2016

LOOKING TO THE FUTURE OF STROKE TREATMENT: COMBINING RECANALIZATION AND NEUROPROTECTION IN ACUTE ISCHEMIC STROKE

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Digital Object Identifier: https://doi.org/10.13023/ETD.2016.394

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LOOKING TO THE FUTURE OF STROKE TREATMENT: COMBINING RECANALIZATION AND NEUROPROTECTION IN ACUTE ISCHEMIC STROKE

Dissertation

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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2016

ABSTRACT OF DISSERTATION

LOOKING TO THE FUTURE OF STROKE TREATMENT: COMBINING RECANALIZATION AND NEUROPROTECTION IN ACUTE ISCHEMIC STROKE

Stroke is the 5th leading cause of death in the U.S. with 130,000 deaths and around 800,000 affected annually. Currently, there is a significant disconnect between basic stroke research and clinical stroke therapeutic needs. Few animal models of stroke target the large vessels that produce cortical deficits seen in the clinical setting. Also, current routes of drug administration, intraperitoneal and intravenous, do not mimic the clinical route of intra-arterial drug administration. To bridge this divide, we have retro-engineered a mouse model of stroke from the current standard of care for emergent large vessel occlusion (ELVO) stroke, endovascular thrombectomy, to include selective intra-arterial pharmacotherapy administration. Using the tandem transient common carotid and middle cerebral artery occlusion (MCAo) model to induce stroke, we threaded micro-angio tubing into the external carotid artery (ECA) towards the bifurcation of the common carotid and internal carotid arteries (CCA/ICA) allowing for the delivery of agents to the site of acute ischemia. Our model was optimized through a flow rate and injection volume study using carbon black ink injected through the intra-arterial model at different flow rates and injection volumes. The purpose of this study was to demonstrate that our injections were arriving at the site of ischemia and to improve injection volumes for future dosing while mitigating systemic side effects by preventing or minimizing systemic distribution. We determined that a flow rate of 2.5 µl/minute and injection volume of 10 µl was optimal. Next, we tested potential neuroprotective compounds nitroglycerin, verapamil, and a combination of verapamil and lubeluzole. Compounds were chosen for drug synergy and to target specific pathways in either an acute or delayed manner. Acute treatments included nitroglycerin and/or verapamil while delayed treatment included lubeluzole. The known mechanism of action for FDA approved nitroglycerin is through vessel dilation that results in increased blood flow to the treated region.

A secondary mechanism of nitroglycerin is the production of nitric oxide, which has demonstrated antioxidant and anti-apoptotic effects when processed and released from cells surrounding the blood vessels. Verapamil, a calcium channel blocker, also FDA-approved for cerebral artery vasospasm: is thought to act by blocking the L-type calcium channels on the cell membrane from opening following membrane depolarization after insult. Finally, lubeluzole, also FDA-approved, is proposed to work as an NMDA modulator inhibiting the release of glutamate and nitric oxide synthase and blocking sodium and calcium channels. Through our stroke model we were able to demonstrate that each drug(s) showed a significant decrease in infarct volume and improved functional recovery while simultaneously minimizing potential systemic side effects suggesting that our stroke model may improve the preclinical validation of potential stroke therapies and help bridge the bench to bedside divide in developing new stroke therapies.

KEYWORDS: Acute Ischemic Stroke, Intra-arterial, Emergent Large Vessel Occlusion, Pharmacotherapy, Neuroprotection

Michael Eric Maniskas

September 8, 2016

LOOKING TO THE FUTURE OF STROKE TREATMENT: COMBINING RECANALIZATION AND NEUROPROTECTION IN ACUTE ISCHEMIC STROKE

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September 8, 2016

This dissertation is dedicated to all those who supported my dreams and encouraged me to never settle, but to always strive for more. To my mother, Chris Maniskas, you have always been an inspiration and the bar to which I measure myself. Your dedication and sacrifice has made me a better person, no amount of thanks can convey my appreciation for all you have done and continue to do. To my father, Eric Maniskas, your commitment to making sure I learned the value of hard work has given me more than you will ever know. It is my hope that when I become a father I will follow in your footsteps, and make sure my children have all the opportunities you provided for me. To my brother, Stephen Maniskas, there is no finer gentleman or best friend.

ACKNOWLEDGMENTS

The following dissertation, while an individual work, benefitted from the insight and direction of several people. First, my Thesis Chairs, Drs. Gregory J. Bix and Justin F. Fraser. They embody the high quality of scholarship to which I aspire. Without their guidance and support none of this would have been possible. Second, Dr. Jill M. Roberts, for providing the time and patience to mentor me in all aspects of good lab practice, surgery, and support at every stage of my project. Third, Ms. Amy A. Gorman. If it wasn't for her never ending support and dedication to helping me with my project, very little would have been accomplished. Fourth, I would like to thank my committee members, Dr. Ed Hall, Dr. Joe Springer and Dr. Steve Estus for agreeing to be on my committee and helping guide me through my PhD career. Lastly, I would like to thank Mary Kathryn McKenna. We started this journey together and it has been an adventure ever since. Here is to hoping the next chapter in our lives is filled with even more excitement.

TABLE OF CONTENTS

List of Tables

List of Figures

Chapter 1: General Introduction

Ischemic stroke is defined as a disruption of blood flow leading to a loss of oxygen and nutrient-rich blood to a region of the brain, which then causes cellular damage to brain tissue. This leads to neurological deficits, brain swelling, and possibly death. There are two forms of stroke; ischemic, which is caused by a clot or thrombus and obstructs blood flow to the brain (responsible for 87% of strokes), and hemorrhagic which is caused by a blood vessel wall rupturing and leakage of blood into the surrounding tissue (responsible for 13 % of strokes). In the United States, ischemic stroke is the 5th leading cause of mortality with 140,000 affected, but the leading cause of long-term morbidity with 800,000 affected annually (1, 2). The incidence of stroke is expected to double by 2050, and affect a wider range of aged individuals, which is related to an increasing aging population and a younger generation becoming sedentary with poor diet and activity (3). More people are surviving stroke due to knowledge of early warning signs, as well as medical care that is readily available and advanced. The most deadly and disabling form of ischemic stroke is emergent large vessel occlusion (ELVO). ELVO is characterized when large vessels of the brain such as the internal carotid artery (ICA), M1 and M2 segment of the middle cerebral artery (MCA), vertebral and basilar artery are occluded (4).

ELVO causes a large territory of brain to lose oxygen and nutrients, causing apoptosis and death to the neural unit. Apoptosis occurs through two pathways, extrinsic or death receptor pathway, and intrinsic or mitochondrial

pathway. ELVO induces the intrinsic apoptotic pathway through nutrient loss and hypoxia (oxygen deficient environment) which leads to a cellular cascade resulting in death. In short, external stimuli signal a change in the inner mitochondrial membrane releasing pro-apoptotic proteins into the cytosol, and these pro-apoptotic proteins activate the caspase dependent pathway that will translocate into the nucleus causing DNA fragmentation, ultimately resulting in cellular death (5). By targeting specific receptors, channels or pathways we hope to prevent further damage. The damaging mechanisms may proceed through rapid nonspecific cell lysis (necrosis) or by active form of cell demise (apoptosis or necroptosis), depending upon the severity and duration of the ischemic insult (6).

There are limited treatments available for ELVO, which consist only of supportive care for downstream effects from stroke (brain edema and herniation, and systemic secondary issues), and thrombolysis (therapies to re-open the vessel and re-establish blood flow). Currently there is only one FDA-approved pharmacotherapy, tissue-plasminogen activator (t-PA), whose mechanism of action is to help break down a clot by fibrinolysis or the activation of plasmin through a series of reactions (7). Due to extensive exclusion criteria such as; a 4.5 hour therapeutic window, diabetes, older than 85 years of age, prior stroke, and use of blood thinners, only about 14 to 38% of patients are eligible (3, 8). Recently, a new therapy, endovascular thrombectomy (ET), was introduced and successfully completed numerous clinical trials (9-11). Now considered standard of care, ET is the mechanical removal of a clot by threading a catheter through

the patient's vasculature to the site of occlusion and removing the clot (9-11). Contrary to intravenous (IV) t-PA, ET is available to a larger portion of the population due to fewer exclusion criteria and wider applicability.

While both ET and IV-tPA have improved patient survival and clinical outcome, the overall improvement beyond treatment has not mirrored the improved ability to rapidly recanalize an occluded vessel (9, 12, 13). This disconnect between successful recanalization using ET and/or IV t-PA and improved clinical outcome highlights the need for adjunctive therapies. Currently there is no adjunctive therapy, t-PA included, that confers neuroprotective or neuroreparative benefits. There have been a number of clinical trials looking at potential neuroprotective compounds ranging from glutamate blockers to free radical scavengers with all showing little to no benefit (14, 15). Many of these compounds showed benefit in the rodent model but failed to translate in a clinical setting (14, 16). Likely causes of failure include administration of drug long after the therapeutic window (up to 48 hours), as well as drug administration to patients whose vessels were still blocked (patients who did not receive successful concomitant thrombolysis) (15, 17). Stroke is an acute disease and treatments that fail to reach the site of ischemia because the vessel remains blocked or the therapeutic window of opportunity has passed are unlikely to succeed clinically (15).

The aim of this project is to use a clinically relevant mouse model of acute ischemic stroke that allows us to guarantee recanalization of the previously

occluded vessel while mimicking the clinical ability to selectively administer a potential neuroprotective agent directly to the site of ischemia. There are numerous mouse models of stroke available; small vessel occlusions include the photothrombotic and embolic models. Large vessel occlusions like ELVO include the suture (suture threaded through vasculature to occlude the Circle of Willis), chemical induction (direct application to the targeted vessel), and filament (ipsilateral tandem occlusion of the common carotid and middle cerebral arteries). Below we will briefly describe each, and discuss the reasons for the model design.

Mouse Models of Stroke - Optimizing a Model

Small Vessel Occlusion

Photothrombotic Model

The photothrombotic model mimics small vessel occlusions in the vessels of the cortex distal to the large vessels of the Circle of Willis, and distal cortical branches. The photothrombotic model allows researchers to induce an ischemic event targeting specific motor or sensory functions in the specific areas of the cortex by occluding vessels supplying the targeted function (18-20). An infarct is induced following the injection of a dye, Rose Bengal, that is then activated using a laser or other light source to cause the dye to coagulate and block the intended vessel on the cortical surface (21). Dye activation and occlusion of the vessel can be achieved in most parts of the cerebrovasculature and is not confined to the motor or sensory cortex but most studies look at deficits from these regions. As

such, the preclinical model correlates to a small vessel infarct in a confined cortical brain region.

Stroke studies using the photothrombotic model have shown it to be representative of a small vessel occlusion by comparing functional and sensory deficits, using an animal model (rat and mouse). Upon dye injection and activation targeting the motor and sensory cortex (middle cerebral artery) a functional and sensory deficit is seen within a few hours affecting the contralateral forelimb and hind limb. Tests used to assess functional deficits are the wire hang, cylinder test, grid walk, reaching, and pole test; while deficits in sensory function use the adhesive removal test (18-20).

Infarct volume measurements for the photothrombotic model show a core and penumbral region around the infarct/laser-dye activated, and infarct volume measurements range from 1 - 2 mm^3 to 8 - 10mm^3 depending on the aforementioned parameters (18, 20). Cellular differences, such as astrocytes and microglial infiltration and decreased neuronal survival, appear in the stroked animals compared to controls (18, 20).

Advantages of the photothrombotic model are minimal surgical impact on the animal for stroke induction, graded lesion volume, ability to target a specific region for motor, sensory or sensorimotor deficit, and high reproducibility coupled with long-term survival. Disadvantages of the model are microvascular insult, small penumbra, permanent occlusion, occlusion and evolution of infarct occurs rapidly, small infarct volume when compared to other stroke models, decreased motor or sensory deficit, adverse effects to blood vessel walls potentially leading

to breakdown and edema in the parenchyma (22). This model is optimal for mimicking small vessel occlusion, particularly the more distal region of the MCA, which present with facial, arm, and leg weakness or inability to move.

Embolic Model

The embolic clot model, another small vessel occlusion model, best mimics the human condition to achieve a clinically relevant thrombus induced infarct. The embolic clot model uses a clot derived from coagulated blood of the animal, and is injected through the ICA into the MCA. The clot circulates into the vasculature of the cortex until it becomes lodged in a cortical vessel where it will obstruct blood flow to a particular region causing an infarction (deficits are representative of where the clot occludes and can have some variability) (23). Briefly describing the procedure, blood from the animal (collected prior to clot injection) is allowed to coagulate into a clot, the clot is loaded into a micro-tubing attached to a micro-syringe and injected into the cervical or cephalic vasculature depending on animal's (rat or mouse) size (24).

The embolic model is considered variable by many due to its non-specific infarct size and placement. This is in direct contrast to mechanical models which allow direct placement of a stroke either in the cortex, subcortical areas or both. While the benefit of the embolic model is to mimic the heterogeneity of thromboembolic stroke in humans, that same heterogeneity is problematic when using *in vivo* preclinical models to study stroke, where the goal is to control the infarct from one animal subject to the next. Because of aforementioned variabilities, the embolic clot model can present with functional, cognitive or a

combination of both deficits depending on clot placement and duration. Successful clot delivery can be tested using a variety of functional and cognitive tests; functional deficits can be tested using the beam walk, adhesive removal, and corner test (25, 26). Cognitive deficits are best measured using the radial arm water maze, and various versions of neurological scores (27-29). Functional or cognitive testing using this model will show differences between stroked and control groups within 5 hours of infarct induction and can persist up to 7 days.

Degree of infarct volume is variable in this model due to varying clot size, location of occlusion, and duration, but does present with a core and penumbral region. Infarct volume measurements range from 3-56 mm^3 depending on aforementioned parameters (30, 31). Inspection of the infarcted region shows astrocytic and microglial infiltration in the core and penumbral region within hours of stroke leading to scar formation over 21 days.

Widely considered the most clinically relevant model, the embolic model requires surgery to inject the clot causing manipulation and permanent occlusion of cervical vasculature to ensure clot delivery via the ICA to MCA. Advantages of this model are both functional and cognitive deficits, true to the clinical condition, and variable infarct volume representing the population of patients. Most importantly, it can be a very relevant model for testing systemic administration of t-PA and other thrombolytics (32, 33). Disadvantages of the model include the need for extensive surgical manipulation, the likelihood of permanent occlusion by the thrombus, and aforementioned variability.

Large Vessel Occlusion Models

Suture Model - MCAO

Large vessel occlusion is produced using mechanical stroke induction and provides a cortical and/or subcortical infarct that manifests in both functional and cognitive deficits through the occlusion of the ICA and MCA. Functional and cognitive deficits require occlusion of the proximal segments (M1/M2) of the MCA with particular attention to the lenticulostriate and anterior cerebral arteries (ACA) of the Circle of Willis. Mechanical induction is achieved through the MCAO model by threading a predetermined length of suture (2-0 or 4-0) with a heat induced rounded end (decreases inflammatory vessel wall response) - through the external carotid artery (ECA) into the ICA at the bifurcation of the common carotid artery (CCA). The suture is threaded through the ICA towards the Circle of Willis and MCA stopping at the transition from ICA to MCA/Circle of Willis. It is important to not thread the occlusion suture beyond the distal most portion of the ICA for risk of puncturing the MCA and causing cerebral bleeding. Indeed, this same model (when used with a non-blunted suture) is used to induce intracerebral hemorrhage for study in preclinical models. Occlusion times of the ICA/MCA/Circle of Willis are variable but 60 - 90 minutes is common in the literature to produce measurable functional and cognitive deficits (34).

The MCAO model is a reliable and reproducible model of stroke causing large cortical and subcortical infarcts spanning the entire occluded hemisphere. Infarct is induced mechanically but does not represent the clinical manifestation of a clot and treatment through intravenous (IV)-tPA or ET but rather restores

blood flow through suture removal from the ICA. Advantages of the MCAO model are its clear functional and cognitive deficits through occlusion of vessels providing blood to both cortical (motor/sensory cortex) but also subcortical (hippocampal/white matter) areas. Infarct volume, cellular inflammatory infiltration and deficits are measurable within days and continue days and weeks post stroke allowing for a variety of tests. Functional deficits are measured using the vertical pole, hanging wire, open field, and corner test. While cognitive deficits can be measured using the Morris or radial arm water maze and novel object recognition (34, 35). Disadvantages of the model are its variability in infarct size, high mortality rate, significant impact on cerebral edema on post-stroke inflammation and outcome, and artificial stroke induction through the manipulation of the CCA/ICA and ECA with permanent occlusion of the ECA.

Endothelin-1

The endothelin-1 model is a vasoconstrictor that can be applied targeting either arterial or venous supply through surface application or intra-cerebral injection. The model is dose dependent and is used to target ischemia in either cortical or subcortical regions. Advantages of the model are minimal tissue edema, localized ischemic infarct, and specific receptor targeting for interruption of astrocyte and neuronal repair. Disadvantages of the model are its lack of clinical correlation, failure to target large vessels in an acute manner and repeated interference with neural repair (22).

Filament Model - MCAo

The tandem transient common carotid/middle cerebral artery occlusion model (MCAo) of large vessel occlusion is similar to the previously described MCAO. It is a mechanical induction of stroke for the cortical region achievable through occlusion of the M1/M2 of the MCA. To achieve an infarct in the cortical region, affecting primary motor function of the forelimb, requires occlusion of the MCA and CCA. A tandem occlusion of the ipsilateral vessels decreases blood flow to the cortical region in question ensuring infarct induction. Mechanical stroke induction begins with exposing the MCA through a burr hole in the skull and using a small wire that is inserted underneath the MCA kinking the vessel closed and then a clamp is placed on the CCA to occlude blood flow. Occlusion times vary between labs but 60 - 90 minutes is common in the literature (20, 36, 37). Reperfusion is achievable with removal of the wire filament and clamp, however this model can also be used as a permanent model. The MCAo model produces a clinically relevant infarct in the cortical region of the MCA and surrounding areas (20, 37-40). It mimics a distal M1/M2 MCA occlusion.

The MCAo model of stroke induction is reliable and reproducible, which yields specific focused infarcts in the cortical region through MCA occlusion. While mechanical induction of stroke provides clinically relevant infarcts of the cortex, it does not mimic the treatment (IV-tPA or ET) but rather restores blood flow through the removal of the MCA filament and CCA clamp. The advantages of the MCAo model are infarct volume, cellular response, and functional manifestations that present within hours of stroke induction and can be measured

days and weeks following. Infarct volume for the MCAo model achieves max volume around 3 days with measurable inflammatory cellular infiltration (astrocyte and microglia) (38). Functional deficits of the forelimb are seen within one day and can be measured using a variety of motor tasks: rotor rod, grip strength, wire hang, open field, beam walk, reaching task, tape removal, and cylinder test (20, 37). The MCAo model is not well studied or defined for cognitive deficit as only the MCA supplying the cortical motor/sensory region is typically affected, but a higher than normal branching of the lenticulostriate arteries can produce memory deficits. This is not typical and should be taken into account when choosing a deficit model of motor, memory, or both following stroke.

The advantages of the MCAo model are a reliable, reproducible, low mortality, clinically relevant infarct in the MCA region affecting motor function with hallmark cellular characteristics and variable occlusion times. Disadvantages of the model are its lack of clinically relevant induction (clot) and treatment (IV-tPA and/or ET), as well as initial difficulty in mastering the surgical technique. Overall, the model provides a number of hallmark measurable characteristics following stroke induction and potential treatments. The MCAo provides the best mouse model of stroke available to mimic the clinical condition of ELVO, with the removal of the clamps as a preclinical correlate to recanalization by thrombectomy, with guaranteed recanalization, similar infarct size and location, and presentation of physical deficits.

Neuroprotective Drug Administration Approach

Intra-arterial model

Our aim was to develop a drug administration model that mimicked selective delivery directly to the site of ischemia while already using the exposed vasculature of the MCAo model. Developing an intra-arterial (IA) model of pharmacotherapy administration allows us to bypass a number of issues that plague the systemic administration. By delivering our drug of choice directly to the site of ischemia, we are able to guarantee the drug reaches its target site, decrease the amount of drug needed, and mitigate any systemic side-effects that may occur (38, 39). An IA model could mimic the opportunity seen in the clinical condition during thrombectomy since after recanalization of the vessel it is possible to selectively deliver targeted drug therapy before completing the procedure, guaranteeing the drug reaches the ischemic region.

By combining the MCAo and IA model, we have developed a clinically relevant mouse model of stroke induction and targeted pharmacotherapy administration. Next, we optimized our model to ensure that our drug of choice reached the site of occlusion following successful recanalization with little to no systemic distribution, and did not produce any systemic side-effects. After optimizing our model we selected three FDA-approved drugs previously tested in both a pre-clinical and clinical setting: nitroglycerin (also known as glyceryl trinitrate, GTN), verapamil, and lubeluzole.

Intra-arterial Nitroglycerin

Nitroglycerin (GTN) is FDA-approved and used as a vasodilator to open collapsed blood vessels in an acute fashion. GTN is commonly used for angina pectoris to vasodilate the vessels of the heart following an ischemic event. It works by relaxing the smooth muscles surrounding the blood vessel, but also by producing nitric oxide (NO_x) which works by blocking apoptotic signaling cascades. GTN has a short half-life (2 – 7 minutes) and possesses a dual mechanism of action, working both acutely (vessel dilation) and delayed (anti-apoptotic) by the conversion of GTN to NO_x (41-43).

Intra-arterial Verapamil

Verapamil, an L-type calcium channel blocker, is FDA-approved and currently used to treat cerebral artery vasospasms following successful recanalization after ELVO. Administered acutely following membrane depolarization, verapamil works by blocking the influx of calcium intracellularly. Verapamil is a one pass drug, meaning it will be degraded once it passes through the liver and loses it bioactivity and effect if not administered upstream of its target site. IA delivery of verapamil ensures it will reach the stroked tissue, have its effect on calcium influx and then be degraded by the liver in venous return from the brain. This is key, because if verapamil is not degraded in the liver and is still bioactive when it reaches the heart it can cause a decreased heart rate and increased vessel dilation (44-46).

Intra-arterial Lubeluzole

Lubeluzole, also FDA-approved, has a less defined mechanism of action but is widely accepted as a NMDA modulator blocking glutamate release. Lubeluzole demonstrated positive effects in a pre-clinical setting but did not translate into the clinical setting and was discontinued due to failure of efficacy (47-50). Pre-clinical testing with lubeluzole (0.63 and 1.25 mg/kg) demonstrated both a decreased infarct volume and improved functional behavior when administered IV within six hours post-infarct (49). Clinically, lubeluzole was administered IV initially (7.5mg) and followed with daily IV doses of 10mg for five days. Two independent clinical trials produced different results, the first showed lubeluzole to be safe and have an improved clinical outcome (51). A second study, showed no differences between groups and demonstrated adverse events (fever, constipation, and headache) (52). Lubeluzole will be administered in an acute fashion with a one-time dose. By delivering the drug selectively to the ischemic region we are bypassing systemic circulation and any potential side effects.

Intra-arterial combinational therapy

By combining an acute drug with a delayed drug we are targeting specific damage that cells are undergoing in the ischemic brain region. Furthermore, as cells within the core (closest to the occluded vessel) are in a different stage of apoptosis than those cells in the penumbra (radiating away from core), a combinational pharmacotherapy approach will allow for a broader range of cells/pathways to be targeted, especially if the drugs work in an acute and

delayed fashion without overlapping mechanisms of action. With this in mind, while both verapamil and GTN are acute drugs, their similar primary mechanism of action (vasodilation) prohibits them from being combined due to potential blood leakage into the parenchyma due to blood brain barrier break down. However, verapamil and lubeluzole working in an acute and delayed setting will target different apoptotic pathways, therefore giving a greater chance of blocking multiple targeted pathways for potential synergistic effect.

Chapter Two: Intra-arterial Model

Maniskas M, Bix G, Fraser J. Selective intra-arterial drug administration in a model of large vessel ischemia. J Neurosci Methods. 2014 Nov 8;240C:22-7. PubMed PMID: 25445249.

Introduction

Emergent large vessel occlusion (ELVO) affects the vital arteries (ICA, MCA, ACA, vertebral and basilar) of the brain. Current therapeutic options for ELVO include IV-tPA and/or endovascular thrombectomy (ET). Intra-arterial ET, involves the mechanical removal of a clot using a retrieval device threaded through the patient's vasculature (10, 53). From 2004 to 2008, the use of IA thrombectomy increased six-fold (54). While advances in medicine such as IV t-PA and mechanical thrombolysis have shown improved restoration of blood perfusion to ischemic brain regions, it has not correlated to improved clinical outcomes in patients (12, 53, 55). To date, there are no therapies, including thrombolysis, that provides either neuroreparative or neuroprotective benefits when administered following successful recanalization of a previously occluded vessel. Due to this disparity, the ability to successfully treat stroke patients and achieve full recovery remains out of reach.

Endovascular thrombectomy begins with a catheter being feed within the femoral artery of the leg superiorly until it reaches the aorta in the abdomen and thorax. Passing through the aorta of the thorax the catheter will feed further superior into the CCA of the neck and into the ICA and MCA of the head. While

the end goal of ET is to successfully guide the catheter to the clot and remove, restoring blood flow, it also provides an opportunity to selectively deliver a potential therapeutic compound that has neuroprotective or neuroreparative benefits. Delivery of targeted stroke treatments guaranteed to reach the site of ischemia provides a number of advantages over systemic administration. These advantages include decreased risk of systemic side-effects (increased intra-cranial pressure, increased heart rate, decreased blood pressure), need for lower concentration of therapeutic compound, and increasing the concentration of the compound to better reach the ischemic brain region.

Years of research and clinical trials has yet to produce a viable compound that can be administered as a neuroprotective treatment following acute ischemic stroke. A bench-to-bedside approach utilizes the knowledge of researcher and clinician to develop new approaches for treating acute ischemic stroke. Modeling ET for selective pharmacotherapy delivery, we retro-engineered a mouse model to mirror the clinical condition of IA thrombectomy following successful recanalization. This allows us to selectively deliver IA pharmacotherapy directly to the site of ischemia while also ensuring restoration of blood flow.

