

2012

Comparing Two Different Statins in a Delayed Pharmacological Treatment for Ischemic Stroke

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COMPARING TWO DIFFERENT STATINS IN A DELAYED
PHARMACOLOGICAL TREATMENT FOR ISCHEMIC STROKE

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

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B.S., University of Dayton, 2010

2012

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

June 12, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Kailyn Marie Hagerty ENTITLED Comparing Two Different Statins in a Pharmacological Treatment for Ischemic Stroke BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Hagerty, Kailyn Marie. M.S. Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2012. Comparing Two Different Statins in a Delayed Pharmacological Treatment for Ischemic Stroke.

Stroke is one of the leading causes of death and the top cause of long term disability. Currently there exists no standardized treatment of care for ischemic stroke patients during the days following stroke other than giving aspirin. A drug combination containing fluoxetine, one of two statins, and ascorbic acid was used to try and improve motor function following strokes in rats. Endothelin induced stroke survival surgery was performed on the right cortex of Long Evans and Sprague Dawley rats. Functional tests were completed pre- and post-surgery and immunohistochemistry was carried out to analyze infarct volume, angiogenesis, and the glial scar. Significant recovery of function was seen after treatment with fluoxetine, simvastatin, and ascorbic acid. Strong trends indicating increased angiogenesis and reduced infarct volume following treatment with fluoxetine, atorvastatin, and ascorbic acid. The glial scar was significantly reduced in animals treated with the atorvastatin combination. Increases in angiogenesis and neurogenesis caused by pharmacological treatment could be helpful in reducing the lifelong implications of stroke.

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I. INTRODUCTION

Background

Someone has a stroke in the United States approximately every 40 seconds (1). Ischemic, hemorrhagic and TIA (transient ischemic attack) are the three types of stroke. Ischemic strokes, caused by blood clot(s) stopping blood flow to certain parts of the brain, make up 87% of all strokes (2). Hemorrhagic strokes occur when a blood vessel ruptures after becoming weakened (2). Lastly, Transient Ischemic Attacks (TIAs) are often thought of as warning signs more so than an actual stroke but should be taken seriously. TIAs are similar to ischemic strokes in that a clot is formed, but the clot is temporary to that seen in a regular stroke (2). The newest statistics report that strokes (ischemic and hemorrhagic combined) are the fourth most common cause of death preceded by diseases of the heart, cancer, and chronic lower respiratory diseases in the United States (1). Strokes account for 1 out of every 18 fatalities (1). Of the approximately 795,000 patients who report having a stroke, 23% are made up of those who are reporting a second attack (1). Of the strokes that do occur each year, only 8-12% of ischemic strokes lead to death in the first month following diagnosis. Hemorrhagic infarcts are much more damaging and lead to death in the first month in 37-44% of cases (1). Mortality rates are age dependent with the mean age of people affected by stroke onset being 79.6 years old (1). It has been estimated that around 7 million Americans aged 20 and above have suffered from a stroke, with 30.7% of them receiving

rehabilitation (1). The high percentage of rehabilitation that patients receive speaks to the fact that stroke is the largest contributor to long term disabilities. Because of this, the cost of stroke is nearly 34 billion dollars annually (1). Ischemic strokes result in a lifetime cost of around 140,000 dollars for patients who suffer from them (1).

Although there are a plethora of risk factors and plenty of warning signs for stroke, a surprisingly low percentage can identify either. When asked to identify three warning signs, such as headache, trouble writing, and memory loss, only 15.7% were able to succeed in 2005 (1). Only half of people who have already suffered from at least one stroke were able to identify one symptom. These facts lead to problems because the only approved treatment, tissue plasminogen activator, has to be administered within the first three hours following stroke (1). The California Acute Stroke Pilot Registry reported that only 23.5% of patients went to the hospital in the first three hours (1). However, although the number of people going to the hospital within the first three hours has not increased, the number of patients who received tissue plasminogen activator has increased which is attributed to better organization at the hospital (1). Still, in 2008 54% of death due to stroke happened outside of a hospital (1). If the population knew that 37% of people show at least two risk factors for stroke (gathered from examinations on diabetes, smoking history, inactivity, obesity, high cholesterol and high blood pressure) then they would probably feel encouraged to learn more about stroke prevention (3). Other than working out and eating healthy, anticoagulants and antiplatelet agents both lead to reduced risk of clotting (4) and can lead to protection against stroke. Estimates report that at least four million more people will suffer from stroke by the year 2030 so

although lack of education could lead to reduce this number, a delayed post-stroke treatment is the biggest contributing factor to getting this number significantly lowered.

Current Treatments

The reason new treatments are needed stems from the fact that the way strokes are treated today doesn't work or isn't appropriate for a majority of patients. Thrombolytic agents are able to dissolve clots that form only within a certain timeframe without danger of subsequent hemorrhage (5). However, a CT scan, physical exam, and medical history all contribute to the decision to receive thrombolytics (6). Pregnancy, surgery, and trauma can also eliminate the ability for patients to receive this drug (6). Clearly, not everyone can receive this drug. Anyone who arrives at a hospital when three hours have passed after the stroke may not qualify for thrombolytics and are also too late to receive tissue plasminogen activator (tPA). Even those that do get to the hospital early enough to receive tPA, it may not work (5). There are only two other options for patients, both invasive surgeries. The MERCI Retrieval System does not have a limit on when it can be used and is a small device similar to a cork screw that enters the body and removes the clot (5). The Penumbra System has been around since 2008 and accomplishes the same thing as the MERCI system but it uses a suction (5). Even with these interventions, the NHLBI's Framingham Study reported several disabilities that people over the age of 65 have struggled with in the six months following a stroke with hemiparesis, aphasia, and depression all being fairly common (7). The future of stroke management needs to see a shift towards delayed and continued treatment to allow more patients to be treated and to see improvement in functional recovery with time. In other words, we need treatments

that can be given later and that can continue to work to repair the brain after injury. These are the ideas that led to the development of this project.

Stroke Animal Model

To work towards finding a delayed treatment, an animal model needed to be developed that would mimic ischemic strokes in humans as closely as possible. Studies have been done that successfully produce an ischemic stroke but they produce far greater infarcts than what is comparable to humans. The techniques used to produce these larger infarcts are typically distal middle cerebral artery occlusion (MCAO) using electrocoagulation or the usage of an intraluminal filament to block the origin of the middle cerebral artery in the Circle of Willis. Both of these take out nearly half of an entire hemisphere of the brain. Not only does the MCAO create infarcts that are too big than what is typically seen in humans, they also damage areas of the brain that are not affected by human MCAO.

Windle et al. succeeded in comparing four different approaches to stroke, including the MCAO using injections or topical treatment with endothelin, a potent vasoconstrictor. An illustration of infarcts created by each stroke model used can be found below in Figure 1 (8). As shown, each procedure produced infarcts varying in size and location. It was decided that the cortical method was most similar to ischemic stroke damage seen in humans and was used to produce the results discussed in this thesis. It consisted of endothelin (a vasoconstrictor) injections into the brain at two difference locations in the motor cortex (8). Although the coordinates and the amount of endothelin

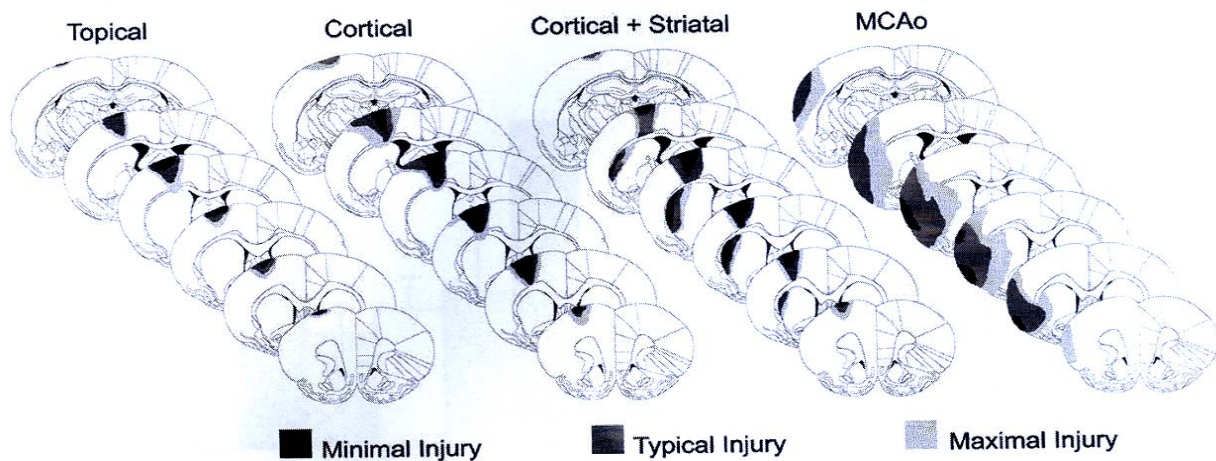


Figure 1 From Windle et al paper, looking at size of infarct produced in 300 gram male rats

injected into each drilled hole were altered from that described in Windle et al.(8), everything else was kept the same because this method had been proven to produce reliable infarcts, and thus deficits, in the motor cortex.

A stroke survival surgery method is not the only thing that goes into producing a useful model for studying stroke. Another problem with most stroke models is that young animals are being used to study stroke. Stroke is a health problem that can affect all ages, but it primarily affects the elderly. Even when it does affect younger people, the recovery rate is increased (9, 10). This could be because younger animals have greater amounts of brain plasticity (9) because neurogenesis decreases as animals age. Using BrdU (which replaces thymidine during cell division), Kuhn et al. successfully showed that the amount of newborn cells seen in aged rats (12-27 months of age) was significantly lower than the number of newborn cells being incorporated into the granule cell layer of young rats (6 months of age) (11). This could explain faster recovery from stroke surgery in younger animals, which was shown in a paper from Wiessner et al (12).

Figure 2 demonstrates that although there was a 90% deficit created compared to pre-stroke function, there was fast recovery seen in control animals in the first four weeks and performance reached almost half that of pre-stroke Montoya Staircase function (Panel C). It was speculated that this would not occur in older animals. Thus, another change made

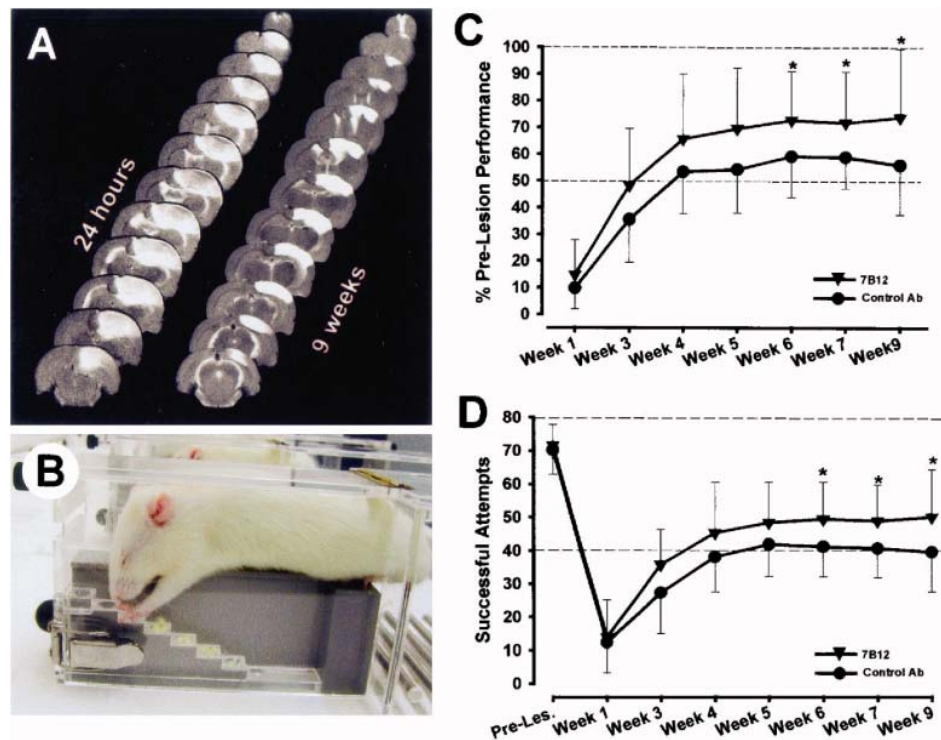


Figure 2 A. Infarct outline; B. rat completing Montoya Staircase; C. % Pre-stroke function; D. Total number of pellets grabbed with left forepaw

from the Windle et al. paper was the age of the animals used. The Windle et al. paper used young rats while the current study used middle aged (12 months old) rats. Long Evans and Sprague Dawley animals were used because both have been shown to be easy to train on Montoya staircase (8, 13, 14).

