ± 8.1 mm^3), which is nearly double the average infarct volume sustained by + 3 h subjects under sodium pentobarbital anesthesia (61.8 ± 3.7 mm^3). Furthermore, in order to obtain comparable whisker functional representation extent values, the threshold of analysis needed to quantify our

functional imaging data was also twice the normal threshold (2.5×10^{-4} to 5.0×10^{-4} FC), further suggesting that an even more explicit relationship exists between the area of activation and infarct volume sustained. Together, these findings further underscore the powerful role that cortical activity plays within the context of cortical ischemia.

Although differences in the amplitude and areal extent of whisker functional representation were observed under isoflurane vs. sodium pentobarbital, several key similarities were also observed that allowed us to resolve the issue of whether this anesthetic is an appropriate and logical alternative to sodium pentobarbital.

First, although fiercely debated, isoflurane administration has been found to be neuroprotective in multiple animal models of stroke (Kawaguchi et al., 2000; Warner, 2000; Sakai et al., 2007). Our laboratory employs a permanent occlusion of the middle cerebral artery (pMCAO), and our data showed that rats that undergo pMCAO and never receive whisker stimulation sustained equal infarct volumes when sedated with either isoflurane or sodium pentobarbital. Therefore, in our model, we concluded that isoflurane is not neuroprotective. Second, stimulation treatment delivered immediately following pMCAO resulted in the complete protection of cortical function and structure as investigated via ISOI and 2,3,5-triphenyltetrazolium chloride histology. The finding that this level of protection occurs irrespective of a particular anesthetic state considerably widens the applicability of this potential treatment strategy.

We have shown previously that every young adult and aged rat that receives stimulation treatment immediately post-pMCAO also demonstrates fully intact behavioral capability and cortical structure when assessed 1 week following ischemic onset (Lay et al., 2010, 2012). We

have also demonstrated that the whisker functional representation, in addition to blood flow, physical capability, and tissue remain completely intact up to 4 months post-pMCAO and immediate stimulation treatment (Hancock et al., 2013). Although these findings strongly indicate that immediate stimulation treatment administered under isoflurane would also confer a similarly long-lasting protective effect, this question is beyond the scope of the current study.

Finally, + 3 h rats anesthetized with isoflurane lose all cortical function and sustain exacerbated infarct in a manner that is very similar to + 3 h rats anesthetized with sodium pentobarbital. The finding that the identical stimulation treatment is protective when delivered early (immediate treatment) and harmful when delivered too late (3 h treatment) indicates that the effects of sensory-induced activity occur irrespective of anesthesia type, and reflects protective processes innate to brain function. This finding would support the notion that the same brain processes described here may be applicable to the human stroke sufferer.

Given the high degree of similarity between these results and those that used sodium pentobarbital, we conclude that, despite the changes in cortical activation patterns compared with sodium pentobarbital, the use of isoflurane anesthesia does not affect outcome in our particular stroke model, at least by 24 h following stroke onset. In our hands, pMCAO results in a consistent infarct size under isoflurane administration. With the addition of stimulation treatment, we found that animals treated immediately post-pMCAO were completely protected from cortical dysfunction and infarct, whereas those treated 3 h post-pMCAO lost all function and sustained significant ischemic damage. Finally, we found a strong correspondence between functional imaging with subthreshold and suprathreshold recordings, and noted a large extent of evoked cortical activation with both techniques. Together, these findings further support the dynamic nature of sensory-induced protection from ischemic stroke, underscore its independence

upon a particular anesthetic state, and allow for future studies to more accurately reflect the state of stroke victims by providing the possibility for awake, behaving study.

CHAPTER 3: Experiment II- Sensory Stimulation-Based Complete Protection from Ischemic Stroke Remains Stable at 4 Months Post-Occlusion of MCA.

INTRODUCTION

Stroke is the fifth leading cause of death in the United States, and is also a leading cause of long-term disability, with annual direct and indirect costs nearing 40 billion dollars (Mozaffarian et al., 2015). The aftermath of a stroke can include hemiparesis, cognitive deficits, depression, dependency on others for daily living, aphasia, and even institutionalization (Mozaffarian et al., 2015; Petrea et al., 2009). Despite the fact that numerous neuroprotective therapies have been tested in rodents over the past 20 years, none have resulted in improved outcome in phase III clinical trials (Sahota and Savitz, 2011). Currently, the only FDA approved drug for ischemic stroke is recombinant tissue plasminogen activator (rt-PA), which can only be given to certain subgroups of patients (Albers, 1997), if the patient quickly arrives at the hospital after the incident and suffers from an ischemic event (NINDS rt-PA Stroke Study Group, 1995; Adams et al., 1996), which comprise 87% of all strokes (Mozaffarian et al., 2015). Even then, this drug can have harmful side effects such as hemorrhagic transformation (Adams et al., 1996; Lapchak, 2002). Clearly, there is a need for a rapid and long-lasting treatment to protect from stroke damage.

We have previously demonstrated that a form of mild sensory stimulation, intermittent mechanical single-whisker stimulation, when delivered immediately (within 1 hour, and in most cases within 2 hours) after permanent middle cerebral artery occlusion (pMCAO), completely protects rodent cortex from impending functional and structural ischemic stroke damage (Lay et al., 2010) (reviewed by Frostig et al., 2013). Treatment consisted of 4.27 minutes of 1-second, 5-

Hz, 9° deflections of a single whisker intermittently during a 120-minute treatment period. Utilizing multiple techniques, such as functional imaging, blood flow imaging, electrophysiological recording, behavioral assessment, and histology, we have confirmed that this mild stimulation results in the gradual recovery of cortical function and reperfusion of the MCA via collateral vessels during the treatment period itself (Lay et al., 2010, 2011). Functional imaging, blood flow imaging, and neuronal recordings showed that cortical function was at or above baseline levels at 24 hours post-pMCAO, while behavioral assessment at 7 days post-pMCAO revealed that rats had no sensorimotor deficits, and histological analysis at 24 hours and 7 days post-pMCAO showed no infarct (Lay et al., 2010). This protection has been observed in young adult rats (3–4 months of age), as well as in aged rats (21–24 months of age) (Lay et al., 2010, 2011, 2012). Non-stimulated control subjects, those that received the pMCAO but no whisker stimulation, showed reduced whisker representations with functional imaging (ISOI), and sustained infarcts according to TTC staining, when assessed 24 hours post-occlusion.

A pivotal question related to the translational potential of these findings is whether this complete protection from impending stroke damage is present for only a short duration, or whether it is truly long-lasting, especially given the major neurovascular plasticity that occurred in these animals enabling reperfusion of the ischemic area. Namely, following whisker stimulation, blood flows in reverse of its normal direction within the permanently occluded MCA, a flow originating from collateral vessels (Lay et al., 2010). To address this question, we assessed rats 4 months post-pMCAO to determine whether cortical function remained intact in rats that received immediate post-occlusion whisker stimulation. Accordingly, we focused solely on protection conferred by whisker stimulation, rather than recovery from ischemic damage in non-stimulated controls, which is qualitatively a different study. Employing functional imaging,

blood flow imaging, behavioral assessment and histology, we demonstrate that in rats receiving a middle cerebral artery occlusion followed immediately by whisker stimulation (+0h subjects, meaning zero hours between time of occlusion and onset of stimulation), cortical function remains intact, blood flow stable, structure undamaged, and behavioral measures are normal compared to a sham-surgery control group, when assessed at 4 months post-pMCAO. Given that 4 months in rats is a significant portion of their lives, equivalent to 10–15 years in humans (Quinn, 2005), the presence of intact cortical function and structure in the +0h subjects at 4 months post-occlusion suggests that this treatment results in a quick and stable protection from ischemic damage following pMCAO.

METHODS

All procedures were in compliance with NIH guidelines and approved by UC Irvine Animal Care and Use Committee (protocol #: 1997-1608, assurance ID#: A3416.01).

Subjects and surgical preparation

Twenty-four experimental subjects, 295–400 g (3–4 months of age) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA), were individually housed in standard cages. At the beginning of each experiment, subjects were injected intraperitoneally with a Nembutal bolus (55 mg/kg b.w.). Supplemental injections of Nembutal (27.5 mg/kg b.w.) were given as necessary. After resection of soft tissue, a ~6.5×8 mm 'imaging' area of the skull over the left primary somatosensory cortex (rostromedial corner positioned approximately 1 mm caudal and 2 mm lateral from bregma) was thinned to ~150 μm using a dental drill. 5% dextrose (3 mL) and atropine (0.05 mg/kg, b.w.) were administered at the beginning of the experiment and every six hours after until the animal was returned to its home cage (the first day of each

experiment typically lasted 8 to 10 hours and the second day, at 4 months post-occlusion, typically lasted 6 to 8 hours). Body temperature was measured via a rectal probe, and maintained at 37° Celsius by a self-regulating thermal blanket. After the completion of the experiment, all animals were returned to their home cage and allowed to recover. All subjects received flumeglumine (2.5 mg/kg b.w.) at the end of surgery, and the health of the animals were monitored daily until their 4 month assessment. Animals remained housed in their home cage throughout the 4-month period.

Overview

Functional imaging, blood flow imaging, and behavior timelines are summarized in Figure 3.1. Using a within subject design that is identical to our previous studies, 24 subjects were randomly assigned to a +0h group or a sham surgical control group. Baseline functional imaging was collected for all subjects at the beginning of surgery. All +0h subjects (n=12) then received a pMCAO, and immediate post-occlusion whisker stimulation. Post-occlusion whisker stimulation consisted of 1 s of 5 Hz deflections of a single whisker (whisker C2). This stimulation was intermittently (with random intervals averaging 21 seconds) delivered 256 times, totaling 4.27 minutes of stimulation, over the course of 2 hours (Lay et al., 2010). Surgical shams (n=12) underwent identical surgery to that of +0h subjects, with the suture needle and thread passing under the MCA, but sutures were not tied around the MCA, leaving the blood vessel intact. Sham surgery was immediately followed by whisker stimulation. After whisker stimulation, all rats were placed back in their home cage for recovery, until 1 to 3 days before their assessment 4 months later, at which point behavioral health was evaluated. For the 4 month assessment, functional imaging, followed by blood flow imaging, was conducted. Rats were then transcardially perfused and brains were sectioned for cresyl violet staining (See below for

detailed methodology and experimental design).

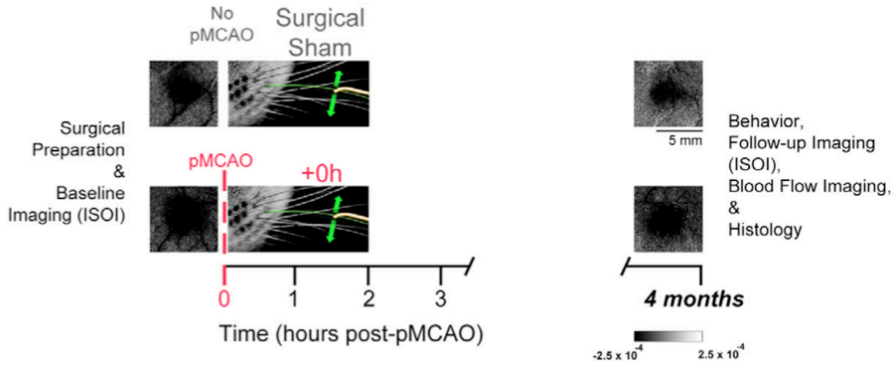

Figure 3.1. Experimental timeline and representative examples of functional imaging findings. Rats underwent baseline ISOI, followed by either a sham occlusion (surgical sham subjects), or a pMCAO (+0h subjects). All rats then received 2 hours of intermittent C2 whisker stimulation treatment. Four months later, all subjects underwent follow-up ISOI, as well as behavioral assessment, blood flow imaging and histology. Images on far left and right are of the ipsi-ischemic C2 whisker functional representation collected before and 4 months after pMCAO. Linear grayscale bar indicates intrinsic signal strength $\times 10^{-4}$. Black and white streaks correspond to large surface blood vessels.

Permanent middle cerebral artery occlusion (pMCAO)

Ischemic conditions were achieved via surgical occlusion of the stem of the left proximal middle cerebral artery (Tamura et al., 1981; Brint et al., 1988; Wang-Fischer, 2009). The skull and dura were carefully removed from a 2×2 mm 'surgical window' just anterior and lateral to the imaging window (over MCA's stem, also known as the M1 segment just distal to MCA's lenticulostriate branch) and a half-curve reverse cutting suture needle and thread (4-0 silk) was passed through the pial layer of the meninges, below MCA and above the cortical surface. To ensure that our pMCAO had completely and permanently obstructed blood flow, we performed a double ligature technique and transection of the MCA, the details of which are outlined by Davis et al. (2013). This preparation did not change for the entire 4 months of the experiment.

Histology

Concluding the 4 month assessment, rats were perfused transcardially with PBS, 1% gelatin, and 4% paraformaldehyde (Wang-Fischer, 2009). Their brains were carefully removed, then post-fixed overnight, and placed in 30% sucrose until ready for sectioning. Brains were embedded in tissue-freezing medium for cryostat sectioning, and were coronally sectioned at 40 μm. Every 5^{th} section was mounted on a slide, stained with cresyl violet, and coverslipped with mounting medium. Images of each section were captured with a 1.5× objective. The Paxinos and Watson rat brain atlas was used to identify anatomical structures (Paxinos and Watson, 1998). A small surgical lesion (~1 mm in diameter) was occasionally apparent at the immediate site of MCA occlusion. This occurred infrequently and equivalently in both +0h and surgical sham groups.

Intrinsic signal optical imaging (ISOI) and analysis

A detailed description of ISOI (Grinvald et al., 1986; Frostig et al., 1990; Ts'o et al., 1990; Frostig and Chen-Bee, 2012) data acquisition and analysis can be found elsewhere (Brett-Green et al., 2001; Chen-Bee et al., 2007). Briefly, a charge-coupled device (CCD) camera (a 12-bit Quantix 0206) equipped with an inverted 50 mm AF Nikon lens (1:1:8) combined with an extender (model PK-13) was used for imaging and controlled by V++ Precision Digital Imaging System software (Digital Optics). The cortex was illuminated with a red light-emitting diode (635 nm maximum wavelength with full width at half height of 15 nm). During each 15-second trial, 1.5 seconds of prestimulus data followed by 13.5 seconds of poststimulus onset data were collected, with a 6 ± 5-second random intertrial interval. Stimulus consisted of a single whisker (whisker C2) being deflected by ~9° in the rostral–caudal direction at a rate of 5 Hz for a total

stimulus duration of 1 second. Data was collected in blocks of 64 stimulation trials over periods of about 30 minutes each. Ratio images were created from calculating fractional change values for each of the four 64 trial blocks by dividing each 500 ms frame of poststimulus signal activity by the 500 ms frame of prestimulus intrinsic signal activity collected immediately before stimulus onset (Chen-Bee et al., 1996). The first phase of the evoked functional representation, the initial dip, was analyzed. This phase is generally associated with the evoked neural activity due to stimulation of a single whisker. As such, it is often referred to as the Whisker Functional Representation (WFR). The ratio image containing the maximal areal extent for this phase was Gaussian filtered, and the areal extent was quantified at a threshold level of 2.5×10^{-4} fractional change away from zero. Peak amplitude was quantified in fractional change units of the peak activity pixel for this intrinsic signal phase.