Attempting to remain as clinically relevant as possible, we modeled our IA process as follows: MCAo is induced for 60 minutes, using the already exposed vasculature of the neck we advance a catheter from the ECA into the bifurcation of the CCA and into the ICA, following 60 minute stroke induction we recanalize and inject IA therapeutics without the need for permanent indwelling micro-ports

or catheters. Similar to the clinical condition, we are able to successfully recanalize, restoring blood flow while also delivering targeted stroke therapeutics. While other models of IA exist, they leave long-term microports (surgically implanted pump for pharmacotherapy delivery) and indwelling catheters which are not clinically relevant for acute ischemic stroke treatment (56, 57). This novel approach allows us to administer IA neuroprotective agents in a clinically relevant fashion with the minimal amount of surgery.

To validate our mouse model of pharmacotherapy administration, it was also essential to show that our model could be effectively utilized to deliver compounds directly and specifically to the site of ischemia. We found that the efficacy of selective delivery of potential pharmacotherapies was dependent upon optimized flow rate and injection volume. The study of flow rate and injection volume through carbon black ink injection allowed us to demonstrate that a compound administered in such a fashion could reach the affected area and its injection rate and volume could be optimized for our model. Through this retro-engineered mouse model, flow rate, and injection volume study we have described a method that can be used to test the hypothesis that local delivery of pharmacologic agents following ischemic stroke will show improved neuroprotection and functional outcomes compared to systemic delivery of therapeutic agents.

Material and Methods

Suture, metal wire, micro-angio tubing and syringe preparation

Two different sized sutures were used: one 2-0 nylon monofilament suture cut two centimeters (cm) in length for occlusion of the CCA and three 6-0 nylon braided silk sutures cut 1.0 cm in length for permanent occlusion of the ECA and securing of the micro-angio tubing. Metal wire (Small Parts, Logansport, IN) 0.0127 cm in diameter were cut to a length of 0.1 - 0.15 cm. Using micro forceps under a dissecting microscope, a 34 gauge needle (Hamilton Syringe Co., Reno, NV) was fitted with a 10 cm length of micro-angio tubing (MRE 010-Braintree Scientific, Braintree, MA), the combined needle and tubing were then attached to a 100 µl Hamilton Gas Tight syringe (Figure 2.1).

Animal preparation for surgery

The Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky approved all procedures, which were conducted in accordance with the principles of animal care and experimentation in the Guide for the Care and Use of Laboratory Animals. Three month old C57/Bl6 male (Jackson Laboratory) mice were anesthetized via intraperitoneal injection with a combination Ketamine/Xylazine mixture in a saline solution using a weight based dosing scale. The mice were shaved on the left temporal region of the head from the lateral corner of the left eye to the medial region of the left ear with the resultant shaved region being 0.75 cm by 0.75 cm (Figure 2.2A). The shaved

INTRA-ARTERIAL SYRINGE, NEEDLE, AND TUBING CONSTRUCTION

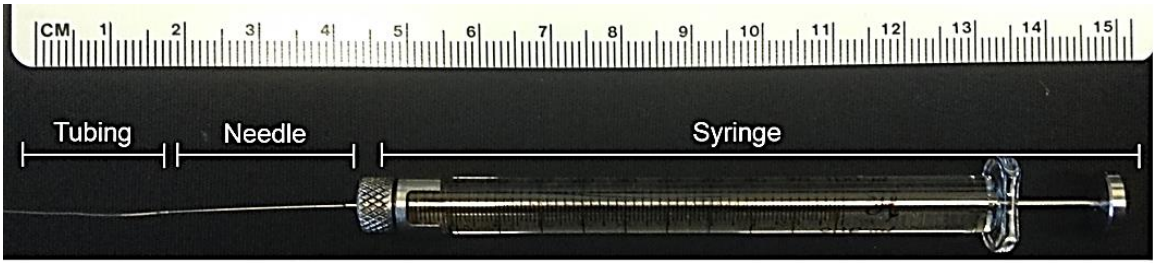

Figure 2.1. Image of 100µl Hamilton Gas Tight syringe with 34 gauge needle and micro-angio tubing attached.

SURGICAL INCISION SITES FOR STROKE SURGERY

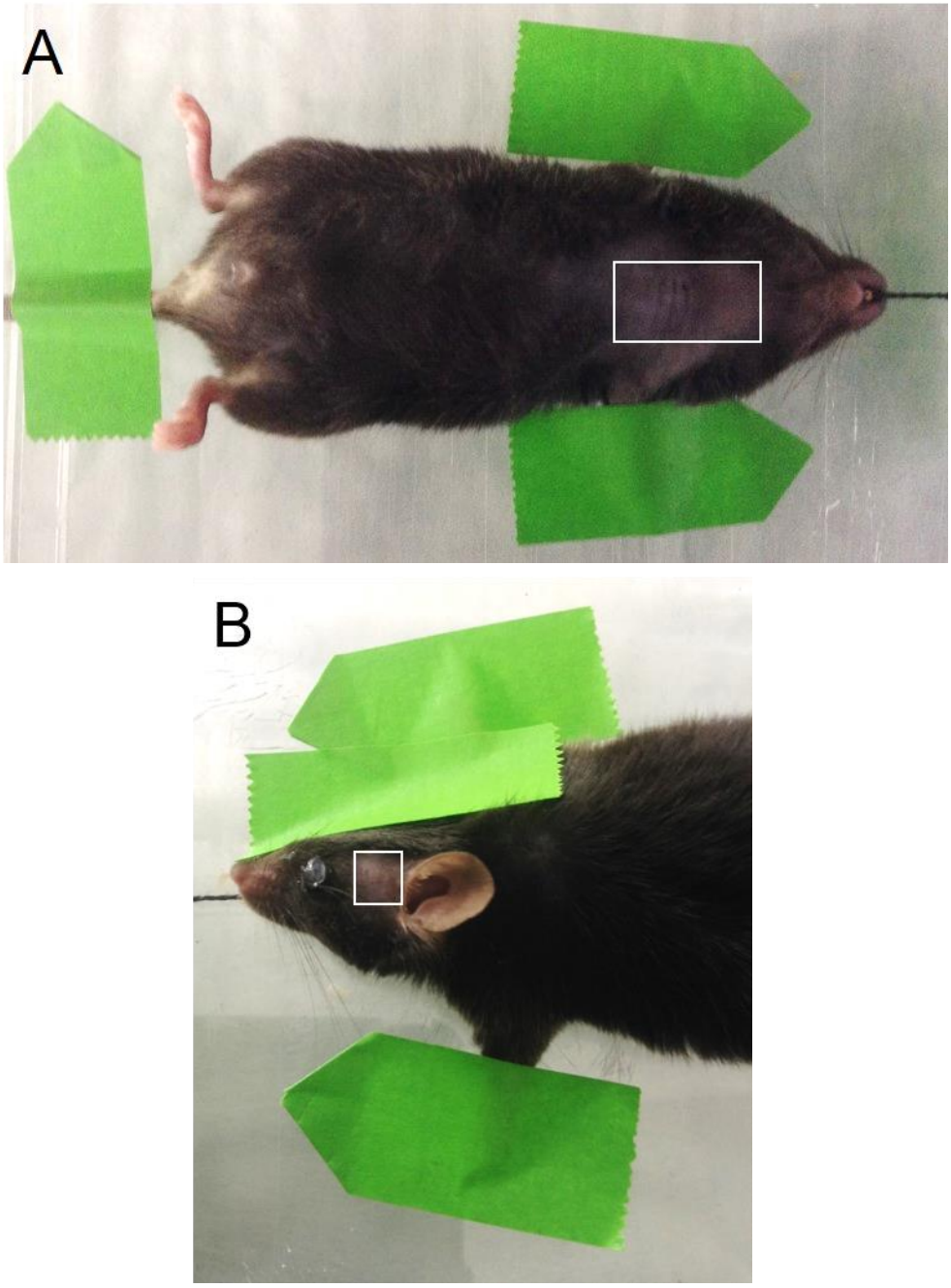

Figure 2.2. A. Mouse in supine position with box outlining shaved thoracic region, a midline incision is made from apex of ribcage to angle of the mandible., B. Mouse in pronated position with box outlining shaved temporal region, temporal incision from lateral corner of left eye to medial region of left ear.

area of the cervical and thoracic region started at the angle of the mandible and extended to the apex of the ribcage with a width spanning from forelimb axillary region to the opposite forelimb axillary region (Figure 2.2B). The shaved areas were cleaned three times with alcohol prep pads followed by a cotton tipped applicator moistened with Betadine.

Surgical Preparation

Once the mouse was fully anesthetized it was placed on a heated (to control body temperature) elevated platform. With the mouse in the supine position, the head was secured using teeth restraints and the forelimbs and tail were secured to the platform using surgical tape (Figure 2.2A & 2.2B).

MCAo stroke surgery

To induce focal cerebral ischemia, we used the previously described transient tandem ipsilateral CCA/MCA occlusion stroke model (MCAo) (37, 40). With the mouse in the supine position a midline incision was made allowing us to isolate and elevate the CCA to place a 2-0 suture underneath. It is important to note that the vagus nerve runs parallel to the CCA and should be separated so that it is not clamped with the CCA. We then released the surgical tape and rotated the mouse so that it was in a pronated position and re-secured. A second incision was made in the left temporal region from the lateral corner of the left eye to the medial region of the left ear exposing the temporal region. The temporalis muscle was reflected, the MCA verified and a small burr hole drilled to allow the metal wire to be placed under the MCA. The mouse was repositioned to

the supine position for temporary clamping of the CCA, (the occlusion time of the CCA/MCA can be varied; our lab occludes for 60 minutes). Before the head was sutured, blood flow measurements using the Laser Doppler and Speckle were taken using the process detailed in the following section. All surgical sites were temporarily sutured with 4-0 pre-needled nylon suture.

Laser Doppler and Laser Speckle for blood flow measurement

To measure blood flow in the ipsilateral MCA, a Perimed Laser Doppler flow meter (Periflux System 5000, Perimed) probe was placed directly over the MCA touching the skull, a reading was taken pre-occlusion (before MCAo) and the probe was placed in the same spot to take the post-occlusion measurements after insertion of the metal wire under the MCA and clamping of the CCA to confirm decreased blood flow. The ipsilateral hemisphere total blood flow was measured using a Laser Speckle (Pericam PSI HR, Perimed) which was positioned 15 cm above the exposed skull of the mouse. The laser sighting system was employed to ensure consistent measurements, a permanent marker was used to make a landmark dot along the sagittal suture to ensure centering of the sighting system to confirm pre and post-occlusion measurements were consistent. Post-occlusion measurements were taken at 5, 10 and 15 minutes post occlusion.

Placement of micro-angio tubing into the ECA

ECA suture placement

With the anesthetized mouse in the supine position the temporary sutures were removed and the previously isolated CCA was exposed, moving superiorly along the CCA (lateral to the trachea) to the bifurcation of the ECA and ICA. With the bifurcation located, it is necessary to further expose the ECA and ICA by 0.50 - 0.75 cm using angled or straight fine tipped micro forceps for suture placement and clamp insertion (Figure 2.3A). Using a pair of fine tipped curved forceps, the ECA was gently lifted allowing three 1.0 cm lengths of 6-0 suture to be threaded underneath, and the sutures were transferred to fine tipped curved forceps from a pair of fine straight tipped forceps. The sutures were then separated so that the first suture was placed at the bifurcation of the CCA, the second suture was placed midway between the bifurcation and the superior most exposed portion of the ECA, and the third suture was placed at the superior most exposed area of the ECA near the muscle (Posterior Belly of the Digastric-PBD) (Figure 2.3B). Once all the sutures were placed, the superior most suture was tied to permanently occlude the ECA. The superior thyroid artery should be identified, if it arises proximal to the distal permanent ECA suture, then a fourth suture should be threaded underneath to achieve permanent occlusion.

Nicking the ECA and micro-angio tubing placement

With the ECA (and superior thyroid) artery permanently occluded, a curved removable clamp (Micro Serrefines-Fine Science, Foster City, CA) was

INTRA-ARTERIAL SUTURE AND TUBING PLACEMENT

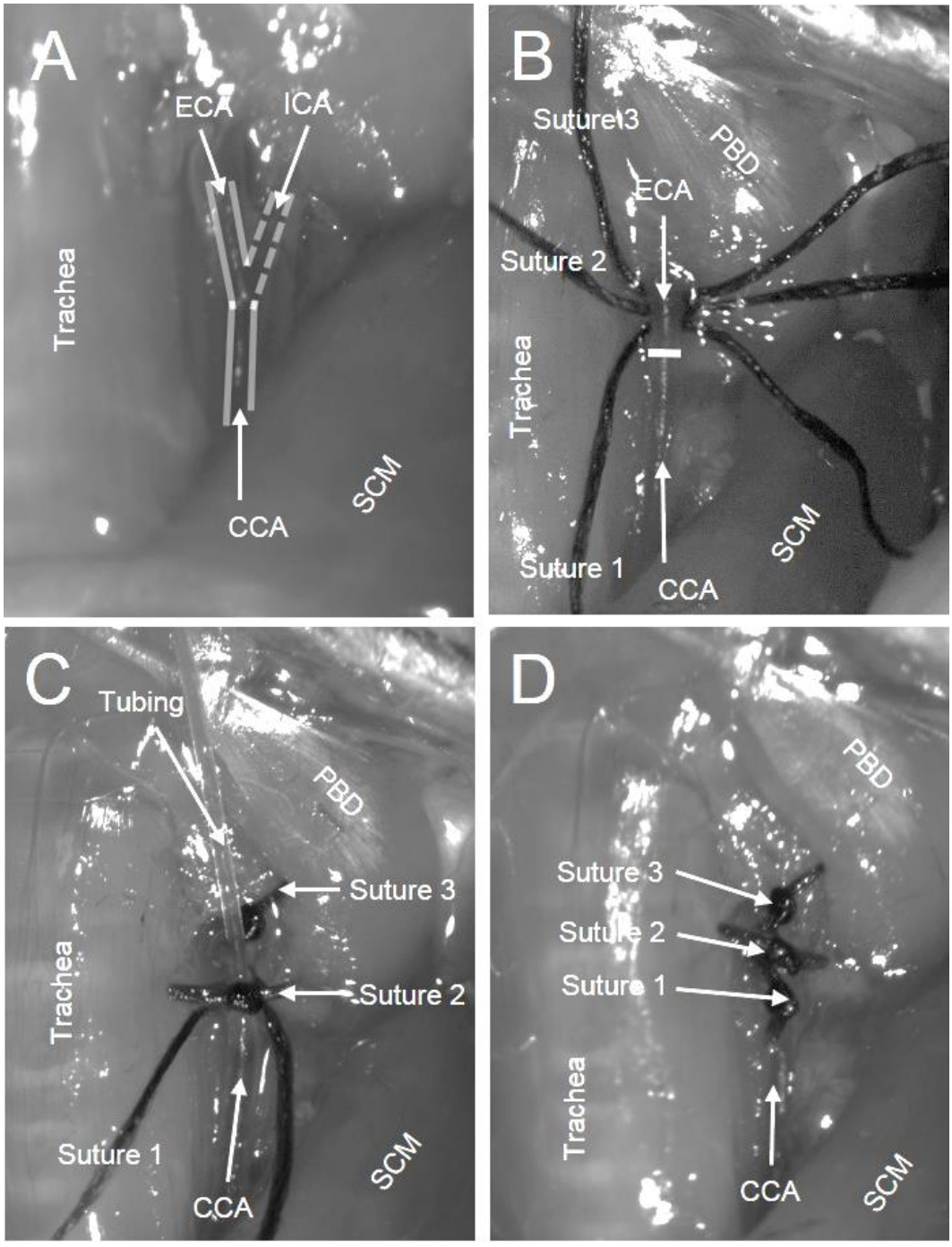

Figure 2.3. A. Outline of CCA/ECA/ICA with trachea and sternocleidomastoid (SCM) as landmarks., B. Sutures 1, 2 and 3 placement and white line marking CCA bifurcation, region framed by trachea, sternocleidomastoid (SCM), and posterior belly of the digastric (PBD)., C. Micro-angio tubing inserted into ECA towards CCA bifurcation and secured with suture 2., D. ECA suture ligated.

attached to the ICA forming a closed system between the clamped CCA, the suture ligated ECA and the clamped ICA. The ECA was then nicked just above the second suture with a pair of micro-scissors (Vannas Spring Scissors-Fine Science, Foster City, CA) to allow for the micro-angio tubing (MRE010 Braintree, Braintree, MA) attached to the Hamilton Syringe to be inserted using a pair of angled forceps (for easier tubing insertion it is recommended to cut the tip of the inserted tubing at a 45° angle) (Figure 2.3C). A pair of angled micro forceps were used to grasp the tubing 0.5 cm from the tip and a pair of curved forceps to grasp the tied suture of the ECA, which helped prevent the ECA from rolling and gave better stability for tubing insertion. Once the tubing was inserted and the tip placed at the bifurcation of the CCA, the second suture was tied securing the tubing and forming a tight seal between the tubing and ECA vessel wall (Figure 3D). Removal of the clamped ICA occurred after ensuring a tight seal around the suture tubing. Given the small blood volume of a mouse, care must be taken to minimize blood loss during these steps, as a significant blood loss can alter stroke volume and study outcomes.

CCA clamp and MCA metal wire removal

At the end of the predetermined occlusion time, the CCA clamp and the metal wire were removed with the mouse in the supine position to restore blood flow to the previously occluded area. To better visualize and remove the temporary sutures and MCA metal wire, the tape securing the left forelimb was removed and the body angled to the contralateral side.

Carbon black ink flow rate and injection volume

After a five minute waiting period for reperfusion, the 100 µl Hamilton syringe with a 34 gauge needle and micro-angio tubing was loaded with a mixture of fountain inks (Pelikan-Fount India & Higgins Fountain Pen India) in a 1:9 ratio (58). The syringe was attached to a syringe pump (BS-8000, Braintree Scientific, Braintree, MA) with flow rate settings of 1 µl, 2.5 µl, 5 µl and 10 µl per minute at volumes of 10 µl, 25 µl, 50 µl and 100 µl for each flow rate.

Removal of micro-angio tubing and permanent ECA occlusion

To remove the micro-angio tubing, the suture on the proximal ECA was first secured at the bifurcation of the CCA ensuring that the ECA was permanently occluded for the remainder of the experiment. With the first suture tied, the tubing was gently removed from the vessel with a pair of fine tipped forceps holding the second suture acting as an anchor to better assist in tubing removal. When the tubing was fully removed from the ECA, the suture was rechecked to ensure no blood leakage and the incision in the neck and head were permanently closed using pre-needled 4-0 nylon monofilament suture.

Reperfusion

Reperfusion of the MCA was confirmed using the Laser Doppler flow meter at 15 minutes post occlusion and Laser Speckle imaging at five minute intervals starting at reperfusion to 15 minutes post occlusion.

Animal Recovery

The mouse was allowed to recover in a heated recovery cage to regulate body temperature until it met or exceeded the guidelines set forth in our approved IUCAC protocol. Once the mouse was fully recovered it was returned to its home cage with its littermates in standard laboratory housing in the University Of Kentucky Division Of Laboratory Animal Resources.

Stroke Volume Assessment

Euthanasia

On post stroke day three or seven, mice were euthanized via cervical dislocation and decapitated for removal of brain (37).

Removal of brain

A midline incision was made using a pair of blunted scissors on the scalp of the decapitated head exposing the calvarium. Next a pair of fine tipped scissors were inserted at the base of the skull and used to cut the calvarium and thereby expose the brain. The brain was lifted from the skull base using a pair of blunted curved forceps and placed in a 1X PBS solution for a quick 10 - 15 second wash.

Brain slicing and staining

The removed brain was transferred to a brain mold and sliced into 2 millimeter (mm) thick sections with a surgical blade. The brain sections were then transferred to a petri dish and stained using a 1% solution of 2,3,5-

triphenyltetrazolium chloride (TTC) for 10 minutes to visualize ischemic infarct (37).

Infarct measurement

TTC stained brain sections were placed horizontally from frontal to occipital in a 3.0 cm wide petri dish and were scanned using a HP Scanjet G4050. The scanned images were input into NIH Image J for infarct analysis (37). Images were analyzed based on pixel density per mm^2. Briefly, a ruler was scanned along with brain sections to determine pixel per millimeter squared. With the known pixel per millimeter squared, scanned TTC brain sections were measured using NIH Image J, measured sections were infarct, ipsilateral and contralateral hemispheres. Infarct volume measurements were assessed using the formula: *Infarct area = Infarct – (Contralateral hemisphere/Ipsilateral hemisphere)*, this formula accounts for edema in both contralateral and ipsilateral hemispheres.

Results

Surgical outcome

The animals tolerated the procedure well with surgical effects (lethargy, decreased grooming, and eating) subsiding and resumption of normal behavior and eating habits within 24 hours. There was a decrease in body weight over two days averaging 2 - 3 grams (if stroke is carried out to post stroke day 7, weight is regained). The MCAo and IA injection models have a low mortality rate of less than 5% with death being attributed to ruptured MCA or CCA. Exclusions from

the IA study occurred when the clamp perforated the CCA upon clamp removal or when the metal wire pierced the MCA as it was being inserted or removed from underneath the MCA. These animals were excluded based on failure to guarantee recanalization and targeted drug delivery.

Laser Speckle and Laser Doppler

Laser Speckle imaging (n = 7) showed a significant ($P < 0.017$) 25.26% decrease in hemisphere blood flow from baseline to occlusion and an increase of 5.23% from occlusion to 15 minutes reperfusion (Figure 2.4A). Laser Doppler (n = 22) showed a significant ($P < 0.001$) 83.81% decrease in MCA flow from baseline to occlusion and an increase of 59.15% from occlusion to 15 minute reperfusion when compared to baseline (Figure 2.4B).

Infarct Volume

Using NIH Image J software, the sectioned and stained brains had an average infarct measurement of 19.89 mm^3, which was significantly ($P < 0.001$) increased when compared to sham measurements (n = 10 and 8, respectively, Figure 4C).

Flow Rate and Injection Volume

Flow rate and injection volume studies (n = 3) were performed to optimize selectivity of infusion to the ipsilateral hemispheric cerebral circulation (Table 1). Injection volumes were 100 microliter (µl), 50 µl, 25 µl and 10 µl with flow rates of 1.0 µl, 2.5 µl, 5 µl, 7.5 µl and 10 µl/minute. The 100µl volume showed

STROKE PERFUSION AND INFARCT VOLUME MEASUREMENTS

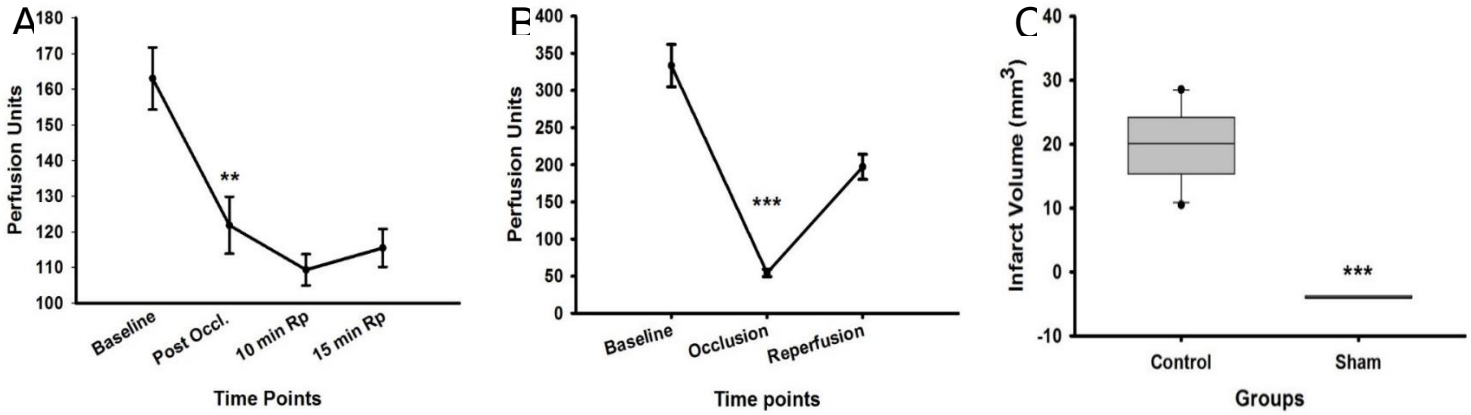

Figure 2.4. A. Laser Speckle (n = 7): Blood flow measurements of the ipsilateral hemisphere at time points Baseline (before occlusion), Post Occlusion (immediately after occlusion), 10 minutes reperfusion (Rp) and 15 minutes Rp. ** indicates P = 0.017., B. Laser Doppler (n = 22) MCA blood flow measurements at time points Baseline (before occlusion), Occlusion (immediately after occlusion) and Reperfusion (5 minutes reperfusion). *** indicates P < 0.001., C. Infarct volume measurements vehicle (n = 10) versus sham (n = 8) after MCAo surgery PSD 3. *** indicates P < 0.001.

INTRA-ARTERIAL FLOW RATE AND INJECTION VOLUME DESIGN

		MCA Staining?		Hemispheric Staining?		
Injection Rate (uL/min)	Injection Volume (uL)	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Liver Staining
10.0	100	Yes	Yes	Yes	Yes	Yes
7.5	100	Yes	Yes	Yes	Yes	Yes
5.0	100	Yes	Yes	Yes	Yes	Yes
2.5	100	Yes	Yes	Yes	Yes	Yes
1.0*	100	N/A	N/A	N/A	N/A	N/A
10.0	50	Yes	Yes	Yes	Yes	Yes
7.5	50	Yes	Yes	Yes	Yes	Yes
5.0	50	Yes	Yes	Yes	Yes	Yes
2.5	50	Yes	Yes	Yes	Yes	Yes
2.5	25	Yes	Slight	Yes	Slight	Slight
2.5	10	Yes	No	Yes	Slight*	No

Table 1. Injection Rates at 10, 7.5, 5.0, 2.5 and 1.0 µl/min at Injection Volumes 100, 50, 25 and 10 µl. Ipsilateral and Contralateral staining of the Middle Cerebral Artery (MCA) at specific injection rates and volumes, no staining at the 1.0 µl/min injection rate due to opposing pressure from the CCA. No further testing of injection rate 1.0 µl/min due to inability to overcome opposing pressure. Little to no contralateral staining was seen at the injection rate 2.5 µl/min at volumes 10 and 25 µl. Slight staining (*) was seen in the frontal region of both hemispheres due to mice having an azygous Anterior Cerebral Artery. Carbon black ink was found throughout the liver and vasculature at injection rates 10, 7.5 and 5.0 µl/min and injection volumes 100 and 50 µl with slight staining at 25 µl and no staining at 10 µl.

contralateral staining at all flow rates except 1 µl/min flow rate which did not provide enough force to overcome the opposing blood pressure of the CCA to begin ink injection immediately. This flow rate was excluded from further testing. The 50 µl volume showed contralateral staining for the 5, 7.5 and 10 µl/min infusion rates but little contralateral staining at 2.5 µl/min. The 25 µl volume showed contralateral staining for the 5.0, 7.5 and 10 µl/min flow rates but little to no contralateral staining at 2.5 µl/min. The 10 µl volume at flow rate 2.5 µl/min showed no contralateral staining, leading us to determine the optimal flow rate as 2.5 µl/min at a volume between 10 and 25 µl. Cross sectional liver images of flow rate 2.5 µl/min at volumes listed above show carbon black ink in the 25 and 50µl but not in the 10µl (Figure 2.5 A - K). It is of note that with a different gauge needle and micro-angio tubing the opposing blood pressure of the CCA could be overcome by varying the above measurements (14, 16).