Pharmacologically Increasing Neurogenesis

The goal with this research was to first pharmacologically increase neurogenesis in older animals, which has been shown to decrease linearly with age (15). It has been shown that adult animals do still have neurogenesis capabilities. Figure 3 shows the migratory pattern of neurogenesis in adult animals. One study showed that transplanting stem cells directly into an adult brain that has suffered from a stroke could work if there wasn't a problem with host rejection (16). Another option, instead of transplanting stem cells directly into the brain, would be to stimulate existing stem cells which could lead to enhanced migration and inclusion in functional circuits (17). This could be possible through drug combinations made up of drugs that have already been shown to be involved in pathways that help with cerebral recovery following stroke or other injury.

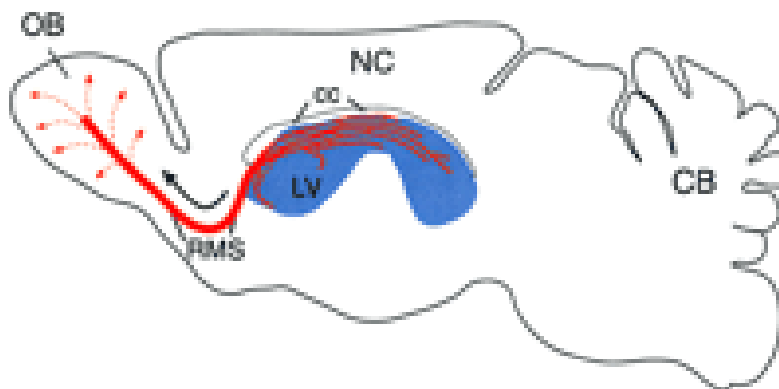


Figure 3 Shows migratory pathway of new neurons in the adult rodent from the subventricular zone to the olfactory bulb.

The statins are FDA approved and widely known as drugs that can lower cholesterol. There are several different types of statins, natural, semi-synthetic, and synthetic. Figure 4 exhibits the structures of some of the statins. As shown, all fermentation-derived statins have similar structures. The synthetic statins have various

structures which are different from each other and also vastly different from the natural statins (18). The difference in structure could mean a difference in downstream effects.

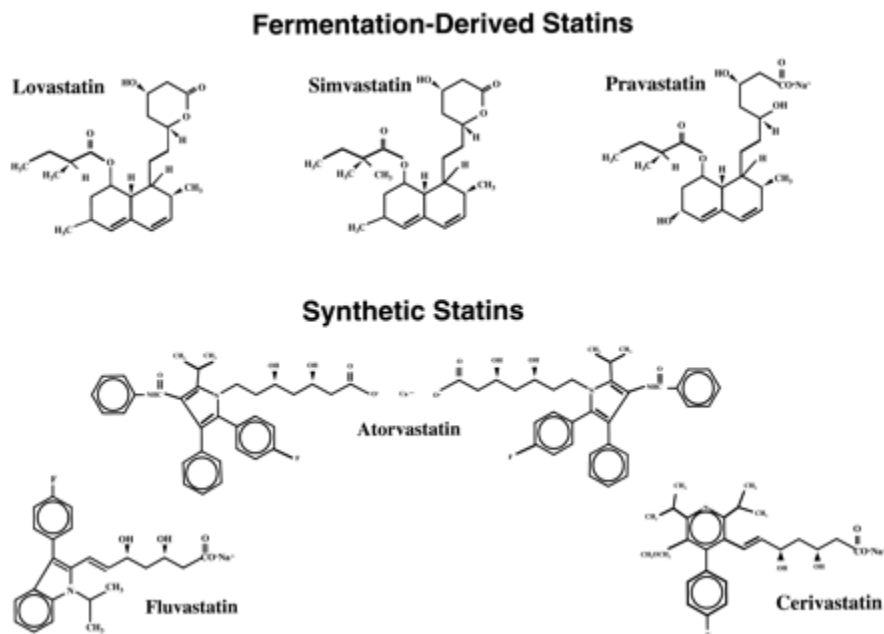


Figure 4 Structures of various statins

Statins have been used in the hopes of finding a delayed treatment for stroke in recent years. One study used simvastatin as a delayed treatment for ischemic stroke in a clinical pilot study. The simvastatin was given 6-12 hours following stroke (which is an additional 3 hours after the tissue plasminogen activator window) and the dose used was 40 mg per day for the first 7 days and then 20 mg per day until day 90. It was shown that patients treated with simvastatin significantly improved by the third day (as shown by at least a 4 point reduction in the NIHSS score, $P=0.022$) (19). However, the author stated that there were increased risks of infection reported along with a non-significant but higher mortality rate (no statistics given). In a more recent study, atorvastatin was used in conjunction with the tissue plasminogen activator in rats with embolic strokes to see if

the tPA window could be broadened (20). It was shown that rats that received atorvastatin at 4 hours post-stroke (at a dosage of 20 mg/kg) and 24 hours post-stroke (again at a dosage of 20 mg/kg) and tPA at 6 hours post-stroke had significantly smaller infarct and smaller neurologic deficits (20). Atorvastatin was able to increase the tPA window by 3 hours. It was discussed that the ability of the statin to prolong the therapeutic window could be because of the upregulation of endothelial nitric oxide synthase (eNOS) caused by the drug (20). Figure 5 below shows the pathway which leads to upregulation of eNOS caused by statins (21). eNOS lines all blood vessels and upregulation of eNOS means increased blood flow (20, 21). If blood flow can be increased following an ischemic stroke then there may be a lesser amount of cerebral damage. Statins have also been shown to increase VEGF which increases angiogenesis.

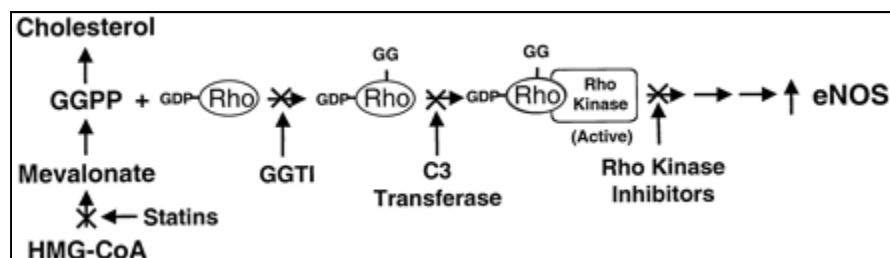


Figure 5 Statins lead to an upregulation of eNOS by inhibiting the production of the isoprenoid geranylgeranyl (which is responsible for attaching to Rho which can inactivate eNOS downstream).

In a study by Wu et al., VEGF and BDNF were increased in the adult rat dentate gyrus and increased activation of the Akt pathway was also seen after treatment with simvastatin (22). Figure 6 below shows the connection between VEGF, eNOS, and

angiogenesis (23). Increased VEGF leads to increased phosphorylation of Akt which leads to phosphorylation of eNOS which ends up causing increased angiogenesis.

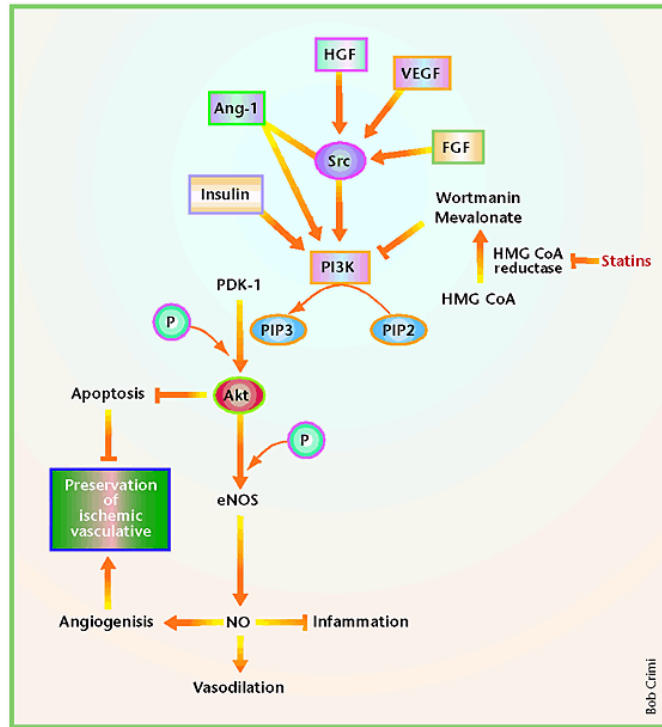


Figure 6 Akt Pathway. This figure demonstrates the role of VEGF and eNOS on increased angiogenesis (23).

This increase in angiogenesis was reported in yet another study in which various doses of simvastatin or atorvastatin were given 24 hours following MCAO to see results of neural function and blood flow. Both statins caused significant improvement in neural function ($P < 0.05$, generalized estimation equation approach) while atorvastatin caused an increase in synaptophysin (a synaptic protein), angiogenesis, and even cell proliferation in areas of the brain associated with neurogenesis ($P < 0.05$, t test) (24). Endothelial dysfunction can be reversed by angiogenesis but also by management in levels of endothelin-1 which is a vasoconstrictor. Luckily, it has also been shown that statins can inhibit the physiological

expression of endothelin-1 (25). Statins also have been shown to promote tissue plasminogen activator (26, 27). Figure 7 below exhibits this pathway (28). Tissue

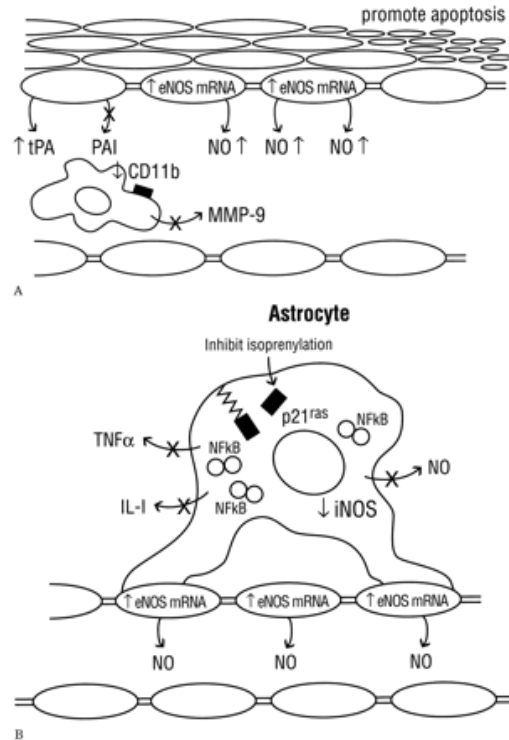


Figure 7 tPA increase

plasminogen activator cleaves pro-BDNF into BDNF, which is highly involved in neurogenesis and has even been shown to increase after statin treatment in rats (29). The multiple effects that statins have on the brain were why simvastatin and atorvastatin were included in the drug treatments discussed later in this paper.

Fluoxetine (Prozac) is an FDA approved selective serotonin reuptake inhibitor (SSRI) that increases serotonin levels in the brain (30). Besides its uses in the treatment of depression and other mental disorders, it has been shown to have effects on neurogenesis. When fetal neural stem cells were treated with fluoxetine, there was a dose-dependent response that led to the proliferation of the cells as well as trends that

indicate fluoxetine could also be responsible for the differentiation of the cells as well (31). Another study using adult mice also reported significant increases in neurogenesis in the hippocampus that they attributed mainly to increased proliferation (as exhibited by increased BDNF levels) but also cited differentiation to mature neurons as a factor as well ($P < 0.05$, ANOVA) (32). Further, when aged Sprague Dawley rats were stained for BrdU (dividing cells) it was shown that there was increased labeling in the hippocampus in response to chronic treatment with the drugs which confirms that the fluoxetine response is not age dependent ($P < 0.05$, ANOVA) (33). It is possible that increased neurogenesis is resulting from the antidepressants upregulation of BDNF (33). SSRI's also have a connection with p21, which is present in immature neurons and neuronal progenitors but not present in mature neurons. It has been shown that when p21 expression is inhibited (by administration of fluoxetine and other reuptake inhibitor drugs), neurogenesis increased in the subventricular zone (34). Still another possibility is that fluoxetine is aiding neurogenesis through its activation of vascular endothelial growth factor (VEGF). In the pathway shown in figure 8 below, it is clear that as serotonin increases in the synaptic cleft, VEGF receptors (as well as BDNF receptors) are activated and VEGF is upregulated which leads to proliferation of progenitor cells (35). To assess the importance of the VEGF receptors to the mechanism of fluoxetine (and other anti-depressants), a study was conducted that efficiently knocked out the VEGF receptors, and subsequent pathways. It was shown that the effect of the drugs on cell proliferation was essentially abolished, indicating that VEGF is altered by the presence of anti-depressants and the connection between the two is imperative (36).