LSI of blood flow and analysis

A detailed description of LSI (Dunn et al., 2001; Choi et al., 2006) data acquisition and analysis can be found elsewhere (Lay et al., 2010). Briefly, a 632.8 nm 15 mW HeNe laser was used as the illumination source. The speckle pattern from the 5.12×5.12 mm imaged region was captured as 512×512 pixel images by a 16-bit CCD camera (Cascade 512F) equipped with a Navitar zoom lens plus extenders such that speckle size matched camera pixel size. Collected images were processed as previously described (Lay et al., 2010). Speckle contrast images were converted to speckle index images by calculating their inverse squares multiplied by the exposure time in seconds, so that larger index values corresponded to faster blood flow. Speckle index images were then averaged to improve signal-to-noise ratio. To quantify blood flow within the MCA, we calculated the mean value within a region of interest (ROI) in MCA cortical

branches as defined according to several criteria described previously (Lay et al., 2010). All flow index values were scaled over a range where 0 flow was set at noise values. Dead animal (noise) values were subtracted from all values.

Behavioral tests

Sensorimotor behavior was assessed at 4 months post-MCAO to evaluate neurological health and determine if any ischemic damage had rendered the rats impaired. All behavioral testing occurred one to three days prior to all 4 month imaging and perfusion. Bederson neurological scores (Bederson et al., 1986) were assigned to each rat to assess the general mobility of subjects, and whisker- and forepaw-guided behavior was assessed as previously performed (Lay et al., 2010). Briefly, forepaw-guided exploration was assessed by placing subjects in the center of a testing cylinder (20 cm in diameter and 45 cm in height) for five minutes, during which initiation of a wall touch was scored, following rearing using the left forepaw, right forepaw, or both paws together. Wall touches were calculated, and forepaw use was expressed as a forepaw asymmetry score (right paw touches minus left paw touches), with a negative score signifying a subject's preference to explore with the left forepaw. In normal subjects, there is a roughly even distribution of usage between left and right paws, while unilateral damage to the somatosensory cortex will result in a greater dependence upon the unaffected limb (Schallert et al., 2000).

Whisker-guided exploration was assessed by placing each subject in a 25-cm-wide rectangular track (120×80 cm, outer diameter) and was allowed 10 s to acclimate before the start of the 5 minute testing session. Whisker scanning was defined as the time spent by the subject touching the walls of the rectangular track with one set of whiskers while locomoting (Chen-Bee

and Frostig, 1996). Care was given to exclude incidents such as rearing and grooming as well as exploration which involved scanning with both sets of whiskers simultaneously, as in the case when a rat is facing perpendicular to a wall surface. Scanning was measured in seconds spent using either the left or right whisker pad by a timer watching the recorded testing session. Each subject was then assigned a thigmotactic scanning score (right score minus left score), with a negative score signifying a subject's preference to scan with the left set of whiskers. While healthy animals occasionally exhibit a whisker set preference, averages across groups of animals do not suggest a disproportionate preference for one whisker set over the other. Animals with unilateral damage to the somatosensory cortex, however, show a preference only for the unaffected whisker set (Luhmann et al., 2005). Observers blind to the rats' experimental conditions performed all behavioral data analysis.

Statistical analysis

Inferential statistics were performed on the raw values of ISOI data, laser speckle velocity and all behavioral data. For ISOI analysis, a repeated measures ANOVA with one between subjects variable (experimental group, +0h vs. sham) and one within subjects variable (time, baseline vs. 4 months) was performed, followed by *post hoc* contrasts to identify which post-occlusion values were significantly different from baseline. Alpha level was set to 0.05 and Bonferroni adjustments were applied to account for multiple contrasts (2 contrasts for an adjusted alpha value of 0.025). For LSI, and forepaw- and whisker-guided behavior, two- sample t-tests were performed with an α-level of significance set at 0.05. Fisher's exact test was performed for Bederson scores. All plotting and statistics were performed using SYSTAT 11 (SYSTAT Software Inc., Chicago, IL, USA).

RESULTS

Cortical function remains stable 4 months post-pMCAO when followed by immediate stimulation treatment

To determine whether this sensory stimulation-based treatment conferred complete, long-lasting protection from ischemic stroke, we first wanted to determine if cortical function remained intact at 4 months post-occlusion of MCA. To assess cortical function, we collected baseline ISOI data. Rats were randomly assigned to one of two experimental groups after baseline imaging. Subjects in the +0h group (n=12) received a permanent MCA occlusion followed immediately by whisker stimulation, while subjects in the surgical sham group (n=12) underwent surgery (leaving the MCA intact), then immediately received whisker stimulation. Four months post-occlusion, the rats underwent imaging again. There was no significant difference between groups ($F_{1,22}$=0.47, P > 0.05, ANOVA) or within subjects ($F_{1,22}$=0.34, P > 0.05, ANOVA) for area, however there was a significant difference between groups ($F_{1,22}$=9.71, P < 0.01, ANOVA) but not within subjects ($F_{1,22}$=2.61, P > 0.05, ANOVA) for amplitude. Post-hoc tests revealed a significant difference in amplitude between surgical controls and shams at 4 months ($F_{1,22}$=9.35, P < 0.01). Thus, ISOI revealed no change in the area or amplitude after 4 months in surgical shams. Normal cortical activity was observed in +0h subjects compared to surgical shams that never received the occlusion (Fig. 3.2), evidenced by the fact that the area and amplitude of the functional representation did not decrease below sham values. In fact, the area and the amplitude of the initial dip were slightly increased in +0h subjects at 4 months compared to their baseline, however neither trends were significant.

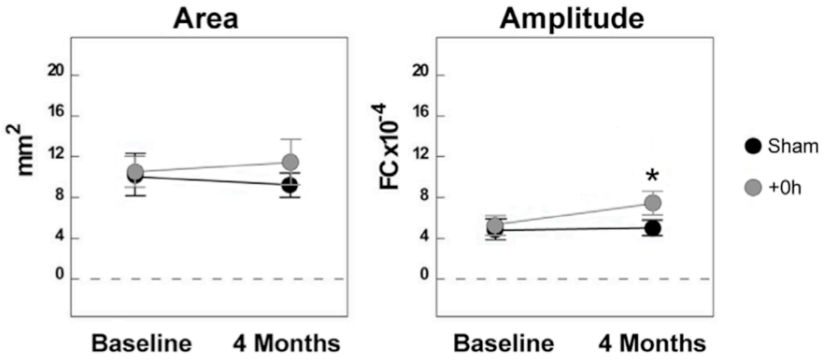

Figure 3.2. Cortical function remains stable at 4 months post-pMCAO. In each graph, group baseline is paired with 4 month data. Means and standard errors are provided for the area and amplitude of the evoked functional representation from the stimulation of the contra-ischemic C2 whisker before and 4 months after pMCAO. A value of zero indicates no response to whisker stimulation. Asterisk indicates a significant difference between +0h and sham subjects at 4 months.

Interestingly, these trends for the increase in area and amplitude in +0h subjects at 4 months compared to baseline, and the significant increase in amplitude at 4 months in +0h subjects compared to shams, are similar to our previous findings of the same parameters for the initial dip when assessed 24 hours post-occlusion (Lay et al., 2010).

Blood flow was assessed with laser speckle imaging (LSI). Given that the MCA was completely and permanently occluded in the +0h subjects, we wanted to confirm that the reversal of blood flow through the occluded MCA that we observe at 24 hours (Lay et al., 2010) was still present at 4 months post-occlusion. Our analysis showed that this reperfusion of the MCA still exists at 4 months post-occlusion and, surprisingly, also revealed a significant difference between the surgical shams and the +0h subjects ($t[22]=-4.13$, $P<0.001$) at this time point (Fig. 3.3).

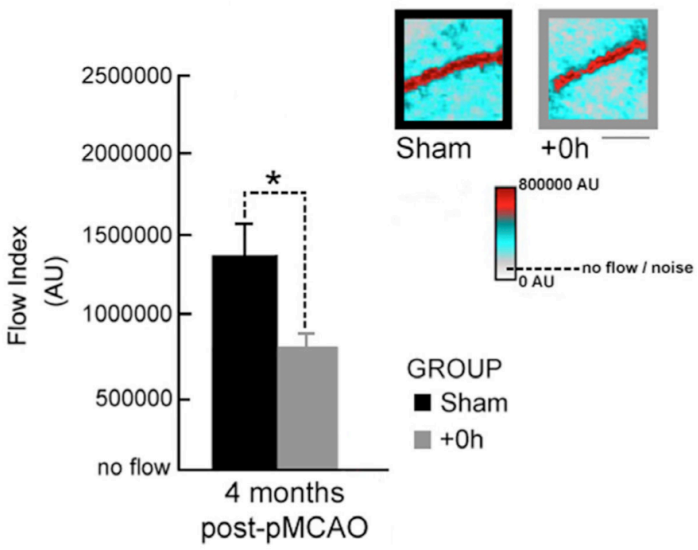

Figure 3.3. LSI demonstrates that at 4 months post-pMCAO, blood flow is maintained in the occluded MCA. Insets, representative linearly color scaled LSI images of the MCA taken at 4 months post-pMCAO for a surgical sham and +0h subject. Blood flow is apparent in both surgical sham and +0h subjects. Flow is expected in shams since the MCA remained intact, while reperfusion of the MCA is maintained in +0h subjects. Scale bar indicates 0.25 mm. Graph, the x-axis crosses the y at the mean noise level, or, 'no flow' level. Means and standard errors for MCA flow at 4 months post-pMCAO. Asterisk indicates a significant difference between flow in surgical shams and +0h subjects at 4 months.

Stimulation treatment results in normal sensorimotor-related behaviors at 4 months

Previously, behavioral assessments at 7 days post-pMCAO reveal no sensorimotor impairments (Lay et al., 2010). In order to determine whether the rats had any sensorimotor-related abnormalities at 4 months post-occlusion, each animal underwent the same behavioral tests as in our previous study: assessment according to the Bederson neurological scale, as well as with forepaw- and whisker-guided exploration. For the Bederson score, rats were assessed for the presence of limb flexion during suspension, a recognized sign of ischemic injury, as well as spontaneous circling behavior, difficulty with gait, and difficulty remaining upright while placed in a large cylindrical chamber and allowed to roam freely for five minutes. Results were then

scored on a 0–4 scale, with 0 representing normal movement, and 4 representing a complete lack of spontaneous movement or stupor (Wang-Fischer, 2009). +0h subjects demonstrated unimpaired behavior on all tasks (Fig. 3.4). No significant difference from surgical shams was observed according to the Bederson scores (p=0.25), where all subjects in both groups had a score of either 0, or a 1 (indicating the presence of limb flexion or circling behavior, but not both). Additionally, asymmetry scores for whisker- (t[22]=-1.53, *P*>0.05) and forepaw-guided exploration (t[22]=-0.38, *P*>0.05) showed equivalent performance for sham and +0h subjects.

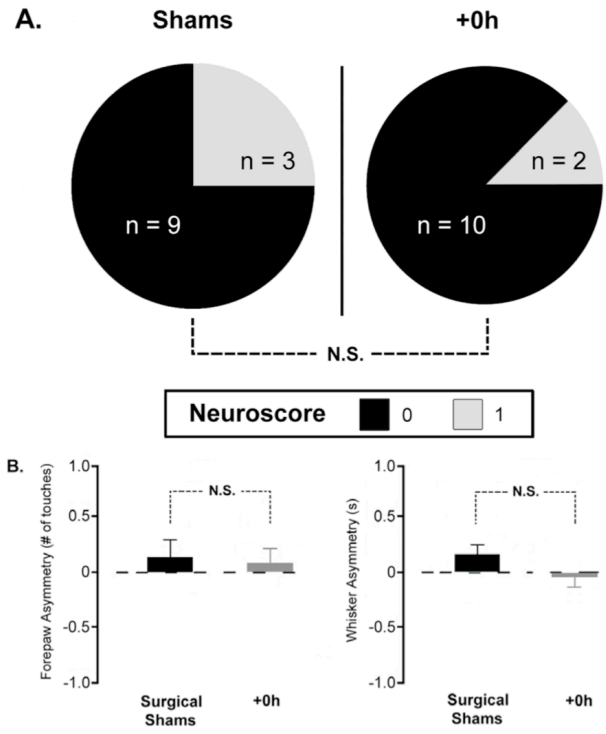

Figure 3.4. Whisker stimulation treatment results in normal sensorimotor behavior at 4 months. A, Neuroscores according to the Bederson scale for sham and +0h subjects. Pie charts represent the number of rats with the corresponding neurological score. All subjects had a score of 0 or 1, with no significant difference between sham and +0h groups. B, Forepaw-guided (left) and whisker-guided (right) asymmetry scores 4 months after pMCAO. Horizontal line indicates "0," or no asymmetry. No significant difference exists between shams and +0h subjects for either type of exploration. All analysis was conducted by blind observers.

+0h subjects remain anatomically intact and histologically equivalent to shams at 4 months post-occlusion

Given that there is a large influx of astrocytes and microglia into the region of infarct as the glial scar forms after an ischemic insult (Nowicka et al., 2008; Fawcett and Asher, 1999; Sofroniew, 2009), the routinely used method of determining stroke-related lesions with 2% 2,3,5-triphenyltetrazolium chloride (Bederson et al., 1986; Tureyen et al., 2004) could not be utilized here, as this method relies on functioning mitochondria to stain healthy tissue red, and glia could therefore show a false positive for healthy cortical neurons. Thus, we employed another widely utilized stain, cresyl violet, to resolve healthy tissue from any glial scar that could be present at 4 months post-occlusion (Tureyen et al., 2004) (Fig. 3.5). Subjects served as internal controls. Histological analysis revealed no glial scar or other ischemic damage, such as abnormal cortical anatomy, in the ipsi-ischemic hemisphere of +0h subjects at 4 months, as compared to the contra-ischemic hemisphere. Additionally, +0h subjects were identical to surgical shams, with no evidence of ischemic damage present.

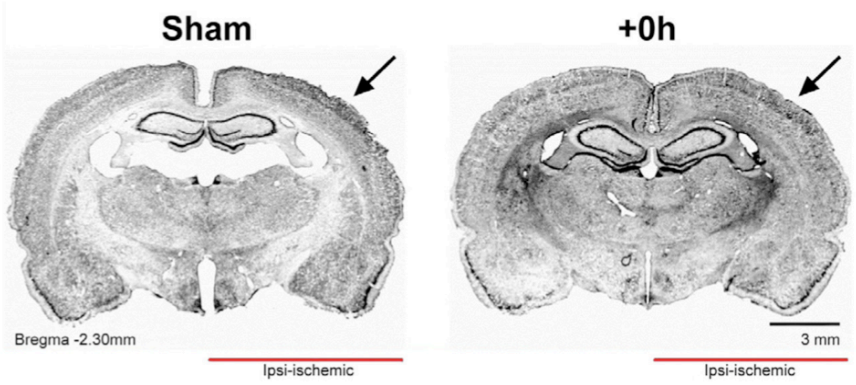

Figure 3.5. Cortical structure in +0h subjects remains equivalent to surgical shams at 4 months post-pMCAO. Representative coronal sections showing primary somatosensory cortex in sham and +0h subjects. Arrows point toward MCA blood supply territory for this cortical region. Cresyl violet staining shows no glial scarring in +0h subjects.