INTRA-ARTERIAL CARBON BLACK STAINING OF CORTEX, CIRCLE OF WILLIS, AND LIVER

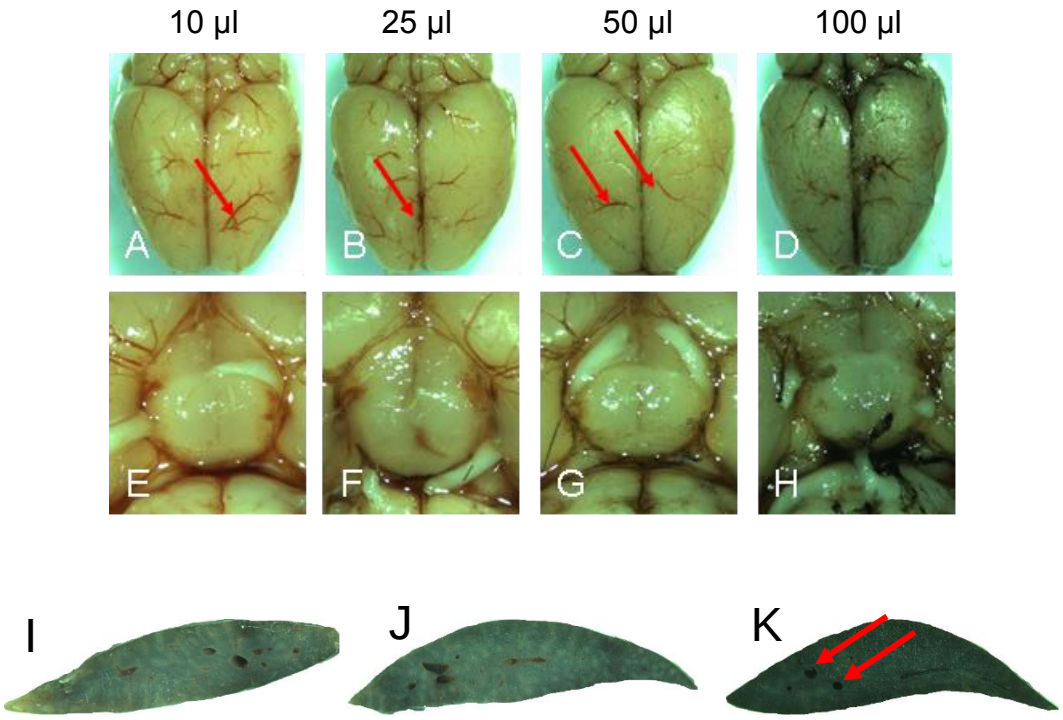

Figure 2.5. Red arrows signify carbon black ink staining in cerebral and liver vasculature., A – D. Dorsal view of brain at injection rate 2.5 µl/min at volume 10 µl, 25 µl, 50 µl and 100 µl., E – H. Circle of Willis at injection rate 2.5 µl/min at volumes 10 µl, 25 µl, 50 µl and 100 µl., Cross section of liver at injection rate 2.5 µl/min at volume 10 µl, 25 µl, and 50 µl.

Discussion

The advancement of neuroprotective pharmacotherapy for ischemic stroke has been complicated by failures in translation from the laboratory to the clinic. Many promising neuroprotective agents have failed in both basic research and clinical trials due to multiple factors, including failure to combine pharmacotherapy with vessel reperfusion, and failure to direct therapy to the affected tissue in a timely fashion. With our modified version of the MCAo model and potential for selective delivery of agents that could be neuroprotective, neuroreparative, barrier-protective, or vasculo-protective, each of these issues is addressed.

Imitating a thrombus in a large vessel occlusion by kinking the MCA and clamping the CCA mimics the large vessel occlusion that is observed clinically. Removing the MCA metal wire and CCA clamp restores blood flow to the occluded vessel as would be seen clinically with the mechanical retrieval of the thrombus. Rapid recanalization of an occluded vessel in acute ischemic stroke is paramount. Indeed, speed of recanalization is required by Comprehensive Stroke Centers to be reported as a ‘core measure’ to the Joint Commission (JCAHO 07-22-2014). Retro-engineering our mouse model from the clinical condition while incorporating flow rate and injection volume optimization allows us to mimic current neurosurgical practices. By bringing our mouse model one step closer to what is seen clinically through the addition of intra-arterial drug delivery, we are demonstrating current practices for stroke treatment. The flow rate and injection

volume studies further mimic what is seen clinically with superselective drug delivery, but through optimization of flow rate and injection volume we also demonstrated that potential therapeutic compounds could selectively reach the site of ischemia for maximal effect and potentially mitigate systemic side effects.

In this study, we employed an easily visible carbon black ink mixture to optimize volume and flow rates to maximally target ipsilateral stroke brain tissue. One potential limitation of this study is that ink is denser than typical pharmacologic agents in solution. While this may confound the applicability of rate/volume studies, lower density solutions would be expected to travel farther with a wider potential distribution (contralateral/systemic); thus, the validated 2.5 µl/min flow rate and 10 - 25 µl volume would serve as an absolute maximum using this technique. Further exploration of our IA model using solutions of different densities and viscosities labeled with a real-time measureable tracer could be employed for further optimization of flow rate and injection volume. Certainly, our flow/distribution experiments do not preclude the need for individual agent-specific localization experiments, but rather provide a starting point for flow rate and volume limitations for future experiments.

Conclusion

By modifying an already well-established mouse stroke model and adapting it to mimic current clinical stroke treatment, we have developed a novel method of IA drug delivery for potential therapeutic compounds that may limit systemic side effects, enable early pharmacotherapy, and provide methodology

for testing the role of acutely administered compounds for neuroprotection and neuro-repair in acute ischemic stroke. In closing, our model of IA pharmacotherapy delivery has shown promise in the MCAo stroke model but could easily be adapted to most mouse models of stroke.

Chapter Three: Intra-arterial Nitroglycerin

Maniskas ME, Roberts JM, Trueman R, Learoyd A, Gorman A, Fraser JF, Bix GJ. Intra-arterial nitroglycerin as directed acute treatment in experimental ischemic stroke. J NeuroInterventional Surgery. (In review)

Introduction

Stroke is defined as the disruption of blood flow to the brain due to vascular occlusion (ischemic) or bleeding (hemorrhagic), and is the second leading cause of death worldwide and a leading cause of long-term disability (59). Emergent large vessel occlusion (ELVO) is the most life-threatening and disabling type of ischemic stroke. Treatment options for ELVO consist of endovascular thrombectomy (ET) and/or intravenous (IV) tissue plasminogen activator (t-PA), but not all patients are eligible due to exclusion criteria (10, 60-62). Furthermore, while ET and IV t-PA have improved patient survival, there is a need for adjunctive therapies to be used in tandem with or as a standalone following successful vessel recanalization (38). These adjunctive therapies could target specific aspects of the apoptotic cascade that occurs when the brain is deprived of oxygen and glucose. Furthermore, any potential pharmacotherapy administration may be most successful and cause fewer systemic side effects if it is targeted to the site of ischemia. We hypothesize that improved treatment for ELVO could include rapid recanalization of the occluded vessel via ET combined with acute pharmacotherapy directed to the site of ischemia. To test such a targeted therapeutic approach, our lab uses an intra-arterial (IA) experimental

model of pharmacotherapy administration in combination with transient tandem ipsilateral common carotid artery (CCA) and middle cerebral artery (MCA) occlusion, hereafter referred to as MCAo (63).

Using the already exposed vasculature of the MCAo we are able to selectively administer our drug of choice IA further mimicking the human clinical condition of pharmacotherapy delivery. Combining the two methods allows us to mimic an acute ischemic stroke but also guarantee successful recanalization followed by drug delivery in a timely manner similar to ET. Thus far, we have successfully optimized our IA model to determine a flow rate of 2.5 µl/min with an injection volume of 10 µl.

In this study, we chose to investigate the therapeutic potential of acute IA administration of nitroglycerin or glyceryl trinitrate (GTN) in our experimental stroke model. GTN is an FDA approved vasodilator for angina pectoris and high blood pressure that was recently studied in the Efficacy of Nitric Oxide in Stroke (ENOS) ischemic stroke clinical trial (41, 43, 64). This study investigated the potential of GTN to reduce blood pressure and improve functional outcome following acute ischemic stroke with treatment through a systemic sustained release transdermal patch. Results showed a reduction in blood pressure on post-stroke days 1 and 7 but no improvement in functional outcome at day 90 (41, 43, 65). Individuals suffering a stroke are at an elevated risk of increased intracranial pressure (ICP); a reduction in ICP either through direct (IA) or indirect (patch) administration to the cerebrovasculature could improve outcome (66).

The ENOS trial acutely reduced blood pressure and by doing so appeared to have a neuroprotective effect but failed to show improvement beyond blood pressure reduction. These results could be directly tied to GTN's short half-life and inability to reach the cerebrovasculature if applied as a patch on the arm or chest.

Material and Methods

Experiments were performed in accordance with protocols on file with the University of Kentucky Division of Laboratory Animal Research, Institutional Care and Use Committee and ARRIVE Guidelines (67). In short, 16 week old C57/Bl6 (25 - 30 grams) male mice from Jackson Laboratories were administered GTN (American Reagents) diluted in vehicle (water - 39.5%, ethyl alcohol - 30%, propylene glycol - 30%) or vehicle only IA four minutes following MCAo. Animals were grouped into naïve (age matched litter mate controls exempted from surgery and anesthesia), control (MCAo surgery with IA injection of vehicle through Internal Carotid Artery (ICA)), and treated (MCAo surgery with IA injection of 3.1, 6.2, 12.5, 25 and 50 µg/µl GTN in 10µl injection volume through the ICA). GTN was administered in a dose escalating fashion to determine safety and efficacy, as there is no standard dose of IA GTN and thus a dose response study was necessary to determine safety. Previous flow rate and injection volume studies determined the optimal flow rate of 2.5µl/min and injection volume of 10µl per mouse (63). Subjects were numerically labelled in a randomized blinded fashion for Laser Doppler flowmetry, infarct volume measurement, functional

outcome assessment and brain immunohistochemistry. Exclusion criteria are as follows: rupture of the MCA following wire placement and/or removal, rupture of CCA following clamp placement and/or removal, inability to place tubing in the ECA, leakage of tubing around the ECA upon pumping, failure of animal to recover from surgery resulting in death, death of animal during study, signs of hemorrhage upon brain extraction (5 animals were excluded based on aforementioned criteria). Our MCAo and IA model of pharmacotherapy administration have a death rate of <5% in both surgery and behavior (63).

Animal Number

The experiment was conducted using 50 animals (5 groups), naïve animals were excluded from Laser Doppler and MouseOx. For Laser Doppler and MouseOx (n = 5/group), infarct volume measurements (n = 10/group), functional outcome assessment (n = 6/group), blood draws (vehicle n = 2, treated n = 5), and brain immunohistochemistry (n = 5/group).

Vital Sign Measurements

Physiological measurements (heart rate – beats per minute (bpm)) and blood pressure (pulse distention – micrometer (μm)) were taken on animals only undergoing the IA surgery (no MCAo surgery) over a 20 minute period, beginning at IA injection and continuous through 20 minute mark. Measurements were taken using the MouseOx Plus (Starr Life Science), where a thigh sensor was placed on the shaved left thigh of an anesthetized mouse.

Perfusion

Blood flow through the MCA was measured using the Laser Doppler Periflux System 5000 with a 2 mm tip (Perimed). Blood flow measurements were taken pre-occlusion (no CCA clamp or MCA metal wire filament insertion), post-occlusion (CCA clamp and MCA metal wire filament insertion) and 15 minutes post-IA injection (pre-occlusion conditions).

MCAo and IA Injection

Using a weight based ratio of Ketamine and Xylazine mixture, animals were anesthetized for the MCAo and IA injection procedure. Focal cerebral ischemia was induced using our previously described method (37, 38, 40, 63). MCAo procedure was for one hour with pre/post-occlusion and recanalization confirmed through Laser Doppler flowmetry. To maintain body temperature, animals were placed on an elevated stage over a heating pad with core temperature measurements taken using the MouseOx Plus (rectal thermometer). Following MCAo and IA injection animals were placed in a recovery cage warmed by a heating pad and overhead lamp until fully recovered.

GTN IA injection followed our previously published protocol (38, 63). In brief, a midline incision was made from mandible to sternum exposing and isolating the CCA, ECA and ICA. The ECA was permanently occluded with 4-0 suture at its distal end and the ICA was temporarily occluded. Using a pair of micro scissors, a small nick was made in the ECA midway between the CCA bifurcation and proximal suture. Micro-angio tubing was inserted into the ECA

through the nick and placed at the bifurcation and suture was used to secure the tubing into the ECA. Following un-occlusion of the MCA, CCA and ICA flow was restored and the vessel was allowed to recanalize for 5 minutes. Next, IA injection of either GTN or vehicle was introduced at a flow rate of 2.5µl/min at a volume of 10 µl (38, 63). Once the injection was complete the ECA was permanently occluded at its proximal end with a third 4-0 suture and the animal was sutured closed.

Behavioral Testing

Animals were subjected to rotor rod (forced motor movement) to determine functional ability following surgery and pharmacotherapy administration. Animals, naïve, control and treated were trained for three days prior to stroke surgery and tested three days following stroke surgery. Rotor rod was used to determine percent change from baseline over a 5 minute interval with an accelerating rod from 0 – 40 rpms for three trials.

Infarct Volume

On post-stroke day (PSD) 7 mice were euthanized via cervical dislocation, the whole brain was removed and flash frozen, sectioned on a cryostat (20 µm) and stained using cresyl violet. Infarct volume was analyzed from scanned cresyl violet sections with Image J software (NIH) (38).

Blood Draws

To measure nitric oxide (NO_x) levels in the blood, submandibular blood draws were performed one day prior to stroke (baseline), post-15 minute IA and PSD 1. In short, animals were placed in the dorsal position with their head tilted either right or left to expose the ramus of the mandible and submandibular vein (68). A 5 mm Golden Rod animal lancet was used to pierce the submandibular vein, blood (10% of total animal blood volume) was collected in an EDTA tube, and centrifuged at 14,000 rpm for 15 minutes. Plasma was collected and analyzed for NO_x levels.

Nitric Oxide (NO_x) Analysis

Nitrate reductase was used to reduce nitrate to nitrite in blood plasma samples. A volume of 5 µl nitrate reductase and 5 µl of co-factor (Nitrite/Nitrate Assay Kit, Sigma-Aldrich, UK) was added to 40 µl plasma and incubated for 2 hours at 25°C. Nitrate/nitrite (NO_x) concentration were then determined using Sievers Nitric Oxide Analyzer (NOA 280i; GE Instruments, UK). During this procedure the nitrite was reduced to nitric oxide by acetic acid. Nitric oxide was then quantified using ozone-chemiluminescence technology.

Immunohistochemistry

Whole brains flash frozen were sectioned on a Leica CM 1950 cryostat at 20 µm and mounted on slides. Glial Fibrillary Activating Protein (GFAP 1:500 antibody dilution, Sigma) immunohistochemistry was used to evaluate astrogliosis. NeuN (1:500 antibody dilution, Abcam) immunohistochemistry was

used to analyze mature neuron survival in the stroke affected region (core and penumbra). Infarct and peri-infarct regions (defined as a 500-μm boundary extending from the edge of the infarct core, medial and lateral to the infarct of the cortex) were identified histologically after cryostat sectioning. Stains were imaged with a Nikon Eclipse T*i* microscope at 20X magnification and images collected with a CCD camera and devoted computer qualified with Adobe Photoshop. Nikon NIS Element BR Analysis imaging software was used to analyze positive pixel density of sectioned brains from naïve, control and treated groups.

Statistical Analysis

Measured variables are shown as mean ± Standard Error of the Mean (SEM). Analysis of results for comparison between treatment groups (infarct volume, immunohistochemistry) was performed using a *Student's* t-test. Time course comparisons (behavior) and NO_x were analyzed using a Two-Way Repeated Measures ANOVA. We defined significance as *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

Compliance with STAIR Criteria

To maximize the applicability of our results, we designed the study with reference to the STAIR recommendations for preclinical neuroprotection research (69). We performed a dose response to determine if there was a deleterious dose and found all animals' survived IA GTN injections.

Results

Vital Sign Measurements

Physiological measurements for heart rate (IA injection only) demonstrated no difference from baseline for all groups except 6.2 µg/µl which showed a significant reduction in beats per minute at post-IA to 15 minutes post-IA. The animals in the 6.2 µg/µl group recovered without incident despite a lowered heart rate. Pulse distention showed no difference between groups from baseline with all animals recovering without incident. Data not shown.

Perfusion

Perfusion measurements using the Laser Doppler were made at baseline, occlusion, and 15 minutes post-IA to determine whether IA GTN had any effect on the MCA perfusion. We demonstrated an 83% reduction in cortical blood flow through the MCA post occlusion. Following un-occlusion and 5 minute recanalization, GTN and vehicle were administered IA and measured 15 minutes post-IA. While all groups increased perfusion through the MCA there was no significant difference between GTN doses and vehicle 15 minutes post-IA injection over the time period analyzed (Figure 3.1).

Infarct Volume

Infarct volume (Figure 3.2 A) was assessed using cresyl violet (Figure 3.2 B) and analyzed on PSD 7 using Image J. Mean infarct volumes (mm^3) demonstrated a dose response effect compared to control, with 12.5 µg/µl

INTRA-ARTERIAL NITROGLYCERIN STROKE PERFUSION MEASUREMENTS

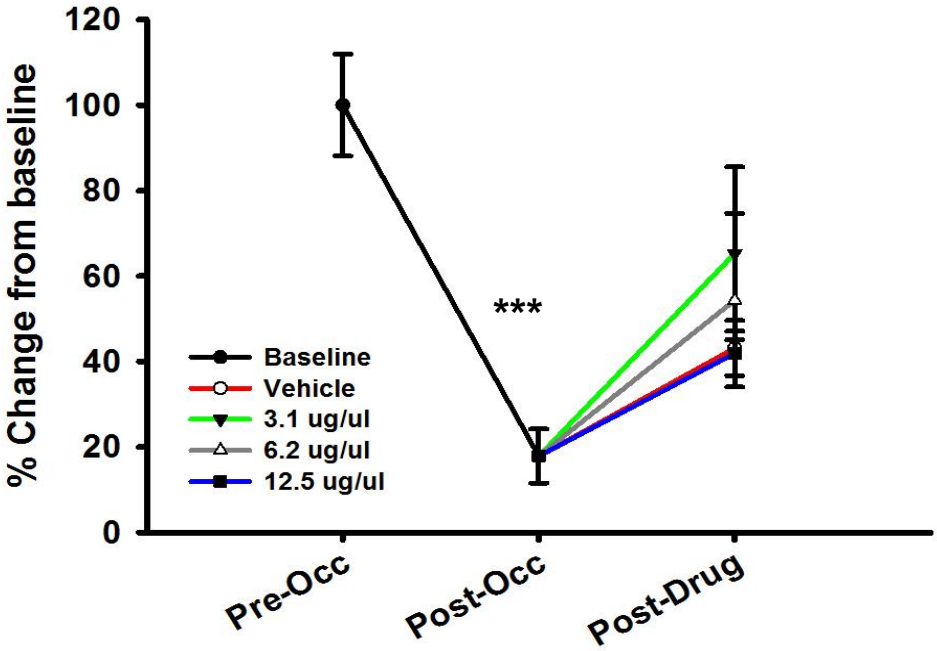

Figure 3.1. Laser Doppler blood perfusion through middle cerebral artery at time point 0 minutes (baseline measurements), 5 minutes (occlusion), and 75 minutes (drug administration/reperfusion). Vehicle (n = 5), 3.1 µg/µl (n = 5), 6.2 µg/µl (n = 5) and 12.5 µg/µl (n = 5)

INTRA-ARTERIAL NITROGLYCERIN INFARCT VOLUME MEASUREMENTS AND IMAGES

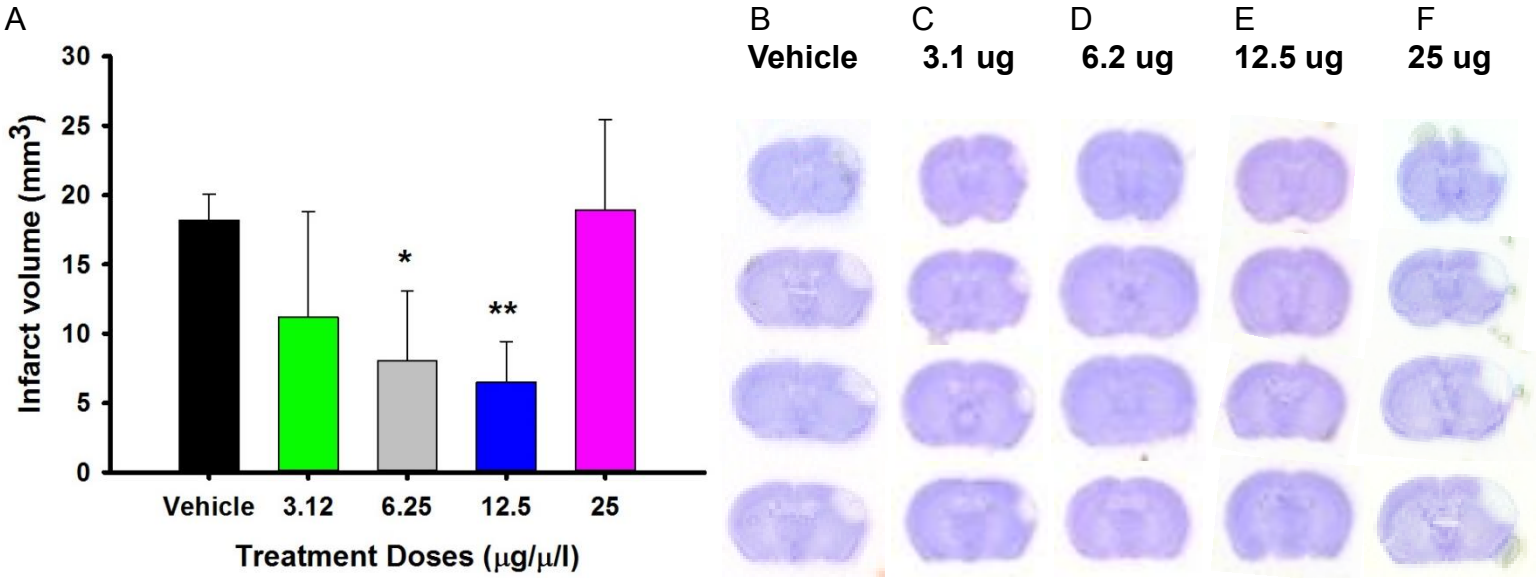

Figure 3.2. A. Infarct volume analysis for vehicle versus GTN treatment (3.1, 6.2, 12.5, and 25 µg/µl) using cresyl violet stained stroked tissue., B. Cresyl violet image for vehicle group., C. Cresyl violet image for 3.1 µg/µl group., D. Cresyl violet image for 6.2 µg/µl group., E. Cresyl violet image for 12.5µg/µl group., F. Cresyl violet image for 25 µg/µl group. Vehicle (n = 20), 3.12 µg/µl (n = 4), 6.2 µg/µl (n = 4), 12.5 µg/µl (n = 4), and 25 µg/µl (n = 4). * indicates $P < 0.05$ and ** indicates $P < 0.001$.

showing a significant decrease in infarct volume. However, the 25 µg/µl dose showed signs of hemorrhage and an infarct volume similar to vehicle control.

Behavioral Testing

In addition to infarct volume assessment, we performed rotor rod functional analysis on all of the groups except the less well-tolerated 25 µg/µl dose. Functional assessment of IA GTN on the rotor rod (forced motor movement) was measured in percent change from baseline on PSD 1, 3 and 7 (Figure 3.3). On PSD 1 the control stroked group showed a decrease from baseline (-27.25 ± 13.42) as expected while the naïve group without any brain injury significantly improved (21.10 ± 31.43) as expected compared to control. GTN doses of 3.1 and 6.2 µg/µl showed a significant ($P < 0.001$) improvement (23.48 +/- 35.30 and 30.51 ± 25.06) from control, while the 12.5 µg/µl GTN dose was slightly less than baseline (-7.36 ± 24.52).

On PSD 3, the control group, while still reduced from baseline (-12.65 ± 32.51), did improve slightly from PSD 1 measurements, while the naïve group continued to significantly increase from baseline (44.30 ± 43.21) and PSD 1 compared to control. However, the GTN dose of 3.1 µg/µl (20.75 ± 19.04) remained above baseline but was slightly less than PSD 1 measurements. GTN group 6.2 µg/µl (36.73 ± 27.2) continued to improve from baseline and PSD 1 measurements, and 12.5 µg/µl (51.77 ± 32.29) improved significantly from PSD 1 measurements.