Ascorbic acid (Vitamin C) is naturally occurring and has antioxidant properties. Much of the damage that results from ischemic stroke is due to oxidative damage by reactive oxygen species (37, 38). Further, the other two drugs used in combination with

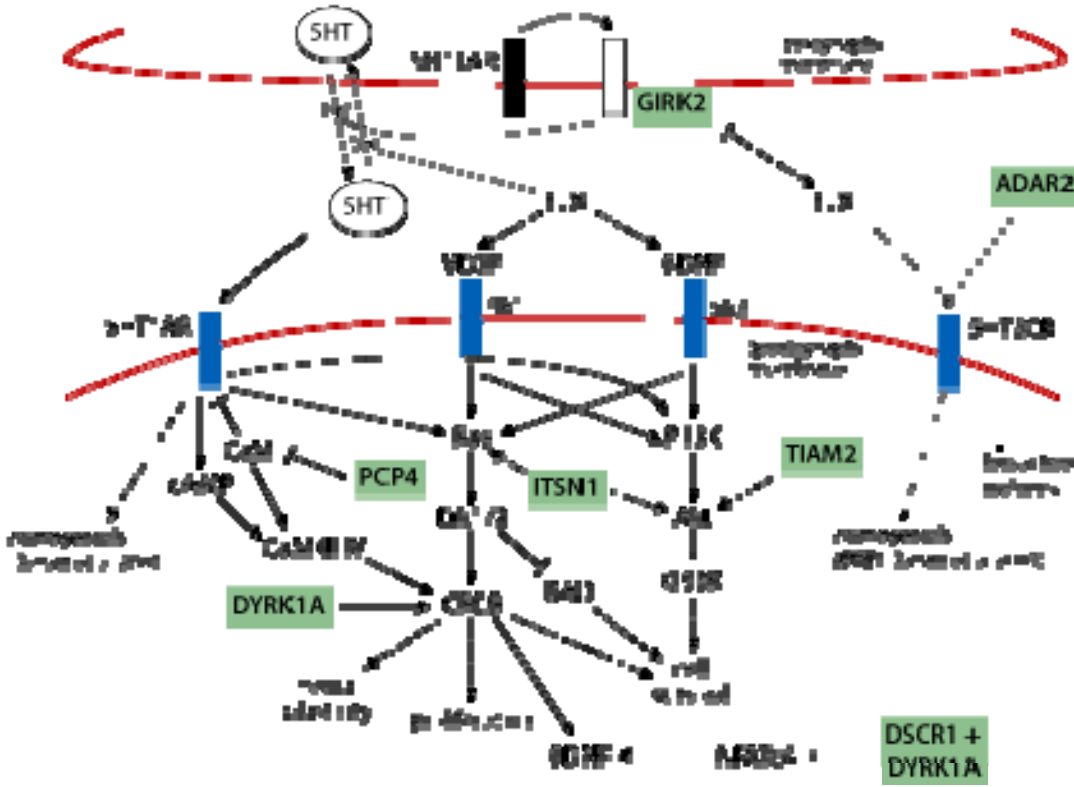
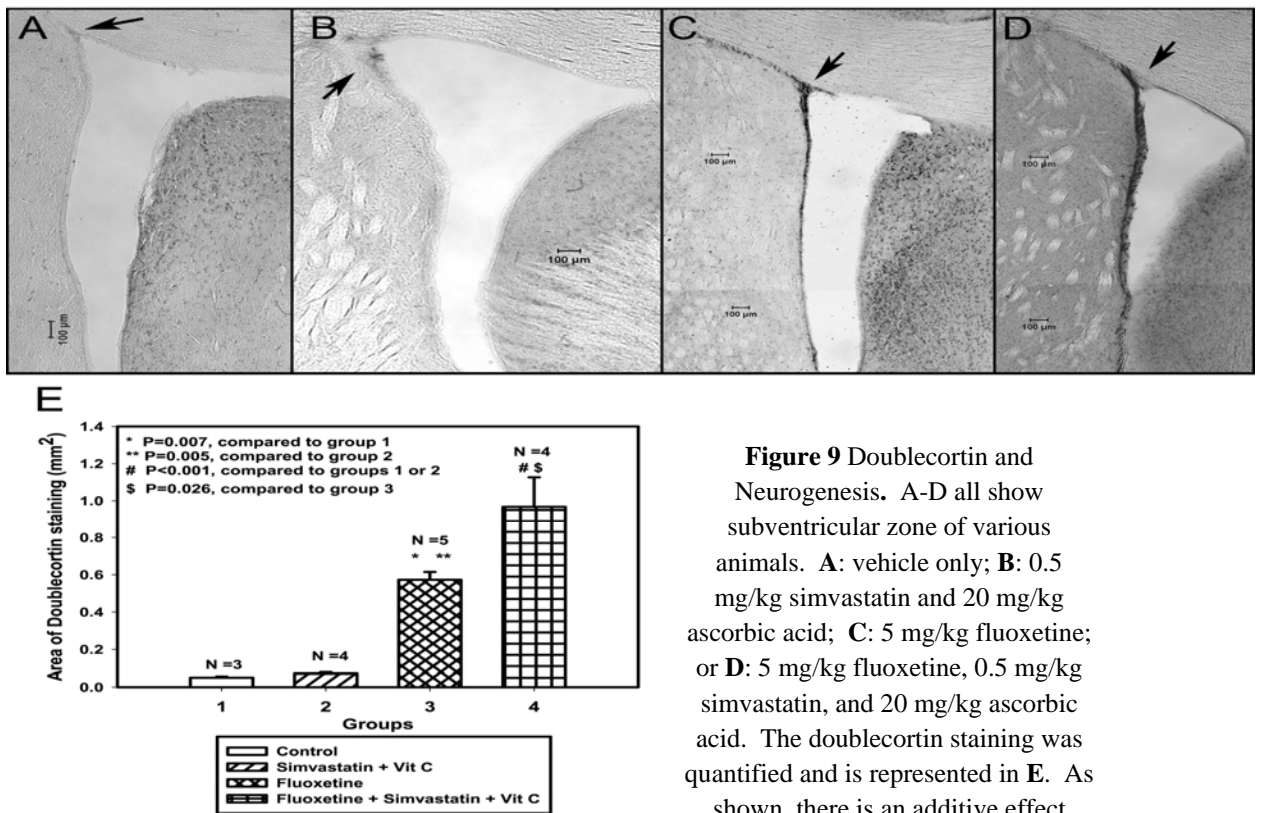


Figure 8 Downstream effects of increased serotonin at the synaptic cleft

ascorbic acid (SSRI; statins) have components that are sensitive to oxidation. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) leads to an increase in serotonin, which is very sensitive to oxidation. The statins used lead to an increase in endothelial nitric oxide synthase (eNOS), which has a component known as tetrahydrobiopterin that is sensitive to oxidation (39). Ascorbic acid has been shown to enhance SSRIs and the activation of eNOS (40, 41).

The initial study testing the drug combination (fluoxetine, simvastatin, ascorbic acid) in adult rats (approximately 1 year old) used doublecortin (which is only found in

newly born, migrating neurons) staining to see if neurogenesis was indeed increased. Drugs were given every day for two weeks. First, there is very little doublecortin staining seen in the subventricular zone of control animals at this age (Figure 9 Panel A and E below). There was no significant difference in neurogenesis when simvastatin and ascorbic acid were given alone compared to control animals (Panels B and E below). There was a 10-fold increase with fluoxetine alone (Panels C and E below). The three drugs combined produced a synergistic effect, with a 19-fold increase in doublecortin staining (Panel D and E below). This initial study showed that the drug treatment was



capable of increasing neurogenesis in the adult rat brain. Thus, the three drug pharmacological treatment was ready to be applied to adult animals that had suffered from an ischemic stroke in the hopes that it would increase neurogenesis after damage and improve functional recovery (42).

II. MATERIALS AND METHODS

Animals and Experimental Design

The strains of rats used in these studies were Long Evans and Sprague Dawley. All rats were between 10 and 12 months of age and were kept in a room that was light for 12 hours and dark for 12 hours. They each had uninhibited access to food/water unless involved in Montoya Staircase training and testing which required food restriction. All procedures listed were approved by WSU IACUC.

Two groups of Long Evans rats were given a vehicle control or a drug combination for 31 days. They were euthanized on day 31. The drug combination consisted of 0.5 mg/kg fluoxetine, 0.05 mg/kg simvastatin, and 20 mg/kg ascorbic acid (FSA). Functional tests were conducted on these rats before and after stroke to see differences in functional recovery in response to drug treatments vs. control.

Two groups of Sprague Dawley rats were given a vehicle control or a drug combination. They were euthanized on day 31 and the brains were collected for immunohistochemistry. The drug combination consisted of 0.5 mg/kg fluoxetine, 0.05 mg/kg atorvastatin, and 20 mg/kg ascorbic acid (FAA). These animals were used to compare differences in angiogenesis, glial scarring, and infarct volume in response to drug treatments vs. control using various primary antibodies. Functional tests were also conducted before and after stroke to see differences in functional recovery in response to drug treatments vs. control.

Prior to stroke surgery, Long Evans and Sprague Dawley rats went through

Montoya Staircase training for two weeks to increase sucrose pellet retrieval and were tested for pre-stroke function in both Montoya and Forelimb Asymmetry. Animals were given a vehicle control or drug combination (previously described) 20-26 hours following stroke surgery via voluntary oral administration. This continued daily for 31 days.

Endothelin-Induced Stroke

Animals were placed in an induction chamber with 5% isoflurane. After anesthesia took effect the head was shaved and the animal was placed in a stereotactic apparatus with ear bars. PuralubeTM ointment was placed in the eyes and anesthesia was continued with 2-3% isoflurane inhalation throughout the duration of the surgery. The head was prepared with povidone, 70% ethanol, and povidone again before a midline incision was made. The incision site was then treated with the analgesic Bupivacaine (0.25%). Bregma was located and a 0.9 mm burr was mounted in a micro-drill (Fine Science Tools) which was used to create two holes. The coordinates used on Long Evans rats were [AP 0.0 mm ML 2.5 mm] and [AP 2.3 mm and ML 2.5 mm]. The coordinates used on Sprague Dawley rats were [AP 0.0 mm ML 2.4 mm] and [AP 1.5 mm ML 2.4 mm]. 1 μ l Endothelin (Human and Porcine, EMD Chemicals) was slowly injected in each hole at a depth of 2.0 mm (400 pmoles/ μ l) over a 2 minute period. The incision site was sutured using Vicryl resorbable sutures and treated with povidone. The drug vehicle (sugar cookie dough in the amount of 4 grams) was given to each rat directly after surgery to allow them to become used to the dough.

Voluntary Oral Drug Administration

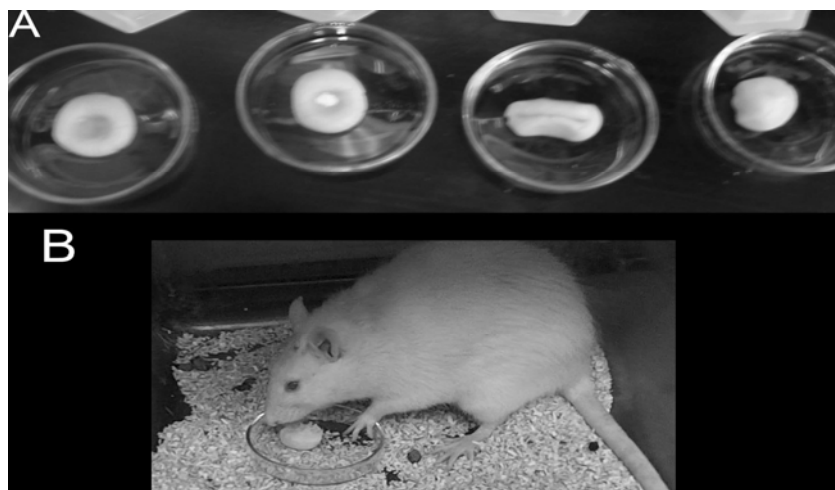


Figure 10 Oral Drug Administration **A.** Demonstrates steps taken to produce dough ball. 4 grams weighed out and depression made, drugs placed in depression, depression edges connected, dough mixed and made into a ball; **B.** Rat consuming dough ball out of Petri dish.