DISCUSSION

Utilizing a rat model of ischemic stroke and a battery of techniques, such as functional imaging, blood flow imaging, behavioral tests and histology, this study demonstrates that the complete protection of cortical function and structure observed 24 hours after pMCAO remains stable even 4 months after a pMCAO. To our knowledge, this is the first time neuroprotection from ischemic damage in rodents has been observed over such a long period of their lives, and has been assessed with multiple measures of cortical function, structure, and health.

One interesting finding arose from the ISOI analysis: the initial dip data for area and amplitude from +0h subjects and shams is similar at both 24 hours (Lay et al., 2010) and 4 months. This alone indicates that the protection of cortical function that exists early on in these +0h subjects remains out to 4 months. At 24 hours post-occlusion, we observe a significant increase in the amplitude of the initial dip compared to baseline (Lay et al., 2010). Although that significance is not present at 4 months, there still exists an increase in +0h subjects at 4 months when compared to baseline, and a significant increase in amplitude when compared to shams at 4 months. This amplitude increase at 24 hours and 4 months compared to baseline possibly represents underlying neuroprotective plastic changes that seem to be maintained, at least when compared to sham controls.

We have demonstrated in our previous studies that the protection observed at 24 hours is due to a massive reorganization of blood flow in the MCA, with blood flowing backwards into the occluded MCA in order to continue reperfusing the ischemic cortical tissue, and that this reversal of flow occurs during the 2 hour treatment period (Lay et al., 2010, 2011). It is possible that this finding could have been an acute response to the ischemic insult, with more

metabolically demanding processes, such as angiogenesis, compensating for reduced blood flow in the long run (Arenillas et al., 2007; Seevinck et al., 2010). However, we did not detect any overt angiogenesis in our +0h subjects at 4 months, but did observe a maintenance of blood flow in the occluded MCA. Not only did we see normal blood flow in +0h subjects, but surgical shams exhibited increased blood flow compared to +0h subjects. Given that this reperfusion of the MCA is constrained by the size of collateral vessels (Lay et al., 2010), it's possible that this collateral reverse flow is slower in +0h subjects when compared to flow in the intact MCA of surgical shams where blood flow was never impeded.

In addition to cortical function remaining fully protected, and reversed blood flow being maintained in the MCA at 4 months, the +0h subjects were behaviorally and histologically equivalent to surgical shams. Many stroke patients suffer from debilitating, and long-lasting effects of ischemia, and despite some patients' recovery over time with rehabilitation, many still do not make a full recovery and can have lifelong damage to their brain. Glial scarring and loss of brain tissue are common after an ischemic event, and can be detected histologically years after the occlusion (Aguilar, 1969; Rolls et al., 2009). In our +0h subjects, no evidence of ischemic damage or glial scarring was found, which would appear as a more densely-stained region due to the high density of glia in the infarct region (Popp et al., 2009). Behaviorally, +0h subjects displayed no preference for an unaffected whisker pad or limbs, and no other sensorimotor deficits were detected, complementing the imaging, blood flow, and histological findings.

In conclusion, the ideal stroke treatment would not only be rapid, but also long-lasting. The sensory stimulation-based treatment herein fits this description. If initiated immediately after ischemic onset, mild sensory stimulation, a no side-effects treatment, confers complete protection from ischemic stroke in rats, and remains stable over a significant portion of the rats'

lifetime. As with the many neuroprotective treatments that have shown promise over the years, it is possible that the phenomenon observed here might be due to unique characteristics of the rodent brain and physiology. Thus, caution should be taken with any new potential treatment for stroke. Nevertheless, this study, involving a new type of neuroprotective treatment for stroke, serves to further highlight the translational potential of this sensory stimulation as a means of neuroprotection from ischemic stroke in humans.

CHAPTER 4: Experiment III- A Sensory Stimulation-Based Collateral Therapeutic Does Not Protect from Ischemic Stroke Damage in The Presence of Hypertension

INTRODUCTION

Approximately 77% of stroke patients have hypertension, which is the number one risk factor for ischemic stroke (Dahlof, 2007; Mozaffarian et al., 2015). In a normal, healthy individual, systolic/diastolic blood pressures are considered to be equal to or less than 119/79 mmHg. However, an individual developing hypertension first experiences a slight increase in their blood pressure, called prehypertension, that ranges from 120-139/81-89 mmHg. Without medical intervention, this can then lead to hypertension with a blood pressure equal to or greater than 140/90 mmHg. After prolonged periods of this increased blood pressure, many physiological changes can occur, including changes to the cerebral vasculature. Importantly, despite extended periods of high blood pressure causing pulsatile stress on the arterial tree, the major complication of hypertension is thrombotic, rather than hemorrhagic (Messerli et al., 2007). This is referred to as the thrombotic paradox of hypertension (Lip and Blann, 2000; Messerli et al., 2007) and could be due to hypercoaguability or a prothrombotic state due to an imbalance between coagulation and fibrinolytic pathways (Lip and Blann, 2000), and/or high pressures damaging vessel walls (Messerli et al., 2007). Additionally, hypertension can lead to stroke by promoting atherosclerosis (when fat, cholesterol, leukocytes, and other substances build up a plaque on arterial walls), as well as arteriosclerosis (a stiffening of the vessel walls) and lipohyalinosis (vessel wall thickening that reduces luminal diameter) in cerebral arteries (Bronner et al., 1995; Harazny et al., 2007) resulting in thrombotic stroke. These factors thereby maintain the elevated blood pressure (Thom, 1997). Atherosclerosis not only occurs in the cerebral vasculature, but can also be promoted throughout the body, increasing the risk of an

embolism originating from the heart, large extracranial vessels, and aortic arch (Johansson, 1999; Harazny et al., 2007). In fact, atherosclerosis usually begins in these larger arteries and aorta, then spreads distally to the circle of Willis and eventually to the smaller intracerebral arteries (Johansson, 1999). Increased atherosclerosis and lipohyalinosis alone are a dangerous combination that can lead to ischemic stroke. However, additional adaptive structural changes can occur due to increased workload, such as growth of smooth muscle cells that can reduce vessel lumen diameter (Johansson, 1999, Faraco and Iadecola, 2013). This results in increased vascular resistance that can reduce collateral blood flow, resulting in ischemia distal to an arterial occlusion (Grabowski et al., 1993).

Despite the fact that most stroke patients present with at least one risk factor, many studies do not take into account the heterogeneity of stroke and comorbidity when developing new treatments, and for good reason as it can introduce multiple confounding factors, making results difficult to interpret. However, because most stroke patients are not young, healthy adults, and often have one or more risk factors, it's important to address a crucial discrepancy between bench and bedside and test potential therapeutics in the presence of these risk factors STAIR, 1999; Fisher et al., 2009), as this is often a reason that clinical trials fail for otherwise promising treatments (Dirnagl et al., 1999).

The development of new stroke therapeutics is imperative given that there is currently no cure, and the only FDA-approved treatment (rtPA) results in positive outcome in only a small portion of stroke patients due to it's limited therapeutic window and the risk of hemorrhagic transformation. Our lab has shown that a collateral-based sensory stimulation treatment is a promising therapeutic treatment, as it is noninvasive, has the potential to be delivered immediately, has long-lasting effects, and completely prevents impending ischemic stroke

damage (Lay et al., 2010, 2011, 2014; Davis et al., 2011; Hancock et al., 2013). Additionally, we have already tested whether rats can be protected in the face of one risk factor, old age, and have observed complete protection in rodents that are equivalent in age to typical stroke patients (65 years old) (Lay et al., 2012). However, to further assess the translational potential of this collateral-based treatment, it is critical to test this protection in cases where the luminal diameter, and other vascular changes are less than ideal, as is the case in hypertension. Thus, in this study we aimed to test whether spontaneously hypertensive rats (SHRs), a widely used model of essential hypertension, could be protected from ischemic damage.

METHODS

All procedures were in compliance with NIH guidelines and approved by UC Irvine Animal Care and Use Committee (protocol #: 1997-1608, assurance ID#: A3416.01).

Subjects and Surgical Preparation

Fourteen experimental subjects, 295-400g (4-5 months of age) male spontaneously hypertensive rats (Harlan Laboratories, Indianapolis, IN, USA) with systolic blood pressures of ~150 mmHg, were individually housed in standard cages. At the beginning of each experiment, subjects were injected intraperitoneally with a Nembutal bolus (55 mg/kg b.w.). Supplemental injections of Nembutal (27.5 mg/kg b.w.) were given as necessary. After resection of soft tissue, a ~6.5 x 8 mm 'imaging' area of the skull over the left primary somatosensory cortex (rostromedial corner positioned approximately 1mm caudal and 2mm lateral from bregma) was thinned to ~150μm using a dental drill. 5% dextrose (3mL) and atropine (0.05 mg/kg, b.w.) were administered at the beginning of the experiment and every six hours after until the animal was

returned to its home cage. Body temperature was measured via a rectal probe, and maintained at 37º Celsius by a self-regulating thermal blanket. Animals were returned to their home cage and allowed to recover overnight prior to all +24 hour experimentation.

Overview

Using a within subject design that is identical to our previous studies, 14 subjects were randomly assigned to a +0h group or a no-stimulation control group. Baseline functional imaging was collected for all subjects at the beginning of surgery. All +0h subjects (n=7) then received a pMCAO, and immediate post-occlusion whisker stimulation. Post-occlusion whisker stimulation consisted of 1 s of 5 Hz deflections of a single whisker (whisker C2). This stimulation was intermittently (with random intervals averaging 21 seconds) delivered 256 times, totaling 4.27 minutes of stimulation, over the course of 2 hours (Lay et al., 2010). No-stimulation controls (n=7) underwent identical pMCAO to that of +0h subjects, but never received whisker stimulation; pMCAO was immediately followed by a 5-hour no-stimulation period. The length of this 5 hour quiet period was chosen by Lay et al. (2010) to match time under anesthesia to subjects in that study that received the two hours of whisker stimulation three hours post-occlusion – this has become the standard for our no-stimulation controls as it produces invariable cortical infarct in these subjects. After whisker stimulation or quiet period, all rats were placed back in their home cage for recovery, until their follow-up assessment at 24 hours post-pMCAO, which consisted of functional imaging and blood flow imaging. Rats were then euthanized and the brains were removed for histological assessment.

Histology (2,3,5-triphenyltetrazolium chloride staining for infarct)

At the conclusion of each experiment, rats were euthanized with sodium pentobarbital (2-3 mL, intraperitoneally), the brain was removed, sectioned into 2 mm coronal slices, and incubated in 2% 2,3,5-triphenyltetrazolium chloride at 37 °C for 20 min in the dark (Bederson et al., 1986). TTC is enzymatically reduced, producing formazan (a bright red byproduct), by dehydrogenases in active mitochondria. Red stain intensity correlates with the number and functional activity of mitochondria, unstained (white) areas are indicative of infarct (Goldlust et al., 1996). The TTC-stained sections are photographed with a digital camera, and images are analyzed using ImageJ software. The total infarct volume is determined by multiplying the infarct area of each slice by the thickness of that slice. An observer blind to experimental condition performs this volume calculation. A small lesion (<1 mm in diameter) is occasionally apparent at the immediate site of MCA occlusion. This occurs infrequently and equivalently in all experimental groups (1–2 subjects per group). The small amount of damage occasionally produced at the surgical site can be readily distinguished from the large ischemic infarct and is excluded from infarct analysis (Tamura et al., 1981).

Permanent Middle Cerebral Artery Occlusion

Permanent ischemic conditions are achieved as follows: The base of the left proximal middle cerebral artery at the M1 segment (Gibo et al., 1981; Tamura et al., 1981; Brint et al., 1988; Wang-Fisher, 2009) is permanently occluded, blocking flow to all MCA cortical branches. To do this, the skull and dura are carefully removed from a 2x2mm ‘surgical window’ just anterior and lateral to the imaging window (over the M1 segment of MCA, just distal to MCA’s lenticulostriate branches and proximal to any cortical branching). A half-curve reverse cutting

suture needle is cut in half and threaded with two 4-0 silk threads and passed through the pial layer of the meninges, below MCA (the needle is kept above the cortical surface to the extent possible to prevent damage). Then the two threads (moved to ~1mm apart after being strung beneath the artery) are both tied and tightened around MCA and the vessel is transected (completely severed) between the two knots. Care is taken to avoid damaging the artery, and experiments are terminated if there are signs of bleeding from MCA. This method of occlusion has been reviewed by Davis et al. (2013).

Intrinsic Signal Optical Imaging (ISOI) and Analysis

A detailed description of ISOI data acquisition and analysis can be found elsewhere (Ts'o et al., 1990; Chen-Bee et al., 2007). Briefly, a charge coupled device (CCD) camera (either a 16-bit Cascade 512F or a 12-bit Quantix 0206, Photometrics, Tucson, AZ, USA) equipped with an inverted 50 mm AF Nikon lens (1:1:8, Melville, NY, USA) combined with an extender (model PK-13, Nikon, Melville, NY, USA) is used for imaging and controlled by V++ Precision Digital Imaging System software (Digital Optics, Auckland, NZ). During each 15-s trial, 1.5 s of prestimulus data followed by 13.5 s of poststimulus data is collected**,** with a 6±5 sec random inter-trial interval. Stimulus consists of a single whisker being deflected by 9° in the rostral-caudal direction at a rate of 5 Hz for a total stimulus duration of 1 second. The cortex is illuminated with a red light emitting diode (635 nm maximum wavelength). Data are collected in blocks of 64 stimulation trials, and a sampled time point (for example pre-pMCAO baseline) is considered complete upon summation of 128 stimulation trials. Ratio images are created from calculating fractional change (FC) values by dividing each 500ms frame of poststimulus signal activity by the 500ms frame of prestimulus intrinsic signal activity collected immediately before

stimulus onset. The ratio image containing the maximum areal extent for the first two intrinsic signal phases (Initial Dip and Overshoot) is Gaussian filtered (half width = 5) and the areal extent quantified at a threshold level of 2.5×10^{-4} away from zero. Peak amplitude is quantified in fractional change units from the pixel with the peak activity within the maximum areal extent for both of the intrinsic signal phases.

Laser Speckle Imaging (LSI) and Analysis

A detailed description of LSI (Dunn et al., 2001; Choi et al., 2006) data acquisition and analysis can be found elsewhere (Lay et al., 2010). Briefly, a 632.8 nm 15 mW HeNe laser was used as the illumination source. The speckle pattern from the 5.12×5.12 mm imaged region was captured as 512×512 pixel images by a 16-bit CCD camera (Cascade 512F) equipped with a Navitar zoom lens plus extenders such that speckle size matched camera pixel size. Collected images were processed as previously described (Lay et al., 2010). Speckle contrast images were converted to speckle index images by calculating their inverse squares multiplied by the exposure time in seconds, so that larger index values corresponded to faster blood flow. Speckle index images were then averaged to improve signal-to-noise ratio. To quantify blood flow within the MCA, we calculated the mean value within a region of interest (ROI) in MCA cortical branches as defined according to several criteria described previously (Lay et al., 2010). All flow index values were scaled over a range where 0 flow was set at noise values. Values were collected from dead animals, and these noise values were subtracted from all other values.