INTRA-ARTERIAL NITROGLYCERIN BEHAVIORAL MEASUREMENTS

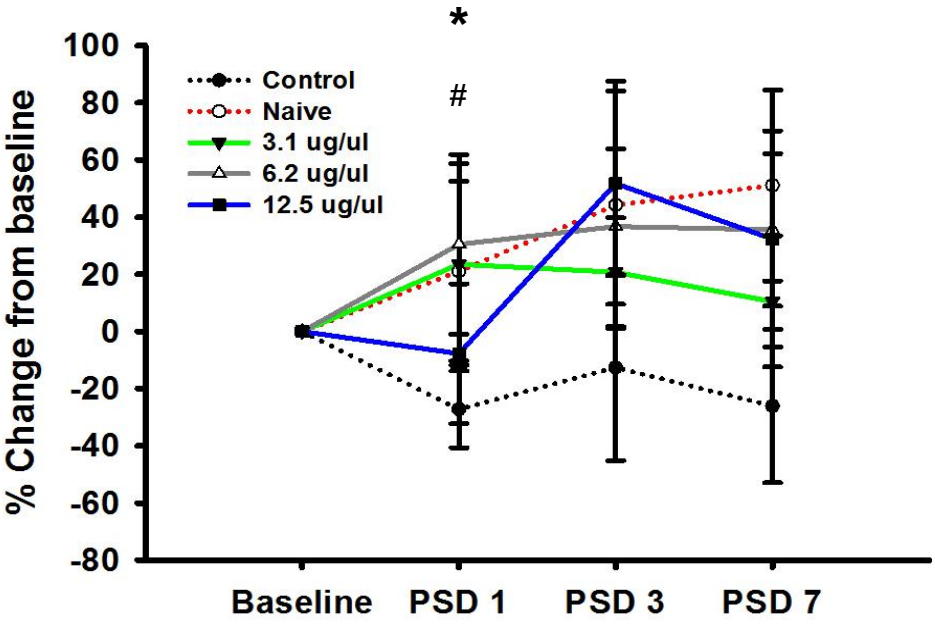

Figure 3.3. Behavioral measurements for Rotor Rod for Control (vehicle), Naïve, 3.1 µg/µl, 6.2 µg/µl and 12.5 µg/µl GTN. Control (black dotted line), Naïve (red dashed line), 3.1 µg/µl (green line), 6.2 µg/µl (grey line), and 12.5 µg/µl (blue line). Control (n = 5), 3.1 µg/µl (n = 5), 6.2 µg/µl (n = 5) and 12.5 µg/µl (n = 5). * indicates $P < 0.05$ (3.1 µg/µl versus vehicle), # indicates $P < 0.05$ (6.2 µg/µl versus vehicle)

On PSD 7, the control group, decreased from both baseline and PSD 3 (-26.11 ± 26.88), while the naïve group continued to significantly increase from baseline (51.10 ± 33.41) and PSD 1 compared to control. However, the GTN dose of 3.1 µg/µl (10.51 ± 22.91) remained above baseline measurements but was slightly less that its PSD 1 and 3 measurements. 6.2 µg/µl (35.59 ± 26.63) and 12.5 µg/µl (32.34 ± 37.79) GTN doses continued to improve from baseline and their PSD1 values. However, none of the GTN dose measurements were significantly greater than the control by PSD 7.

Blood Nitric Oxide (NO_x) Measurements

Next, as one of the goals of the IA administration of GTN after stroke was to limit systemic effects, we investigated whether such IA GTN administration had any effect on blood nitric oxide (NO_x) levels. Compared to baseline blood draws, blood NO_x levels were elevated 15 minutes after either IA vehicle or GTN treated groups, the latter being slightly, but not significantly, more elevated. By PSD 1, blood NO_x remained similarly elevated from baseline pre-stroke levels in both groups (Figure 3.4).

Immunohistochemistry

Antibodies specific to proteins associated with mature neuron survival (NeuN) (Figure 3.5 A -E), and astrocytic activation (GFAP) (Figure 3.5 F -J). Compared to control, GTN treated animals showed a dose response stair step effect with a significant ($P < 0.001$) increase in NeuN positive pixels at doses 3.1,

6.2, and 12.5 µg/µl. Likewise, a similar stair step dose response was seen in GFAP with control having a significantly ($P < 0.001$) increased number of GFAP positive pixels compared to GTN doses.

INTRA-ARTERIAL NITROGLYCERIN BLOOD NITRIC OXIDE ANALYSIS

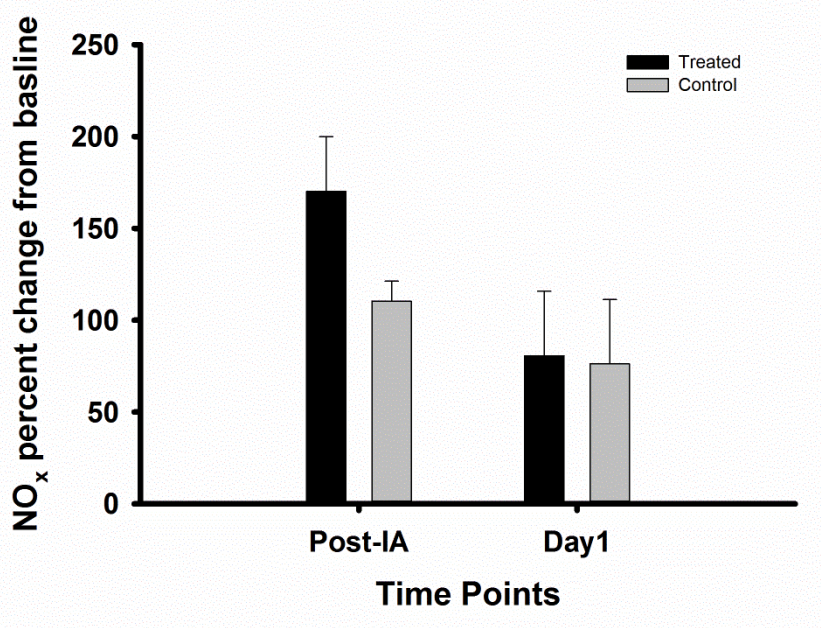

Figure 3.4. Blood NO_x levels of GTN (12.5 µg/µl) versus control post-IA and Day 1. Treated (n = 2), Control (n = 5)

INTRA-ARTERIAL NITROGLYCERIN IMMUNOHISTOCHEMISTRY GRAPH AND IMAGES

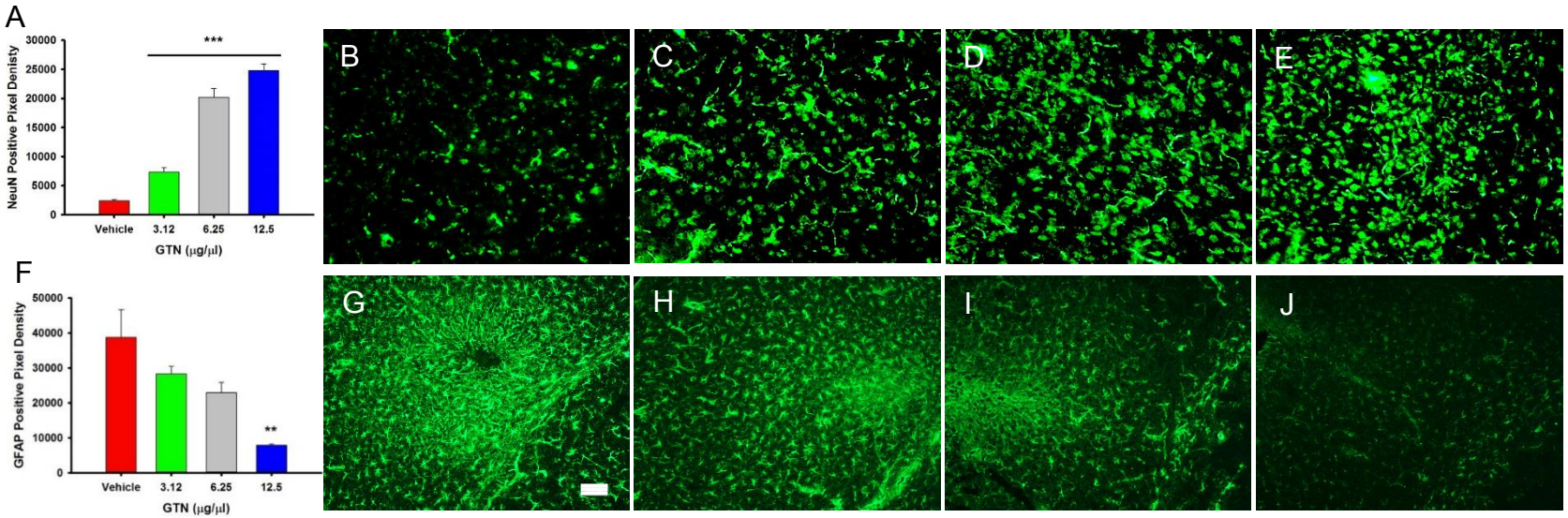

Figure 3.5. Graphs and images for immunohistochemistry magnification at 20X with quantification of positive pixel density. A. Graph depicting positive pixel for NeuN stain of vehicle versus GTN (3.1, 6.2, and 12.5 µg/µl) in infarcted region., B. Image of vehicle NeuN stain from infarcted region at magnification 20X., C. Image of 3.1 ug/ul NeuN stain from infarcted region at magnification 20X., D. Image of 6.2 µg/µl NeuN stain from infarcted region at magnification 20X., E. Image of 12.5 µg/µl NeuN stain from infarcted region at magnification 20X., F. Graph depicting positive pixel density for GFAP stain of vehicle versus GTN (3.1, 6.2, and 12.5 µg/µl) in infarcted region., G. Image of vehicle GFAP stain from infarcted region at 20X., H. Image of 3.1 ug/ul GFAP stain from infarcted region at magnification 20X., I. Image of 6.2 µg/µl GFAP stain from infarcted region at magnification 20X., J. Image of 12.5 µg/µl GFAP stain from infarcted region at magnification 20X. Vehicle (n = 4), 3.1 µg/µl (n =4), 6.2 µg/µl (n = 4) and 12.5 µg/µl (n = 4) White scale bar, 100µm. ** indicates $P < 0.001$, *** indication $P < 0.001$

Discussion

Stroke remains a world-wide leading cause of mortality and morbidity with limited therapeutic options. Currently, IV t-PA is the only approved pharmacotherapy available but extensive exclusion criteria limits its use to less than 16% of those affected by stroke. With the recently completed and successful ET trials for ELVO such as MR Clean trial, ET can be used to rapidly recanalize stroke through IA access. This provides a clinical/translational opportunity to co-administer potential neuroprotective compounds. Using a clinically relevant mouse model of stroke (MCAo), we have developed a novel method of IA (similar to ET) pharmacotherapy delivery that allows us to deliver selected compound directly to the site of ischemia while mitigating systemic side-effects (38).

Glyceryl trinitrate or GTN was used in the recently completed ENOS trial and shown to be safe when administered in patch form following stroke. The outcome of the trial was an acute reduction in blood pressure, but no improvement in functional outcome (41, 65, 66). GTN is a known vasodilator and is commonly used to treat ischemic heart disease, but we and others believe it may also have significant neuroprotective properties as discussed below. Therefore, our goal in this study was to target GTN directly to the site of the stroke via our IA model of pharmacotherapy administration following MCAo to evaluate these potential neuroprotective properties.

Since there is not a standard IA dose of GTN available we started with a dose-response evaluation (3.1, 6.2, 12.5 and 25 µg/µl) to determine if IA GTN

was safe (no bleeding into the parenchyma) and had an effect on infarct volume. Our study showed the mice tolerated and survived IA GTN with no overt deleterious effects, and there was a positive dose-dependent effect on infarct volume with a significant reduction noted with the 12.5 µg/µl dose. However, the 25 µg/µl dose had bleeding into the ventricles and an infarct volume similar to vehicle control. Therefore, we conclude that IA GTN is a safe potential neurotherapeutic with a specific toxicity profile, which includes intraventricular hemorrhage at the highest dose tested. Additional studies should further delineate the effective dose-response therapeutic window.

We evaluated the potential effect of IA GTN on functional outcome. While the lower doses (3.1 and 6.2 µg/µl) had initial significant positive effects immediately following infarct induction on PSD 1, these effects disappeared by PSD 7. Overall, there was no difference among groups in functional outcome. This may be due, in part, to GTN's very short half-life (1.5 - 7.5 minutes) such that noted beneficial effects on infarct volume and neuronal viability (and decreased reactive gliosis) were not sufficiently pervasive to result in long-term functional benefit. In further support of such a conclusion, one potential mediator of GTN neuroprotection, NO_x (discussed in greater detail below (70-72)), also has as extremely short half-life of 2 - 6 seconds. We expect that further studies with post-stroke IA GTN, or perhaps combining IA-GTN with other potential, longer lasting neuroprotective agents that more thoroughly test functional outcome using multiple assessment tools may demonstrate functional benefits.

While we saw no effects of IA GTN on animal pulse distention, there needs to be further examination of potential systemic-effects from IA GTN administration; therefore, we measured post-stroke blood NO_x levels at several points after IA GTN or vehicle administration. NO_x is a good measure of IA GTN administration because GTN is converted to NO_x by endothelial nitric oxide synthase (eNOS) and released into the blood stream from the vascular endothelium. Ultimately, we noted a transient, but insignificant increase in blood NO_x levels with IA GTN treatment, results that are consistent with efficacious GTN delivery and suggest a potential mechanism of action, while simultaneously further demonstrating a relative lack of systemic effects with IA GTN treatment.

It has been known for decades that GTN has both neuroprotective and neurotoxic properties, depending upon whether it is present in physiologic or pathologic concentrations, respectively. Post-stroke GTN mediated neuroprotection may result from vasodilation of the effected vasculature which leads to increased blood flow and return of nutrients. Nitric oxide has been shown to be neuroprotective through vessel dilation, anti-apoptotic mechanisms as a reactive oxygen species scavenger, and through inhibition of lipid peroxidation sustaining cellular membrane integrity (71, 73-75). In our study, we noted transient, but insignificant increases in both MCA vasodilation and blood NO_x levels with IA GTN treatment. While IA GTN's mechanism of action may be a combined effect (vasodilation and production of nitric oxide species), one additional possibility is that IA GTN may have increased local NO_x levels in the stroke-targeted brain, an analysis that should be performed in additional studies.

Collectively, despite the limitations of this study, our results suggest a novel therapeutic modality of GTN that, independent of its blood pressure lowering effects, may be of benefit after ischemic stroke with further study.

Chapter Four: Intra-arterial Verapamil

Maniskas ME, Roberts JM, Aron I, Fraser JF, Bix GJ. Stroke neuroprotection revisited: Intra-arterial verapamil is profoundly neuroprotective in experimental acute ischemic stroke. J Cereb Blood Flow Metab. 2015 Oct 2. PubMed PMID: 26661189.

Introduction

The only FDA-approved pharmacotherapy for acute ischemic stroke is t-PA, but with a therapeutic window of 4.5 hours and extensive exclusion criteria, few patients receive treatment (55, 61). A second and more accessible form of therapy is ET that in addition to IV t-PA is now the standard of care (10, 60, 61). While both treatments have improved patient survival and rate of good clinical outcomes, the magnitude of outcome advancement has not mirrored the increasing improvements in the ability to re-open occluded vessels (9, 13, 60). This disconnect highlights an urgent need for adjunctive therapies to further improve outcomes after effective thrombolysis with t-PA and thrombectomy (12, 60, 76).

Using the MCAo to model ischemic stroke and IA technique to model pharmacotherapy administration in mice, we have developed a model that mimics the human clinical stroke condition to better test potential neuroprotective compounds (37, 40, 63). This method mirrors the clinical scenario of a recanalized artery immediately following thrombectomy; it allows us to test the feasibility, safety, and efficacy of IA pharmacotherapy immediately following thrombectomy, and provides an animal model to reflect the present clinical opportunity to infuse agents IA immediately following successful thrombectomy

with the patient still on the angiography procedure table. Verapamil, a calcium channel blocker (CCB), is FDA approved for angina and arrhythmia (77). Previous verapamil stroke studies had mixed results; while some studies support its neuroprotective potential, other experiments failed to show a benefit (44-46, 78). However, in experiments that failed to show benefit, animal models were induced with permanent vessel occlusion and/or given the drug in a time interval far beyond its likely therapeutic potential (44, 46). Clinically, we hypothesize that treatment for ELVO strokes should consist of rapid recanalization of the occluded vessel combined with selective pharmacotherapy, i.e. IV or IA t-PA administration directly to the site of ischemia (79, 80). Such a stroke-targeted approach could greatly optimize the therapeutic potential of neuroprotective agents. Therefore, in this study we investigated the therapeutic potential of IA verapamil in our experimental ischemic stroke.

Material and Methods

All experiments conformed to protocols on file with the University of Kentucky Division of Laboratory Animal Research, Institutional Animal Care and Use Committee and ARRIVE Guidelines (67). Briefly, 16 week old male C57/Bl6 mice (25 – 30 gram) from Jackson Laboratories were separated into 3 groups (10 per group for control and treated, 5 for naïve) in a blinded fashion using randomized selection. Groups were divided into naïve (age matched litter mate controls excluded from anesthesia and surgery), control (MCAo surgery with 10 µl saline injection at 2.5 µl/minute IA through ICA) and treated (MCAo surgery

with 10 µl saline + verapamil injection IA through ICA). Verapamil was administered at a dose of 0.15 milligram (mg) per kilogram (kg) (81, 82). This dose was calculated to be similar to 10 mg in a 70 kg person, which is the most clinically relevant current IA dose. Prior studies determined the optimal flow rate of 2.5 µl/minute with an injection volume of 10 µl per animal (63). Physiological measurements, blood flow measurements, behavioral, infarct volume and immunohistochemistry analyses were conducted in a blinded fashion with experimental subjects numerically labeled. Animals were excluded from the study if the MCA or CCA was punctured during wire and clamp insertion or removal, died following surgery in recovery or were euthanized before the end of the study due to poor health. Overall, there is <5% death rate for our stroke model following both surgery and during behavior.

Animal Numbers

The experiment was conducted using 65 animals (3 groups), naïve animals were excluded from Laser Doppler and MouseOx. For Perfusion, MouseOx and infarct volume (n = 25 for treated and n = 20 for control), and brain immunohistochemistry (n = 10 for both control and treated). Additionally, an n of 5 naïve age matched controls was used for behavioral testing.

Vital Signs

Vital signs were monitored using the MouseOx Plus (Starr Life Science). A thigh sensor, placed on the shaved left thigh, was used to measure heart rate (beats per minute – bpm) and pulse distention or blood pressure (micrometers -

μm) with measurements taken from 0 - 5 minutes for baseline measurements, at 65 minutes (drug administration) after un-occlusion/reperfusion following 60 MCAo surgery, at 70 minutes and 75 minutes reperfusion.

Perfusion

Blood flow through the MCA was measured pre and post-occlusion and after reperfusion using the Laser Doppler Periflux System 5000 with a 2 mm tip and Laser Speckle PeriCam PSI System – Blood Perfusion Imager High Resolution (Perimed). To measure occlusion and reperfusion of the MCA, measurements were taken before drilling the burr hole (0 minutes), after metal wire insertion under MCA (5 minutes) and following 60 minute occlusion with un-occlusion/reperfusion measurements at 70 (drug administration), 75 and 80 minutes. Care was taken to ensure the probe was placed in the same location for each measurement. To measure hemispheric blood flow the Laser Speckle was positioned 8 cm above the exposed pronated mouse skull and set to measure at Regions on Interest (ROI) and Time Points of Interest (TOI). The laser guidance system positioned along the sagittal suture acted as a landmark to ensure consistent measurements. Measurements were taken before occlusion at 0 minutes, at 5 minutes after occlusion and after reperfusion at 80 minutes which included drug administration and 10 minutes of reperfusion.

MCAo and IA injection

Animals were anesthetized using a Ketamine and Xylazine mixture in a weight based dosing scale (100 μl of anesthesia for every 10 grams). To induce

focal cerebral ischemia, we used the previously described MCAo stroke model (37, 40). The occlusion was for a one-hour interval, and recanalization was confirmed via Laser Doppler and Speckle. Animals were warmed on a heating pad throughout surgery and core temperature (rectal thermometer) monitored using the MouseOx Plus (Starr Life Science) to ensure body temperature did not drop below recommended levels. Post-surgery, animals were warmed on a heating pad with overhead light and checked every 5 minutes to ensure hyper/hypothermia did not occur.

To infuse IA verapamil, we followed our previously published protocol (63). Briefly, we exposed the CCA bifurcation and its branches the ICA and ECA. The distal ECA was permanently occluded, the ICA was temporarily occluded, and a nick was made with micro scissors in the ECA to allow for micro-angio tube insertion and secured with suture. Following the one-hour occlusion the CCA and ICA clamps and metal filament under the MCA were removed restoring blood flow. After 5 minutes of reperfusion, the study agent (saline or verapamil) was infused IA at a rate of 2.5 µl/minute at a volume of 10 µl (63). After the injection the proximal ECA was permanently occluded and the incisions in the thoracic and temporal region were sutured.

Behavioral Testing

Two behavioral tests were performed to assess functional outcome after surgery and pharmacotherapy administration: the rotor rod (used to measure forced movement) and open field (used to measure free roam). For the rotor rod,

animals, naïve (age matched controls, no anesthesia or surgery), control (MCAo surgery with IA injection of saline), and treated (MCAo surgery with IA injection of verapamil) were trained for 3 days prior to stroke surgery and tested for 3 days following stroke surgery: the mice were placed on the rotor rod for 5 minutes with an increasing acceleration from 0 – 40 rpm for three trials. Distance covered by each animal was measured in percent change from baseline. The open field consists of a squared box measuring 50 cm by 50 cm by 30 cm with an infrared camera positioned above the box, which tracks the movement of the animals. Our parameters were set to measure percent change from baseline. Behavioral training occurred on the rotor rod for 3 days prior to stroke (-3, -2 and -1) and on post stroke day (PSD) 1, 3, and 7, while the open field testing occurred 1 day prior to stroke (-1) and testing on PSD 1 and 7.

Infarct Volume Analysis

The mice were euthanized via cervical dislocation on PSD 7, and the whole brain was removed, sectioned using a brain mold into 2 mm sections and stained using Triphenyl Tetrazolium Chloride (TTC) for 10 minutes. Infarct volume was analyzed from scanned TTC stained brain sections with Image J software (NIH). Whole brains were flash frozen, sectioned on the cryostat and stained with Cresyl Violet for additional infarct volume analysis as described below under immunohistochemistry.

Immunohistochemistry

Flash frozen whole brains were sectioned on a Leica CM 1950 cryostat at 20 µm and mounted on slides. Infarct volume and overall neuronal health was assessed by TUNEL stain (Millipore). Astrogliosis was assessed with Glial Fibrillary Activating Protein (GFAP 1:1000 antibody dilution, Sigma) immunohistochemistry. Mature neuron survival within the stroke affected area was assessed via NeuN (1:1000 antibody dilution, Abcam) immunohistochemistry. Area of infarct analysis corresponds to cortical region that was the epicenter of the stroke morphologically identified based on cryostat sectioning to include greatest affected area. Stains were visualized with a Nikon Ecliplse T*i* microscope at 20X magnification and images collected via CCD camera and attached to computer qualified with Adobe Photoshop. Nikon NIS Element BR Analysis imaging software was used to analyze brain sections from control and treated groups, sections were plated 4 to a slide with 10 slides per animal for a total of 40 sections.

Statistical Analysis

All measured variables are presented as mean ± SEM. We selected rotor rod as the primary endpoint for power calculations because it had the smallest anticipated effect size. Based on an anticipated effect size of 1.0 (Cohen's d) and significance level of 0.05, we expected to have 81% power with 17 animals per group to detect improvement from day 1 to day 7 post-surgery. We increased the sample sizes in each group to bolster power and account for potential losses.

Based on preliminary data, we expected close 100% power to detect differences in infarct volume (anticipated Cohen's d = 3.4) using these same groups. Analysis of results for comparison between treatment groups (infarct volume, immunohistochemistry) was performed using a *Student's* t-test. For time course comparisons (behavior, vital signs and perfusion), a Two-Way Repeated Measures ANOVA was used. Significance is defined as a * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Compliance with STAIR Criteria

To maximize the applicability of our results, we designed the study with reference to the STAIR recommendations for preclinical neuroprotection research (69). With regard to dose of drug, we selected a weight-based dose-equivalent to 10 mg in a 70-kg human. This is the dose used currently by endovascular neurointerventionalists to treat patients in the angiography suite. We have not yet conducted preclinical dose-response studies. In terms of window of opportunity, we administered the drug in immediate sequence after vessel reperfusion in order to simulate the clinical scenario of IA administration immediately following thrombectomy. We conducted the experiments in a blinded fashion, with randomization of assignment to treatment groups. We conducted a power analysis to ensure adequate subject numbers as detailed. We performed physiologic monitoring as detailed, and achieved Laser Doppler flow reduction ≥60% as detailed in results. Regarding outcome measures, we evaluated functional outcome and infarct volume, as well as immunohistochemistry analysis

of infarct. We did not analyze gender or age differences in this study, but such analysis is planned for future study.

Results

Vital Signs

Measurements of baseline heart rate (bpm) for control and treated groups (Figure 4.1A), combined prior to drug injection at 0 minutes (227 +/- 2.92) and 5 minutes (210 +/- 8.08), demonstrated a non-significant decrease of 67 bpm over this time period. Five minutes after recanalization and initiation of IA drug administration (i.e. at 70 minutes), the verapamil treated group had a significantly ($P < 0.001$) higher heart rate (218.37 +/- 3.83 vs. 134.00 +/- 1.02 in controls), a difference that largely persisted at the time (75 min) of final measurements (202.20 +/- 7.04 vs. 125.70 +/- 1.36).

Similarly, combined baseline pulse distention (μm) (Figure 4.1B) measurements (μm) at 0 minutes (34 +/- 0.23) and 5 minutes (32 +/- 0.61) decreased slightly by 2 μm over this time period. At 70 minutes the control group had a non-significant increase in pulse distention (24.15 +/- 0.68 vs. 21.60 +/- 0.69), a difference that became significant ($P < 0.001$) at 75 minutes (31.45 +/- 1.54 vs. 22.23 +/- 1.51).