The fluoxetine and both statins were of generic origin (atorvastatin, 80 mg tablet; simvastatin, 80 mg tablet; fluoxetine HCl, 20 mg capsule) and the weights of active vs. inactive ingredients were compared. The pure form of Ascorbic acid (Vitamin C, Fluka) was used. Four grams of sugar cookie dough was used as the drug vehicle (Pillsbury brand). After it was weighed out to 4 grams it was rolled into a ball and a small depression was made in the dough ball. The drugs were weighed (based on previously mentioned amounts) and placed in the depression. The edges of the depression were then connected and the dough ball was manually mixed so all drugs were completely encased and incorporated in the dough ball. Each dough ball contained drugs or was a control and thus had no drugs and each dough ball was specific for a single animal. The dough was placed in a Petri dish and given to rats, which were housed individually, at approximately 12 noon each day. The dough was consumed very quickly in most cases and the criteria for inclusion in the study required the rats not go longer than 2 days without consuming

the dough ball or not have more than 3 days of incomplete ingestions of the dough ball. Leftover dough was recorded and counted against the total ingestion which allowed for a measure of the reliability of the voluntary oral administration.

Montoya Staircase

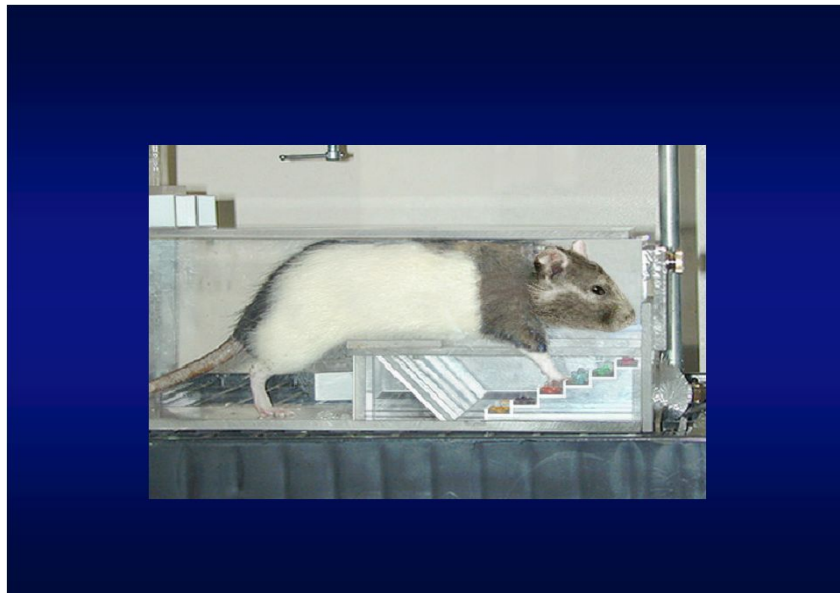


Figure 11 Montoya Staircase. Long Evans rat completing Montoya Staircase, right forepaw.

Pre-stroke Montoya Staircase training required that the animals fast overnight and then be placed on restrictive diets throughout training. When animals arrive their daily food intake is measured and is reduced to 85% of that ad lib intake (restrictive diet) during training. Training was conducted in the dark phase and each animal was allowed to explore the chamber for 15 minutes each day for two weeks. Each depression in the stair of the chamber had a total of three 50 mg sucrose pellets that were coated with maple extract that was allowed to dry previous to being used in training or testing. The results from the last three days of training were used to establish the baseline pre-stroke function. The best performance (most pellets retrieved) from those three days was used.

If two or more days produced the same number of pellets retrieved for the best performance but each forepaw produced different numbers an average was taken. Although feed was reduced to 85% of ad lib feed the weight of each animal was kept about 90% of their ad lib feed weight. Post-stroke Montoya Staircase tests were conducted on post-stroke days 8, 9, and 10 and also on post-stroke days 29, 30 and 31. An overnight fast preceded each post-stroke 3 day testing period and these tests were again completed in the dark phase. During these three days, the total food ingestion was 10 grams (including the 4 gram dough ball) for each rat. Sucrose pellets retrieved did not count towards total food administered. The required criteria that needed to be met in order for the rats to be included in the Montoya functional analysis were collection of at least 9 pellets by each forepaw. Similarly, if any animals failed to retrieve any pellets during post-stroke tests they were not included in Montoya functional analysis. Long Evans rats (n=4 for control, n=4 for FSA) were required to show a 30% deficit when compared to pre-stroke function to be included in functional analysis. Sprague Dawley rats (n=8 for control, n=7 for FAA) were required to show a 20% deficit when compared to pre-stroke function to be included in functional analysis.

Forelimb Asymmetry

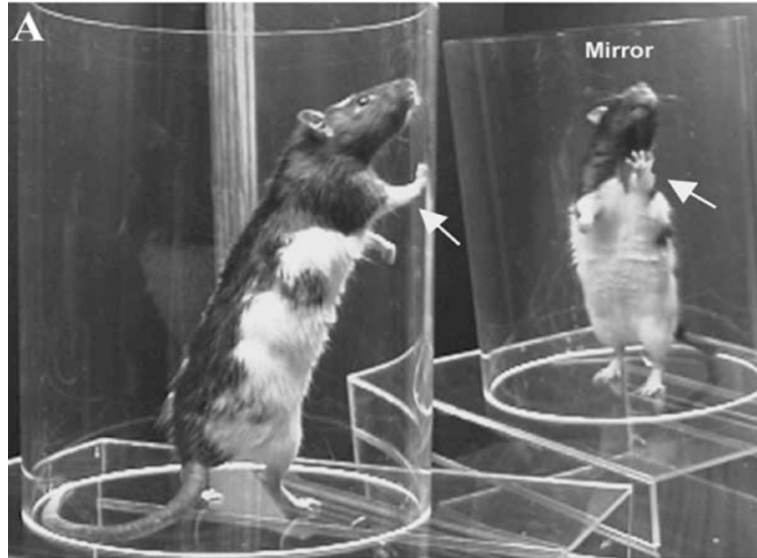


Figure 12 Forelimb Asymmetry. Shows Long Evans rat in Forelimb Asymmetry chamber. Picture shows mirror on back side of chamber to allow for counting of forepaw wall contacts on all sides of chamber.

A vertical, circular chamber 9 inches in diameter and 18 inches in height made of clear acrylic was made and maple extract was applied to the top of the chamber. It was mounted on a stand and each animal was placed inside and recorded for a total of 5 minutes. The videos were analyzed for the total number of wall contacts made with each forepaw in slow motion. Forelimb Asymmetry tests were conducted on post-stroke days 3, 16 and 28. Long Evans rats (n=6 for control, n=7 for FSA) were required to show a 30% deficit when compared to pre-stroke function to be included in functional analysis. Sprague Dawley rats (n=8 for control, n=6 for FAA) were required to show a 20% deficit when compared to pre-stroke function to be included in functional analysis.

Euthanization and Cardioperfusion

Animals were treated with an IP injection of 100 mg/kg pentobarbital (Euthasol) and cardioperfusion was completed with at least 150 mls PBS once the anesthesia took effect. Then animals were cardioperfused with 150 ml of 4% paraformaldehyde in PBS.

<i>Antigen</i>	<i>Stains for</i>	<i>Label on Secondary Ab</i>	<i>Host</i>	<i>Dilution of Primary Ab</i>	<i>Source</i>
<i>8-hydroxy G</i>	<i>Infarct Area</i>	<i>Rhodamine Red X</i>	<i>Mouse</i>	<i>1:500</i>	<i>AbCAM ab62623</i>
<i>eNOS</i>	<i>Angiogenesis</i>	<i>FITC</i>	<i>Rabbit</i>	<i>5 µg/mL</i>	<i>AbCAM ab66127</i>
<i>GFAP</i>	<i>Astrocytes</i>	<i>Rhodamine Red X</i>	<i>Mouse</i>	<i>1:200</i>	<i>AbCAM ab4648</i>

Table 1 Information on primary antibodies

Immunohistochemistry and Immunofluorescence

After cardioperfusion all brains from Sprague Dawley rats were collected and placed in 4% paraformaldehyde in PBS at 5°C to be blocked and postfixed for 24 hours. Brains were then transferred into 30% sucrose in PBS at 5°C for an additional three days. 50 micron sections were then made on the cryostat. Sections were placed into 3 different vials each containing PBS. Sections in vial 1 were then blocked for an hour with PBS containing 1% Triton and 3% secondary antibody host serum then reacted with 8-hydroxy guanosine primary antibody (1:500 dilution, AbCAM antibody) overnight at 5°C. Sections in vial 2 were also blocked for an hour with PBS containing 1% Triton and 3% secondary antibody host serum and then reacted with endothelial nitric oxide synthase (eNOS, 5 µg/mL, AbCAM antibody) and glial fibrillary acidic protein (GFAP, 1:200 dilution, AbCAM antibody) primary antibodies overnight at 5°C. Vial 3 was a

control and thus was not blocked or treated with any primary antibodies. All vials were washed three times with PBS with 0.1% Triton and incubated with secondary antibodies (Rhodamine Red X-labeled Donkey anti-mouse IgG, FITC-labeled Donkey anti-rabbit IgG; Jackson Immuno) in the dark at room temperature for 2.5 hours. All secondary antibodies were at a dilution of 1:100 in the blocking solution. Finally, all sections were mounted on gel subbed slides.

Image Analysis

All sections with infarcts from vial 1 (8-hydroxy guanosine) were analyzed and digital images of infarcts and areas of interest were collected and montages were compiled using Adobe Photoshop. Infarct volume was collected by outlining infarct area (as shown in Figure 13) using NIH *ImageJ* program and multiplying the resulting infarct area value by 3 to determine the full infarct volume since we only sampled every third section for this analysis. The value was then multiplied again by 0.05 mm to account for the depth of each section (decided by the cryostat thickness for each section, 50 microns).

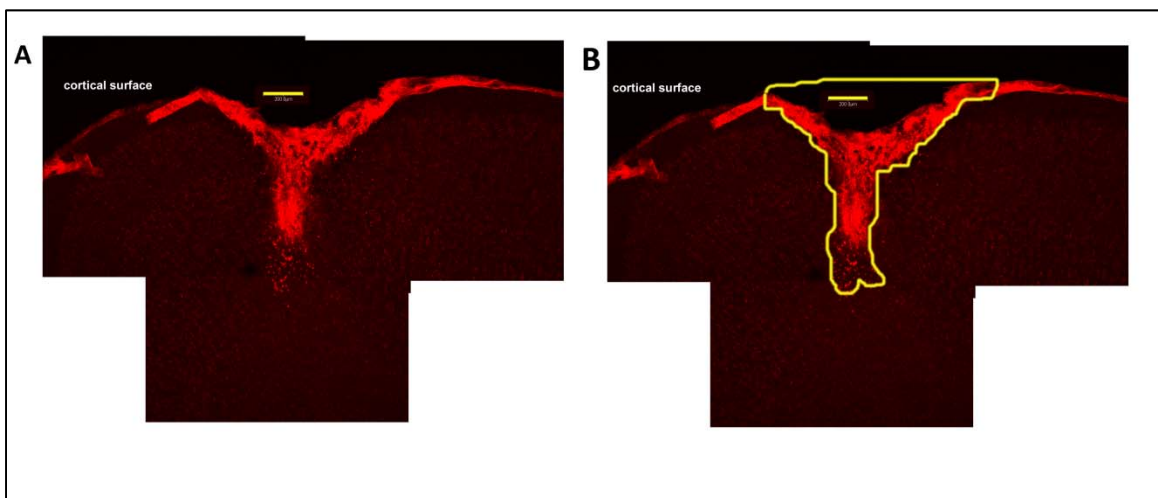


Figure 13 Infarct Area Measurement. **A.** Example of a typical infarct montage. **B.** Representation of the outlining of the infarct that took place using *ImageJ* program. Scale bar is 200 μ m.