Statistical Analysis

For imaging data, ANOVA were run on baseline values to ensure no significant differences before pMCAO. Because there were no responses to quantify at 24 hours, post-

pMCAO imaging evoked area and amplitude were converted to difference score values (postocclusion - baseline) with values away from 0 signifying a change from baseline. A constant was added to difference values, which were then transformed with a natural log function to better satisfy the assumptions of an analysis of variance (ANOVA) and inferential statistics were performed on the transformed data. Raw values of laser speckle velocity were used for analysis. After ANOVA, specific contrasts were performed to identify which groups differed from baseline. Alpha level was set to 0.05 and Bonferroni adjustments were applied to account for multiple contrasts. Infarct volume comparisons were performed by employing two-sample t-tests. All plotting and statistics were performed using SYSTAT 11 (SYSTAT Software Inc., Chicago, IL, USA).

RESULTS

Treatment does not protect cortical function in hypertensive rats

Before pMCAO, there was no significant difference between groups for either the area ($mean_{treated}$=5.01 ± 1.11; $mean_{untreated}$ =4.18 ± 0.80; $F_{1,12}$=0.19, P > 0.05, ANOVA) or amplitude ($mean_{treated}$ =4.43±0.28; $mean_{untreated}$ =4.13 ± 0.26; $F_{1,12}$=0.55, P > 0.05, ANOVA) of the whisker functional representation (WFR). At twenty-four hours post-pMCAO, neither treated or untreated groups were protected and therefore had no whisker functional representation at this time point (Fig. 4.1). As such, there were no significant differences between groups at 24 hours for either area ($mean_{treated}$ =0.02 ± 0.02; $mean_{untreated}$ =0.01 ± 0.01; $F_{1,12}$=0.79, P > 0.05, ANOVA) or amplitude ($mean_{treated}$ =0.24±0.17; $mean_{untreated}$ =0.24±0.16; $F_{1,12}$=0.37, P > 0.05, ANOVA). The lack of cortical activity at twenty-four hours post-pMCAO resulted in a significant reduction in area and amplitude compared to baseline activity for treated subjects (n=7; area: $F_{1,12}$=13.97, P

< 0.005; amplitude: $F_{1,12}$=83.02, P < 0.001) as well as untreated subjects (n=7; area: $F_{1,12}$=10.14, P < 0.01; amplitude: $F_{1,12}$=68.08, P < 0.001) (Fig. 4.2). Thus, treated and untreated subjects were equivalent, as treatment did not have a protective effect.

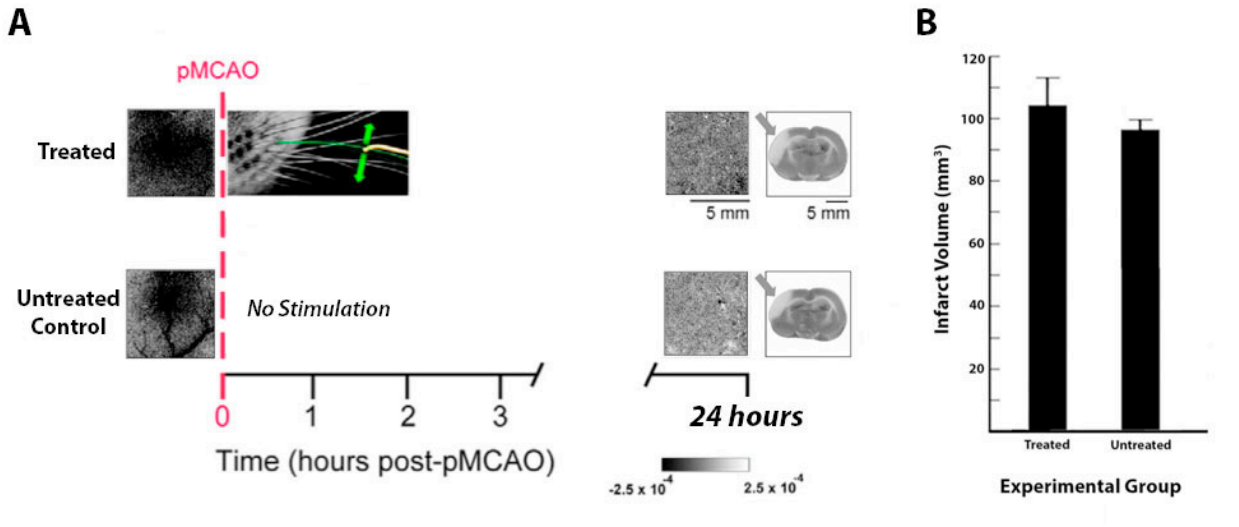

Figure 4.1. Treated hypertensive rats lack cortical activity and sustain large infarcts at 24 hours post-pMCAO. A, Experimental schema with ISOI representative cases for treated (top) and untreated (bottom) subjects, before (left) and 24 hours after (right) pMCAO. Treated and untreated subjects have normal whisker functional representations (WFR) at baseline. After imaging, all subjects received a pMCAO which was followed immediately by 2 hours of whisker stimulation treatment or a no-stimulation period. At 24 hours post-pMCAO, all subjects lacked a WFR and sustained large infarcts (arrow). B, Quantification of infarct for treated and untreated subjects. No difference in infarct size existed between the groups.

Hypertensive rats do not have collateral flow feeding the MCA at 24 hours post-pMCAO

A subset of subjects underwent blood flow imaging to assess whether the MCA was capable of being reperfused via the collateral vasculature. There were no significant differences between groups either before ($F_{1,5}$=3.83, P > 0.05, ANOVA) or twenty-four hours ($F_{1,5}$=0.05, P > 0.05, ANOVA) after pMCAO. We found that treated (n=4; $mean_{baseline}$=1,517,107 AU±204,357; $mean_{24hours}$=481,894 AU±88,249) and untreated (n=3; $mean_{baseline}$=2,689,382 AU±655,340; $mean_{24hours}$=442,614 AU±182,628) subjects all had reduced blood flow within the MCA post-

occlusion, and this was a significant reduction compared to baseline flow for both treated ($F_{1,5}$=16.96,P<0.01) and untreated subjects ($F_{1,5}$=35.25,P<0.005) (Fig.4.3).

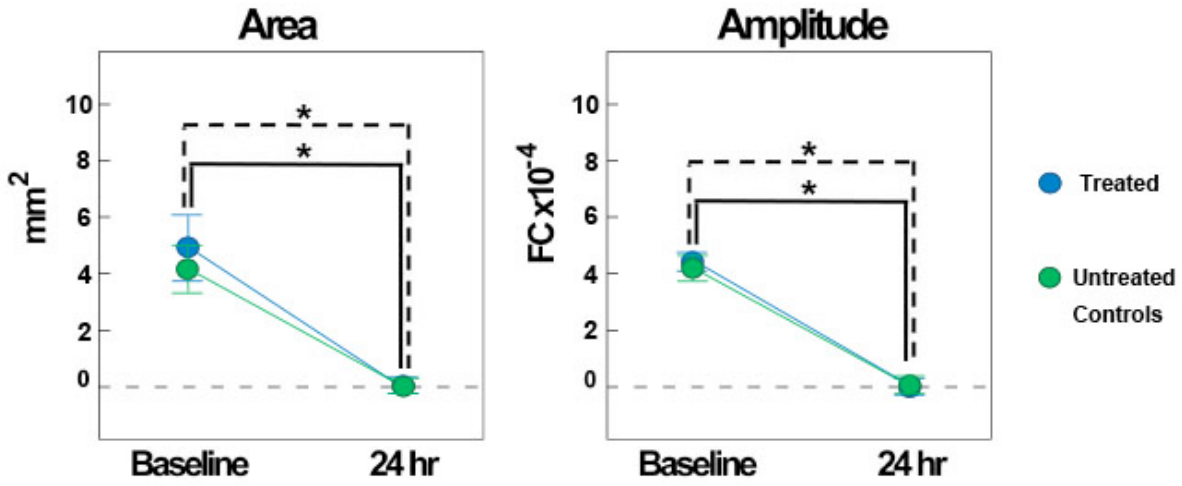

Figure 4.2. Treated and untreated hypertensive subjects show a reduction of the WFR 24 hours after pMCAO. Quantification of the area (left) and amplitude (right) of the whisker functional representation at both baseline and 24 hours for treated and untreated subjects. Treated and untreated subjects showed a significant decrease in both parameters at 24 hours post-pMCAO compared to baseline (dashed and solid lines).

Hypertensive rats sustain large cortical infarcts after pMCAO despite receiving immediate treatment

Finally, we assessed ischemic damage with TTC staining and found that the hypertensive rats sustained large infarcts regardless of whether they received immediate treatment or not (Fig. 4.1). There was no effect of treatment on infarct size (t[12]=0.76, *P*>0.05), with treated subjects sustaining infarcts ranging from 74.27–137.94 mm^3 (mean = 104.05±9.37 mm^3), and infarcts for untreated subjects ranging from 83.96 – 107.74 mm^3 (mean = 96.53±3.32 mm^3).

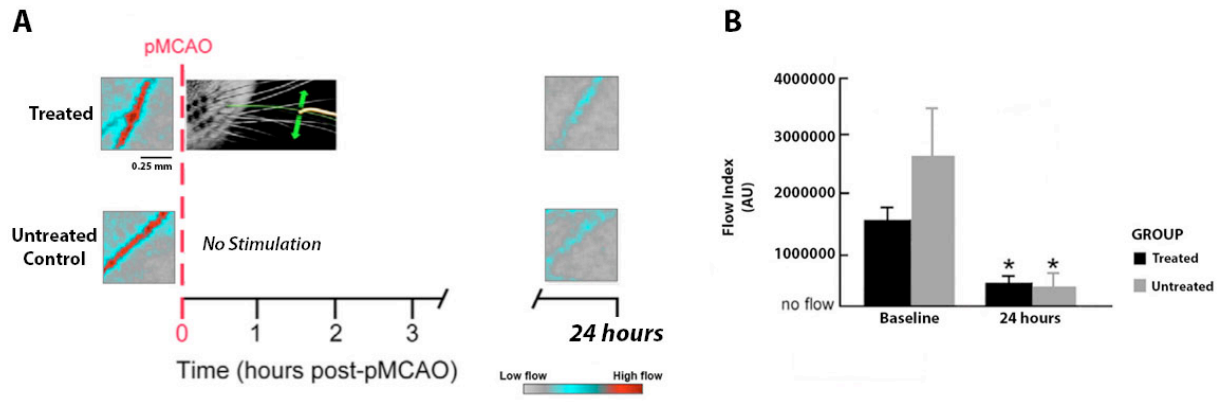

Figure 4.3. Treatment resulted in minimal retrograde blood flow within the MCA at 24 hours post-pMCAO. A, Experimental schema with LSI representative cases for treated (top) and untreated (bottom) subjects, before (left) and 24 hours after (right) pMCAO. Treated and untreated subjects are similar in that there was reduced blood flow at 24 hours post-pMCAO). B, Quantification of laser speckle velocity for both groups. The reduction in blood flow was significant for all subjects.

DISCUSSION

Our previous research has shown that in normotensive rats, cortical activity is maintained in early-treated, protected subjects at 24 hours post-pMCAO (Lay et al., 2010, 2011; Davis et al., 2011). This finding stands in stark contrast to the results presented here. It is clear from the ISOI, LSI and TTC data that these hypertensive rats are not protected from impending ischemic stroke damage when given immediate collateral-based sensory stimulation treatment. The whisker functional representation for treated subjects was completely eliminated at 24 hours post-pMCAO. Importantly, this is identical to what we observed for untreated control subjects that never received treatment. Additionally, these treated subjects lacked significant retrograde blood flow within the MCA at 24 hours post-pMCAO as we normally observe in normotensive treated, protected subjects. Finally, TTC staining confirmed that these subjects did in fact have infarct. Not only did treated subjects sustain massive infarcts similar to the untreated controls, but these values are much larger than what we have observed in any of our previous normotensive subjects when anesthetized with pentobarbital. Normotensive untreated controls typically sustain infarcts

of 28.4±2.4 mm^3, while subjects that receive treatment 3 hours post-pMCAO (+3h) sustain larger infarcts of 61.4±2.4 mm^3 (Lay et al., 2010). The only damage that treated subjects tend to experience comes from surgical damage (<1mm in diameter) and is observed infrequently.

Hypertension is the single most important risk factor for the development of stroke (Air and Kissela, 2007), as it promotes atherosclerosis and various types of structural remodeling as compensatory mechanisms for dealing with chronic high blood pressure. Unfortunately, all of these vascular stresses can result in stenosis and increase the risk of ischemic stroke. To further compound the issue, hypertension can alter cerebrovascular autoregulation and reduce vascular responsiveness to endothelium-dependent vasodilators such as nitric oxide (Didion et al., 2000; Capone et al., 2011) and functional hyperemia (Jennings et al., 2005; Capone et al., 2012; Calcinaghi et al., 2013; Iadecola and Davisson, 2008; Faraco and Iadecola, 2013). In some cases, this impaired functional hyperemia is due to stenosis, and thickening and hardening of vessel walls, but it can also be attributed to high angiotensin II levels (Kazama et al., 2003). These effects are present both in animal models and in patients with hypertension. A study by Jennings et al. (2005) showed that increased cerebral blood flow as a result of functional hyperemia from brain activation was reduced in patients with chronic hypertension. Additionally, several studies on humans have also shown that a history of hypertension is more frequently associated with fewer leptomeningeal collaterals in CT angiography (Lima et al, 2010) and with poor collateral flow (Liebeskind et al., 2008).

Given that hypertension is known to negatively impact the brains vasculature in humans and animal models of hypertension, including the collateral vessels that are critical to the efficacy of our treatment, it is not surprising that these hypertensive rats were not protected from impending ischemic stroke damage. The model used here, the spontaneously hypertensive rat

(SHRs), was first developed in 1963 by Okamoto and Aoki as a model for essential hypertension, which is the most common form (i.e., persistent high blood pressure of unknown causation), affecting approximately 95% of hypertensive patients. SHRs are currently the only, and thus most widely used, model for human essential hypertension (Doggrell and Brown, 1998), since it occurs without any treatment to induce hypertension (Okamoto and Aoki, 1963). SHRs also exhibit hypertension in stages similar to humans, with pre-hypertension existing for the first 6-8 weeks of their lives (with systolic blood pressures around 100-120 mmHg), and then hypertension developing over the next 12-14 weeks, where systolic blood pressure remains over 150 mmHg (Doggrell and Brown, 1998). The cerebral vasculature of these rats is known to have similar characteristics to that of hypertensive individuals. This includes decreased distensibility of cerebral arteries due to an increase in collagen but not elastin content (Baumbach et al., 1988), and increased thickness of the vascular wall, specifically the intima media (which comprises the middle smooth muscle, and inner endothelial layers of a blood vessel) (Mangiarua and Lee, 1992; Tice et al., 1996; Air and Kissela, 2007). This is in contrast to large amounts of smooth muscle, and small amounts of elastin, basement membrane and collagen in pial arteries in normotensive rats.