Cerebral Blood Flow

Five minutes after vessel occlusion, Laser Doppler (Figure 4.2A) blood flow measurements (Perfusion Units) through the MCA were reduced to approximately 17% of the pre-occlusion value. Reperfusion at 65 minutes

INTRA-ARTERIAL VERAPAMIL PHYSIOLOGICAL MEASUREMENTS

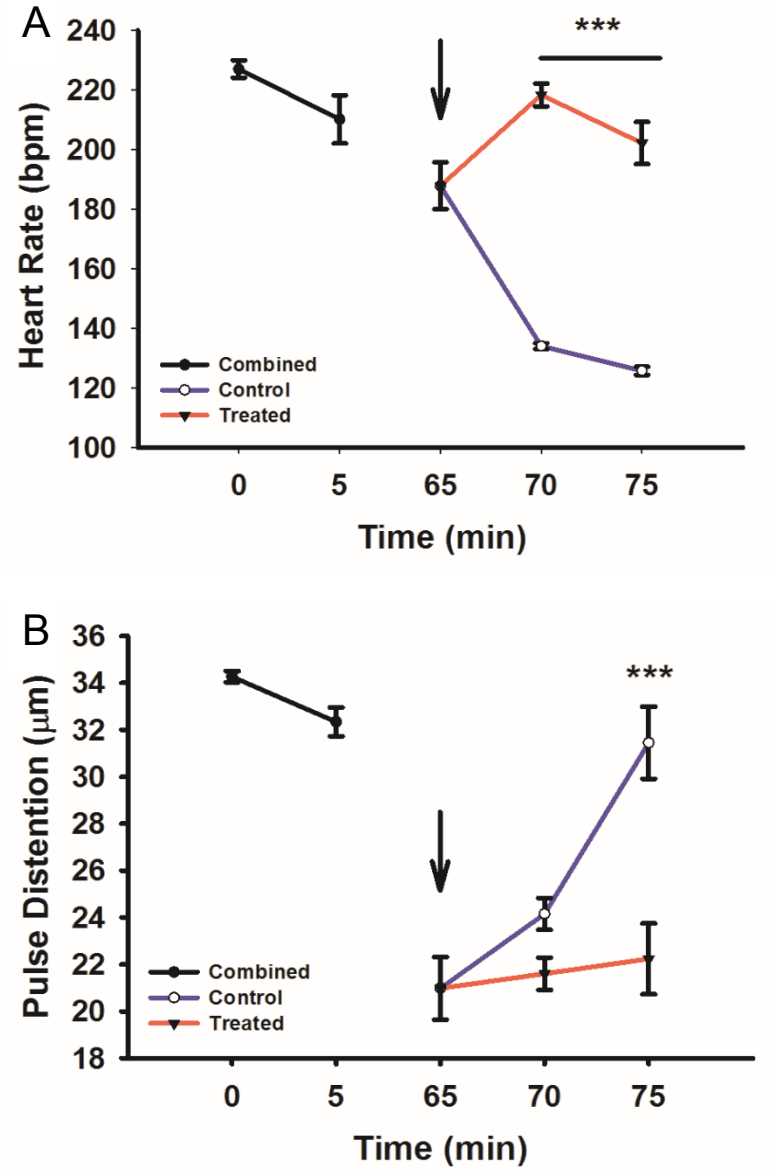

Figure 4.1. MouseOx Plus Physiological Measurements for Combined (black), Treated (red) and Control (blue) groups. Arrow indicates drug administration after reperfusion/un-occlusion for 5 minutes. A. Heart Rate in beats per minute (bpm), 0 - 5 minutes (baseline measurements), 65 minutes (reperfusion/un-occlusion and drug administration), 70 and 75 minutes (reperfusion)., B. Pulse Distention measuring vessel diameter (micrometers or μm), 0 - 5 minutes (baseline), 65 minutes (reperfusion/un-occlusion and drug administration), 70 and 75 minutes (reperfusion). Combined (N = 30), Control (n = 15), Treated (n = 15).

demonstrated a 78.71% restoration of flow with a gradual and similar (i.e. not significantly different) return to pre-occlusion levels by 75 minutes after occlusion regardless of control or verapamil treatment.

Similarly, Laser Speckle images (Figure 4.2B) and corresponding graph (Figure 4.2C) for equilateral cerebral blood flow measurements (Perfusion Units) demonstrated an expected reduction of the ipsilateral cerebral hemisphere 5 minutes after occlusion with non-significantly different restoration in blood flow measurements in the two treatment groups at 80 minutes post occlusion.

Behavioral Testing

Behavioral testing for forced motor (rotor rod) movement (Figure 3.3A) was measured using percent change from baseline. On the day following surgery and drug administration (PSD 1), the two treatment groups showed no significant difference in performance (control 0.86 ± 13.92 vs. treated -10.28 ±- 15.35), but were significantly ($P < 0.05$) different from naïve (23.71 ± 5.48). Treatment groups separated and were significantly ($P < 0.01$ and $P < 0.001$) different on PSD 3 (control -31.68 ±- 15.96 vs. treated 16.70 ± 9.81), and PSD 7 (control -60.20 ± 20.72 vs treated 24.70 ± 8.23). Naïve was not significantly different from treated on PSD 3 (25.31 ± 12.51) and PSD 7 (29.31 ± 19.51) but was significant from control ($P < 0.01$ and $P < 0.001$).

In the free roam motor (open field) movement (Figure 3.3B), analysis was measured in percent change from baseline (measurements for 5 minutes). Both groups had a significant ($P < 0.001$) decrease in free roam movement

INTRA-ARTERIAL VERAPAMIL PERFUSION MEASUREMENTS

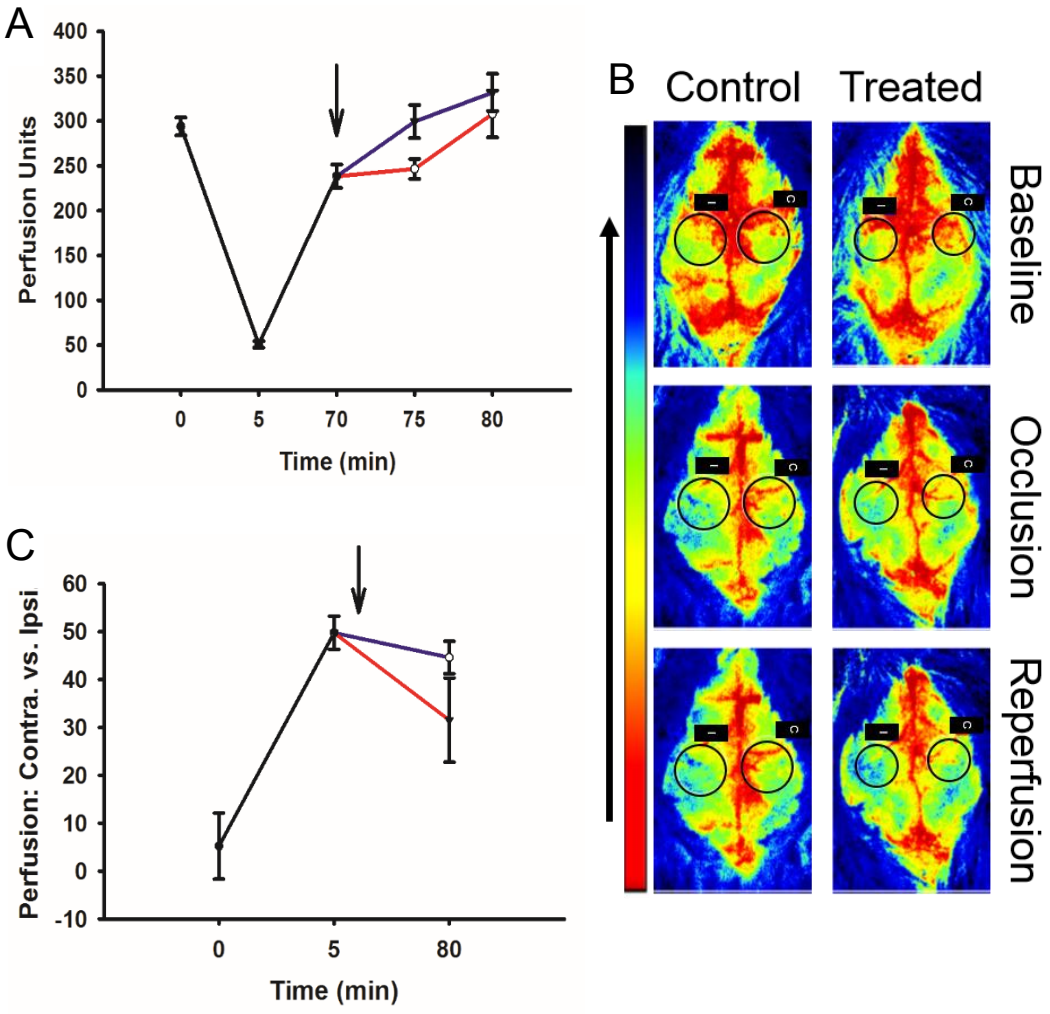

Figure 4.2. Perfusion Measurements for Combined (black), Treated (red) and Control (blue) groups. Arrow indicates drug administration after reperfusion/un-occlusion for 5 minutes. A. Laser Doppler blood perfusion through middle cerebral artery at time points 0 minutes (baseline measurements), 5 minutes (occlusion), 70 minutes (reperfusion/un-occlusion) and 75 and 80 minutes (reperfusion) Treated (n = 9). Control (n = 9)., B. Laser Speckle whole brain perfusion images through contralateral and ipsilateral cortex at time points 0 minutes (Baseline), 5 minutes (MCA occlusion) and 80 minutes (Reperfusion) for Treated (n = 3) and Control (n = 3). Black circles indicate Regions of Interest, Ipsilateral (I) and corresponding Contralateral (C) for MCA occlusion at aforementioned time points. C. Laser Speckle graph for whole brain perfusion through contralateral and ipsilateral cortex at time points 0 minutes (baseline measurements), 5 minutes (MCA occlusion) and 80 (15 minutes reperfusion/un-occlusion) Treated (n = 3), Control (n = 3).

INTRA-ARTERIAL VERAPAMIL BEHAVIORAL MEASUREMENTS

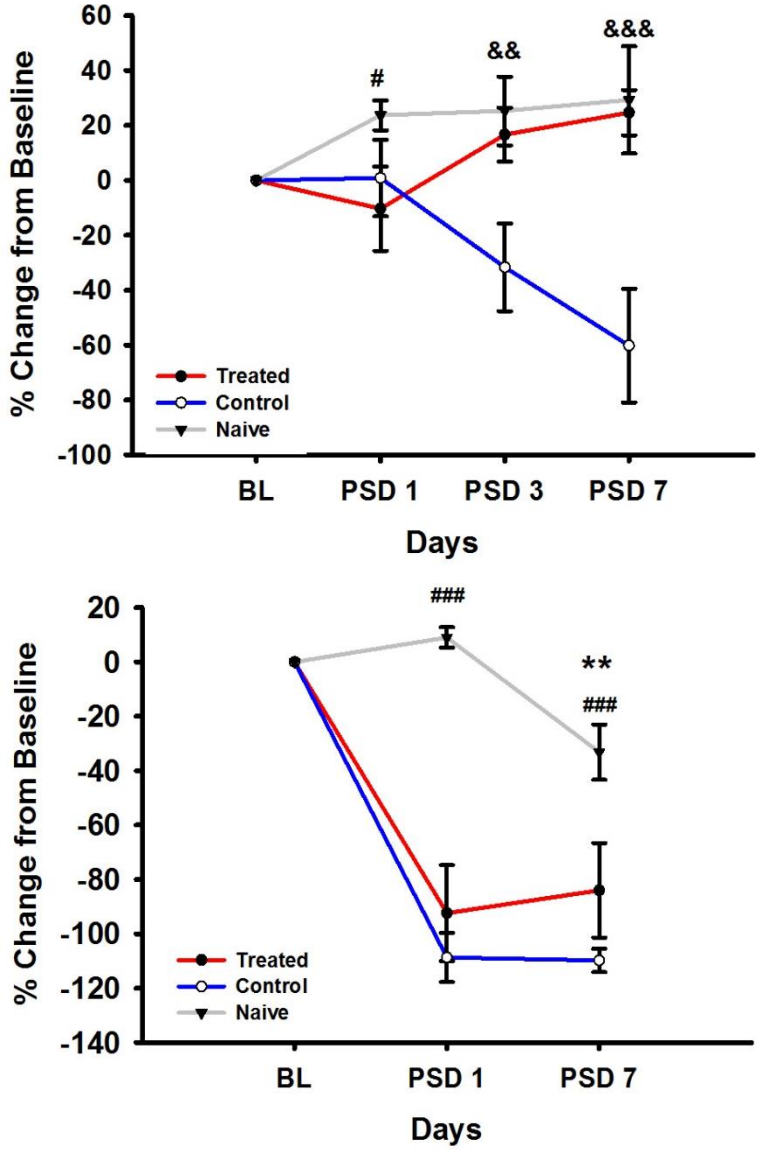

Figure 4.3. Behavioral measurements for Rotor Rod and Open Field for Naive (gray), Treated (red) and Control (blue) groups. A. Rotor Rod is a forced motor movement test and recorded in percent change from baseline, treatment groups separated following stroke surgery into control, treated and naive and were tested on days (1, 3, and 7)., B. Open Field free roam movement, groups separated into control, treated and naive groups on PSD 1 and 7. Combined (N =49), Treated (n = 25), Control (n = 20), Naive (n = 5). # indicates $P < 0.05$ for naïve versus treated/ control, && indicates $P < 0.01$ for treated/naïve versus control, &&& indicates $P < 0.001$ for treated/naïve versus control, ### indicates $P < 0.001$ for naïve versus treated/control, ** indicates $P < 0.01$ for treated versus control.

on PSD 1 (control -108.73 ± 9.03 vs. treated -92.39 ± 17.67) when compared to baseline and naïve (9.01 ± 3.75). By PSD 7 the treated group moved significantly ($P < 0.05$) more (control -109.81 ± 4.36 vs. treated -84.00 ± 17.41) but both groups were significantly ($P < 0.01$) less than naïve (-33.17 ± 10.18).

Both groups significantly ($P < 0.001$) decreased in overall distance covered on PSD 1 compared to baseline and naïve, there was no significant difference between baseline and naïve (9%) from baseline to PSD 1. Finally, while control stroked mice open field performance remained significantly ($P < 0.001$) worse than naïve, aged matched control mice on PSD 7, IA verapamil treated group performance recovered but was still significantly ($P < 0.01$) decreased from naïve.

Infarct Volume

Infarct volume was analyzed using two forms of measurements; TTC and Cresyl Violet infarcts were combined and analyzed on PSD 7 (Figure 4.4). Mean infarct volumes (mm^3) were significantly different ($P < 0.001$) between the control group (17.46 +/- 2.66) and treated (0.64 +/- 0.35).

Immunohistochemistry

Specific antibodies were used for proteins related to overall cellular health (TUNEL stain) (Figure 3.5 A – C), astrocytic inflammation/infiltration (GFAP) (Figure 3.5 D - F) and neuron survival (NeuN) (Figure 3.5 G - I). Compared to

INTRA-ARTERIAL VERAPAMIL INFARCT VOLUME ANALYSIS

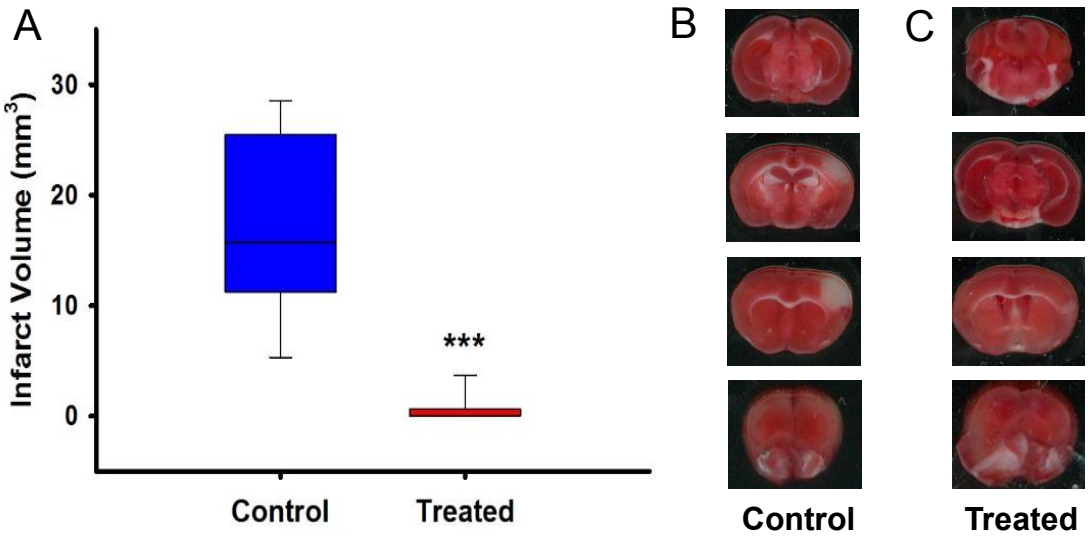

Figure 4.4. A. Infarct volume analysis for control versus treated using TTC and cresyl violet stained stroked tissue. Treated (n = 18) Control (n = 15)., B. TTC image for control group., C. TTC image for treated. *** indicates P ≤ 0.001.

INTRA-ARTERIAL VERAPAMIL IMMUNOHISTOCHEMISTRY

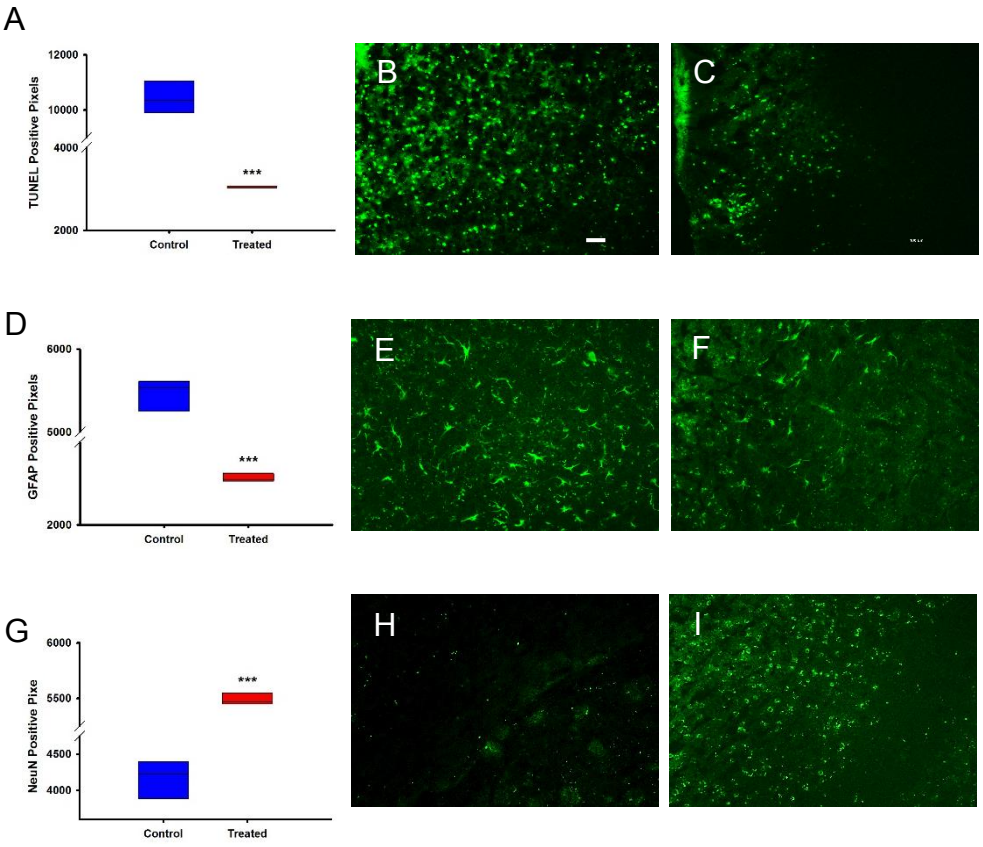

Figure 4.5. Graphs and images for immunohistochemistry, magnification at 20X with quantification of positive pixel density. A. Graph depicting positive pixel for TUNEL stain of control versus treated in infarcted region., B. Image of control TUNEL stain from infarcted region at magnification 20X., C. Image of treated TUNEL stain from infarcted region at magnification 20X., D. Graph depicting positive pixels for GFAP stain of control versus treated in infarcted region., E. Image of control GFAP stain from infarcted region at magnification 20X., F. Image of treated GFAP stain from infarcted region at magnification 20X., G. Graph depicting positive pixels for NeuN stain of control versus treated in infarcted region., H. Image of control NeuN stain from infarcted region at magnification 20X., I. Image of control and treated NeuN stain from infarcted region at magnification 20X. Treated (n = 10), Control (n = 10). White scale bar, 100μm. *** indicates $P \leq 0.001$.

treated animals, there was a significantly greater increase in TUNEL positive pixel density in control groups ($P < 0.001$). Likewise, GFAP immunohistochemistry demonstrated significantly more GFAP immunoreactivity in the infarcted brain of control animals ($P < 0.001$). Lastly, NeuN immunohistochemistry demonstrated significantly decreased NeuN immunoreactivity in the infarcted brains of control animals ($P < 0.001$). Importantly, as mentioned in the methods section, the area of brain analyzed in these studies corresponds to the cortical region that was the epicenter of the stroke morphologically identified based on cryostat sectioning to include the greatest affected area

Discussion

Stroke remains a leading cause of morbidity and mortality in the United States. However, with advances in vessel recanalization with t-PA and endovascular thrombectomy there is a greater chance of stroke survival and opportunity to decrease long-term disability. We believe that targeted IA delivery of a potential neuroprotective compound into the just recanalized large cerebral blood vessel is the next logical step in clinically acute ischemic stroke care. Our lab uses a clinically relevant mouse model of stroke to induce a focused, reliable and reproducible cortical infarct that similarly affects motor function as seen clinically. Following vessel recanalization we IA administered the potentially neuroprotective compound verapamil, to mimic IA administration as a potential clinical therapeutic approach following endovascular thrombectomy. This was

done at a specific flow rate (2.5 µl/min) and injection volume (10 µl) to maximize ipsilateral hemispheric targeting of the agent of interest while minimizing systemic exposure (no significant effects were noted in heart rate and pulse distention between baseline measurements and the treated group post drug injection). This is an important result inasmuch as intravenous, systemically administered verapamil, an anti-hypertensive medication, would be expected to lower heart rate and pulse distention which can potentially cause bradycardia and hypotension by way of its venous return to the heart. Thereby further exacerbate stroke symptoms by decreasing post-recanalization cerebral blood flow and negating verapamil's intended neurological effects.

Verapamil is routinely administered IA to the brain by neurointerventionalists to treat cerebral artery vasospasm after subarachnoid hemorrhage. Given its recognized safety profile in the clinical setting, along with speculation of its neuroprotective properties, we chose to study its potential as an adjunctive therapeutic in stroke models. While its proposed primary mechanism has been vasodilation of intracranial arteries, we propose that it may have direct neuroprotective effects through L-type calcium channels beyond the vascular bed. To address this question of mechanism, we examined perfusion using Laser Doppler (testing the focal MCA) and Laser Speckle (testing whole brain cortex perfusion). Following IA administration, the Laser Doppler showed transiently higher perfusion in the control group. However, overall, the administration of verapamil did not significantly affect perfusion whether focally in the MCA or globally for cortical perfusion in the short term. This contrasted with the control

group after drug administration that showed a significant drop in heart rate but a significant increase in perfusion units (blood pressure). Thus, the Laser Doppler and perfusion units correlate in the control group, showing an elevation relative to the verapamil treated group. Given the role in maintaining adequate cerebral perfusion in ischemic stroke, we would not expect an elevation in blood pressure after reperfusion to explain the highly significant difference between the control and treatment group. While reperfusion injury is a recognized issue after recanalization, the transient differences (relative tachycardia and lower pulse distention) seen in the treated group do not adequately explain the benefit of verapamil in neuroprotection. One limitation of this assessment was a lack of follow-up assessments in mice 12 - 24 hours after the procedure. Performing such an assessment would be challenging, but could provide additional data on the perfusion mechanisms for neuroprotection by verapamil in the setting of recanalized ischemic stroke.

Acute stroke therapies are aimed at preventing death and minimizing functional disability. In our experimental strokes, death rates were not significantly affected with treatment, but were universally low in all conditions (less than 5% in both groups), a particular advantage of this experimental stroke model over others. Functional outcome was measured using two behavioral tests, rotor rod for forced motor movement and open field for free roam to determine the effect of IA verapamil on post-stroke function. Similarly, in the open field testing, although both groups reduced their movement on PSD 1, the treated group improved by PSD 7, while the control group continued to decline.

Collectively, these results suggest that acutely-administered IA verapamil is very effective in preventing behavioral decline in experimental stroke. However, this experiment may be confounded on PSD 1 by the recent surgical procedure. Recovery can be seen when comparing naïve, treated and control PSD 1 rotor rod measurements to baseline measurements; we found variable rates of improvement from baseline for the naïve and control groups (23% and 0.86%, respectively) while the treated control group decreased (not significantly) 10% from baseline. However, such an effect should be dissipated by PSD 7. Finally, this analysis is somewhat limited by a lack of more long-term (> 30 day) functional assessment which is planned for future study. Therefore, the possibility that spontaneous functional recovery in the control stroked mice might eventually reach or plateau at the level of the stroked verapamil treated mice cannot be excluded. However, in the setting of our planned methods for gross and immunohistochemistry assessment, we feel that seven day post-stroke testing was reasonable for initial assessment of verapamil's treatment effect.

Verapamil was further tested to determine its effect on brain tissue preservation. Brain TTC and immunohistochemistry showed a large reduction in infarct, with reduction in cellular apoptosis and preservation of mature neurons. We chose these experiments as markers of step-wise processing in ischemic stroke – ischemic volume, inflammatory cell activation and infiltration, cell death and apoptosis, and neuronal survival. In each case, our data supports the hypothesis that verapamil is neuroprotective on brain tissue when administered early and focally after reperfusion. Verapamil's neuroprotective mechanism(s) of

action remain to be elucidated. Indeed, as it is clear from these experiments that verapamil has significant potential as a therapeutic adjunct to thrombectomy in ischemic stroke, further study is needed to focus on the mechanism by which verapamil acts in this setting as well as its efficacy in different clinical models of ischemia such as the embolic model.

Indeed, while there are a number of animal models to mimic stroke with their differing advantages and disadvantages, our model mimics distal M1/M2 MCA vessel occlusion and produces the hallmark motor and physiological deficits one would expect to see in a patient presenting with an occlusion in the M1/M2 MCA. By comparison, injected thrombus models with t-PA have variable rates of recanalization, and technology does not exist to perform mechanical intracranial thrombectomy in a rodent model. While we do not inject a clot or administer t-PA in our ischemic stroke model, we are able to significantly decrease the flow of blood through the MCA as is seen clinically and un-occlude guaranteeing recanalization. For these reasons, we conclude that our experimental approach reasonably mimics selective IA neuroprotective administration after clinical ELVO thrombectomy.