The first ten sections with infarcts from vial 2 (eNOS, GFAP) were analyzed and digital images of infarcts and areas of interest were collected and montages were compiled using Adobe Photoshop. Total tissue area was collected by outlining all of the tissue on the entire montage using NIH *ImageJ* program. Fluorescence was collected by converting images to grayscale in Adobe Photoshop and adjusting the threshold using *ImageJ* (progression shown in Figure 15). When adjusting threshold, although each montage had a variable amount of fluorescence expression, the threshold was kept to the right side of the threshold curve as shown in Figure 14. This allowed for intense areas of

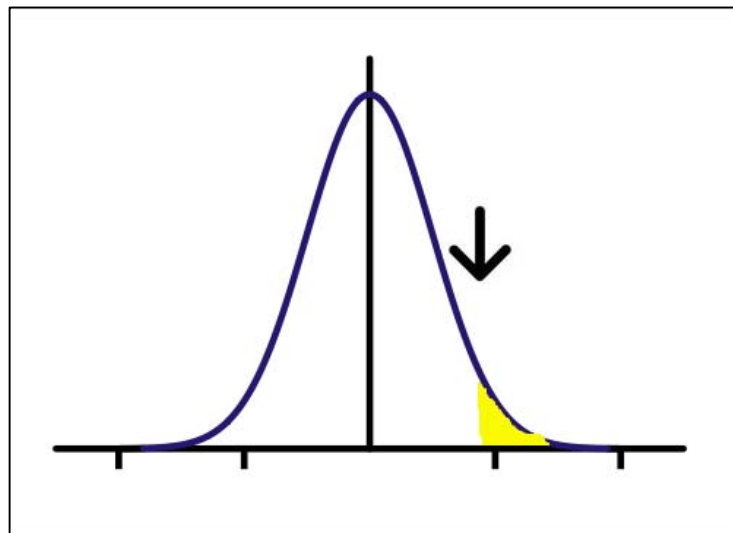


Figure 14 Threshold. Example of threshold adjustment in *Image J*. No matter what kind of curve was produced, only the far right side of the curve was selected (as shown by arrow and shading).

fluorescence to be measured while excluding background expression from the measurements. By doing this, each montage threshold value was able to be standardized. The GFAP montages were also analyzed for varying morphology. The montages seemed to have intense GFAP expression near the infarct (indicative of a glial scar) or spread out

and in diffuse areas away from the area of injury. It should also be noted that one rat that was included in GFAP analysis only had six sections with an infarct instead of ten sections. Because of this, the average tissue area and average GFAP staining per montage went into calculating the GFAP density (staining/mm² tissue) to account for the varying number of sections analyzed to produce the results. However, all of the rats analyzed for eNOS had ten sections with an infarct so the summed tissue area and the summed eNOS expression went into calculating the eNOS density (staining/mm² tissue).

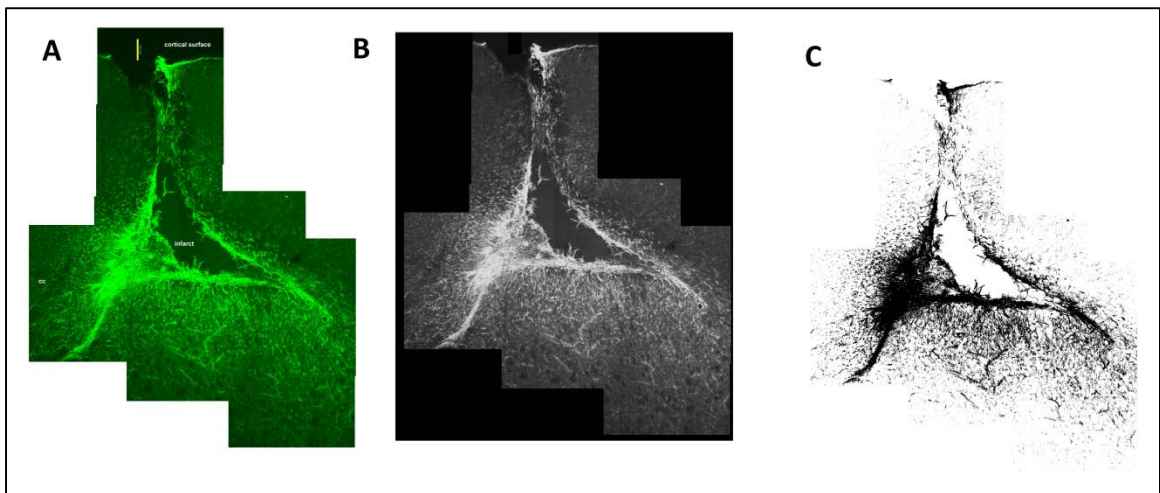


Figure 15 Fluorescence Measurement Progression. **A.** Example of montage in full color (eNOS stain). **B.** Grayscale of montage used to adjust threshold. **C.** Mask of montage. This shows the areas of staining that went into producing the fluorescence measurements for this montage. Scale bar is 200 μ m.

Statistical Analysis

Two Way Repeated Measures ANOVA was used to analyze the functional data (both Forelimb and Montoya). Simple t-tests were used to analyze the fluorescence measurements. Fischer's exact test was used to analyze GFAP morphology.

Exclusions

There were several animals that were not included in analysis for various tests. Five Long Evans animals did not meet criteria for Montoya Staircase. Two animals that did

meet criteria were left out of analysis because they failed to eat their medicine for 3 or more days. Additionally, one animal was excluded because it failed to show a 30% deficit (this animal received half the endothelin as the other animals). Two animals were not analyzed for forelimb asymmetry because they missed their drug dosage. Two were excluded from forelimb because they received half of the amount of endothelin in comparison to the other animals. One animal was excluded because it did not meet criteria. All the rest of the animals showed at least a 30% deficit for forelimb.

Table 2 has each Sprague Dawley animal and information on their inclusion in functional and immunofluorescence tests. The required criteria to be included in Montoya Staircase analysis was that each rat grab at least 9 sucrose pellets with each forepaw. If they failed to do so they were eliminated from that section of the study. As shown in the table, 9% of Sprague Dawley rats failed to meet criteria for Montoya Staircase. The required deficit for Sprague Dawley animals to be included in Montoya Staircase or Forelimb Asymmetry was 20%. This means that the pre-stroke performance needed to be reduced by at least 20% for the animals to be included in the functional analysis. As shown by the table, 25% of those rats that met criteria for Montoya failed to meet the deficit requirement and were thus excluded. Finally, while all of the Sprague Dawley animals met criteria for Forelimb Asymmetry (which was at least one wall contact), 31% failed to meet the required deficit and were thus excluded.

There were also problems that removed the Sprague Dawley rats from immunofluorescence analysis. Two animals (360, 361) were excluded because of problems with cardioperfusion. Two animals (374, 275) were excluded because of problems cutting on the cryostat. One animal (396) was excluded because when

cardioperfusion was to be completed it was noted that the tissue had undergone extreme degeneration and would therefore not represent damage only caused by the stroke surgery. There were also two rats (377, 384) that seemed to have suffered from secondary hemorrhagic strokes (possibly because a blood vessel was nicked by the needle during surgery) and these animals were also excluded from immunofluorescence analysis. Examples of infarcts caused by ischemic stroke and by secondary hemorrhagic stroke can be seen in Figures 16 and 17, respectively. Hemorrhagic infarcts usually stay near the surface of the cortex and continue on for a long distance while ischemic infarcts normally go into the cortex toward the lateral ventricles and are normally smaller. All montages in this thesis will be oriented in the same manner as the cortical diagram shown in Figure 16 so as to understand the various landmarks that surround the infarcts. Finally, five animals (387-393) were left out of the eNOS analysis because the stain failed to adhere to the tissue. Examples of a robust eNOS stain and a weak eNOS stain can be seen below in Figure 18. The last thing to be noted from Table 2 is that rat 392 was included in GFAP analysis but there were only 6 infarcts found instead of the usual 10 that all the other animals analyzed for GFAP contributed. As such, certain measures had to be taken to account for the difference in infarct number.

Rat ID #	Forelimb Assymetry	Montoya Staircase	8-DOG Analysis	eNOS Analysis	GFAP Analysis
360	included	criteria not met	Unable to cardioperfuse	Unable to cardioperfuse	Unable to cardioperfuse
361	included	included	Not a hard fix	Not a hard fix	Not a hard fix

364	included	included	completed	completed	completed
		criteria not			
366	deficit not met	met	completed	completed	completed
		deficit not			
368	deficit not met	met	completed	completed	completed
		deficit not			
369	deficit not met	met	completed	completed	completed
371	included	included	completed	completed	completed
		deficit not			
372	deficit not met	met	completed	completed	completed
			Sections	Sections	
374	included	included	shattered	shattered	Sections shattered
			Sections	Sections	
375	included	included	shattered	shattered	Sections shattered
377	included	included	Hemorrhagic	Hemorrhagic	Hemorrhagic
		deficit not			
378	included	met	completed	completed	completed
		deficit not			
380	included	met	completed	completed	completed
381	deficit not met	included	completed	completed	completed
383	deficit not met	included	completed	completed	completed

384	included	included	Hemorrhagic	Hemorrhagic	Hemorrhagic
387	included	included	completed	stain failed	completed
388	included	included	completed	stain failed	completed
391	included	included	completed	stain failed	completed
					completed, only 6
392	deficit not met	included	completed	stain failed	infarcts found
393	included	included	completed	stain failed	completed
			tissue	tissue	
396	included	included	degenerated	degenerated	tissue degenerated

Table 2 Information on individual rat inclusion in functional, infarct volume, and florescence analyses

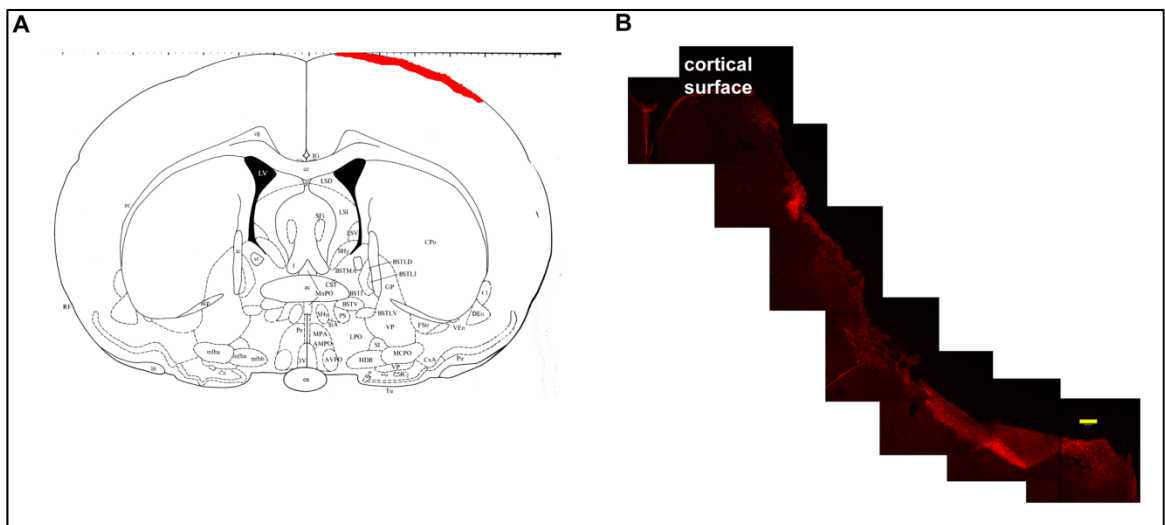


Figure 16 Example of a secondary hemorrhagic infarct. A. Outlined representation of damage caused by a hemorrhagic event. B. Actual picture of a hemorrhagic infarct seen in Rat 384. As shown, damage stays near surface of the cortex and continues on around the edge of the right hemisphere. Scale bar is 200 μ m.

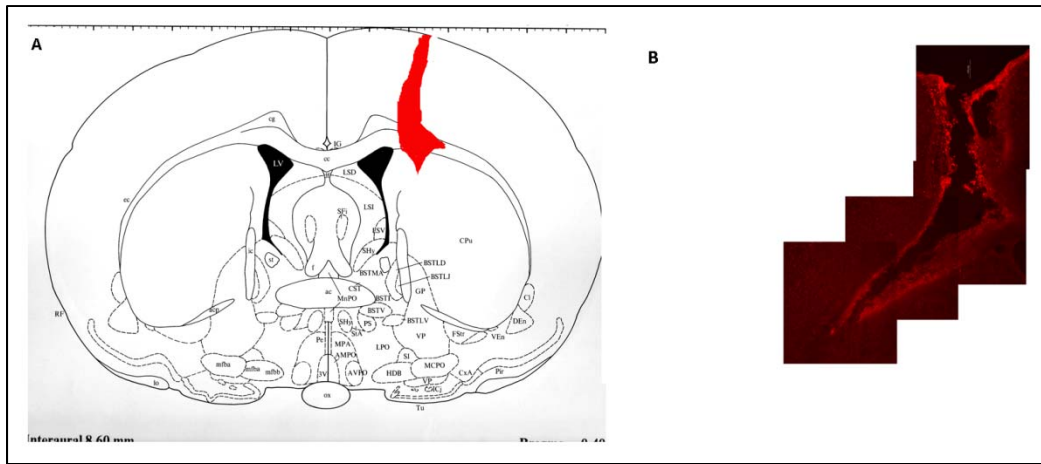


Figure 17 Example of a typical ischemic infarct. **A.** Outlined representation of damage caused by our stroke surgery in the adult rat brain **B.** Actual picture of an ischemic infarct in Rat 392. As shown, it dips down into cortex toward lateral ventricle (not shown). All other montages will be oriented the same as this cortical diagram. Scale bar is 200 μ m.