Due to these hypertension-induced vascular impairments, spontaneously hypertensive rats sustain infarcts of greater volume, but with less variability, than normotensive controls (Barone et al., 1992; Nishimura et al., 2000). This is likely the result of cortical collateral flow that's reduced to an unsustainable level due to the reduced luminal diameter and reduced vasodilation and autoregulation capabilities rather than a lack of collaterals (Coyle and Jokelainen, 1982; Coyle and Heistad, 1986; Jacewicz, 1992; Faraco and Iadecola, 2013). The severely impaired vasculature in these rats could thus explain the larger infarct size compared to

normotensive untreated and +3h subjects from our lab. +3h subjects may have large infarcts due to enhanced blood flow through functioning collaterals at a late time point, thereby exacerbating the damage that has already occurred, while the hypertensive rats may have a general reduction in blood flow post-occlusion due to increased vascular resistance as a result of brain-wide vascular impairments and reduced vessel lumen diameters. This could result in a larger portion of cortex remaining hypoperfused after pMCAO.

The findings presented here further corroborate the existing literature. Regardless of whether or not rats received early treatment, we observed severely reduced collateral flow in hypertensive rats as demonstrated by significantly reduced blood flow in the MCA at 24 hours post-pMCAO. This reduction in retrograde blood flow in the MCA, which we have shown originates from the pial collaterals in treated, protected subjects (Lay et al., 2010), resulted in the development of ischemic damage as is evident by the lack of evoked cortical activity and large infarcts.

The collateral vasculature is known to be responsible for rescuing penumbral tissue and reducing infarct size. However, our results show that cerebrovascular impairments in these hypertensive rats did not permit viable levels of collateral blood flow and led to ischemic damage. When considering the translational potential of this collateral-based treatment for ischemic stroke patients, it is important to keep in mind that these rats may not be an optimal model, as they begin developing hypertension from a relatively young age. Thus, although it appears that this treatment might not be a viable solution to prevent ischemic damage in this population of stroke patients, it is possible that the effects of hypertension in these rats may impair the cerebral vasculature more severely compared to patients with hypertension. Additionally, this model does not take into account the fact that many patients are on

medications to control blood pressure, which may also reduce the severity of the vascular impairments depending on how long the patient has had hypertension and the length of time that they've been taking medications. Thus, an effective solution for patients with hypertension and minimal or impaired collateral flow may be to combine this collateral-based sensory stimulation treatment with recanalization therapy (see chapter 6 for further discussion). To conclude, hypertension-induced cerebrovascular impairments prevent ischemic stroke protection via the collateral-based treatment described by our lab in a rat model of essential hypertension. Further work is deemed necessary to determine whether this treatment may be useful for patients with hypertension, and in what capacity it might prevent ischemic damage.

CHAPTER 5: Experiment IV- A Sensory Stimulation-Based Collateral Therapeutic Does Not Protect Either of Two Mouse Strains from Ischemic Damage

INTRODUCTION

The success and translational capability of preclinical ischemic stroke research relies on the use of the appropriate models, and this is not an easy task given the heterogeneity of the patient population. Ischemic stroke patients often present with comorbidities, and there are many risk factors that can affect individuals differently based on genetic and environmental factors, and which can also affect the cerebral vasculature in a non-uniform manner. Thus, it is challenging to develop a model that would encompass all of these factors and no single model is sufficient to assess all the variables that might reduce a potential treatments success. Rather, models displaying single, specific risk factors/comorbidities can be useful in dissecting their role in ischemic stroke outcome after the delivery of interventions. The Stroke Therapy Academic Industry Roundtable has recommended testing potential treatments in the presence of comorbidities, as well as in additional species, in order to guide preclinical research such that translational capability can be effectively assessed (STAIR, 1999; Fisher et al., 2009; Turner et al., 2011; Howells et al., 2014). There is increasing evidence that poor pial collateral vessel flow is a critical predictor of stroke severity as it has been linked to poor outcome even in the event of recanalization (Bang et al., 2008, 2011; Hussein et al., 2010; Lima et al., 2010; Liebeskind, 2008b; Winship, 2015).

Previously our lab has shown that the collateral vessels that anastomose (connect) with the distal middle cerebral artery (MCA) branches are critical to reperfusion of the occluded MCA, and protection of cortex. When these distal branches were occluded in addition to the standard MCA occlusion at the M1 segment, rats were not protected despite having received

immediate whisker stimulation (Lay et al., 2010). Therefore, reperfusion of the MCA occurs through these existing patent (open) collaterals, ensuring that the viability of neurons in the potentially ischemic region is maintained. Given that the basic mechanism (collateral blood flow) behind cortical protection has been elucidated, investigation of the underlying molecular mechanisms of protection would be a logical next step.

The use of mice as animal models has flourished in recent decades due to the availability of genetic manipulations that enable the dissection of molecular mechanisms. The C57BL/6J strain is known to have numerous pial collaterals (Chalothorn, 2007; Chalothorn and Faber, 2010; Wang et al., 2010), and in fact were shown to have high numbers of collaterals and larger collateral vessel diameters than 14 other mouse strains (Zhang et al., 2010). Additionally, Zhang et al. (2010) found that infarct volume appears to be strongly correlated inversely with collateral number and diameter.

Thus, our main goal was to test whether immediate delivery of the collateral-based sensory stimulation treatment used in our lab could protect another species, the inbred C57BL/6J mouse strain, from impending ischemic stroke damage with the hopes that if protected, these mice would open new doors for exploring the mechanisms underlying the protection we observe. This strain was chosen given that it is widely used in stroke research and they are known to have abundant pial collaterals. We hypothesized that these mice would be protected from ischemic damage when used in our stroke model. However, early results indicated this was not the case, so a small group of outbred CD1 mice, a popular and slightly larger mouse strain that is also known to have functioning collaterals (Chalothorn et al., 2009), were assessed for protection.

METHODS

All procedures were in compliance with NIH guidelines and approved by UC Irvine Animal Care

and Use Committee (protocol #: 1997-1608, assurance ID#: A3416.01).

Subjects and Surgical Preparation

Twenty-three experimental subjects, 25-30g 10-12 week old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), and eight experimental subjects, 30-40g 10-12 week old male CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were individually housed in standard cages. At the beginning of each experiment, subjects were injected intraperitoneally with a Nembutal bolus (50 mg/kg b.w.). Supplemental injections of Nembutal (27.5 mg/kg b.w.) were given as necessary. After resection of soft tissue, the parietal bone was thinned to ~150μm using a dental drill to create an 'imaging' area in the skull over the left primary somatosensory cortex. 5% dextrose (.3mL) and atropine (0.05 mg/kg, b.w.) were administered at the beginning of the experiment and every six hours after until the animal was returned to its home cage. Body temperature was measured via a rectal probe, and maintained at 37º Celsius by a self-regulating thermal blanket. Animals were returned to their home cage and allowed to recover overnight prior to all +24 hour experimentation.

Overview

Using a within subject design that is identical to our previous studies, 23 C57BL/6J mice were randomly assigned to a +0h group, a no-stimulation control group or a surgical sham group, and 8 CD1 mice were randomly assigned to a +0h group or a no-stimulation control group. Baseline functional imaging was collected for all subjects at the beginning of surgery. All +0h subjects (n=8, C57BL/6J; n=4, CD1) then received a pMCAO, and immediate post-occlusion whisker stimulation. Post-occlusion whisker stimulation consisted of 1 s of 5 Hz deflections of a single whisker (whisker C2). This stimulation was intermittently (with random intervals

averaging 21 seconds) delivered 256 times, totaling 4.27 minutes of stimulation, over the course of 2 hours (Lay et al., 2010). No-stimulation controls (n=8, C57BL/6J; n=4, CD1) underwent identical pMCAO to that of +0h subjects, but never received whisker stimulation; pMCAO was immediately followed by a 5-hour no-stimulation period. Surgical shams (n=7, C57BL/6J) underwent identical surgery to that of +0h subjects, with the suture needle and thread passing under the MCA, but sutures were not tied around the MCA, leaving the blood vessel intact. Sham surgery was immediately followed by whisker stimulation. After whisker stimulation or quiet period, all mice were placed back in their home cage for recovery, until their follow-up assessment at 24 hours post-pMCAO, which consisted of functional imaging and blood flow imaging. Mice were then euthanized and the brains were removed for histological assessment.

Histology (2,3,5-triphenyltetrazolium chloride staining for infarct)

At the conclusion of each experiment, mice were euthanized with sodium pentobarbital (0.2-0.3 mL, intraperitoneally), the brain was removed, sectioned into 2 mm coronal slices, and incubated in 2% 2,3,5-triphenyltetrazolium chloride at 37 °C for 20 min in the dark (Bederson et al., 1986). The TTC-stained sections are photographed with a digital camera, and images are analyzed using ImageJ software. The total infarct volume is determined by multiplying the infarct area of each slice by the thickness of that slice. An observer blind to experimental condition performs this volume calculation. A small surgical lesion is occasionally apparent at the immediate site of MCA occlusion. This occurs infrequently and equivalently in all experimental groups. The small amount of damage occasionally produced at the surgical site can be readily distinguished from the large ischemic infarct and is excluded from infarct analysis (Tamura et al., 1981).

Permanent Middle Cerebral Artery Occlusion

Permanent ischemic conditions are achieved as follows: The base of the left proximal middle cerebral artery at the M1 segment (Gibo et al., 1981; Tamura et al., 1981; Brint et al., 1988; Wang-Fisher, 2009) is permanently occluded, blocking flow to all MCA cortical branches. To do this, the skull and dura are carefully removed from a 2x2mm 'surgical window' placed in the bottom left portion of the imaging window, directly over the M1 segment of MCA, just distal to MCA's lenticulostriate branches and proximal to any cortical branching. A half-curve reverse cutting suture needle is cut in half and threaded with two 4-0 silk threads and passed through the pial layer of the meninges, below MCA (the needle is kept above the cortical surface to the extent possible to prevent damage). Then the two threads (moved to ~1mm apart after being strung beneath the artery) are both tied and tightened around MCA and the vessel is transected (completely severed) between the two knots. Care is taken to avoid damaging the artery, and experiments are terminated if there are signs of bleeding from MCA.

Intrinsic Signal Optical Imaging (ISOI) and Analysis

A detailed description of ISOI data acquisition and analysis can be found elsewhere (Ts'o et al., 1990; Chen-Bee et al., 2007). Briefly, a charge coupled device (CCD) camera (either a 16-bit Cascade 512F or a 12-bit Quantix 0206, Photometrics, Tucson, AZ, USA) equipped with an inverted 50 mm AF Nikon lens (1:1:8, Melville, NY, USA) combined with an extender (model PK-13, Nikon, Melville, NY, USA) is used for imaging and controlled by V++ Precision Digital Imaging System software (Digital Optics, Auckland, NZ). During each 15-s trial, 1.5 s of prestimulus data followed by 13.5 s of poststimulus data is collected**,** with a 6±5 sec random inter-trial interval. Stimulus consists of a single whisker being deflected by 9° in the rostral-

caudal direction at a rate of 5 Hz for a total stimulus duration of 1 second. The cortex is illuminated with a red light emitting diode (635 nm maximum wavelength). Data are collected in blocks of 64 stimulation trials, and a sampled time point (for example pre-pMCAO baseline) is considered complete upon summation of 128 stimulation trials. Ratio images are created from calculating fractional change (FC) values by dividing each 500ms frame of poststimulus signal activity by the 500ms frame of prestimulus intrinsic signal activity collected immediately before stimulus onset. The ratio image containing the maximum areal extent for the first two intrinsic signal phases (Initial Dip and Overshoot) is Gaussian filtered (half width = 5) and the areal extent quantified at a threshold level of 2.5×10^{-4} away from zero. Peak amplitude is quantified in fractional change units from the pixel with the peak activity within the maximum areal extent for both of the intrinsic signal phases.

Laser Speckle Imaging (LSI) and Analysis

A detailed description of LSI (Dunn et al., 2001; Choi et al., 2006) data acquisition and analysis can be found elsewhere (Lay et al., 2010). Briefly, a 632.8 nm 15 mW HeNe laser was used as the illumination source. The speckle pattern from the 5.12×5.12 mm imaged region was captured as 512×512 pixel images by a 16-bit CCD camera (Cascade 512F) equipped with a Navitar zoom lens plus extenders such that speckle size matched camera pixel size. Collected images were processed as previously described (Lay et al., 2010). Speckle contrast images were converted to speckle index images by calculating their inverse squares multiplied by the exposure time in seconds, so that larger index values corresponded to faster blood flow. Speckle index images were then averaged to improve signal-to-noise ratio. To quantify blood flow within the MCA, we calculated the mean value within a region of interest (ROI) in MCA cortical

branches as defined according to several criteria described previously (Lay et al., 2010). All flow index values were scaled over a range where 0 flow was set at noise values. Dead animal (noise) values were subtracted from all values.

Statistical Analysis

For imaging data, ANOVA were run on baseline values to ensure no significant differences before pMCAO. Because there were no responses to quantify at 24 hours, post-pMCAO imaging evoked area and amplitude were converted to difference score values (postocclusion - baseline) with values away from 0 signifying a change from baseline. A constant was added to difference values, which were then transformed with a square root function to better satisfy the assumptions of an analysis of variance (ANOVA) and inferential statistics were performed on the transformed data. Raw values of laser speckle velocity were used for analysis. After ANOVA, specific contrasts were performed to identify which groups differed from baseline. Alpha level was set to 0.05 and Bonferroni adjustments were applied to account for multiple contrasts. Infarct volume comparisons were performed by employing either two-sample t-test or ANOVA. All plotting and statistics were performed using SYSTAT 11 (SYSTAT Software Inc., Chicago, IL, USA).

RESULTS

Treatment does not protect cortical activity in mice

Before pMCAO, there were no significant differences between the three C57BL/6J groups for either area ($mean_{treated}$ = 3.28 ± 0.71, $mean_{untreated}$ = 2.93 ± 0.63, $mean_{surgical\ control}$ = 4.31 ± 0.55; $F_{2,20}$=1.38, P > 0.05, ANOVA) or amplitude ($mean_{treated}$ = 5.07 ± 0.52, $mean_{untreated}$ = 5.73 ± 0.65, $mean_{surgical\ control}$ = 6.94 ± 0.54; $F_{2,20}$=3.03, P > 0.05, ANOVA) of the whisker

functional representation (WFR). However, at 24 hours post-pMCAO there was a significant difference between C57BL/6J groups for both area ($F_{2,20}$=6.26, $P < 0.01$, ANOVA) and amplitude ($F_{2,20}$=7.46, $P < 0.001$, ANOVA). Post-hoc Tukey's HSD tests showed that the area and amplitude for surgical controls were significantly different from the other two groups at 24 hours. Despite receiving immediate treatment, the treated C57BL/6J mice (n=8) were equivalent to untreated C57BL/6J subjects (n=8), with both groups exhibiting a reduction in both area ($mean_{treated}$ = 0.1 ± 0.1, $mean_{untreated}$ = 0.3 ± 0.3) and amplitude ($mean_{treated}$ = 0.26 ± 0.11, $mean_{untreated}$ = 0.16 ± 0.14) at 24 hours compared to baseline. For treated subjects, this reduction was significant for both area and amplitude (area: $F_{1,20}$=10.85, $P < 0.005$; amplitude: $F_{1,20}$=41.54, $P < 0.001$), and the same was true for untreated subjects (area: $F_{1,20}$=9.96, $P < 0.005$; amplitude: $F_{1,20}$=59.49, $P < 0.001$). Surgical controls (n=7), although trending for an increase in area, didn't have a significant change in area ($mean_{surgical\ control}$ = 5.36 ± 1.22) or amplitude ($mean_{surgical\ control}$ = 5.52 ± 0.32) at 24 hours post-pMCAO (area: $F_{1,20}$=0.51, $P > 0.05$; amplitude: $F_{1,20}$=4.49, $P < 0.05$, not significant with Bonferonni correction) (Fig. 5.1, Fig. 5.3A).