Recent clinical trials such as MR CLEAN, ESCAPE, and EXTEND-IA have shown that endovascular thrombectomy is the new standard of care for ELVO stroke to ensure recanalization of the vessel and reperfusion of the ischemic area (12, 13, 60, 61). Incorporating IA administration of a potential neuroprotective agent following recanalization and reperfusion, would be a simple adjunctive

process, as the thrombectomy procedure inherently provides timely and selective arterial access to the cerebrovascular bed. While multiple neuroprotective agents have shown promise in animal studies, a lack of positive clinical data limited their adoption in patient care (15). These past studies did not have rapid access to selective arterial infusion after recanalization; our model provides this translational testing ground. Using our mouse model of acute ischemic stroke with IA administration we have shown verapamil is physiologically safe, improves functional outcome, decreases infarct volume, decreases astrogliosis activation, and increases mature neuron survival. The exact mechanism of these actions for verapamil is not yet known, and will be the focus of future studies that further elucidate additional efficacy for this drug and for neuropharmacology in ischemic stroke. However, this study provides the necessary data to support clinical evaluation of this therapy.

Chapter Five: Combinational Therapy

Introduction

While IV t-PA and ET remain the gold standard for treatment of ELVO they are methods of recanalization and do not confer neuroprotection or neurorepair. Restoration of blood flow to the previously occluded area is the first step in treating ELVO. Even though recanalization rates have increased and times to recanalization have decreased, there has been a disconnect between recanalization rates and improved functional recovery.

Despite the fact that clinical trials looking at pharmacotherapy administration following stroke have largely failed, they have demonstrated the ability to target therapies at a specific receptor or signaling molecule. Using the IA model of drug administration we are looking to incorporate a two drug approach to target the acute and late phase of cellular degeneration following stroke. For the acute phase we selected verapamil, an L-type calcium channel blocker, due to its ability to regulate calcium influx in the early period of apoptosis. To treat later phases of apoptosis we selected lubeluzole, which regulates the NMDA receptors, to disrupt downstream glutamate. Administering two drugs with different mechanisms of action allows cells undergoing different phases of cellular degeneration to receive treatments specific to their level apoptosis.

Verapamil and lubeluzole were selected because of their preclinical and clinical history. Verapamil is used clinically to treat cerebral artery vasospasms

following successful recanalization but recently demonstrated in a preclinical setting to be neuroprotective when administered acutely following stroke. Lubeluzole also demonstrated neuroprotection in a preclinical setting but did not show benefit in clinical trials. While both drugs demonstrated neuroprotective effects in a preclinical setting, the same effect was not seen in the clinical model of ELVO. Here we present a method of combinational pharmacotherapy delivery that is clinically relevant but also addresses issues that might have caused both drugs to fail in clinical trials. First is successful recanalization because without restored flow the drugs will not be able to reach the target tissue. Second, we will administer one dose of each drug IA following successful recanalization since we feel a selective administration directly to the site of ischemia will provide a better outcome than broad dosing through IV. Our goal is to mitigate any systemic side effects while also ensuring the drug reaches the stroked tissue, verapamil if administered IV will cause vasodilation and could have significant effects on heart rate and blood pressure. Lubeluzole was serially delivered in the clinical setting but was halted because no effect was seen in mortality between treatment groups. By administering a one-time dose we are limiting the peripheral effects of the drugs but also enhancing their intended effects by selectively administering acutely.

Using the MCAo mouse model of stroke and IA pharmacotherapy delivery we tested a combinational therapy approach looking at overall neuronal health and functional outcome. Here we present the methods and results outlining our

case for why a combinational therapy approach for stroke treatment is safe and effective.

Material and Methods

Experiments conformed to protocols and guidelines set forth by the University of Kentucky Division of Laboratory Animal Research, Institutional Animal Use and Care Committee and ARRIVE Guidelines (83). In brief, 16 week old C57/Bl6 (25 - 30 gram) male mice from Jackson Laboratories were separated into 3 groups (control, treated and naïve) in a blinded fashion using randomized selection. Groups were divided into naïve (age matched litter mate controls), control (MCAo surgery with 10 µl saline (APP Pharmaceuticals) injected at 2.5 µl/min IA), and treated (MCAo surgery with 10 µl + verapamil/lubeluzole injected IA). Verapamil (Hospira) was administered at a dose of 0.15 milligram (mg) per kilogram (kg) and lubeluzole (Sigma Aldrich) was administered at a dose of 0.63 mg per kg (49, 50, 81, 82). Doses were extrapolated from both preclinical and clinical doses using an intravenous/intraperitoneal method of drug delivery. Previous studies for flow rate and injection volume allowed us to determine the optimal flow rate of 2.5 µl/min with an injection volume of 10 µl per mouse (36, 63). Perfusion measurements, physiological measurements, behavioral measurements, infarct volume and immunohistochemistry were conducted in a blinded fashion. Exclusion criteria was applied to any animal that had a ruptured MCA or CCA during MCAo surgery, died during the study, or was shown to have

poor health and was euthanized due to study protocol. Our MCAo and IA model had a death rate of <5% in both surgery and behavior.

Animal Number

We conducted the experiment with a sample size of 23 animals; 12 (7 treated and 5 control) were used for perfusion measurements and physiological measurements, 11 (5 treated, 3 control) behavioral assessment (historical controls included), infarct volume and immunohistochemistry. Groups were divided into naïve, control and treated.

Perfusion

Blood flow through the MCA was measured at baseline and occlusion using a Laser Doppler Periflux System with a 2 mm tip (Perimed).

Physiological Measurements

Vital signs were monitored with the MouseOx Plus (Starr Life Science). To monitor pulse distention (blood pressure) and heart rate a thigh sensor was placed over a shaved section of the left thigh. Measurements were taken at baseline (0 – 5 minutes), reperfusion/drug administration (5 minutes post reperfusion) and post drug administration (10 and 15 minutes reperfusion).

MCAo and IA injection

Animals were anesthetized using a weight based ratio of Ketamine and Xylazine mixture for both the MCAo and IA procedures. Induction of focal cerebral ischemia through the MCAo model was induced using our previously

described method (36, 37, 63). MCAo was for one hour with baseline and occlusion confirmed with Laser Doppler, previous studies have demonstrated a 60% recanalization at 15 minutes post occlusion (36, 63). Animal body temperature was maintained on a heating pad throughout MCAo and IA procedure, to ensure the core body temperature did not drop below recommended levels a rectal thermometer was attached to the MouseOx Plus. Post MCAo and IA procedure, animals were moved to a heated recovery cage and checked at 5 minute intervals until fully awake and able to be returned to the animal facility.

Intra-arterial injection of combinational drug or saline followed our previously published protocol (36, 63). In short, during the MCAo occlusion we further exposed the CCA superiorly until reaching the bifurcation into the ICA and ECA. The ICA and ECA are exposed allowing for distal permanent occlusion of the ECA and temporary occlusion of the ICA, a small nick was made midway between the proximal and distal end of the ECA. Micro-angio tubing was placed into the ECA and threaded until it reached the bifurcation and secured with temporary suture. Following the one hour occlusion the CCA and ICA clamp as well as the MCA metal wire filament were removed restoring blood flow to the cerebrovasculature. After five minutes of recanalization a syringe pump injected the study agent (combinational drug or saline) over a period of four minutes at the previously described flow rate and injection volume. Once the injection was complete the ECA was suture ligated at the proximal end (closest to the CCA bifurcation) and the temporal and cervical region was sutured.

Behavioral Testing

Animals underwent two behavioral tests, rotor rod for forced motor movement and open field for free roam, to determine functional outcome after MCAo and IA pharmacotherapy administration. For rotor rod, animals were grouped, naïve (age matched litter mate controls), treated (MCAo surgery with IA injection of verapamil/lubeluzole), and control (MCAo surgery with IA saline injection). Training occurred three days prior to MCAo and baseline measurements were taken one day prior to MCAo, and tested three days following stroke surgery, post-stroke day 1, 3 and 7. Rotor rod consisted of three trials with mice being placed on a rotating rod for five minutes with increasing acceleration from 0 - 40 rpm. Measurements are reported as percent change from baseline for individual animals and grouped together. Open field contained the same groups and consists of four squared boxes (50 cm by 50 cm by 30 cm) with an top down mounted infrared camera to track animal movement. Open field parameters were set to measure percent change from baseline. Measurements were taken at baseline (day prior to MCAo) and PSD 1 and 7.

Infarct Volume

Mice were euthanized on PSD 7 via cervical dislocation and whole brains removed, flash frozen, sectioned on a cryostat (20 µm) and stained using cresyl violet. Infarct volume analysis from scanned cresyl violet images was analyzed using NIH Image J software (36, 37, 63).

Immunohistochemistry

Whole flash frozen brains were sectioned on a Leica CM 1950 cryostat at 20 µm and mounted. NeuN (1:500 antibody dilution, Abcam) immunohistochemistry was used to measure mature neuron survival in the stroked region (penumbra and core). Glial Fibrillary Activating Protein (GFAP 1:500 antibody dilution, Sigma) immunohistochemistry was used to assess astrogliosis. Infarct corresponds to the core and penumbra in the cortex or the epicenter of the stroke affected region, morphologically identified to include the greatest area. Fluorescent stains were visualized using a Nikon Eclipse T*i* microscope at 20X magnification with images collected using an attached CCD camera to a dedicated computer and quantified with Adobe Photoshop. Sectioned brains from sham, treated and control were analyzed using Nikon NIS Element BR Analysis imaging software. Brains were sectioned and plated four slices to a slide with a total of 40 slices over 10 slides.

Statistical analysis

All measured variables are shown as ± Standard Error of the Mean (SEM). For comparison between treatment groups (infarct volume, immunohistochemistry) a ***Student's*** t-test was used for analysis. Time course comparisons (physiological measurements, behavior) were analyzed using a Two-way repeated measures ANOVA. Significance was defined as * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

Compliance with STAIR

Study design was implemented with reference to STAIR recommendations for preclinical neuroprotection trials and to maximize the applicability of our results. Drug doses were selected on previously published preclinical and clinical data currently used by neurointerventionalists to treat patients in the angiography suite (49, 50, 81, 82). To better determine optimal and deleterious doses a future dose response study has been planned. To best mimic the clinical condition, drug administration followed successful recanalization (five minutes post) to maximize the window of opportunity for each drugs targeted effects. The study was conducted in a blinded fashion with drug treatment randomized. A power analysis was conducted to confirm adequate number of subjects per group. For safety we monitored animal's vital signs (pulse distention and heart rate) but also demonstrated a 77% reduction of blood flow through the MCA as stated in the results section. Drug effect following MCAo, successful recanalization and IA injection was evaluated using infarct volume, behavior and immunohistochemistry. Future studies are planned to determine differences in gender and age.

Results

Perfusion

Laser Doppler perfusion measurements were taken at baseline (anesthetized animal, no surgery) and after MCAo (MCA and CCA occluded) induction. Laser Doppler perfusion measurements from baseline (265.75 ±

16.23) to occlusion (60.22 ± 5.09) demonstrated a 77% reduction in flow through the MCA (Figure 5.1).

Physiological Measurements

Baseline heart rate (beats per minute, bpm) measurements for control and treated groups (Figure 5.2A), measurements were combined prior to MCAo and drug injection at zero minutes (227.03 ± 2.92) and five minutes (210.17 ± 8.08), showed a non-significant decrease of 17 bpm over a five minute period. At reperfusion (following MCAo and successful recanalization) the heart rate for combined groups decreased significantly (147.78 ± 0.15) from baseline. At drug administration both treated (144.29 ± 0.15) and control (147.33 ± 0.01) remained significantly decreased from baseline (five minutes) but not from each other. Post 15 minutes drug administration both treated (149.55 ± 0.22) and control (149.67 ± 0.06) remained significantly reduced from baseline (five minutes) but no different from reperfusion or drug administration against each other.

Pulse distention (blood pressure) baseline measurements (Figure 5.2B) for control and treated groups were combined at zero minutes (34.27 ± 0.23) and five minutes (32.34 ± 0.61), showed a non-significant decrease of 1.93 µm over five minutes. Following reperfusion pulse distention decreased for combined groups (28.40 ± 0.06) 3.94 µm from baseline (five minutes). Groups separated at drug administration with treated increasing (35.99 ± 0.15) from both baseline and control, control decreased (27.06 ± 0.03), neither group was significantly different from baseline (five minutes). Post 15 minutes drug administration, the treated

INTRA-ARTERIAL COMBINATIONAL THERAPY PERFUSION MEASUREMENTS

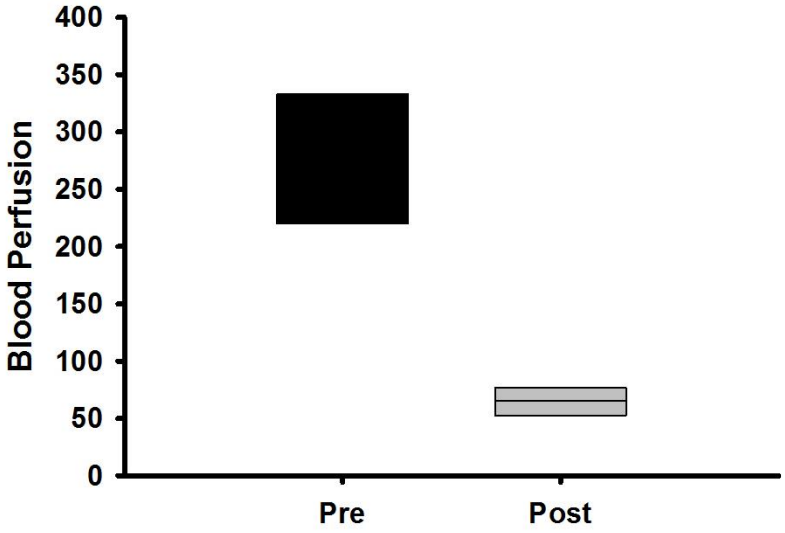

Figure 5.1. Laser Doppler blood perfusion through middle cerebral artery Pre and Post occlusion, reduction for combined groups. Reduction of 78%. (n = 37)

INTRA-ARTERIAL COMBINATIONAL THERAPY PHYSIOLOGICAL MEASUREMENTS

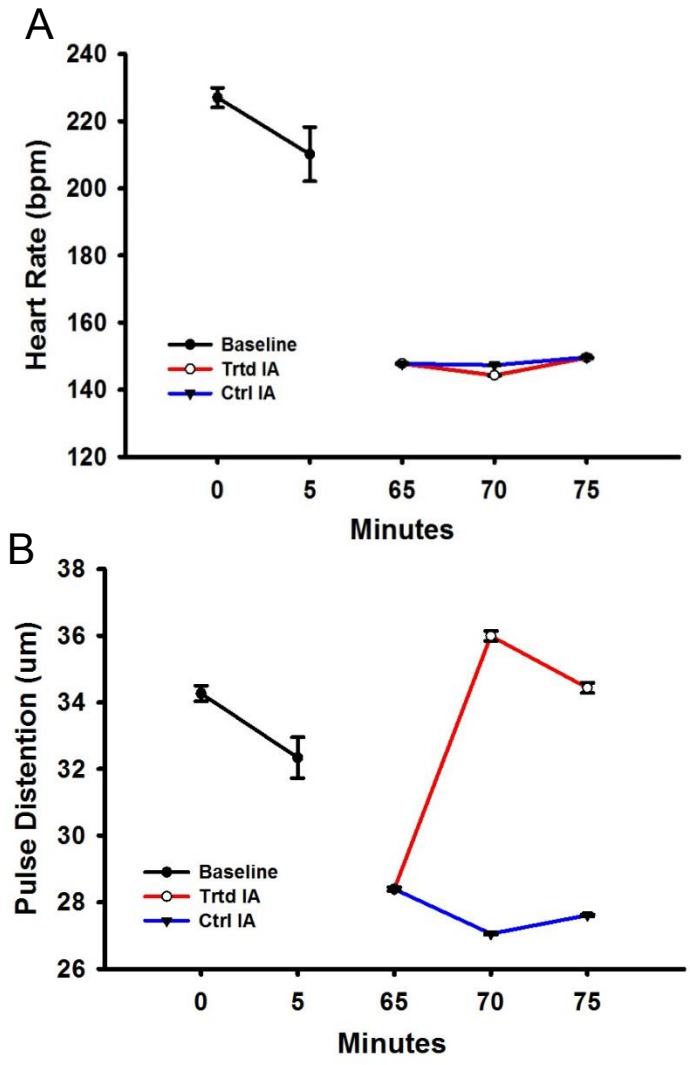

Figure 5.2. MouseOx Plus Physiological Measurements for Combined (black), Treated (red) and Control (blue) groups., A. Heart rate in beats per minute (bpm), 0 - 5 minutes (baseline measurements), 65 minutes (reperfusion/un-occlusion and drug administration), 70 and 75 minutes (reperfusion)., B. Pulse Distention measuring vessel diameter (micrometer or µm), 0 - 5 minutes (baseline), 65 minutes (reperfusion/un-occlusion and drug administration), 70 and 75 minutes (reperfusion), Combined (n = 36), Control (n = 5), Treated (n = 5)

group remained elevated (34.44 ± 0.15) from baseline but the control (27.61 ± 0.03) remained decreased from baseline and treated.

Behavioral Testing

Animals were tested using forced motor (rotor rod) movement (Figure 5.3A) measured in percent change from baseline, groups were trained for three days and tested for baseline measurements one day prior to stroke. Following stroke and IA drug treatment administration on post-stroke day (PSD) 1, the control (-0.95 ± 11.86) and treated (-3.61 ± 19.90) showed no difference between groups. However on PSD 3 the groups separated, with treated (31.24 ± 10.08) being significantly different ($P < 0.01$) from control (-31.20 ± 14.07). The trend continued on PSD 7 with the treated group (34.69 ± 13.65) again being significantly different ($P < 0.001$) from control (-54.78 ± 29.31). Naïve treated animals showed improvement (not significant) on PSD 1 (23.71 ± 5.48) compared to treated and control. The naïve group continued to improve on PSD 3 (25.31 ± 12.51) and was significantly ($P < 0.01$) from control but not treated, a trend which continued on PSD 7 (29.31 ± 19.51) ($P < 0.001$).

Animals were again tested using free roam (open field) movement (Figure 5.3B) with percent change from baseline measured over a five minutes, both groups significantly ($P < 0.001$) decreased from baseline on PSD 1 (control -129.00 ± 27.21 vs. treated -181.73 ± 39.53) but were not different from each other. On PSD 7 the treated group (-65.18 ± 11.20) and control group (-109.83 ±

14.90) both improved but not significantly from each other ($P < 0.055$). The naïve group at both PSD 1 (9.01 ± 3.75) and PSD 7 (-33.17 ± 10.18) was significantly ($P < 0.001$ and $P < 0.01$)) improved from both treated and control.

Infarct Volume

Analysis of infarct volume (Figure 5.4A) was assessed from cresyl violet staining (Figure 19B) on PSD 7 with Image J. Infarct volumes (mm^3) are significantly different ($P \leq 0.009$) between control (21.94 ± 5.26) and treated (4.54 ± 1.91).

Immunohistochemistry

Antibodies specific to proteins associated with mature neuron survival (NeuN) and astrocytic inflammation/infiltration (GFAP) were used (Figure 5.5 A – F). Compared to control, combinational therapy treated animals showed a significant ($P < 0.001$) increase in NeuN positive pixel density. Similarly, combinational therapy treated animals had a significant ($P < 0.001$) decrease in GFAP positive pixel density compared to control.

INTRA-ARTERIAL COMBINATIONAL THERAPY BEHAVIORAL MEASUREMENTS

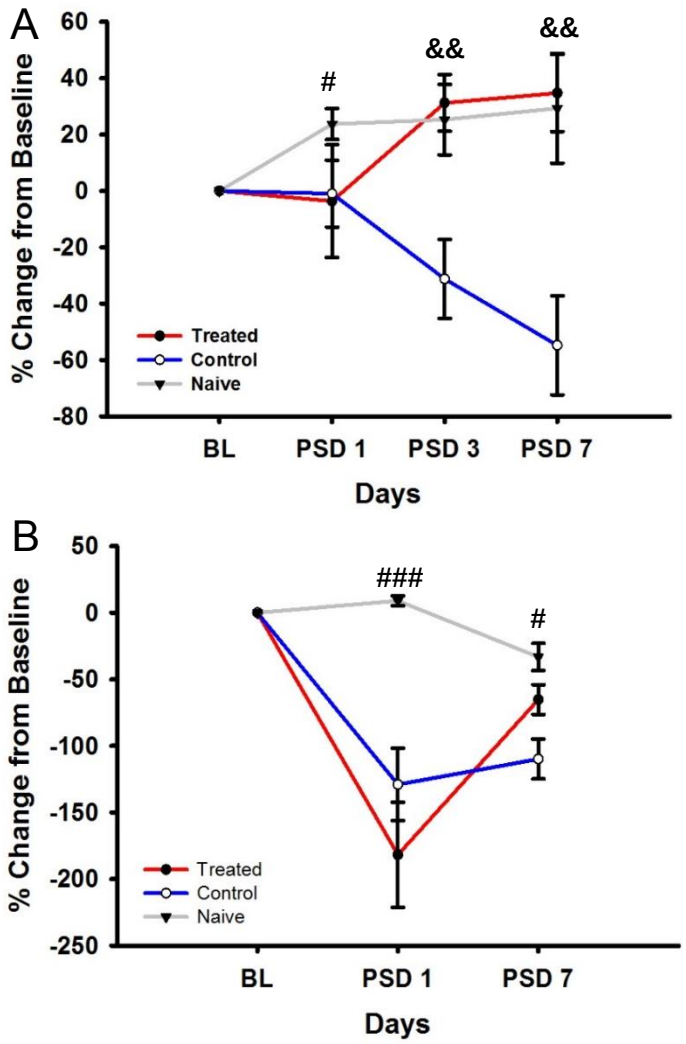

Figure 5.3. Behavioral measurements for Rotor Rod and Open Field for Treated (red), Control (blue) and Naïve (gray)., A. Rotor Rod forced motor movement test for percent change from baseline, groups separated on post-stroke day (PSD) 1 into treated, control and naïve and were tested on PSD 1, 3 and 7., B. Open Field free roam movement separated into treated, control and naïve and were tested on PSD 1 and 7. Rotor Rod: Treated (n = 5), Control (n = 23) and Naïve (n = 4), Open Field: Treated (n = 5), Control (n = 16) and Naïve (n = 4). # indicates $P < 0.05$ for naïve versus treated/control, ### indicates $P < 0.001$ for naïve versus treated/control, && indicates $P < 0.001$ for treated/naïve versus control.

INTRA-ARTERIAL COMBINATIONAL THERAPY INFARCT VOLUME ANALYSIS

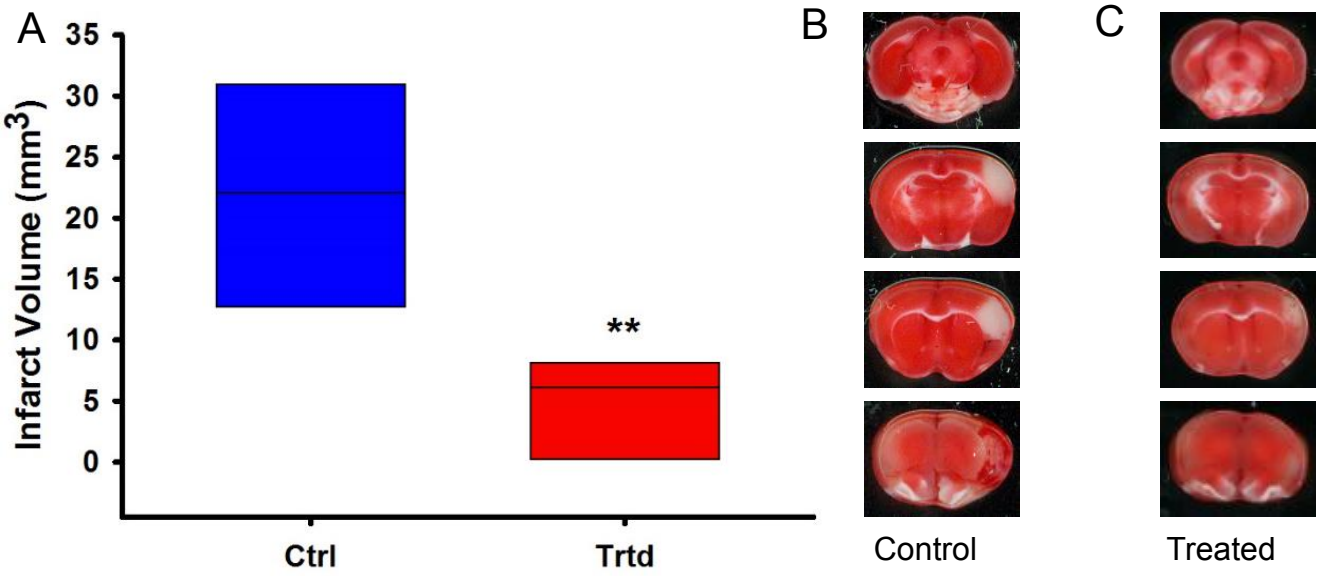

Figure 5.4. A. Infarct volume analysis for control versus treated using TTC and cresyl violet stained stroked tissue. Control (n = 6) Treated (n = 6)., B. TTC Image for control group., C. TTC image for treated group.