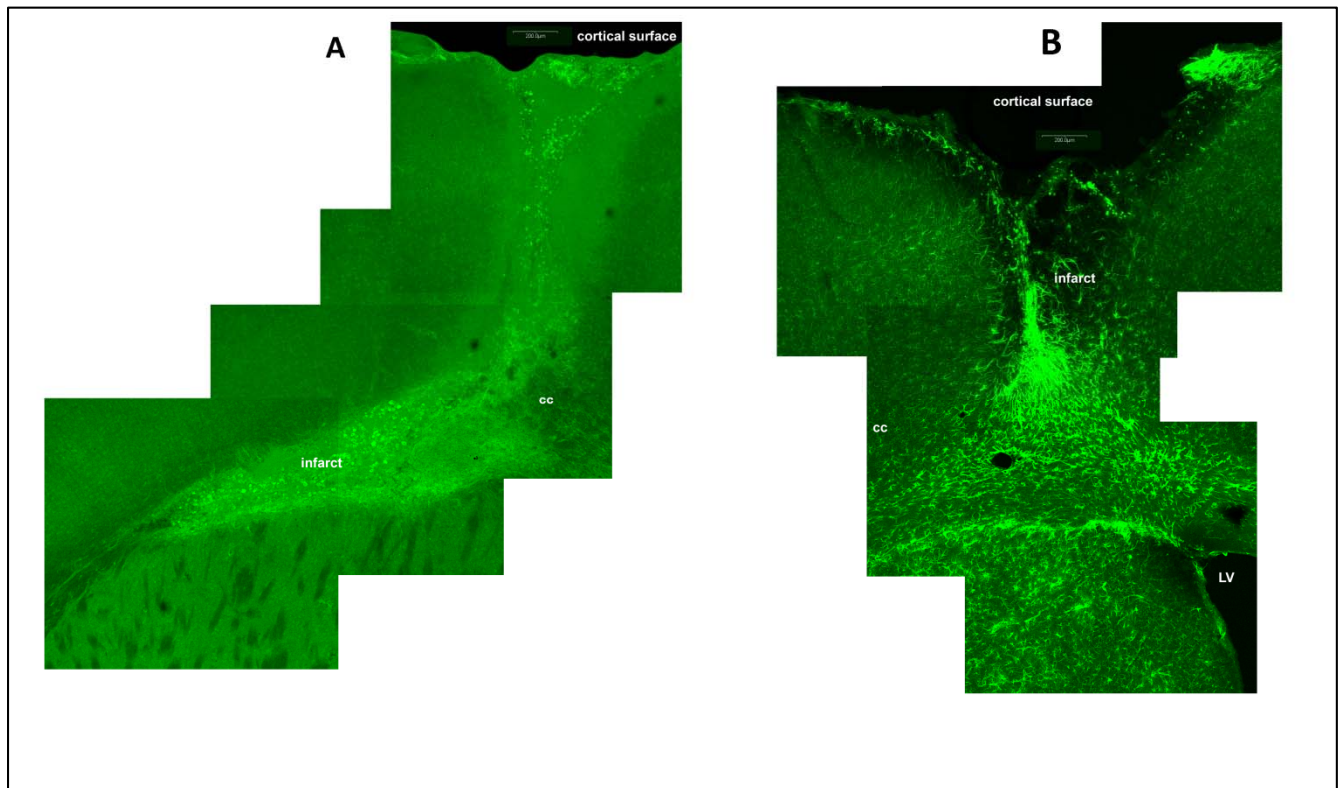


Figure 18 Weak vs. Robust eNOS stain. **A.** Representative montage showing a very weak eNOS stain seen in Rat 392. Animals that showed signs of this were not included in the eNOS analysis. **B.** Sample montage exhibiting a very robust eNOS stain from Rat 369. This is typically what is supposed to be seen with a good eNOS stain. Scale bar is 200 μ m.

III. RESULTS

Montoya Staircase

The two drug combinations (1. FSA- 5.0 mg/kg fluoxetine, 0.05 mg/kg simvastatin, 20.0 mg/kg ascorbic acid; 2. FAA- 5.0 mg/kg fluoxetine, 0.05 mg/kg atorvastatin, 20.0 mg/kg ascorbic acid) were compared to a vehicle control to observe the effect of functional recovery in Montoya Staircase. Long Evans and Sprague Dawley rats (10-12 months old) were given the drug treatment or control vehicle every day for 31 days beginning 20-26 hours after stroke induction. Functional recovery was examined by comparing pre- and post-stroke performance on Montoya Staircase. Long Evans results are shown in Figure 19 (n=4 for control, n=4 for FSA). The figure shows percentage of pre-stroke performance on the contralateral (left) side. Any values that reach 1.0 on the y axis represent function that has recovered to 100% pre-stroke performance. Any value less than one represents a functional deficit. As mentioned earlier, testing was conducted pre-stroke and on post-stroke days 8-10 and 29-31 and the best performance from each three day set was recorded. The FSA drug combination did significantly increase functional recovery from post-stroke day 8, 9, 10 to post-stroke day 29, 30, 31 ($P= 0.002$) and returned function to approximately 85% pre-stroke function, as shown by the bar on the right hand side of the graph. Further, there was a significant difference between drug and control vehicle on post-stroke day 29, 30, 31 ($P=0.023$). It should also be noted that the deficit seen in control animals on post-stroke day 8, 9, 10 was nearly 80%.

Montoya Staircase: Contralateral Side

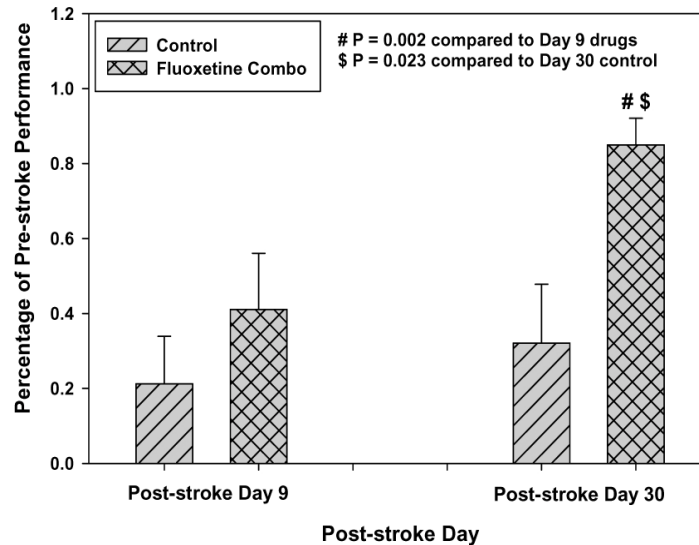


Figure 19 Long Evans Montoya Staircase Results. Training occurred for two weeks pre-stroke and post-stroke at days 8, 9, and 10 and days 29, 30, and 31. Post-stroke contralateral (left paw) performances were compared to pre-stroke performance to get a percentage of pre-stroke function. There was a significant increase in function for FSA treated animals from the best performance on Days 8, 9, 10 to Days 29, 30, 31 ($P=0.002$) and there was also a significant difference between FSA treated animals and control animals on post-stroke days 29, 30, 31. As shown on right side of graph, on post-stroke days 29, 30, 31 the FSA treated animals had reached nearly 90% of pre-stroke function. Animals not analyzed included those which did not show at least a 30% functional deficit. $n=4$ for control, $n=4$ for FSA

Sprague Dawley results for Montoya Staircase are shown in Figure 20 ($n=8$ for control, $n=7$ for FAA). The figure shows deficit on the contralateral side (post-stroke/pre-stroke). Any values that approach 1.0 on the y axis represent function that has recovered to 100% pre-stroke performance. Any values less than 1.0 represent a functional deficit. First, note that the mean deficit seen in control Sprague Dawley animals on post-stroke days 8, 9, 10 was much less than that seen with the Long Evans strain (~53% deficit compared to ~80% deficit respectively). However, like the Long Evans strain, control animals do not show much spontaneous functional recovery from

the stroke. The FAA drug combination did not significantly increase functional recovery from post-stroke days 8-10 to post-stroke days 29-31 ($P=0.459$). Additionally, there was not a significant difference between drug and control vehicle groups ($P=0.82$).

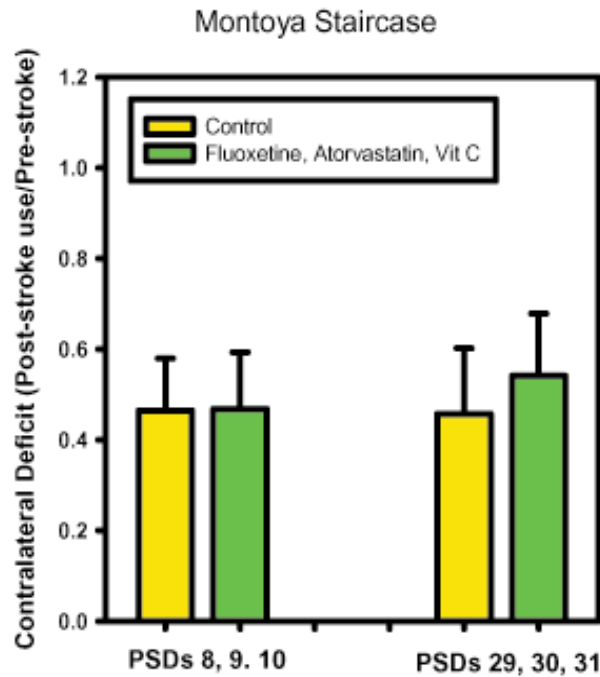


Figure 20 Sprague Dawley Montoya Staircase Results, Training occurred for two weeks pre-stroke and post-stroke at days 8, 9, and 10 and days 29, 30, and 31. Post-stroke contralateral (left paw) performances were divided by pre-stroke performance to get a percentage of pre-stroke function. There was not a significant increase in function for FAA treated animals from Day 9 to Day 30 ($P=0.459$) and there was not a significant difference between FAA treated animals and control animals on post-stroke day 30 ($P=0.82$). As shown on right side of graph, the FAA treated animals had only reached about 55% of pre-stroke function at the end of the treatment period. Animals not analyzed included those which did not show at least a 20% functional deficit. $n=8$ for control, $n=7$ for FAA

Forelimb Asymmetry

The two drug combinations (1. FSA- 5.0 mg/kg fluoxetine, 0.05 mg/kg simvastatin, 20.0 mg/kg ascorbic acid; 2. FAA- 5.0 mg/kg fluoxetine, 0.05 mg/kg atorvastatin, 20.0 mg/kg ascorbic acid) were also compared to a vehicle control to observe the effect of functional recovery in Forelimb Asymmetry. Long Evans and

Sprague Dawley rats (10-12 months old) were given the drug treatment or vehicle control every day for 31 days. Functional recovery was examined by comparing pre- and post-stroke performance on Forelimb Asymmetry. Long Evans results are shown in Figure 21 (n=6 for control, n=7 for FSA). The figure shows percentage of contralateral usage vs. pre-stroke function (post-stroke/pre-stroke). Any values that reach 1.0 on the y axis represent function that has recovered to 100% pre-stroke function. Notice that the deficit seen in forelimb asymmetry was much less than what was seen in Montoya Staircase for Long Evans. With the forelimb, we see that the deficit was only around 40%. It should also be noted that the control animals do not show spontaneous recovery from post-stroke day 3 onward. The FSA drug combination did significantly increase functional recovery from post-stroke day 16 to post-stroke day 28 (P= 0.041) and returned function to approximately 90% of pre-stroke performance. Further, there was a significant difference between drug and control vehicle on post-stroke day 28 (P=0.05).