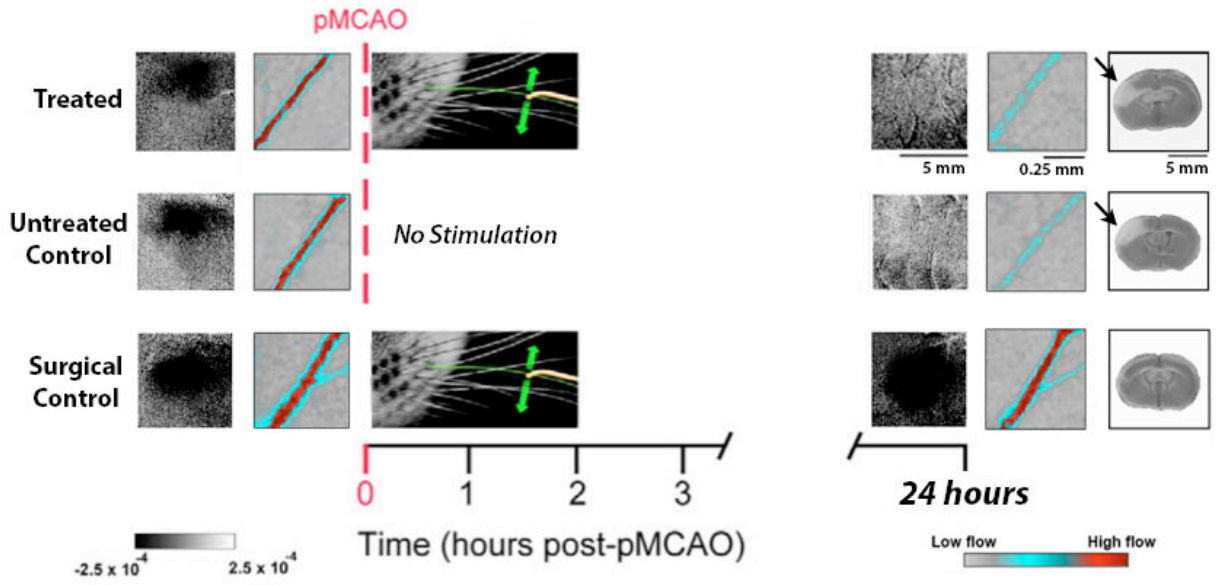

Figure 5.1. Treated C57BL/6J mice are not protected from ischemic damage. Experimental schema with ISOI, LSI and TTC representative cases for C57BL/6J mice: treated (top), untreated (middle), and surgical control (bottom) subjects, before (left) and 24 hours after (right) pMCAO. All subjects had whisker functional representations (WFRs) and blood flow within the MCA at baseline. When assessed at 24 hours post-pMCAO, treated and untreated subjects lacked WFRs and retrograde blood flow within the MCA, and TTC staining revealed infarct (right). Surgical controls, however, maintained both WFRs and MCA blood flow, and did not sustain infarct since these subjects never received pMCAO.

For CD1 subjects, there was no significant difference between treated (n=4) and untreated (n=4) groups at baseline for area ($mean_{treated}$ = 4.16 ± 2.10, $mean_{untreated}$ = 4.25 ± 1.20; $F_{1,6}$=0.03, P > 0.05, ANOVA), or for amplitude ($mean_{treated}$ = 4.37 ± 1.05, $mean_{untreated}$ = 3.97 ± 0.92; $F_{1,6}$=0.08, P > 0.05, ANOVA). Likewise, at 24 hours post-pMCAO, there was no significant difference between groups for area ($mean_{treated}$ = 0.04 ± 0.04, $mean_{untreated}$ = 0.02 ± 0.02; $F_{1,6}$=0.02, P > 0.05, ANOVA) or for amplitude ($mean_{treated}$ = 0.10 ± 0.10, $mean_{untreated}$ = 0.19 ± 0.13; $F_{1,6}$=0.12, P > 0.05, ANOVA). Similar to the C57BL/6J treated and untreated subjects, all CD1 subjects showed a drastic reduction in area and amplitude at 24 hours post-pMCAO. This reduction was significant for both area and amplitude for untreated subjects (area: $F_{1,6}$=14.75, P < 0.01; amplitude: $F_{1,6}$=15.55, P < 0.01), but for treated subjects the reduction was

only significant for amplitude (area: $F_{1,6}$=4.57, P > 0.05; amplitude: $F_{1,6}$=14, P < 0.01) (Fig. 5.2, Fig. 5.3B).

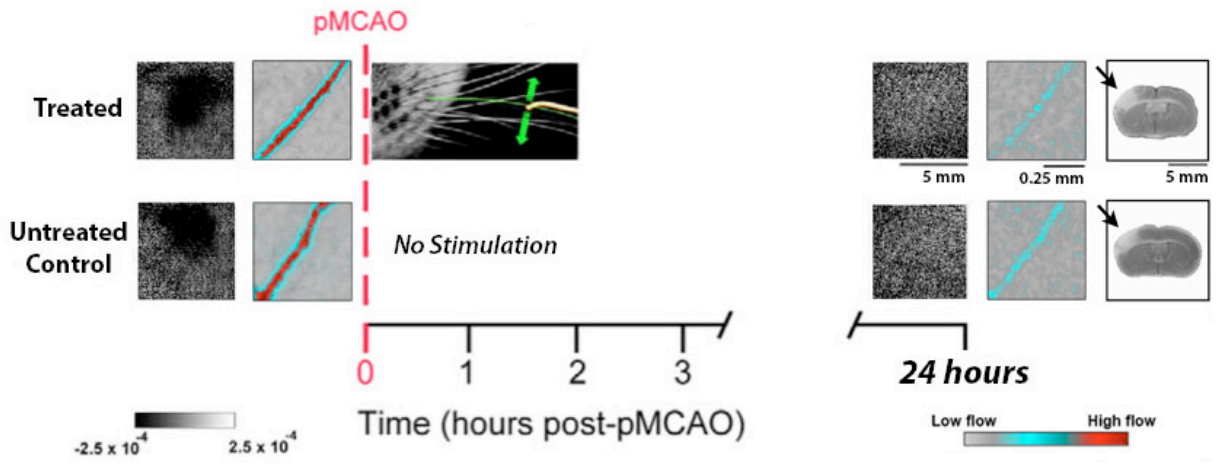

Figure 5.2. Treated CD1 mice are not protected from ischemic damage and are equivalent to untreated mice. Experimental schema with ISOI, LSI and TTC representative cases for CD1 mice: treated (top), and untreated (bottom) subjects before (left) and 24 hours after (right) pMCAO. At baseline, all subjects had WFRs and MCA blood flow, however at the 24 hour assessment all subjects lacked WFRs and had a significant reduction in blood flow. TTC revealed that all subjects sustained infarct (far right).

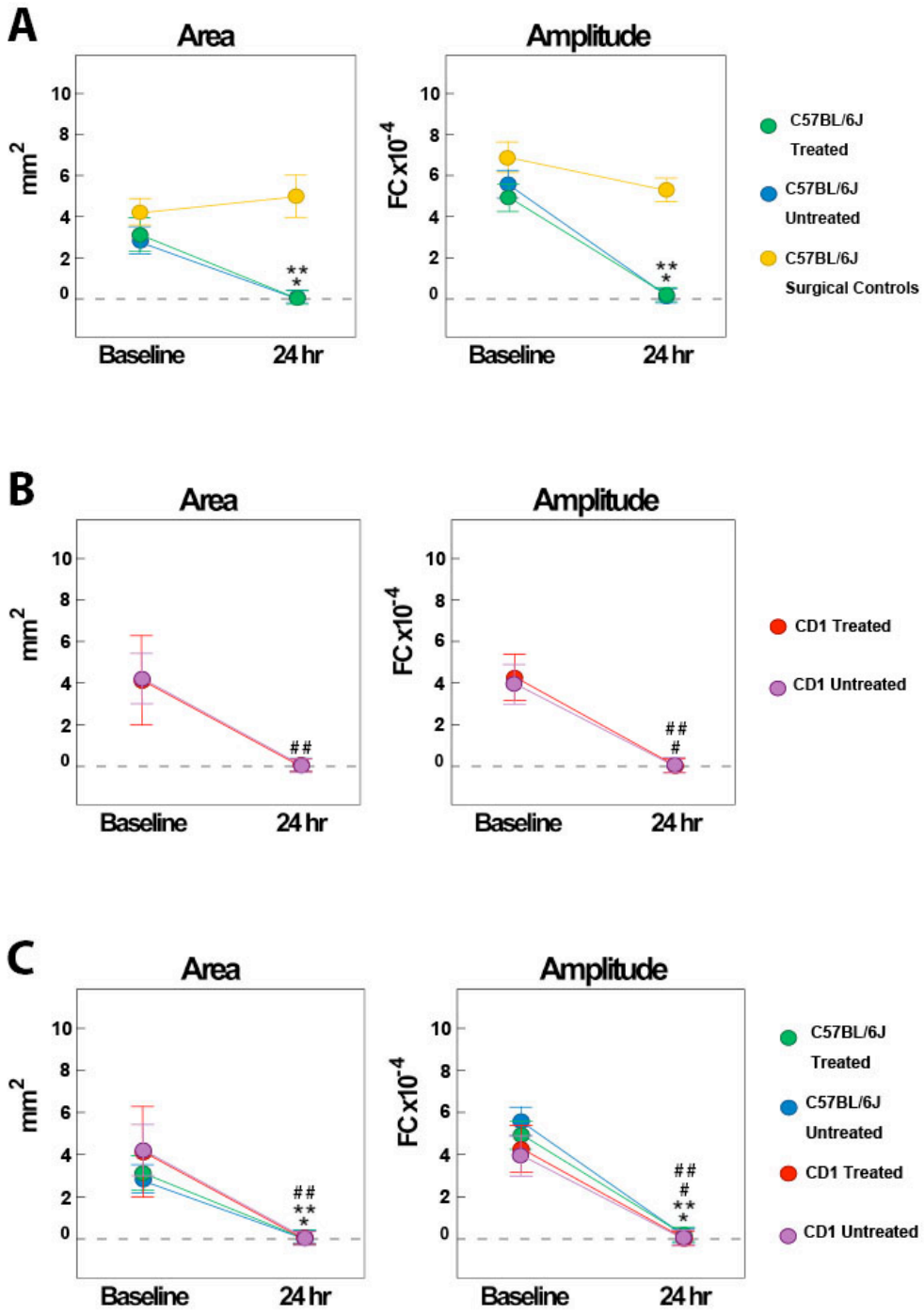

Figure 5.3. WFR quantification for C57BL/6J and CD1 mice. WFRs were quantified in terms of their area and amplitude at baseline and 24 hours post-pMCAO. A, WFR quantification for C57BL/6J mice. Treated and untreated subjects had significant reductions in WFRs at 24 hours, while surgical controls maintained cortical activity. B, CD1 treated and untreated subjects both had reductions in area and amplitude of WFRs at 24 hours compared to baseline. Despite the area for CD1 treated subjects being drastically reduced, it did not reach significance, however area for untreated subjects and amplitude for both experimental groups showed a significant reduction at 24 hours. C, No differences were found between treated and untreated mice for C57BL/6J and CD1 strains, indicating these groups

(Figure 5.3 cont.) were equivalent at baseline and 24 hours. (All significant differences are between baseline and 24 hours: * = C57BL/6J treated, ** = C57BL/6J untreated, # = CD1 treated, ## = CD1 untreated).

We also wanted to determine whether there were any differences between the treated and untreated groups of both C57BL/6J and CD1 strains. At baseline, we observed no difference among experimental groups ($F_{1,20}$=0.04, P > 0.05, ANOVA) or strains ($F_{1,20}$=0.92, P > 0.05, ANOVA) for area. Similarly, there was no difference among experimental groups ($F_{1,20}$=0.02, P > 0.05, ANOVA) or strains ($F_{1,20}$=3.12, P > 0.05, ANOVA) for amplitude. The same was true at 24 hours post-pMCAO, with no difference among experimental groups ($F_{1,20}$=0.10, P > 0.05, ANOVA) or strains ($F_{1,20}$=1.29, P > 0.05, ANOVA) for area or amplitude (no difference among experimental groups: $F_{1,20}$=0.05, P > 0.05, ANOVA; no difference among strains: $F_{1,20}$=2.11, P > 0.05, ANOVA). Therefore, we can conclude that treated and untreated subjects were equivalent between both strains of mice (Fig. 5.3C).

Mice lack sufficient collateral blood flow to the MCA post-occlusion

C57BL/6J mice underwent laser speckle imaging to assess whether there was retrograde blood flow in the MCA 24 hours post-occlusion. First, no significant difference was found between treated (n=8; $mean_{baseline}$ = 2,069,184 AU ± 172,271), untreated (n=8; $mean_{baseline}$ = 2,012,626 AU ± 145,488) and surgical control (n=7; $mean_{baseline}$ = 1,678,293 AU ± 198,882) C57BL/6J groups at baseline ($F_{2,20}$=1.45, P > 0.05, ANOVA), however there was a significant difference between groups at 24 hours post-pMCAO ($F_{2,20}$=34.82, P < 0.001, ANOVA). After pMCAO, both treated and untreated subjects showed a drastic reduction in blood flow within the MCA, indicating a lack of collateral blood flow. Treated (n=8, $mean_{24\ hours}$ = 253,497 AU ± 38,389; $F_{1,20}$=7.45, P < 0.02) and untreated (n=8, $mean_{24\ hours}$ = 256,975 AU ± 48,360; $F_{1,20}$=7.43, P < 0.02) C57BL/6J mice had significant reductions compared to baseline, while

C57BL/6J surgical controls (n=7, $mean_{24\ hours}$ = 1,570,480 AU ± 229,170; $F_{1,20}$=1.73, P > 0.05), which did not receive pMCAO, had no significant difference from baseline (Fig. 5.1, Fig 5.4A).

Additionally, CD1 mice underwent blood flow imaging. At baseline, there was no significant difference between treated (n=4, $mean_{baseline}$ = 1,861,146 AU ± 245,979) and untreated (n=4, $mean_{baseline}$ = 1,403,031 AU ± 360,767) groups ($F_{1,6}$=1.10, P > 0.05, ANOVA), and this was true at 24 hours post-pMCAO as well ($F_{1,6}$=0.91, P > 0.05, ANOVA). Similar to the C57BL/6J mice, treated and untreated groups both showed a reduction in MCA blood flow when assessed 24 hours post-pMCAO, meaning there was no retrograde blood flow through collaterals into the MCA. This reduction was significant for both CD1 treated (n=4, $mean_{24hours}$ = 298,609 AU ± 104,047; $F_{1,6}$=15.07, P < 0.01) and untreated groups (n=4, $mean_{24hours}$ = 195,073 AU ± 31,686; $F_{1,6}$=10.43, P < 0.02) (Fig. 5.2, Fig 5.4B).