INTRA-ARTERIAL COMBINATIONAL THERAPY IMMUNOHISTOCHEMISTRY

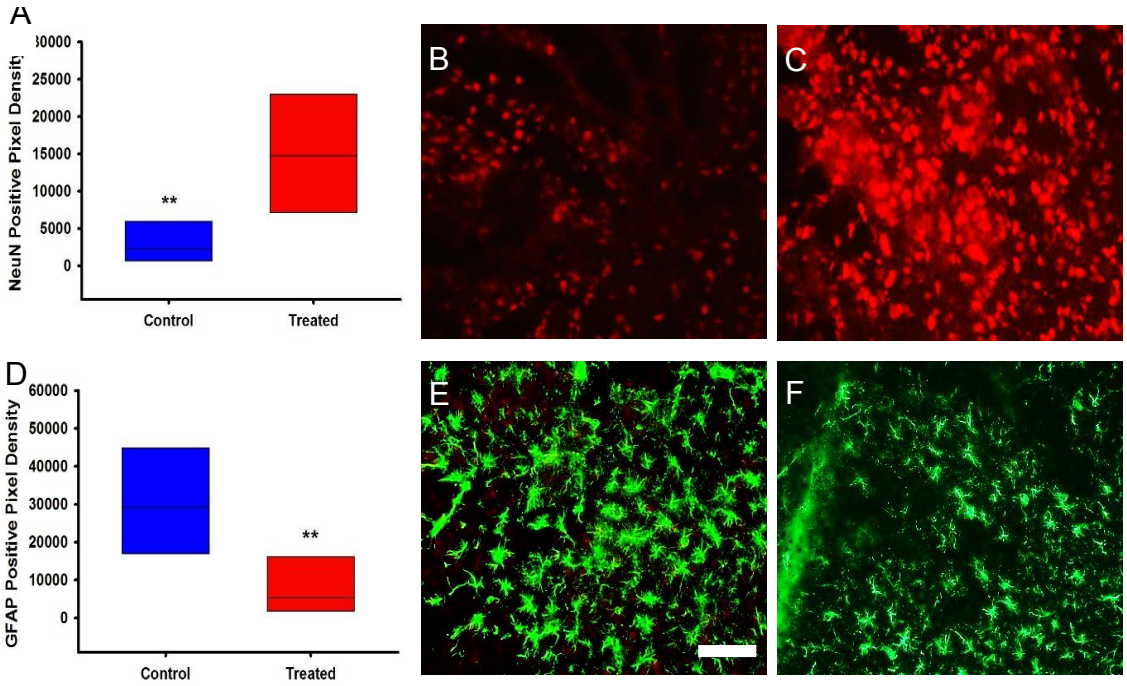

Figure 5.5. Graphs and images for immunohistochemistry magnification at 20X with quantification of positive pixel density. A. Graph depicting positive pixel for NeuN stain of control versus treated in infarcted region., B. Image of control NeuN stain from infarcted region at magnification 20X., C. Image of treated NeuN stain from infarcted region at magnification 20X., D. Graph depicting positive pixel for GFAP stain of control versus treated in infarcted region., E. Image of control GFAP stain from infarcted region at 20X., F. Image of treated GFAP stain from infarcted region at magnification 20X.,Control (n = 6), and Treated (n = 6) White scale bar, 100μm. ** indicates $P < 0.001$

Discussion

Stroke continues to affect millions of individuals annually and with the incidence expected to double by 2050 the need for new therapies is vital. Current therapies of IV t-PA and ET offer improved recanalization rates but this does not correlate with improved functional outcomes. Adjunctive therapies to be used following successful recanalization targeted at specific cellular pathways will assist in preserving neuronal tissue. While a number of pre-clinical trials demonstrated positive effects following animal models of stroke, the transition to the clinical practice did not show the same success. We feel the breakdown between pre-clinical and clinical stroke trials can be attributed to a few key areas; (1) successful recanalization of the previously occluded vessel, (2) administering potential neuroprotective drugs in a representative time frame that will allow the drug to affect the targeted pathway, (3) selective administration, guaranteeing the drugs reach the ischemic tissue by selectively administering directly to the site of occlusion.

Using a clinically relevant mouse model of stroke we incorporated the above factors of successful recanalization, timely drug delivery and targeted administration to test a combinational therapy approach. We selected verapamil and lubeluzole as our neuroprotective agents, both are FDA-approved and have shown pre-clinical and clinical neuroprotection. Verapamil is an L-type calcium channel blocker currently used for cerebral artery vasospasms but in a recent pre-clinical study demonstrated neuroprotective benefits. Lubeluzole is an NMDA

modulator and successfully completed a number of pre-clinical studies before being halted in clinical trials. Our goal is to select a combination of drugs that can be used to activate or downregulate specific cellular pathway's during and following stroke. Because the infarcted tissue is undergoing different stages of apoptosis the ability for one drug to treat multiple pathways is unlikely. We will administer our drugs IA following successful recanalization to target the acute and delayed phases of cellular apoptosis. Verapamil will be used for the acute phase when cells undergo a membrane depolarization and allow L-type calcium channels to open increasing the amount of calcium intracellularly. Lubeluzole will be used for the delayed phase, after cells have undergone a membrane depolarization and an intracellular cascade is triggered. We hope to regulate NMDA receptors and glutamate release intracellularly, by doing so we will inhibit the lipid peroxidation and DNA degeneration that are directly related to increased intracellular glutamate.

To determine if our combinational therapy was effective we looked at a variety of measures to determine drug safety and efficacy both functionally and histologically. First we determined whether our drug combination had an effect on physiological measurements (heart rate and pulse distention), we showed no difference in heart rate between controls and treated following recanalization, drug administration and reperfusion. Next we looked at pulse distention (blood pressure) and found the treated group returned to baseline measures following drug administration and reperfusion but the control group remained significantly lower than both baseline and treated. To determine if there was a functional

difference we tested our groups on rotor rod (forced motor movement) and open field (free roam movement) and found in both tests that the treated group improved but it was significant in the rotor rod and trending towards ($P < 0.055$) significance in the open field.

Following a successful functional test we looked at infarct volumes between treated versus control, and found the treated group to have a significantly ($P < 0.05$) lower infarct volume (mm^3) than the control group. The reduction in infarct volume correlates well with the improved functional outcome at later time points (PSD 7) in the treated group.

Lastly we looked at histological measures of neuronal health and astrogliosis; in both measures we found the treated group to have significant improvement over the control group. Looking at neuronal health, we used NeuN to measure the amount of healthy neurons in the infarcted and penumbral region and found a greater percentage of healthy neurons in treated compared to control. This could be attributed to the acute and delayed drug treatment of verapamil and lubeluzole, by targeting early calcium channels and NMDA receptors, we are selectively treating specific cellular cascades. Next we looked at astrogliosis or the infiltration/activation of astrocytes, the control group which received IA saline had a greater number of astrocytes in the infarcted/core region than the treated groups. A decrease in astrogliosis correlates with decreased infarct volumes and insults to the brain, again we targeted an early and late stage therapy that has demonstrated positive results.

As a whole, a combinational approach post stroke allows for a greater opportunity to treat tissue of varying degrees of cellular death. Starting from the core (area closest to occluded vessel) and spreading to the penumbra (area receiving watershed blood flow), the amount of degradation is highest in the core and decreases as it radiates outward. The advantage of a combinational approach allows for cells in the core and penumbra to receive treatment at their specific stage of cellular degeneration and halt further damage allowing the cells to begin reparative processes. A two drug approach allows for a more broad therapeutic approach, and demonstrates a neuroprotective benefit in both a functional and histological approach. Results from this study while positive should be applied to female mice, aged mice and used under different models of stroke to fulfill the STAIR criteria.

Chapter Six: General Discussion

Worldwide stroke remains a leading cause of mortality and long-term morbidity; with an aging population, the number of individuals suffering a stroke is expected to double by 2050 (1-3). Ischemic stroke (>80% of strokes) occurs when blood flow to the brain is occluded by a clot, which typically occurs within the large vessels of the brain. A subset of ischemic stroke, emergent large vessel occlusion (ELVO) is the most disabling and life-threatening form of ischemic stroke. To date, the only FDA-approved pharmacotherapy for ischemic stroke is intravenous (IV) tissue plasminogen activator (t-PA), but due to extensive exclusion criteria, relatively few are eligible (3). The mechanism of action for IV t-PA is to break down a clot through a series of chemical reactions. While this process restores blood flow to the previously occluded portion of the brain it does not confer neuroprotective or neuroreparative benefits. Without a steady blood flow, the brain and more specifically the neurovascular unit under goes a hypoxic (lack of oxygen and nutrients) event leading to cellular death. This cellular death is triggered by, the intrinsic/mitochondrial apoptotic pathway, causing a cascade of cellular signals both extracellular and intracellular. The intrinsic pathway begins with an insult leading to membrane depolarization and cellular membrane channels opening causing an increase in chemical transport across the cell membrane disturbing the balance between intra- and extracellular environments. This imbalance can be restored if aid is rendered before the cell reaches a point of no return; up until this point the ability of administering therapy and restoring proper cellular function remains possible.

To date, there are only two approved therapies for ELVO, IV t-PA and endovascular thrombectomy (ET), and while both have improved recanalization rates, there is disconnection with positive functional outcome (12). An adjunctive therapy following successful recanalization that targets cell-specific pathways in the neurovascular unit could improve outcome. Drugs conferring neuroprotective benefits have performed well in pre-clinical testing, but there is currently no pharmacotherapy option (t-PA aside) that has successfully transitioned from the pre-clinical models to the clinic; as all failed to demonstrate positive effects following stroke. This highlights a disconnection between the bench and the bedside, and can be attributed to a number of causes. Previous pre-clinical and clinical trials demonstrated a deficiency in experimental design evaluating heterogeneous stroke patients, a lack of co-administration of neuroprotectants with thrombolytics, delay in drug initiation up to 48 hours, and an in ability to ensure that the treatment agent reached ischemic tissue (15).

Endovascular thrombectomy uses a catheter threaded through the patient's vasculature to mechanically remove a clot restoring blood flow, but it also allows for targeted pharmacotherapy treatment directly to the site of ischemia following recanalization. Delivering a drug selectively has many advantages: lower concentration of drug is needed, systemic side effects can be minimized, the agent can be directly given to the pathological tissue, and it can piggyback on an approved therapy (ET).

While there are a number of mouse models of stroke, the tandem transient common carotid/middle cerebral artery occlusion (MCAo) model is representative of large vessel occlusion. This model mimics ELVO in that it guarantees successful recanalization, generates a cortical infarct similar to the clinical condition and allows for a catheter to be threaded into the cervical vasculature to intra-arterially deliver pharmacotherapy (20, 36, 37, 63). Using the already exposed cervical vasculature of the MCAo model, the CCA is isolated superiorly until the CCA bifurcation is reached and the ECA and ICA exposed. Micro-angio tubing was inserted into the ECA toward the ICA and a series of carbon black dye injections were administered to optimize the flow rate and injection volume. Working from max needle flow and syringe volume we were able to determine the optimal flow rate to be 2.5 µl/min with an injection volume of 10 µl. The use of carbon black ink allowed us to determine staining throughout the brain and systemic circulation and help narrow the optimal range. Though carbon black ink has a greater density than potential neuroprotective compounds (typically dissolved in saline) it provides a starting point that will allow future studies to test potential neuroprotective compounds.

Translational projects strive to mimic the clinical condition; our model of IA administration allows us to bypass threading a catheter from the femoral artery of a mouse but limits us to administering the drug into the ICA and not directly into the MCA. If the micro-angio tubing was to be threaded into the ICA and MCA it would occlude the recanalized CCA and ICA during injection. This would hinder the injected drug from being carried by the blood to the ischemic tissue. By

injecting into the ICA directly we are using the recanalized blood vessel to carry our agent of choice to the target region. Carbon black staining demonstrated that even when injecting into the ICA and not directly into the MCA we are able to reach the ischemic tissue while not hindering the recanalized vessel or overshooting our target region.

Improvements of the model would be smaller tubing threaded into the ICA to the MCA without significantly hindering flow, to date the needle gauge and tubing diameter (internal bore and external diameter) needed are not available. If the IA model were used in a larger animal model (rabbit or dog) the current needle and tubing setup would allow for more direct administration to the MCA. This would require optimization as the flow rate through a larger animal's vasculature will be different than a mouse. STAIR criteria recommends testing a method in different models and animal species.

Intra-arterial Model

The IA model will work in most stroke models but caution must be used in the suture model (MCAO) as it uses the same vasculature to thread the suture and occlude as we use to thread our catheter. The ability to transition between a suture and catheter following MCAO without significant blood loss is major factor. The IA model would work well in the thrombin/embolic clot model as it could be used to administer IA t-PA in smaller doses directly to the occluded vessel and mimic ELVO and ET. The photothrombotic model would not be a good stroke model as it is a permanent occlusion and recanalization and IA administration to

the site of ischemia cannot be achieved. Overall the IA model will work in a number of stroke models and could be scaled up to larger animal species but first optimization of flow rate and injection volume is needed.

Nitroglycerin

A potential neuroprotective compound, nitroglycerin or glyceryl trinitrate (GTN) is a known vasodilator used in clinic to treat angina and other heart conditions. Starting in the early 2000's, GTN was evaluated clinically as a potential therapy for stroke, applying a GTN patch for seven days to an individual suffering stroke following recanalization (41, 43, 84). The hypothesis behind applying the patch was a decrease in systemic blood pressure would relate to neuroprotection and better functional scores at 90 days post stroke. The recently published ENOS trial showed an acute reduction in blood pressure following patch placement but no significant difference between treated and control groups at 90 days post-stroke (41, 43). The primary objective of improved functional outcome (shifting the Modified Rankin Scale-clinical measure of functional stroke assessment) was not met but a reduction in acute blood pressure was achieved. Reducing blood pressure during and following stroke will help decrease the chance an individual will suffer increased ICP, a common symptom. While improved functional outcome was not met overall, a sub analysis of ENOS patients receiving a GTN patch within six hours had a beneficial shift in functional outcome and reduced death (84). Though GTN is a known vasodilator it also has a proposed secondary mechanism of action as a nitric oxide (NO_x) donor. Nitric

oxide has been shown to reduce inflammation, maintain vascular tone and reduce infarct volume (anti-apoptotic) (74). GTN is converted to NO_x through a series of reactions following its uptake into the vascular endothelium and released into the blood stream where it works on smooth muscles surrounding the vessel and directly on neurons through inhibition of nitric oxide synthase (NOS).

Similar to verapamil being used to treat cerebral artery vasospasms, GTN is used following ET to open collapsed cortical vessels following recanalization. GTN's neuroprotective potential following ELVO and ET was tested in our preclinical MCAo and IA model to determine if it had protective benefits. As with verapamil, most believe any beneficial effect will come from restored/increased blood flow to the brain. Because there is no known IA dose of GTN, five doses were selected to test for safety (physiological measurements) and from those doses three were selected (3.1, 6.2 and 12.5 μg/μl) for treatment. Using the Laser Doppler, MCA perfusion measurements following recanalization and GTN administration were taken. There was no significant difference between GTN treatment and vehicle groups. Nitroglycerin groups did show a dose dependent increase in perfusion with the 3.1 μg/μl having the highest perfusion and the 12.5 μg/μl having the lowest. This is contrary to what was expected, and possible reasons are: (1) higher doses of GTN are more concentrated upon IA injection and will saturate the ICA endothelium whereas lower IA GTN doses are diluted and will work transiently throughout the ICA and MCA, (2) GTN is converted into NO_x and released back into the blood stream, (3) we missed the full effect of

GTN (half-life of 2.5 - 7.5 minutes) by measuring outside its effective range (started measuring 10 minutes post-IA injection).

As with all potential neuroprotective compounds, the primary outcome measure is improved functional recovery over time. GTN was administered following MCAo and successful recanalization with mice being tested using forced motor movement over seven days (PSD 1, 3 and 7) and compared to baseline (day prior to stroke). Similar to perfusion measurements, lower doses of GTN (3.1 and 6.2 µg/µl) had an acute effect with significant improvement on PSD 1 compared to control but this effect dissipated on PSD 7 with both groups beginning to decline. The acute effect seen in lower doses could be from increased blood flow and a transient increase of NO_x production helping spare neurons in the motor cortex region. The effect dissipates over time as neurons farther from the treatment site (penumbra) which did not receive NO_x continue through cellular degeneration leading to decreased functional ability over time. The 12.5 µg/µl group decreased from baseline on PSD 1 but significantly improved by PSD 7, overall the higher dose of GTN improved during the test but had variability initially. This effect could be related to greater NO_x production, as GTN is administered IA it is converted into NO_x and circulates through the ischemic region. Circulating NO_x will be taken up by neurons in the ischemic region undergoing apoptosis and block NOS, hindering further cellular degradation. This effect can be seen in improved functional movement from PSD 1 to 7. Nitric oxide may preserve neurons in the motor cortex and as they begin to recover so will the functional outcome.

Increased blood NO_x levels (12.5 µg/µl) were confirmed through blood draws 15 minutes post stroke and on PSD 1 compared to baseline (day prior to stroke). While the effect on both time points was not significantly different than control, NO_x levels did show an elevation beyond normal levels following stroke. Previous studies have shown that NO_x levels are acutely elevated (30 minutes) following stroke and drop below baseline during subsequent days (74). This increase in NO_x levels beyond physiological normal measurements might be the reason GTN treated mice performed better compared to control (all doses) in the rotor rod. While NO_x is increased following stroke, GTN's conversion to NO_x will boost the concentration of NO_x levels available. Increased circulating NO_x will increase vascular tone, decrease inflammation and work through anti-apoptotic mechanisms to preserve neuronal health in the infarcted region.

Lastly we looked at histological measures of mature neuron survival and astrogliosis in the infarcted region and found a stair step dose response effect. Mature neuron survival was observed least at dose 3.1 µg/µl and greatest with the 12.5 µg/µl dose. The opposite trend was seen in astrogliosis with the 3.1 µg/µl having the greatest expression and the 12.5 µg/µl dose having the lowest. The two trends correlate, fewer neurons dying leads to less astrocyte infiltration/activaton to clean up and support the infarcted region. As was seen in infarct volume, IA GTN works in a dose escalating fashion showing protective effects at higher doses. The preservation of neurons in the infarcted region can be attributed to a return of nutrient rich blood and NO_x, both provide key components a neuron needs to survive. NO_x will work to preserve neuronal

health by stopping the apoptotic cascade and the return of glucose will allow the cell to resume energy production. With fewer neurons dying, the need for astrocytes to infiltrate and support/clean up cellular debris is lessened.

The use of GTN as a neuroprotective compound has demonstrated positive effects in a sub analysis of ENOS, but the primary measure of better functional outcome was not met for the main study. This could be related to GTN patch application over a period of seven days and having greater systemic effects than targeted effects in the cerebrovasculature. While ENOS did show an acute reduction in blood pressure this was not enough to alter 90 day functional scores between groups. Using GTN in our model of IA administration, it was shown to be physiologically safe, have little effect on vessel dilation, have a functional effect, decrease infarct volume and preserve neuronal health. These positive results are attributed to successful recanalization of the occluded vessel and conversion of GTN to NO_x. Neuroprotection is two-sided, neuroprotective drugs are beneficial to stopping cellular cascades associated with apoptosis, but a neuron needs nutrients to continue energy production. Working together, recanalization and IA GTN demonstrated neuroprotective effects following ELVO.

Verapamil

Verapamil has a long history in cerebrovascular disease but is not currently used for stroke treatment. It is administered following successful recanalization when a previously occluded MCA vessel wall spasms causing turbid blood flow through the cerebrovasculature. Verapamil was selected

because it is FDA-approved, currently used in the clinic, and at one time was suspected of having neuroprotective benefits. Previous pre-clinical stroke experiments testing verapamil suffered from experimental study design flaws; most notable is not successfully recanalizing the occluded vessel and delayed drug administration (44-46). Verapamil has two proposed mechanism of actions, first, blocking L-type calcium channels on the cell membrane, and second, vasodilation of the blood vessel through blocking calcium release and smooth muscle relaxation. It is noteworthy that verapamil is used outside of the neuroprotection field to block p-glycoprotein pumps (PGP), thereby inhibiting the efflux of target species from intracellular to extracellular.

Using the MCAo and IA models, we tested verapamil for safety and neuroprotective benefit and to determine if the protection was from vasodilation or blocking of L-type calcium channels. Administration of vasodilators can cause a decrease in heart rate (decreased muscle contractions) and drop in blood pressure (vessel dilation). Following stroke, a series of physiological responses known as Cushing's triad (increased intracranial pressure-ICP, decreased blood pressure and increased heart rate) can occur causing a worse outcome. Making sure potential neuroprotective agent(s) do not exacerbate these symptoms is key. Verapamil administered IA following successful recanalization demonstrated a heart rate similar to baseline and lowered blood pressure (not significant), while the control group (IA saline) had an increased pulse distention (blood pressure) and lowered heart rate 10 - 15 minutes post drug administration. Physiological measurements demonstrate that verapamil preserved heart rate and pulse

distention following MCAo but the control group demonstrated 2/3 of Cushing's triad which could mean the mouse suffered increased ICP following recanalization.

To dispel the myth that verapamil's neuroprotective effect is through vasodilation we looked at two measures of vessel dilation, global and local. Laser Doppler was used to measure perfusion through the ipsilateral MCA and Laser Speckle was used to measure hemispheric flow comparing contralateral versus ipsilateral. Results from Laser Doppler and Speckle show there is no difference in perfusion through either the ipsilateral MCA or ipsilateral hemisphere when compared to contralateral. Laser Doppler showed greater flow (not significant) for IA control through the MCA at drug administration, 10 and 15 minutes post administration compared to IA verapamil. Laser Speckle demonstrated an increased (not significant) return to global perfusion (contralateral versus ipsilateral perfusion measurements) for IA verapamil versus IA control. Here we dispel the notion that verapamil's main mechanism of action is vasodilation, and while some effect can be observed, it is not significant between groups. This can be attributed to blood vessels of the brain already being maximally dilated, trying to return blood supply to the effected region. This could be tested by looking at a known vasodilators such as nitroglycerin and measuring cerebrovascular perfusion both locally and globally.

In the clinic, the goal for stroke patients is to return to normal function or return to functional ability one day prior to stroke. To test whether verapamil

conveyed neuroprotective benefits, mice were tested on the rotor rod (forced motor movement) and open field (free roam movement) on specific days. The reasoning behind the functional test lies in the MCAo model, because the MCA supplies blood to the motor cortex of the brain responsible for forelimb movement. Occluding the MCA should produce a functional deficit on the contralateral forelimb making forward mobility difficult. Both behavioral tests showed the control group having decreased mobility on testing days (open field PSD 1 saw no difference between groups) when compared to verapamil treated. The difference in functional performance with the treated group performing better than control would lend support to the theory that verapamil was protective of the neurons in the motor cortex of the brain, thus allowing for better control and movement of the forelimb.

Next, we examined infarct volume to determine if there was any difference between groups. Infarct analysis showed the treated group had a significantly smaller infarct than the control meaning verapamil conveyed a protective effect. While the decrease in infarct correlates to improved functional outcome, it was the histological data that helps support verapamil's claim as neuroprotective.

Histologically, groups were stained looking at mature neuron survival, astrogliosis, and apoptosis. Neurons cannot produce glucose and require nutrient rich blood for a continuous supply. If glucose is deficient, mitochondria in the cell will no longer make adenosine tri-phosphate (ATP) causing a breakdown in cellular function leading cellular degeneration. The mitochondrial or intrinsic

pathway of apoptosis begins with membrane depolarization leading to the opening of L-type calcium channels on the cellular membrane surface. This opening leads to calcium influx and eventual imbalance between intracellular and extracellular environments. With restored blood flow, glucose returns to the nutrient deficient region, is taken up by glucose receptors returning the cell to normal concentrations, but not altering the apoptotic cascade already in motion. If verapamil is administered IA, it will stabilize the flow of calcium by blocking the L-type calcium channel inhibiting downstream effects by increased intracellular calcium. This was demonstrated with preservation of mature neurons in the infarct region of the treated group versus the control group. Next we looked at astrogliosis which results from dying cells releasing cytokines that attract/activate astrocytes to the damaged region. Astrocytes will clean up cellular debris and form a "wall" around the infarct helping support those neurons that are still viable. Increased astrocyte infiltration/activation is a marker of cell distress. Control brains showed a greater number of astrocytes in and surrounding the infarcted region compared to treated brains. This correlates with the mature neuron data, as greater neuronal death in the infarcted region will lead to increased astrocyte infiltration/activation peri-infarct. Using a marker of apoptosis we again looked to see if verapamil preserved cells in the infarcted region and found fewer positive apoptotic cells in the infarcted region of the treated group than the control. This demonstrates with both mature neuron survival and decreased apoptosis that verapamil treatment following MCAo and IA administration has a neuroprotective benefit on a cellular level.

Verapamil used as an adjunctive therapy following successful recanalization in a mouse model of stroke has been demonstrated to be safe, have a non-significant effect on blood perfusion, improve functional outcome, preserve neuronal health, and decrease astrocyte infiltration, and apoptosis. Here verapamil is shown to have neuroprotective benefits, but it is through the mechanism of action as an L-type calcium channel blocker that we propose its neuroprotective effect and not through vasodilation.

Combinational Therapy

Clinical neuroprotection trials of the past have focused on one drug to treat all aspects of stroke (15). Based on current literature we know that a brain undergoing a hypoxic event displays a myriad of cellular events (5, 85). The expectation of one drug being able to treat multiple pathways is unrealistic, where a more practical approach would be to use a combination of drugs to target specific cellular pathways. As the brain experiences a stroke, cells undergo different stages of degeneration depending on their location in the neurovascular unit. Neurons in the core, those that surround and receive nutrients from the occluded MCA will begin degradation earlier than those cells in the penumbra that receive nutrients via a watershed effect from collateral vessels.

Utilizing a multi drug approach will allow for broader treatment of cells undergoing varying stages of cellular degeneration. Potential neuroprotective compounds should be targeted to specific pathways, not have overlapping

effects (vasodilation), FDA-approved, and work synergistically. Potential neuroprotective compounds were selected based on the above parameters. Verapamil and lubeluzole (indirect NMDA antagonist) have both demonstrated neuroprotective effects in pre-clinical and clinical stroke models. Verapamil, as mentioned above, works acutely through blocking calcium channels on the membrane surface helping restore extra/intracellular calcium homeostasis. Lubeluzole works in a more delayed fashion and has a proposed mechanism of action through inhibiting the release of glutamate, nitric oxide synthase, and Na^+ and Ca^{2+} channels (47-50, 86, 87).

Testing the synergistic effect of IA combinational therapy following ELVO using physiological measures showed a decreased heart rate but a pulse distention similar to baseline at 5 and 10 minutes post-drug injection. Compared to control, heart rate measurements were not significantly different but pulse distention showed a significant increase. Decreased heart rate was seen in both groups (control and treated) and could be attributed to anesthesia as is seen in baseline measurements. There is no difference between groups 5 and 10 minutes post-IA administration compared to control, if combinational therapy has an effect on heart rate it is negligible from control animals receiving saline. Increased pulse distention for the treated group, while significant from the control group, demonstrates rescuing effect returning measurements to baseline, animals were followed beyond graphed measures and all animals survived the surgery with no complications.