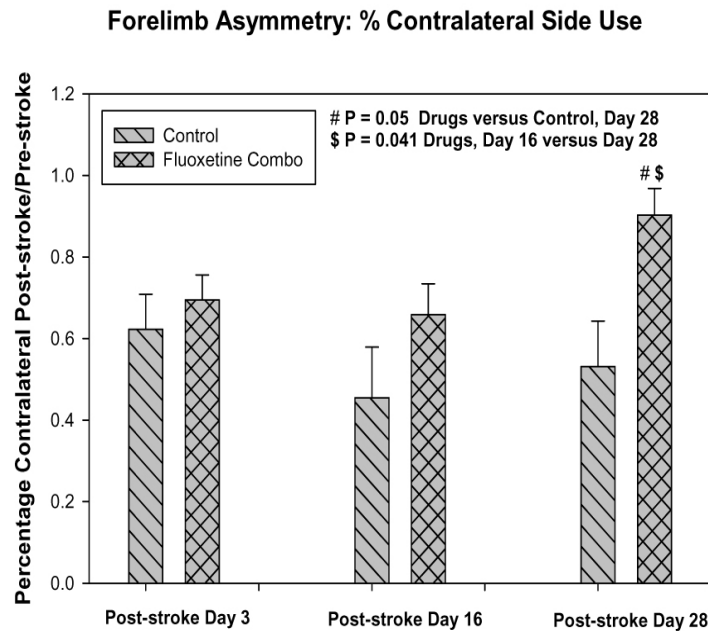


Figure 21 Long Evans Forelimb Asymmetry Results, Post-stroke contralateral wall contacts were divided by pre-stroke contralateral wall contacts to show percentage of pre-stroke contralateral performance. Testing occurred on post-stroke days 3, 16, and 28. There was significant recovery from post-stroke day 16 to day 28 ($P=0.041$). There was also a significant difference between control and FSA treated animals on post-stroke day 28 ($P=0.05$). FSA treated animals had reached nearly 90% pre-stroke function by post-stroke day 28. Animals not analyzed included those which did not show at least a 30% functional deficit. $n=6$ for control, $n=7$ for FSA

Sprague Dawley results are shown in Figure 22 ($n=8$ for control, $n=6$ for FAA). The figure shows percentage of contralateral usage relative to pre-stroke function (post-stroke/pre-stroke). Any values that reach 1.0 on the y axis represent contralateral forelimb function that has recovered to 100% pre-stroke performance. Notice that the control Sprague Dawley animals show an even smaller deficit (30%) than the Long Evans animals (40%). However, although the deficit was smaller with these animals, we still see that there was not spontaneous recovery in the control animals. The FAA drug combination did not significantly increase functional recovery when the different time points were compared ($P=0.059$, power=0.394). Further, there was not a significant difference between drug and control vehicle on any testing day ($P=0.364$).

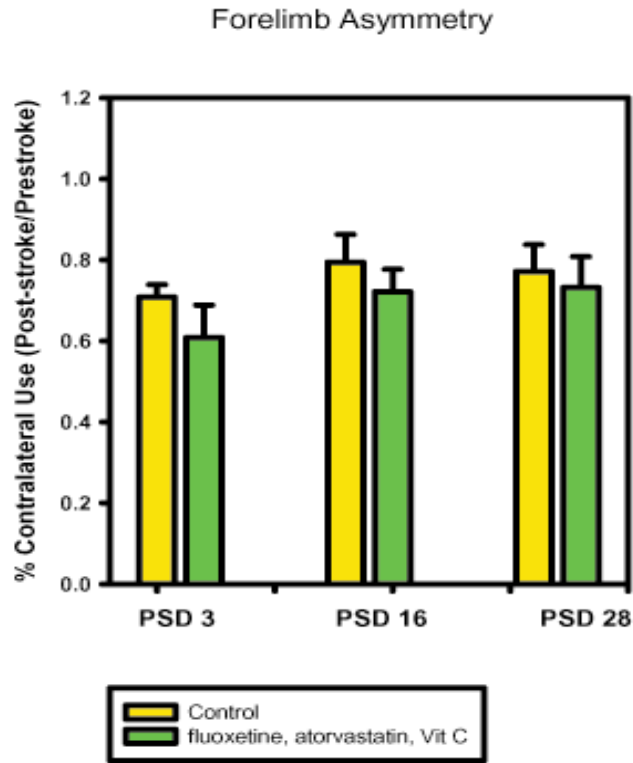


Figure 22 Sprague Dawley Forelimb Asymmetry Results, Post-stroke contralateral wall contacts were divided by pre-stroke contralateral wall contacts to show percentage of pre-stroke contralateral performance. Testing occurred on post-stroke days 3, 16, and 28. There was not significant recovery for FAA treated animals between post-stroke day 3, 16 and 28 ($P=0.059$, power=0.394). There was not a significant difference between control and FAA treated animals on post-stroke day 28 ($P=0.364$). FAA treated animals had only reached 75% pre-stroke function by post-stroke day 28 and the control animals reached an even higher % of pre-stroke function at nearly 80%. Animals not analyzed included those which did not show at least a 20% functional deficit. $n=8$ for control, $n=6$ for FAA

Sprague Dawley results for total number of wall contacts are shown in Figure 23 ($n=8$ for control, $n=6$ for FAA). The figure shows the total number of wall contacts for both the left and right forepaws. There is a significant difference in wall contacts when the different times points are compared ($P=0.002$, power=0.904). As shown, there is a habituation with time and the number of wall contacts does decrease over time. There was not a significant difference between drug and control ($P=0.222$). Although the

number of wall contacts did decrease over time there were still a high number of contacts on the final testing day (post-stroke day 28) which proves that the drug treatment did not alter exploration within the chamber.

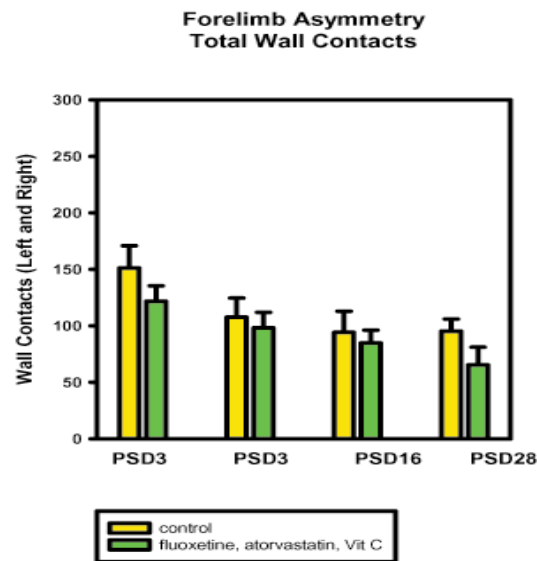


Figure 23 Sprague Dawley Forelimb Asymmetry wall contacts, control vs. FAA drug treated animals. This graph is meant to show that the FAA drug treatment did not alter the exploration of the animals within the forelimb asymmetry chamber when compared to animals not treated with the FAA drug combo. There is a habituation with time (shown by the decrease in wall contacts, $P=0.002$) but the animals still showed a large number of wall contacts on post-stroke day 28. There was not a significant difference between wall contacts for FAA treated animals and control animals ($P=0.222$) which is what we wanted to prove. Animals not analyzed included those which did not show at least a 20% functional deficit. $n=8$ for control, $n=6$ for FAA

Infarct Volume (8-hydroxy guanosine)

8-hydroxy guanosine primary antibody, which is indicative of oxidative damage to DNA, was used to compare the differences in infarct volume. Results for individual Sprague Dawley animals are shown in Table 3. Those values are represented in Figure 24 ($n=8$ for control, $n=7$ for FAA). It shows that, although there is definitely a trend showing smaller infarct volumes (in mm^3) for drug treated animals, there was no

statistical difference between control and FAA drug treated animals ($P=0.072$, power=0.223) when using t-test statistical analysis. Since the power of the test is below 0.800, we can use these results to determine the number of animals that should be used to test for differences in infarct volume. Figure 25 shows montages for representative 8-hydroxyguanosine expression for an infarct in an animal that received the control vehicle (left panel) and an infarct in an animal that received the FAA drug treatment (right panel).

Rat	Average Infarct Volume in mm^3
364	1.051
368	3.968
371	1.222
380	7.852
383	0.430
387	1.362
391	3.513
393	3.146
366	1.128
369	1.705
372	0.728
378	0.662
381	1.911
388	3.670
392	1.088

Table 3 Infarct Volume for individual Sprague Dawley animals, control animals shown in yellow at top of table.

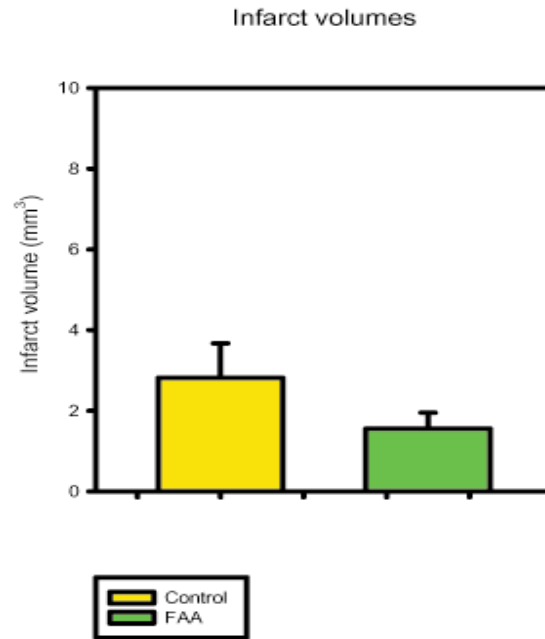


Figure 24 Sprague Dawley Infarct Volume Results. There is a strong trend suggesting that the FAA drug treatment did reduce the infarct volume when compared to the control vehicle ($P=0.072$, power=0.223) after stroke survival surgery.

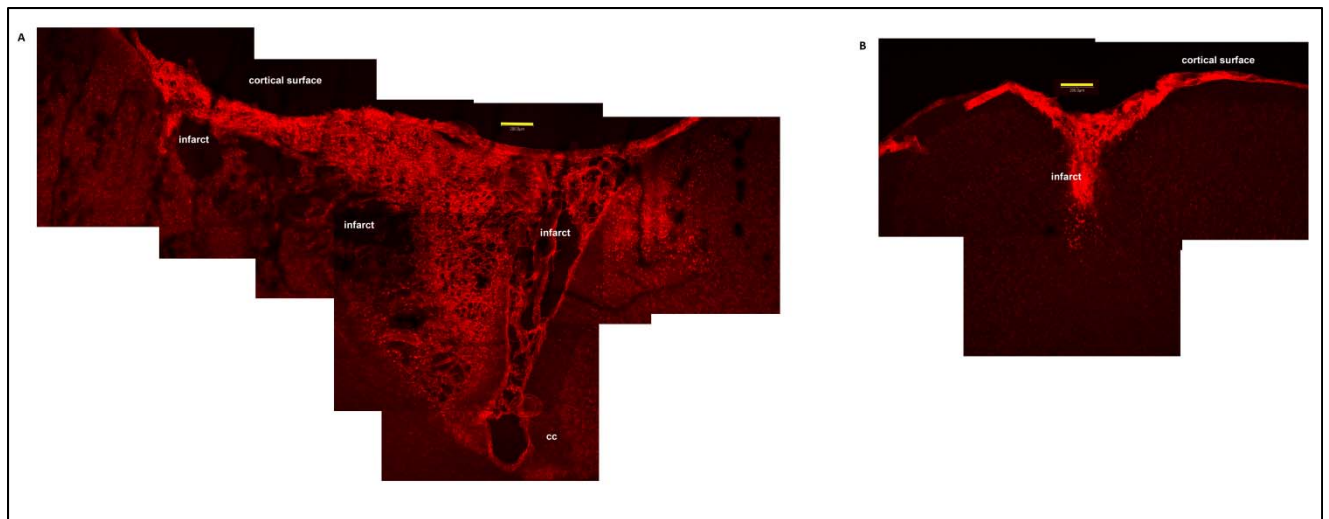


Figure 25 Infarct Area Montages. **A.** This is a representative of infarct of control Rat 380. As shown, it dips down deeper into the cortex and is much larger than what is seen on the right. Cortical surface is towards top of the image. **B.** This is an example of an infarct seen in a rat that received the FAA drug treatment, Rat 372. Cortical surface is shown at the top of the image. Scale bar (yellow) is 200 μ m.