Finally, we assessed whether differences existed between C57BL/6J and CD1 strains for both the treated and untreated groups. At baseline, there was no difference between experimental groups ($F_{1,20}$=1.38, P > 0.05, ANOVA) or strains ($F_{1,20}$=3.47, P > 0.05, ANOVA), and the same was true at 24 hours post-pMCAO, with no effect of experimental group ($F_{1,20}$=0.75, P > 0.05, ANOVA) or strain ($F_{1,20}$=0.02, P > 0.05, ANOVA). Thus, the treated and untreated C57BL/6J and CD1 subjects were equivalent at both time points (Fig 5.4C).

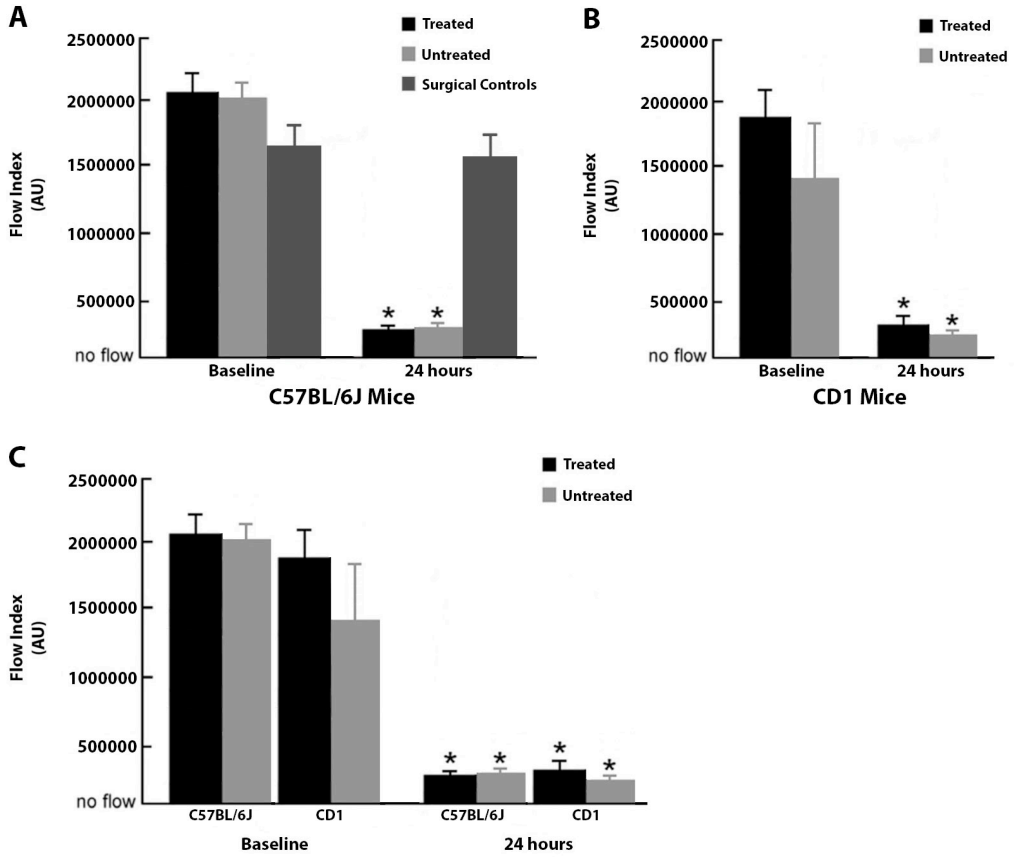

Figure 5.4. Blood flow quantification for C57BL/6J and CD1 mice at baseline and 24 hours post-pMCAO. A, At baseline, C57BL/6J treated, untreated and surgical control groups were equivalent, but at 24 hours post-pMCAO, treated subjects were equivalent to untreated subjects and had significantly reduced blood flow within the MCA, indicating that there was not sufficient collateral blood flow and retrograde flow within the MCA. Surgical controls maintained baseline levels of flow at 24 hours. B, Similar to C57BL/6J mice, CD1 treated and untreated subjects were equivalent and had significant reductions in blood flow at 24 hours. C, No differences were found between strains for treated and untreated subjects at either baseline or 24 hours post-pMCAO.

Histology revealed that mice sustain ischemic damage despite receiving immediate treatment

Ischemic damage was assessed with TTC staining and revealed that immediate treatment did not prevent infarct in C57BL/6J mice (Fig. 5.1). There was a significant difference in infarct size between C57BL/6J groups (treated: n=8, range=8.86–34.69 mm^3, mean = 16.32 ± 3.20 mm^3; untreated: n=8, range=10.81–21.05 mm^3, mean = 15.26 ± 1.25 mm^3; surgical controls: n = 7, no infarcts but occasionally there would be evidence of surgical damage of <1mm^3) ($F_{2,20}$=18.45, $P < 0.001$, ANOVA), and post-hoc Tukey's test showed the significant difference

lied only between surgical controls and the other two C57BL/6J groups (Fig. 5.5A). For treated and untreated subjects, this is 6.93 ± 1.23% and 6.94 ± 0.52% of the ipsi-ischemic hemisphere, respectively. Thus, treated and untreated C57BL/6J groups sustained infarcts of equivalent size, while the surgical controls had no ischemic damage since they did not receive pMCAO. CD1 mice also underwent histological assessment and no significant difference existed between infarct size for the CD1 treated (n=4, range=14.36–29.86 mm^3, mean = 20.84 ± 3.47 mm^3) and untreated control groups (n=4, range=11.45–18.48 mm^3, mean = 15.35 ± 1.47 mm^3) ($t[6]=1.46$, $P>0.05$) (Fig. 5.2, Fig. 5.5B). When represented as a percentage of ipsi-ischemic hemisphere, treated and untreated subjects had infarcts of 9.34 ± 1.64% and 6.97 ± 0.84%, respectively. Finally, when comparing treated and untreated groups for both C57BL/6J and CD1 strains, there was no effect of experimental group ($F_{1,20}=1.37$, $P > 0.05$, ANOVA) or strain ($F_{1,20}=0.68$, $P > 0.05$, ANOVA) on infarct size (Fig. 5.5C).

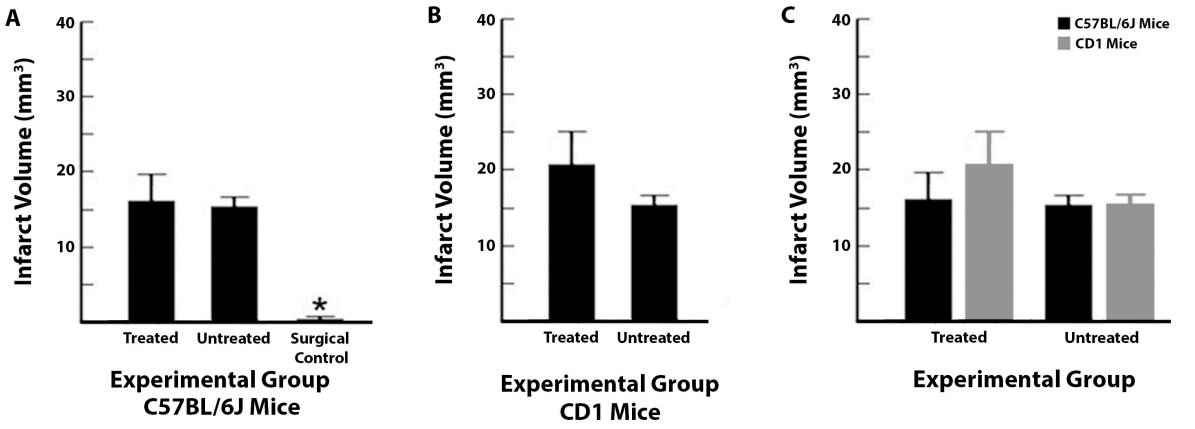

Figure 5.5. TTC revealed no protective effect of treatment for either C57BL/6J or CD1 subjects. A, Infarct quantification for C57BL/6J treated, untreated and surgical control groups. Despite receiving immediate treatment, treated subjects sustained infarct equivalent in size to untreated subjects. Surgical controls were significantly different from treated and untreated subjects as they did not sustain ischemic damage, but occasionally subjects would have evidence of surgical damage that was less than 1 mm^3. B, Treatment did not prevent ischemic damage in CD1 mice, as treated and untreated subjects had similar infarct volumes. C, C57BL/6J and CD1 groups were equivalent for infarct volume.

DISCUSSION

This study assessed whether our previous findings of complete protection from ischemic stroke damage in rats could be replicated in an additional species. Mice were chosen since, if findings had been replicated, this would allow us to begin dissecting the molecular mechanisms underlying this treatment. Although both C57BL/6J and CD1 mice are known to have functioning collaterals, we did not observe protection from ischemic damage in either strain with this collateral-based single-whisker stimulation treatment. Additionally, a small group of subjects that received full-whisker array stimulation treatment were not protected from ischemia either (data not shown). Despite receiving single-whisker stimulation treatment immediately post-occlusion, treated and untreated subjects from both strains exhibited drastic and significant reductions in cortical activity and blood flow 24 hours post-pMCAO, while C57BL/6J surgical controls confirmed that the surgical manipulations themselves (aside from pMCAO) were not responsible for the impairments observed. Ischemic damage was assessed histologically with TTC staining, confirming the presence of infarct in untreated and treated subjects in both mouse strains. It is helpful to think about infarct size in these mice in terms of percent infarct of the ipsi-ischemic hemisphere so comparisons can be made to our previous studies in rats. As mentioned above, infarcts in C57BL/6J treated subjects were 6.93 ± 1.23% of the ipsi-ischemic hemisphere, while infarcts in C57BL/6J untreated subjects were 6.94 ± 0.52%, and CD1 treated and untreated subjects had infarct volumes that were 9.34 ± 1.64% and 6.97 ± 0.84% of the ipsi-ischemic hemisphere, respectively. These percentages fall within the range of what we have observed in our rat studies (9-35mm^3, 3-12% of hemisphere), and they are also in line with what is observed in humans (28-80 cm^3, 4.5-14% of hemisphere) (Carmichael, 2005).

The minimal retrograde MCA blood flow observed here indicates the main potential problem may be that pial collaterals in these mice are not recruited in the same manner as in rats that do show protection when receiving immediate treatment (Lay et al., 2010). In these mouse strains, the significantly reduced blood flow at 24 hours post-pMCAO is not sufficient to protect cortex from impending ischemic damage. It's important to keep in mind that laser speckle blood flow images the surface vasculature, thereby limiting our ability to determine the role of any additional factors. Thus, in addition to impaired pial collateral function, we can postulate that the lack of sufficient blood flow and ischemic stroke protection could be due to several factors, such as an incomplete circle of Willis, impaired neurovascular coupling and functional hyperemia, and impaired function of penetrating arterioles. These will be discussed further below.

Although pial collaterals have been shown to be critical to reducing infarct size, the circle of Willis in C57BL/6J mice is known to be highly variable in terms of the extent of its primary collateralization (Doyle et al., 2012), with many mice lacking either one or both posterior communicating arteries, leaving about 10% with a complete circle of Willis (McColl et al., 2004). It's important to note here that this percentage is much smaller than the percentage of humans with a complete circle of Willis, and that rats are similar to humans in this respect (Lee, 1995; Wang-Fisher, 2009). Thus, the reduced redundancy in blood flow at this early level of cerebral vascularization in these mice may set the stage for the decreased ability to sustain sufficient pial collateral flow after an ischemic event. Additionally, C57BL/6J mice are known to have fewer pial collaterals between the PCA and MCA compared to collaterals interconnecting the ACA and MCA trees (Zhang et al., 2010). It is not clear from our previous work in rats whether collaterals from the ACA or PCA trees are involved to an equal extent or whether one is more involved than the other in protection from ischemic damage. If collaterals from the PCA

are recruited to a larger extent than those from the ACA, this could help explain the lack of protection in mice, however further work is necessary to determine the involvement of collaterals from different arterial trees.

Despite this deficit, there is evidence that these mice do have some pial collateral flow after MCA occlusion. As Zhang et al. (2010) showed, C57BL/6J mice had large numbers of pial collaterals and larger collateral vessel diameters compared to fourteen other inbred mouse strains, and this was associated with smaller infarcts than in other strains. Similarly, Li and Murphy (2008) observed spontaneous retrograde flow in pial collaterals of C57BL/6J mice from the Anterior Cerebral Arterial (ACA) tree 19 minutes after a temporary filament occlusion of the MCA, however, this flow decreased closer to the core of the MCA tree and this damaged region did not recover after reperfusion as a result of removal of the occlusion. Thus, despite having this spontaneous reperfusion via pial collaterals in both of these studies, it was not sufficient to completely protect from ischemic damage under their conditions. Additionally, Cristofaro et al. (2013) found that transgenic CD1 mice that had increased density of pial collaterals also sustained ischemic damage, as not all collaterals were functional. It becomes clear from these results, along with our data, that the impact of collateral flow on infarct size, or complete protection from ischemic damage in our case, relies not just on collateral vessel numbers but importantly vessel functionality.

One would imagine, then, that if these mice received immediate collateral-based sensory stimulation treatment, they may be completely protected from damage as this treatment seems to enhance blood flow in the affected region beyond the minimal blood flow resulting from spontaneous reperfusion in the aforementioned studies. However, both mouse strains were clearly not protected. One possible explanation is that perhaps the mechanisms of neurovascular

coupling that mediate the functional hyperemia response differs between rats and mice. Spontaneous reperfusion occurs as a result of the pressure drop after MCA occlusion, which leads to the pial collateral anastomoses dilating to allow retrograde flow from the ACA and PCA (Brozici et al., 2003; Zhang et al., 2010; Winship, 2015), thereby potentially reducing infarct size but not resulting in complete protection from damage. However, functional hyperemia during evoked cortical activity results in dilation of the local vasculature in order to meet the increased energy demands of the tissue. Our sensory stimulation treatment takes advantage of this phenomenon, resulting in enhanced collateral flow. Although we have shown that enhanced collateral flow has resulted in complete protection from ischemic stroke in rats (Lay et al., 2010, 2011, 2012; Davis et al., 2011), this was not observed in mice. Neurovascular coupling is known to be impaired to varying degrees under ischemic conditions, and targeting this coupling mechanisms has been suggested as a strategy for reducing damage that may succeed in translation to humans (Lo et al., 2003). Thus, it would not be too surprising if this impaired coupling response were the reason for the lack of protection in mice. Further studies are necessary to determine to what extent functional hyperemia and pial collateral dilatation may be impaired in order to understand the inability of the vasculature in these mice to respond to our collateral-based sensory stimulation treatment in a similar manner as in rats.