Results from combinational therapy versus control using rotor rod and open field demonstrated a protective effect for the treated group in both tests. Control and treated groups decreased from baseline on PSD 1 and are not different and subsequent testing days show the treated group improving significantly when compared to control. The positive functional effect seen between groups is attributed to preservation of neurons in the motor cortex, allowing the mouse to regain forelimb mobility. While rotor rod tests forced motor movement and the animals ability to stay on an accelerating rotating rod, open field tests both distance covered and health. When a mouse undergoes stroke they limit their movement as can been seen on PSD 1, but as the mouse recovers they will begin to explore (increase distance covered) the box moving from corner to corner. The combinational therapy group significantly improved from PSD 1 measurements to PSD 7, while it's not significant from control PSD 7 it shows increased exploration and distance covered (two measures of overall health).

Histological measures were analyzed to determine if combinational therapy had a protective cellular effect. Infarct volume analysis demonstrated a significant reduction (75%) in the treated group compared to control. This extended to both mature neuron survival and astrogliosis in the infarcted region. Combinational treatment conferred neuroprotective benefit at PSD 7 both for decreased infarct volume, significantly increased mature neurons, and significantly decreased astrogliosis. Cellular health in the neurovascular unit following stroke is measured by mature neuron survival and astrogliosis. With a

decreased infarct, the treatment group improved neuronal health leading to a decreased need for support and debris clean up from astrocytes. Verapamil is an acute therapy blocking L-type calcium channels which are opened once the membrane becomes depolarized. Neurons undergoing this stage of apoptosis will be found in the penumbral regions, they receive a watershed of blood supply and are not directly affected by the MCA. Lubeluzole works in a delayed fashion and targets those neurons with a depolarized membrane (past verapamil's mechanism of action) to inhibit glutamate release and NMDA receptor influx of calcium. Because of its delayed mechanism of action, lubeluzole will target those neurons surrounding the MCA and receiving direct blood supply. These neurons will be farther along in cellular degeneration and in the range for targeted treatment for lubeluzole.

While preservation of neurons is the goal, a secondary measure of brain health is astrocyte infiltration/activation. Astrocytes are routinely found throughout the brain providing support, but following injury astrocytes migrate towards the site of injury and take on roles of cellular debris cleanup and support. Following ischemia, additional astrocytes will infiltrate the ischemic region and surrounding area to help mitigate the damage, a lower density of astrocytes marks less cellular damage. Comparing groups, there are significantly less astrocytes in and around the infarcted region for the treatment group. Combined with decreased infarct volume and increased mature neuron survival lends credence to verapamil and lubeluzole working together in a neuroprotective fashion.

Stroke is associated with a wide variety of symptoms, which are directly related to the effected cerebrovasculature and surrounding tissue. When a brain undergoes a hypoxic event there is a gradient of cell death, with cells closest to the MCA losing nutrients first and start down the apoptotic pathway before those receiving nutrients from watershed vessels (penumbra). This leads to neurons undergoing various stages of apoptosis, thus the need for a drug(s) that can target various stages of cell death. Using a combinational approach, we selected one acute acting drug (verapamil) and one delayed (lubeluzole). Both drugs are FDA-approved and have shown benefit in pre-clinical and clinical trials. Drug synergy is important when combining therapies and care must be taken to account for physiological measures during and following drug administration. Using an IA model mitigates systemic side-effects commonly associated with oral, patch or intravenous drug administration. Heart rate and pulse distention measurements show no deleterious effects during IA drug administration.

Primary outcomes for many clinical trials are improved functional scores 90 days post stroke, in a pre-clinical stroke setting animals are tested acutely (PSD 1) and long-term (PSD 7+) to determine functional recovery. Intra-arterial combinational therapy demonstrated a beneficial motor outcome at PSD 7 for both a forced motor and free roam movement. This was correlated to decreased cell death in the effected MCA and surrounding territory, simply; brain tissue was preserved and had improved function compared to control.

Selecting drugs that target different apoptotic pathways, a broader range of cells undergoing various stages of apoptosis can be treated and returned to normal function. Presented here are two such drugs that are FDA-approved and either underwent or are undergoing clinical trials. Whether they are the drugs that will help future stroke patients regain normal function or simply demonstrate drug synergy it is a step in the right direction.

Summary

Stroke remains a leading cause of mortality and morbidity worldwide. Here we focus on a subset of ischemic stroke, ELVO, and a novel method of delivering potential neuroprotective compounds. Drugs were selected based on pre-clinical and clinical testing, FDA-approval, and drug compatability. Studies have shown that GTN, verapamil, and verapamil and lubeluzole conferred a neuroprotective benefit. Each drug tested worked through a specific pathway either acutely or in a delayed fashion. Combining potential therapies requires drugs having independent mechanisms of action targeting a variety of cellular processes. Here we present a stepping stone for future researchers to expand upon our work and eventually develop a therapeutic for the treatment of stroke.

Reference List

1. Thacker C. Stroke falls to No. 5 cause of death in U.S. American Heart Association Media Alert. 12/30/2014 ed2014.
2. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation. 2014;129(3):e28-e292.
3. Chapman SN, Mehndiratta P, Johansen MC, McMurry TL, Johnston KC, Southerland AM. Current perspectives on the use of intravenous recombinant tissue plasminogen activator (tPA) for treatment of acute ischemic stroke. Vascular health and risk management. 2014;10:75-87.
4. Tsivgoulis G, Safouris A, Katsanos AH, Arthur AS, Alexandrov AV. Mechanical thrombectomy for emergent large vessel occlusion: a critical appraisal of recent randomized controlled clinical trials. Brain and behavior. 2016;6(2):e00418.
5. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35(4):495-516.
6. Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain research reviews. 2007;54(1):34-66.
7. Lijnen HR, Collen D. Fibrinolytic agents: mechanisms of activity and pharmacology. Thrombosis and haemostasis. 1995;74(1):387-90.
8. Lichtman JH, Watanabe E, Allen NB, Jones SB, Dostal J, Goldstein LB. Hospital arrival time and intravenous t-PA use in US Academic Medical Centers, 2001-2004. Stroke; a journal of cerebral circulation. 2009;40(12):3845-50.
9. Fransen PS, Beumer D, Berkhemer OA, van den Berg LA, Lingsma H, van der Lugt A, et al. MR CLEAN, a multicenter randomized clinical trial of endovascular treatment for acute ischemic stroke in the Netherlands: study protocol for a randomized controlled trial. Trials. 2014;15:343.
10. Saver JL, Jahan R, Levy EI, Jovin TG, Baxter B, Nogueira RG, et al. Solitaire flow restoration device versus the Merci Retriever in patients with acute ischaemic stroke (SWIFT): a randomised, parallel-group, non-inferiority trial. Lancet. 2012;380(9849):1241-9.
11. Saver JL, Goyal M, Bonafe A, Diener HC, Levy EI, Pereira VM, et al. Stent-Retriever Thrombectomy after Intravenous t-PA vs. t-PA Alone in Stroke. N Engl J Med. 2015.
12. Fargen KM, Meyers PM, Khatri P, Mocco J. Improvements in recanalization with modern stroke therapy: a review of prospective ischemic stroke trials during the last two decades. Journal of neurointerventional surgery. 2013;5(6):506-11.
13. Campbell BC, Mitchell PJ, Kleinig TJ, Dewey HM, Churilov L, Yassi N, et al. Endovascular therapy for ischemic stroke with perfusion-imaging selection. The New England journal of medicine. 2015;372(11):1009-18.
14. Lees KR, Zivin JA, Ashwood T, Davalos A, Davis SM, Diener HC, et al. NXY-059 for acute ischemic stroke. The New England journal of medicine. 2006;354(6):588-600.

15. Grupke S, Hall J, Dobbs M, Bix GJ, Fraser JF. Understanding history, and not repeating it. Neuroprotection for acute ischemic stroke: from review to preview. Clinical neurology and neurosurgery. 2015;129:1-9.
16. Savitz SI. A critical appraisal of the NXY-059 neuroprotection studies for acute stroke: a need for more rigorous testing of neuroprotective agents in animal models of stroke. Experimental neurology. 2007;205(1):20-5.
17. Saver JL, Kidwell C, Eckstein M, Starkman S, Investigators F-MPT. Prehospital neuroprotective therapy for acute stroke: results of the Field Administration of Stroke Therapy-Magnesium (FAST-MAG) pilot trial. Stroke; a journal of cerebral circulation. 2004;35(5):e106-8.
18. Li H, Zhang N, Lin HY, Yu Y, Cai QY, Ma L, et al. Histological, cellular and behavioral assessments of stroke outcomes after photothrombosis-induced ischemia in adult mice. BMC neuroscience. 2014;15:58.
19. Clarkson AN, Lopez-Valdes HE, Overman JJ, Charles AC, Brennan KC, Thomas Carmichael S. Multimodal examination of structural and functional remapping in the mouse photothrombotic stroke model. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2013;33(5):716-23.
20. Bix GJ, Gowing EK, Clarkson AN. Perlecan domain V is neuroprotective and affords functional improvement in a photothrombotic stroke model in young and aged mice. Translational stroke research. 2013;4(5):515-23.
21. Schmidt A, Hoppen M, Strecker JK, Diederich K, Schabitz WR, Schilling M, et al. Photochemically induced ischemic stroke in rats. Experimental & translational stroke medicine. 2012;4(1):13.
22. Carmichael ST. Rodent models of focal stroke: size, mechanism, and purpose. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics. 2005;2(3):396-409.
23. Zhang Z, Chopp M, Zhang RL, Goussev A. A mouse model of embolic focal cerebral ischemia. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 1997;17(10):1081-8.
24. Chen Y, Zhu W, Zhang W, Libal N, Murphy SJ, Offner H, et al. A novel mouse model of thromboembolic stroke. Journal of neuroscience methods. 2015;256:203-11.
25. Morris DC, Chopp M, Zhang L, Lu M, Zhang ZG. Thymosin beta4 improves functional neurological outcome in a rat model of embolic stroke. Neuroscience. 2010;169(2):674-82.
26. Michalski D, Kuppers-Tiedt L, Weise C, Laignel F, Hartig W, Raviolo M, et al. Long-term functional and neurological outcome after simultaneous treatment with tissue-plasminogen activator and hyperbaric oxygen in early phase of embolic stroke in rats. Brain research. 2009;1303:161-8.
27. Nedelmann M, Wilhelm-Schwenkmezger T, Alessandri B, Heimann A, Schneider F, Eicke BM, et al. Cerebral embolic ischemia in rats: correlation of stroke severity and functional deficit as important outcome parameter. Brain research. 2007;1130(1):188-96.

28. Andersen M, Overgaard K, Meden P, Boysen G, Choi SC. Effects of citicoline combined with thrombolytic therapy in a rat embolic stroke model. Stroke; a journal of cerebral circulation. 1999;30(7):1464-71.
29. Dinapoli VA, Rosen CL, Nagamine T, Crocco T. Selective MCA occlusion: a precise embolic stroke model. Journal of neuroscience methods. 2006;154(1-2):233-8.
30. Langhauser FL, Heiler PM, Grudzenski S, Lemke A, Alonso A, Schad LR, et al. Thromboembolic stroke in C57BL/6 mice monitored by 9.4 T MRI using a 1H cryo probe. Experimental & translational stroke medicine. 2012;4(1):18.
31. Ansar S, Chatzikonstantinou E, Wistuba-Schier A, Mirau-Weber S, Fatar M, Hennerici MG, et al. Characterization of a new model of thromboembolic stroke in C57 black/6J mice. Translational stroke research. 2014;5(4):526-33.
32. Busch E, Kruger K, Hossmann KA. Improved model of thromboembolic stroke and rt-PA induced reperfusion in the rat. Brain research. 1997;778(1):16-24.
33. Ansar S, Chatzikonstantinou E, Thiagarajah R, Tritschler L, Fatar M, Hennerici MG, et al. Pro-inflammatory mediators and apoptosis correlate to rt-PA response in a novel mouse model of thromboembolic stroke. PloS one. 2014;9(1):e85849.
34. Manwani B, Liu F, Xu Y, Persky R, Li J, McCullough LD. Functional recovery in aging mice after experimental stroke. Brain, behavior, and immunity. 2011;25(8):1689-700.
35. Pettigrew LC, Kryscio RJ, Norris CM. The TNFalpha-Transgenic Rat: Hippocampal Synaptic Integrity, Cognition, Function, and Post-Ischemic Cell Loss. PloS one. 2016;11(5):e0154721.
36. Maniskas ME, Roberts JM, Aron I, Fraser JF, Bix GJ. Stroke neuroprotection revisited: Intra-arterial verapamil is profoundly neuroprotective in experimental acute ischemic stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2016;36(4):721-30.
37. Lee B, Clarke D, Al Ahmad A, Kahle M, Parham C, Auckland L, et al. Perlecan domain V is neuroprotective and proangiogenic following ischemic stroke in rodents. The Journal of clinical investigation. 2011;121(8):3005-23.
38. Maniskas ME, Roberts JM, Aron I, Fraser JF, Bix GJ. Stroke neuroprotection revisited: Intra-arterial verapamil is profoundly neuroprotective in experimental acute ischemic stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2015.
39. Maniskas M, Bix G, Fraser J. Selective intra-arterial drug administration in a model of large vessel ischemia. J Neurosci Methods. 2014;240C:22-7.
40. Aronowski J, Cho KH, Strong R, Grotta JC. Neurofilament proteolysis after focal ischemia; when do cells die after experimental stroke? Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 1999;19(6):652-60.
41. Investigators ET, Bath PM, Woodhouse L, Scutt P, Krishnan K, Wardlaw JM, et al. Efficacy of nitric oxide, with or without continuing antihypertensive

treatment, for management of high blood pressure in acute stroke (ENOS): a partial-factorial randomised controlled trial. Lancet. 2015;385(9968):617-28.
42. El-Zammar ZM, Latorre JG, Wang D, Satyan S, Elnour E, Kamel A, et al. Intra-arterial vasodilator use during endovascular therapy for acute ischemic stroke might improve reperfusion rate. Annals of the New York Academy of Sciences. 2012;1268:134-40.
43. Bath PM, Houlton A, Woodhouse L, Sprigg N, Wardlaw J, Pocock S, et al. Statistical analysis plan for the 'Efficacy of Nitric Oxide in Stroke' (ENOS) trial. International journal of stroke : official journal of the International Stroke Society. 2014;9(3):372-4.
44. Roy MW, Dempsey RJ, Meyer KL, Donaldson DL, Tibbs PA, Young AB. Effects of Verapamil and Diltiazem on Acute Stroke in Cats. J Neurosurg. 1985;63(6):929-36.
45. Ueda T, Yamamoto YL, Diksic M. Transvenous perfusion of the brain with verapamil during focal cerebral ischemia in rats. Stroke; a journal of cerebral circulation. 1989;20(4):501-6.
46. Berger JR, Busto R, Ginsberg MD. Verapamil - Failure of Metabolic Amelioration Following Global Forebrain Ischemia in the Rat. Stroke; a journal of cerebral circulation. 1984;15(6):1029-32.
47. Maiese K, TenBroeke M, Kue I. Neuroprotection of lubeluzole is mediated through the signal transduction pathways of nitric oxide. Journal of neurochemistry. 1997;68(2):710-4.
48. Lesage AS, Peeters L, Leysen JE. Lubeluzole, a novel long-term neuroprotectant, inhibits the glutamate-activated nitric oxide synthase pathway. The Journal of pharmacology and experimental therapeutics. 1996;279(2):759-66.
49. De Ryck M, Keersmaekers R, Duytschaever H, Claes C, Clincke G, Janssen M, et al. Lubeluzole protects sensorimotor function and reduces infarct size in a photochemical stroke model in rats. The Journal of pharmacology and experimental therapeutics. 1996;279(2):748-58.
50. Culmsee C, Junker V, Wolz P, Semkova I, Krieglstein J. Lubeluzole protects hippocampal neurons from excitotoxicity in vitro and reduces brain damage caused by ischemia. European journal of pharmacology. 1998;342(2-3):193-201.
51. Grotta J. Lubeluzole treatment of acute ischemic stroke. The US and Canadian Lubeluzole Ischemic Stroke Study Group. Stroke; a journal of cerebral circulation. 1997;28(12):2338-46.
52. Diener HC, Cortens M, Ford G, Grotta J, Hacke W, Kaste M, et al. Lubeluzole in acute ischemic stroke treatment: A double-blind study with an 8-hour inclusion window comparing a 10-mg daily dose of lubeluzole with placebo. Stroke; a journal of cerebral circulation. 2000;31(11):2543-51.
53. Investigators IIT. The Interventional Management of Stroke (IMS) II Study. Stroke; a journal of cerebral circulation. 2007;38(7):2127-35.
54. Hassan AE, Chaudhry SA, Grigoryan M, Tekle WG, Qureshi AI. National trends in utilization and outcomes of endovascular treatment of acute ischemic

stroke patients in the mechanical thrombectomy era. Stroke; a journal of cerebral circulation. 2012;43(11):3012-7.
55. Broderick JP, Palesch YY, Demchuk AM, Yeatts SD, Khatri P, Hill MD, et al. Endovascular therapy after intravenous t-PA versus t-PA alone for stroke. The New England journal of medicine. 2013;368(10):893-903.
56. Chen L, Swartz KR, Toborek M. Vessel microport technique for applications in cerebrovascular research. Journal of neuroscience research. 2009;87(7):1718-27.
57. Van Winkle JA, Chen B, Lei IF, Pereira B, Rajput PS, Lyden PD. Concurrent middle cerebral artery occlusion and intra-arterial drug infusion via ipsilateral common carotid artery catheter in the rat. J Neurosci Methods. 2013;213(1):63-9.
58. Hasan MR, Herz J, Hermann DM, Doeppner TR. Visualization of macroscopic cerebral vessel anatomy--a new and reliable technique in mice. J Neurosci Methods. 2012;204(2):249-53.
59. Feigin VL, Forouzanfar MH, Krishnamurthi R, Mensah GA, Connor M, Bennett DA, et al. Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. Lancet. 2014;383(9913):245-54.
60. Goyal M, Demchuk AM, Menon BK, Eesa M, Rempel JL, Thornton J, et al. Randomized Assessment of Rapid Endovascular Treatment of Ischemic Stroke. The New England journal of medicine. 2015;372(11):1019-30.
61. Parsons MW, Albers GW. MR RESCUE: is the glass half-full or half-empty? Stroke; a journal of cerebral circulation. 2013;44(7):2055-7.
62. Goyal M, Demchuk AM, Menon BK, Eesa M, Rempel JL, Thornton J, et al. Randomized assessment of rapid endovascular treatment of ischemic stroke. The New England journal of medicine. 2015;372(11):1019-30.
63. Maniskas M, Bix G, Fraser J. Selective intra-arterial drug administration in a model of large vessel ischemia. J Neurosci Methods. 2015;240:22-7.
64. Jin RC, Loscalzo J. Vascular Nitric Oxide: Formation and Function. Journal of blood medicine. 2010;2010(1):147-62.
65. Bath PM, Robson K, Woodhouse LJ, Sprigg N, Dineen R, Pocock S, et al. Statistical analysis plan for the 'Triple Antiplatelets for Reducing Dependency after Ischaemic Stroke' (TARDIS) trial. International journal of stroke : official journal of the International Stroke Society. 2015;10(3):449-51.
66. Woodhouse L, Scutt P, Krishnan K, Berge E, Gommans J, Ntaios G, et al. Effect of Hyperacute Administration (Within 6 Hours) of Transdermal Glyceryl Trinitrate, a Nitric Oxide Donor, on Outcome After Stroke: Subgroup Analysis of the Efficacy of Nitric Oxide in Stroke (ENOS) Trial. Stroke; a journal of cerebral circulation. 2015;46(11):3194-201.
67. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS biology. 2010;8(6):e1000412.
68. Golde WT, Gollobin P, Rodriguez LL. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. Lab animal. 2005;34(9):39-43.

69. Stroke Therapy Academic Industry R. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke; a journal of cerebral circulation. 1999;30(12):2752-8.
70. O'Mahony D, Kendall MJ. Nitric oxide in acute ischaemic stroke: a target for neuroprotection. J Neurol Neurosurg Psychiatry. 1999;67(1):1-3.
71. Godinez-Rubi M, Rojas-Mayorquin AE, Ortuno-Sahagun D. Nitric oxide donors as neuroprotective agents after an ischemic stroke-related inflammatory reaction. Oxid Med Cell Longev. 2013;2013:297357.
72. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci. 2007;8(10):766-75.
73. Mao M, Sudhahar V, Ansenberger-Fricano K, Fernandes DC, Tanaka LY, Fukai T, et al. Nitroglycerin drives endothelial nitric oxide synthase activation via the phosphatidylinositol 3-kinase/protein kinase B pathway. Free radical biology & medicine. 2012;52(2):427-35.
74. Terpolilli NA, Moskowitz MA, Plesnila N. Nitric oxide: considerations for the treatment of ischemic stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2012;32(7):1332-46.
75. O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. Circulation research. 2001;88(1):12-21.
76. Berkhemer OA, Fransen PS, Beumer D, van den Berg LA, Lingsma HF, Yoo AJ, et al. A randomized trial of intraarterial treatment for acute ischemic stroke. The New England journal of medicine. 2015;372(1):11-20.
77. Brodsky SJ, Cutler SS, Weiner DA, McCabe CH, Ryan TJ, Klein MD. Treatment of stable angina of effort with verapamil: a double-blind, placebo-controlled randomized crossover study. Circulation. 1982;66(3):569-74.
78. Wauquier A, Melis W, Janssen PA. Long-term neurological assessment of the post-resuscitative effects of flunarizine, verapamil and nimodipine in a new model of global complete ischaemia. Neuropharmacology. 1989;28(8):837-46.
79. Investigators IMSS. Combined intravenous and intra-arterial recanalization for acute ischemic stroke: the Interventional Management of Stroke Study. Stroke; a journal of cerebral circulation. 2004;35(4):904-11.
80. del Zoppo GJ, Higashida RT, Furlan AJ, Pessin MS, Rowley HA, Gent M. PROACT: a phase II randomized trial of recombinant pro-urokinase by direct arterial delivery in acute middle cerebral artery stroke. PROACT Investigators. Prolyse in Acute Cerebral Thromboembolism. Stroke; a journal of cerebral circulation. 1998;29(1):4-11.
81. Sung RJ, Elser B, McAllister RG, Jr. Intravenous verapamil for termination of re-entrant supraventricular tachycardias: intracardiac studies correlated with plasma verapamil concentrations. Annals of internal medicine. 1980;93(5):682-9.
82. Waxman HL, Myerburg RJ, Appel R, Sung RJ. Verapamil for control of ventricular rate in paroxysmal supraventricular tachycardia and atrial fibrillation or

flutter: a double-blind randomized cross-over study. Annals of internal medicine. 1981;94(1):1-6.
83. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. Journal of pharmacology & pharmacotherapeutics. 2010;1(2):94-9.
84. Bath PM, Woodhouse L, Krishnan K, Anderson C, Berge E, Ford GA, et al. Effect of Treatment Delay, Stroke Type, and Thrombolysis on the Effect of Glyceryl Trinitrate, a Nitric Oxide Donor, on Outcome after Acute Stroke: A Systematic Review and Meta-Analysis of Individual Patient from Randomised Trials. Stroke research and treatment. 2016;2016:9706720.
85. Tang Q, Han R, Xiao H, Shen J, Luo Q, Li J. Neuroprotective effects of tanshinone IIA and/or tetramethylpyrazine in cerebral ischemic injury in vivo and in vitro. Brain research. 2012;1488:81-91.
86. Marrannes R, De Prins E, Clincke G. Influence of lubeluzole on voltage-sensitive Ca++ channels in isolated rat neurons. The Journal of pharmacology and experimental therapeutics. 1998;286(1):201-14.
87. Ashton D, Willems R, Wynants J, Van Reempts J, Marrannes R, Clincke G. Altered Na(+)-channel function as an in vitro model of the ischemic penumbra: action of lubeluzole and other neuroprotective drugs. Brain research. 1997;745(1-2):210-21.

Vita

Date Prepared: 09-08-2016

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I. Education

07/05	Marshall University	BS
12/12	Marshall University	MA
05/16	University of Kentucky	MS

II. Professional Experiences

05/07 – Present	Recreational Staff	Stewart Home School
06/07 – Present	Coach	Special Olympics
11/08 – 05/12	Aquatics	YMCA

III. Teaching Activities

08/10 – 06/11	Teaching Assistant	Dept. of Chemistry
01/11 – 05/16	Teaching Assistant	Dept. of Ana. & Neuro.
08/16 – 05/17	Teaching Assistant	Dept. of Ana. & Neuro.

IV. Research, Abstracts and/or Scholarships

Research Projects:

> "Superselective Intra-arterial Administration of Verapamil is Profoundly Neuroprotective in Experimental Ischemic Stroke" 08/13 - Present

Abstracts:

2014	International Stroke Conference	Poster
2014	Southern Society of Neurosurgery	Presentation
2014	Markesbery Symposium	Poster

2014	BGSFN & CCTS Research Day	Poster
2014	Neuroprotection & Neurorepair	Poster
2014	Society for Neuroscience	Poster
2014	KAARN	Poster
2015	AANS/CNS	Presentation
2015	Markesbery Symposium	Poster
2015	International Stroke Conference	Poster
2015	BGSFN & CCTS Research Day	Poster
2016	Southern Society of Neurosurgery	Presentation
2016	Neuroprotection & Neurorepair	Presentation
2016	National Neurotrauma Society	Poster

Scholarships:

Dean's Scholarship for Anatomy & Neurobiology 2012-2013

NIH T32 Training Grant 5T32 NS077889

V. Awards

Posters Awards:

Bluegrass Society for Neuroscience 2014 & 2015 Poster Award

Markesbery Symposium Outstanding Poster 2014 & 2015

VI. Publications

Maniskas M, Bix G, Fraser J. Selective intra-arterial drug administration in a model of large vessel ischemia. Journal of Neuroscience Methods. 2015; 240:22-7.

Maniskas M, Roberts J, Aron I, Fraser J, Bix G. Stroke neuroprotection re-visited: Intra-arterial verapamil is profoundly neuroprotective in experimental acute ischemic stroke. Journal of Cerebral Blood Flow and Metabolism. 2015. (In Press)