Related to the idea of impaired neurovascular coupling and functional hyperemia, it's also important to consider that the penetrating arterioles may not have dilated sufficiently to feed the capillaries, and thus the parenchyma (Baran et al., 2015; Shih et al., 2009). Penetrating arterioles can be thought of as bottlenecks, and their responsiveness has been shown to ultimately regulate the rescuing of penumbral tissue since there is no blood flow between neighboring penetrating arterioles as they are not interconnected. Additionally, their dilation is

associated with dilation of upstream pial vessels (Iadecola, 1993), and in fact, the dilation initiated by active neurons can be propagated retrogradely to pial arterioles (Segal, 2005; Girouard and Iadecola, 2006). In C57BL/6J mice, Baran et al. (2015) showed that penetrating arterioles connected to the MCA that are close to numerous pial collaterals between MCA and ACA dilate, whereas those penetrating arterioles further away from collaterals constrict. They concluded that to have dilation of penetrating arterioles to support protection of the tissue from ischemia, there must be blood flow in the pial collaterals through the anastomoses between the MCA and ACA. The coupling of the dilation of pial collaterals and penetrating arterioles after ischemic stroke is clearly a complex process. In our model, it is possible that the evoked cortical activity from treatment did not result in adequate dilation and retrograde blood flow through the pial collaterals, leading to impaired dilation of penetrating arterioles, further compounding the issue and resulting in infarct. Our LSI results would also support this interpretation since minimal blood flow would be observed in MCA if penetrating arterioles were not adequately dilated. In future studies, it would be important to assess perfusion in the penetrating arterioles, perhaps with functional ultrasound (Urban et al., 2015), in order to dissect their role in protection from or deterioration to damage.

The difference in outcome between rats and mice begs the question of which one is a better model and may be more similar to humans. We believe that both can be relevant models and can represent different stroke patient populations. Significant differences in the amount and functionality of cerebral collateralization are documented in humans (Christoforidis et al., 2005; Meier et al., 2007; Bang et al., 2008, 2011; Lima et al., 2010). Thus, our rat studies may represent humans that have well-developed pial collaterals and less impaired neurovascular coupling, while results from the mice may indicate the potential outcome for stroke patients that

lack functioning collaterals without comorbidities. This work further highlights the importance of functional collaterals in the protection from ischemic stroke damage. The collateral-based sensory stimulation treatment described by our lab is a promising treatment for ischemic stroke patients, however the determination of patients that are most likely to benefit should be routinely performed prior to its administration by assessing the extent of cerebral collateralization.

CHAPTER 6: Experimental Summary and Discussion

The overarching theme of this dissertation is the further characterization of a promising collateral-based sensory stimulation treatment that is noninvasive, non-pharmacological, and has the potential to be delivered rapidly. We have previously shown that the early delivery of this treatment can completely protect rats from impending ischemic stroke damage when delivered within the first two hours after pMCAO (Lay et al., 2010, 2011; Davis et al., 2011). However, if treatment is delivered 3 hours post-pMCAO, rats sustain larger infarcts than if they never received treatment (non-stimulated controls). We found that this early treatment relies on retrograde blood flow into the occluded MCA via functioning pial collaterals (Lay et al., 2010). Additionally, we have found that aged rats equivalent in age to the typical stroke patient can be completely protected (Lay et al., 2012), and our original findings have also been replicated by an independent group that showed sensory stimulation could protect cortical function after induction of ischemia (Liao et al., 2015). The Stroke Therapy Academic Industry Roundtable (STAIR) has developed criteria (STAIR, 1999; Fisher et al., 2009; Lapchak et al., 2013) for guiding preclinical research in animal models of ischemic stroke such that the translational capability of potential treatments can be effectively assessed. The studies mentioned above by Lay et al. (2012) and Liao et al. (2015) address concerns by the STAIR, as they tested our treatment in the presence of a critical risk factor (old age) and showed reproducibility of our positive results, respectively. In keeping with STAIR criteria (STAIR, 1999), the work set forth in this dissertation serves to determine the efficacy of this treatment under various conditions, and identify potential limitations in order to define its translational potential.

Chapter 2 assessed whether this treatment resulted in complete protection from ischemic stroke under isoflurane anesthesia, and importantly, whether this type of anesthesia aided in

protection from damage. Our original experiments were conducted under pentobarbital anesthesia, which results in subjects not waking up for several hours after surgery. This prolonged time under anesthesia presents a potential issue, as this does not accurately reflect the conditions under which humans have strokes. Isoflurane, however, allows subjects to wake up within minutes after cessation of anesthesia delivery, which would more closely model the human condition. We found that untreated subjects sustained infarcts equivalent in size to those in rats anesthetized with pentobarbital, and treated subjects were completely protected regardless of which anesthesia was used. Additionally, subjects anesthetized with isoflurane that received treatment 3 hours post-pMCAO sustained very large infarcts. Thus, isoflurane did not have any protective effects in our model. The importance of this study lies in the fact that it set the groundwork for us to test whether sensory stimulation could protect awake, behaving rats from ischemic damage. In this study by Lay and Frostig (2014), the use of isoflurane anesthesia for the induction of stroke permitted animals to awaken within minutes after pMCAO. The results indicate that sensory stimulation via exploration of an enriched environment immediately after pMCAO can prevent impending ischemic damage, and protection was not dependent on whisker stimulation as whisker-barbered rats, like rats that retained their whiskers, were also protected. These findings are of great clinical importance, as most human stroke patients are typically awake, and speak to the relevance of sensory stimulation as an ischemic stroke treatment.

In addition to stating the importance of testing treatments in conscious animals, the STAIR criteria recommend assessing long-term outcomes, testing treatments in the presence of comorbidities, and testing additional species (STAIR, 1999; Fisher et al., 2009). Chapters 3-5 address these topics. In chapter 3, we sought to determine whether protection from ischemic damage was simply delayed or whether it was truly long lasting. Functional and blood flow

imaging, behavior and histology all provide evidence that rats remain protected from ischemic damage at 4 months post-pMCAO, which is equivalent to 10-15 years in humans (Quinn, 2005). This is an important finding as viable stroke treatments should not only be fast-acting, but also long lasting as stroke is currently the leading cause of long-term disability.

Chapter 4 tested the efficacy of this treatment in the presence of the most important risk factor for ischemic stroke, hypertension. Hypertension is known to negatively impact the cerebral vasculature, including pial collaterals, in both humans and a commonly used model for hypertension, the spontaneously hypertensive rat. As such, it was not surprising that we did not observe protection from ischemic damage with our collateral-based sensory stimulation treatment. Although testing this treatment in naïve hypertensive rats was an essential first step, it is important to keep in mind that there are limitations with this model. First, hypertension in these rats is genetic, and they are known to develop pre-hypertension and hypertension at an early age. However, the genetic basis of hypertension in humans is not clear and environmental factors play a large role in its development. Secondly, many hypertensive patients may be on antihypertensive medications to control blood pressure either prior to stroke, or they may initiate treatment soon after admission to the hospital, and there is clinical evidence of antihypertensive agents having beneficial cerebrovascular effects (Gorelick, 2002). These factors allude to spontaneously hypertensive rats potentially having more severe cerebrovascular impairments than humans with hypertension, and could be why we did not observe protection in them. Future research could include interaction studies to determine whether commonly used medications could ameliorate the vascular impairments due to hypertension and increase the likelihood that our collateral-based treatment may result in protection from ischemic damage. Additionally, we should consider that the length of time an individual has had hypertension, and the

environmental risk factors they've been exposed to could have varying effects on the severity of vascular impairment. Chronic hypertension can accelerate the arteriosclerotic and atherosclerotic processes, and these structural changes in vessels can compromise the collateral circulation. Over time, however, new collaterals may develop (Liebeskind et al., 2011) and other compensatory changes may occur to combat the negative effects of hypertension and/or progressive atherosclerosis. These compensatory processes could include the thickening and stiffening of the vessel walls in order to withstand the increased pressure, making them potentially less responsive to our treatment, or could result in new collaterals developing to compensate for reduced flow in the case of progressive atherosclerotic stenosis, and may increase the likelihood of a positive outcome with our treatment. Clearly, hypertension and its role in ischemic stroke is a complex issue and further research is needed to determine under what conditions collateral-based therapeutics may be useful in this patient population.

Finally, chapter 5 addresses the effectiveness of our treatment in another species. After initial rodent studies, the STAIR recommends testing treatments in gyrencephalic species such as nonhuman primates and dogs (Fisher et al., 2009), and even rabbits have been suggested for use since a rabbit embolic clot model has actually resulted in an effective therapy in human acute ischemic stroke – tPA (Lapchak, 2010, 2013; Turner et al., 2011). However, our goal was to replicate our findings in mice as this would open new doors for us to explore the mechanisms underlying protection with our treatment. Two mouse strains, C57BL/6J and CD1 mice, were tested for protection from ischemic damage when receiving our collateral-based sensory stimulation treatment immediately post-MCAO. Although both strains are known to have functioning pial collaterals, we did not observe protection from ischemia with our treatment. The main potential problem may be impaired function of pial collaterals, however other factors such

as an incomplete circle of Willis, impaired neurovascular coupling and functional hyperemia, and impaired function of penetrating arterioles should also be considered. As discussed in chapter 5, these results are important as they provide support for these mice representing a specific stroke patient population that lack functioning collaterals in the absence of major stroke risk factors.

The work presented in this dissertation not only characterizes the conditions in which this collateral-based sensory stimulation treatment may be protective, but it also highlights the challenges associated with the development of a viable treatment given the heterogeneity of the stroke population. No two patients that suffer from ischemic stroke are alike. Between a variety of risk factors that can be altered by genetic and environmental factors, and variability in time from stroke onset to the patients' arrival at the hospital, ischemic stroke is difficult to treat; a treatment that seems promising in some patients may not be ideal for others. Additionally, the goal of current treatments is to reduce ischemic damage by rescuing penumbral tissue through increased blood flow to this region. This may reduce damage and improve recovery but it does not result in complete protection from ischemia. It is becoming apparent that collateral flow may be critical to the prevention of ischemic stroke damage, and the efficacy of current treatments, such as rt-PA and endovascular recanalization techniques, may rely on the extent of collateralization. Spontaneous reperfusion through pial collaterals can occur in stroke patients as a result of the change in pressure after an occlusion, but it's clear that this blood flow is not sufficient to completely protect the brain from ischemic damage. Collateral therapeutics, treatments that can enhance this retrograde blood flow through collaterals, are therefore a promising target for the treatment of ischemic stroke.

Since the level of cerebral collateralization is known to vary in humans (Christoforidis et al., 2005; Meier et al., 2007; Bang et al., 2008, 2011; Lima et al., 2010), it's increasingly clear that imaging patients to assess the extent of collateralization may be crucial in order to determine the best course of treatment as it could help predict the likelihood of a positive or negative outcome (Bang et al., 2008, 2011; Liebeskind, 2014; Liebeskind et al., 2014). Conventional angiography has been the gold standard for assessing collateral perfusion, however indirect assessment with noninvasive, high resolution CT angiography may be a good alternative for quickly assessing patients with acute stroke (Lima et al., 2010). In fact, pial collaterals visible on CT angiography are not only associated with lower in-hospital mortality, but are also a good predictor of outcome (Lima et al., 2010; Menon et al., 2011a, 2011b, 2013). Several studies have shown that the degree of pre-treatment collateral circulation revealed by angiography correlates with infarct growth, with good circulation resulting in lesser infarct growth than those with poor collateral circulation (Bozzao et al., 1989; Kucinski et al., 2003; Mohammad et al., 2008). Importantly, these studies, and others (Christoforidis et al., 2005), indicate that collaterals can potentially be more important than recanalization in some patients, since if good collaterals were observed in patients but recanalization was not achieved, there was still no growth of the infarcted region. Poor outcome after recanalization therapy with rtPA in humans could be due to re-occlusion of the artery and/or poor collateral flow (Winship, 2015; Hussein et al., 2010; Liebeskind, 2008b). Thus, a combined treatment plan with a collateral therapeutic and recanalization therapy may be an excellent option to increase the likelihood of a positive outcome in individuals with good pre-treatment spontaneous reperfusion through pial collaterals, as collateral flow may not only aid in the early rescue of penumbral tissue, but it could also assist in the delivery of fibrinolytics to the clot and facilitate its dissolution (Lima et al., 2010).

Several collateral flow grading systems currently exist for pretreatment diagnostic cerebral angiography (Liebeskind et al., 2011). The most extensive, however, is the American Society of Interventional and Therapeutic Neuroradiology/Society of Interventional Radiology (ASITN/SIR) Collateral Flow Grading System (Higashida et al., 2003), and it can effectively differentiate five degrees of collaterals and incorporates spatial and temporal features of collateral flow (Liebeskind et al., 2011). With improved imaging techniques and the development of methods for thoroughly recording the extent of collateralization, patients can be more routinely assessed for collateral flow and treatment can be optimized depending on the extent of this flow. For individuals with good collateral flow, collateral therapeutics could be the treatment of choice for reducing ischemic damage. Several collateral therapeutics, such as transient aortic occlusion and sphenopalatine ganglion stimulation, show promise for increasing cerebral perfusion after ischemic stroke, however they are invasive, don't result in complete protection from ischemia, and may not be widely available outside stroke centers (Suzuki et al., 1990; Lylyk et al., 2005; Noor et al., 2010; Oluigbo et al., 2011; Levi et al., 2012; Winship et al., 2014, 2015). Therefore, a noninvasive intervention that could be delivered quickly, such as our collateral-based sensory stimulation treatment, may be more desirable.

The work in this dissertation supports the clinical data discussed here, highlighting the importance of functioning collaterals for positive outcome, or in our case for complete protection from ischemic damage. The discrepancy between clinical results that show only reduced infarct and our results that show complete protection from ischemic damage could be due to several factors. Importantly, many patients may not arrive at the hospital within the window of opportunity to receive effective treatment, increasing the likelihood of larger infarcts and poor outcome. Furthermore, it's possible that the spontaneous reperfusion via collaterals in humans is

not above the threshold necessary to maintain viable tissue, and current treatments may not sufficiently enhance this flow or reestablish anterograde flow. Our collateral-based sensory stimulation treatment relies on the functional hyperemia response to evoked cortical activity, and enhances pial collateral flow, resulting in complete protection in rats (Lay et al., 2010, 2011, 2012; Davis et al., 2011). Additionally, this treatment is noninvasive and non-pharmacological, and it has the potential to be delivered quickly. The data presented in this dissertation builds off of this previous work by showing complete protection via collateral-based sensory stimulation treatment is not dependent on the type of anesthesia used, is long-lasting, and heavily relies on functioning collateral vessels to quickly bring blood back to the ischemic region. We have identified a potential group of ischemic stroke patients, those with hypertension, that may not benefit from this treatment as they have impaired cerebral vasculature and collateral flow. Finally, the mouse study may indicate the outcome for patients without major risk factors that lack functioning collaterals, further emphasizing their importance for complete protection from ischemic damage.

Taken together, the work presented here and the clinical data suggest that collateral therapeutics, including the one describe by our lab, are promising treatments. Although we show a lack of protection in the presence of hypertension, patients with this major risk factor may still be able to receive our treatment as the severity of vascular impairments and extent of collateral flow may vary between patients. Similarly, it may not be beneficial for patients without major risk factors to receive this treatment as they may have poor collateral flow. Given the heterogeneity of the ischemic stroke population and the continued development of collateral therapeutics, it would be beneficial to determine the extent of collateralization prior to delivery of treatments. Thus, prompt imaging of collateral flow, perhaps best elucidated with CT

angiography, should be routine as patients arrive at the hospital in order to determine the best course of action. Future work would be required to determine what grade of collateral flow may be necessary for positive outcome with our treatment. However if translational, the collateral-based sensory stimulation treatment described by our lab is noninvasive, non-pharmacological, fast-acting and long-lasting, making it a desirable treatment option that warrants further research.

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