Endothelial Nitric Oxide Synthase Expression

Endothelial nitric oxide synthase (eNOS) primary antibody was used to observe changes in angiogenesis between control and FAA drug treated animals. It was hypothesized that FAA treated animals would show increased eNOS staining because there would be increased angiogenesis (because eNOS lines all blood vessels). Table 4 shows results for each individual rat that went into producing the following figures (n=4 for control, n=5 for FAA). The tissue area sum is a measure of the total tissue area in the montages of each rat. These results were gathered to be able to account for total area when observing fluorescence. The eNOS sum is the total sum of eNOS fluorescence in montages for each individual rat. The final column, eNOS staining/mm was created by dividing the eNOS sum value by the total tissue area sum. This is the eNOS density per rat within this montage. Figure 26 shows total tissue area found in the montages for eNOS fluorescence (P= 0.497). The yellow bar is for control animals. The green bar is for the FAA treated animals. The mean total tissue area for control is 38.225 mm. The mean total tissue area for FAA treated animals was 32.841 mm. Although the control animals had slightly larger tissue areas (because they generally had larger infarcts), the statistics support the fact that there is no significant difference (P=0.497). Figure 27 shows the total sum of eNOS staining (P= 0.622). This is the total eNOS fluorescence for all montages per rat. The yellow bar represents control animals and the green bar represents FAA treated animals. The mean eNOS sum for control animals is 4.749 mm while the mean eNOS sum for FAA treated animals is 5.710 mm. There is not a statistical difference between control and drug treated animals for eNOS sum. Figure 28 shows the eNOS density in staining/mm² (P= 0.070, power= 0.365). The yellow bar

represents control animals. The green bar represents FAA animals. The mean eNOS density for control animals was 0.113/mm² while the mean eNOS density for FAA treated animals was 0.174/mm². There is a strong trend that shows increased eNOS expression, and thus increased angiogenesis, in drug treated animals. Tables 5-13 are results for each montage for each individual rat so each table has the tissue area sum, eNOS sum, and eNOS density for each of the ten montages that were analyzed. The sums and averages from each column can be found at the bottom of each table. This is to show the variation found within each rat because the averages from each rat were used to produce the eNOS figures (figures 26-28). Tables with a yellow bar at the top represent control animals. Lastly, Figure 29

Rat	Tissue Area Sum (mm)	eNOS Sum (mm)	Average eNOS staining/mm
364	35.638	3.051	0.084
368	37.933	3.581	0.097
371	22.349	2.148	0.093
380	56.981	10.216	0.175
366	39.248	5.590	0.144
369	29.303	6.087	0.208
372	25.879	5.511	0.211
378	26.039	3.150	0.124
381	43.740	8.213	0.179

Table 4 Various eNOS results for individual animals, control animals highlighted in yellow at top of table. Column 1 shows rat ID number. Column 2 shows the total tissue area sum for all montages from each rat. Column 3 shows the total eNOS sum for all montages from each rat. Column 4 shows the eNOS density which was gathered by dividing the eNOS sum for each animal by the total tissue area sum for each animal. All values in mm.

shows montages for representative endothelial nitric oxide synthase expression for an infarct in an animal that received the control vehicle (left panel) and an infarct in an animal that received the FAA drug treatment (right panel).

Endothelial Nitric Oxide Synthase Summed Area (10 sections)

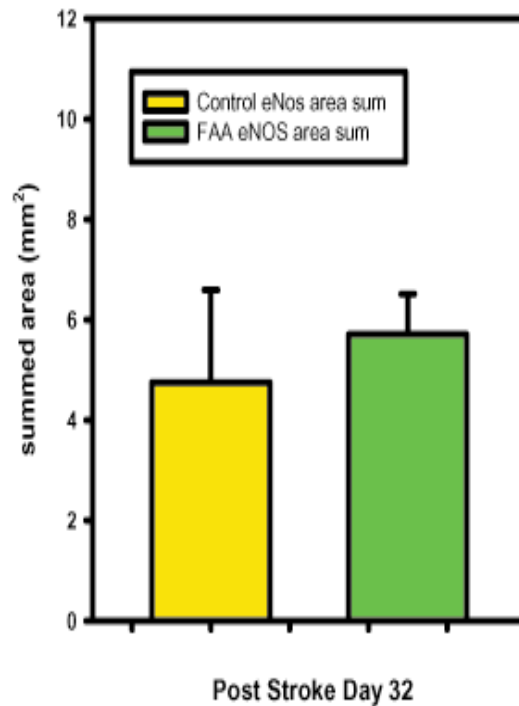


Figure 26 eNOS Total Summed Tissue Area. This graph shows that there was not a statistical difference between the total tissue area sum for control and FAA treated animals. It was thought that because the control animals generally had larger infarcts that the total tissue area in montages would be larger as well. With a P value of 0.497, this is not the case. n=4 for control, n=5 for FAA

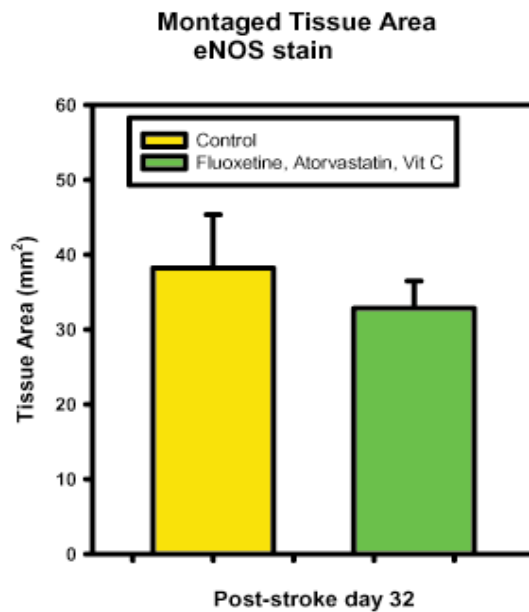


Figure 27 eNOS Sum. This graph shows that there was not a statistical difference between the eNOS sum from all montages for control and FAA treated animals. There is not yet a statistical difference shown between eNOS expression between the two groups ($P=0.622$). $n=4$ for control, $n=5$ for FAA

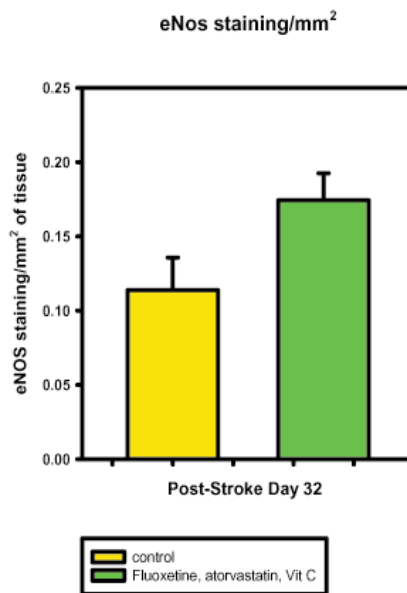


Figure 28 eNOS density (staining/mm²). This graph shows a strong trend towards increased eNOS staining for FAA treated animals, which points to increased angiogenesis ($P=0.070$, power=0.365). This graph account for the total tissue area sum from each montage because the eNOS sum was divided by the tissue area sum. This is the most accurate representation of the eNOS staining for each group (control, FAA). $n=4$ for control, $n=5$ for FAA

RAT 364			
Section #	Tissue Area	eNOS Sum	eNOS Density
1	4.650	0.523	0.112
2	2.502	0.289	0.115
3	3.363	0.262	0.077
4	3.272	0.334	0.102
5	4.349	0.436	0.100
6	2.616	0.175	0.066
7	2.684	0.131	0.049
8	4.988	0.388	0.077
9	3.554	0.260	0.073
10	3.660	0.253	0.069
Sum	35.638	3.051	0.844
Average	3.5638	0.305	0.084

Table 5 eNOS Results for Rat 364, control animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 368			
Section #	Tissue Area	eNOS Sum	eNOS Density
1	7.013	0.472	0.067
2	2.618	0.204	0.077
3	5.707	0.432	0.075
4	2.566	0.150	0.058
5	3.287	0.230	0.069
6	2.675	0.306	0.114
7	4.344	0.422	0.097
8	3.600	0.478	0.132
9	3.534	0.494	0.139
10	2.589	0.393	0.151
Sum	37.933	3.581	0.979
Average	3.7933	0.3581	0.097

Table 6 eNOS Results for Rat 368, control animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 371			
Section #	Tissue	eNOS Sum	eNOS
	Area		Density
1	2.496	0.165	0.066
2	2.794	0.318	0.113
3	2.393	0.220	0.091
4	3.164	0.390	0.123
5	2.320	0.192	0.082
6	1.180	0.098	0.083
7	3.110	0.320	0.102
8	1.081	0.123	0.113
9	1.451	0.104	0.071
10	2.360	0.218	0.092
Sum	22.349	2.148	0.936
Average	2.2349	0.214	0.093

Table 7 eNOS Results for Rat 371, control animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 380			
Section #	Tissue	eNOS Sum	eNOS
	Area		Density
1	5.355	0.776	0.144
2	7.017	1.070	0.152
3	6.532	1.670	0.255
4	4.455	0.900	0.202
5	7.058	1.230	0.174
6	7.165	1.490	0.207
7	4.521	0.777	0.171
8	3.732	0.472	0.126
9	6.618	1.100	0.166
10	4.528	0.731	0.161
Sum	56.981	10.216	1.758
Average	5.6981	1.021	0.175

Table 8 eNOS Results for Rat 380, control animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 366

Section #	Tissue		
	Area	eNOS Sum	eNOS Density
1	5.168	0.685	0.132
2	2.470	0.387	0.156
3	3.242	0.490	0.151
4	3.867	0.605	0.156
5	3.207	0.383	0.119
6	3.031	0.622	0.205
7	3.855	0.581	0.150
8	4.382	0.544	0.124
9	3.407	0.492	0.144
10	4.314	0.441	0.102
Sum	36.943	5.230	1.439
Average	3.6943	0.523	0.144

Table 9 eNOS Results for Rat 366, FAA treated animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 369

Section #	Tissue Area	eNOS Sum	eNOS Density
1	2.704	0.521	0.192
2	2.069	0.327	0.158
3	2.980	0.578	0.193
4	3.539	0.660	0.186
5	3.338	0.625	0.187
6	2.940	0.874	0.297
7	2.737	0.522	0.190
8	2.759	0.824	0.298
9	3.479	0.586	0.168
10	2.758	0.570	0.206
Sum	29.303	6.087	2.075
Average	2.9303	0.608	0.208

Table 10 eNOS Results for Rat 369, FAA treated animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 372

Section #	Tissue Area	eNOS Sum	eNOS Density
1	2.750	0.739	0.268
2	3.393	1.300	0.383
3	1.680	0.563	0.335
4	3.323	0.693	0.208
5	3.358	0.633	0.188
6	1.935	0.354	0.182
7	1.287	0.205	0.159
8	3.420	0.388	0.113
9	1.043	0.147	0.140
10	3.690	0.489	0.132
Sum	25.879	5.511	2.108
Average	2.5879	0.551	0.211

Table 11 eNOS Results for Rat 372, FAA treated animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 378

Section #	Tissue Area	eNOS Sum	eNOS Density
1	1.381	0.113	0.081
2	2.212	0.584	0.264
3	2.517	0.260	0.103
4	2.392	0.333	0.139
5	1.979	0.264	0.133
6	3.122	0.290	0.092
7	2.205	0.257	0.116
8	2.571	0.270	0.105
9	4.511	0.425	0.094
10	3.149	0.354	0.112
Sum	26.039	3.150	1.239
Average	2.6039	0.315	0.124

Table 12 eNOS Results for Rat 378, FAA treated animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

RAT 381

Section #	Tissue Area	eNOS Sum	eNOS Density
1	3.057	0.336	0.109
2	3.091	0.353	0.114
3	4.575	1.960	0.428
4	3.957	0.689	0.174
5	3.463	0.527	0.152
6	5.777	0.983	0.170
7	4.407	0.497	0.112
8	4.171	0.877	0.210
9	3.481	0.411	0.118
10	7.761	1.580	0.203
Sum	43.740	8.213	1.790
Average	4.3740	0.8213	0.179

Table 13 eNOS Results for Rat 381, FAA treated animal. Results for the total tissue area, eNOS Sum, and eNOS Density for each of the ten montages analyzed for this animal. Sum and average for each column shown at bottom of table.

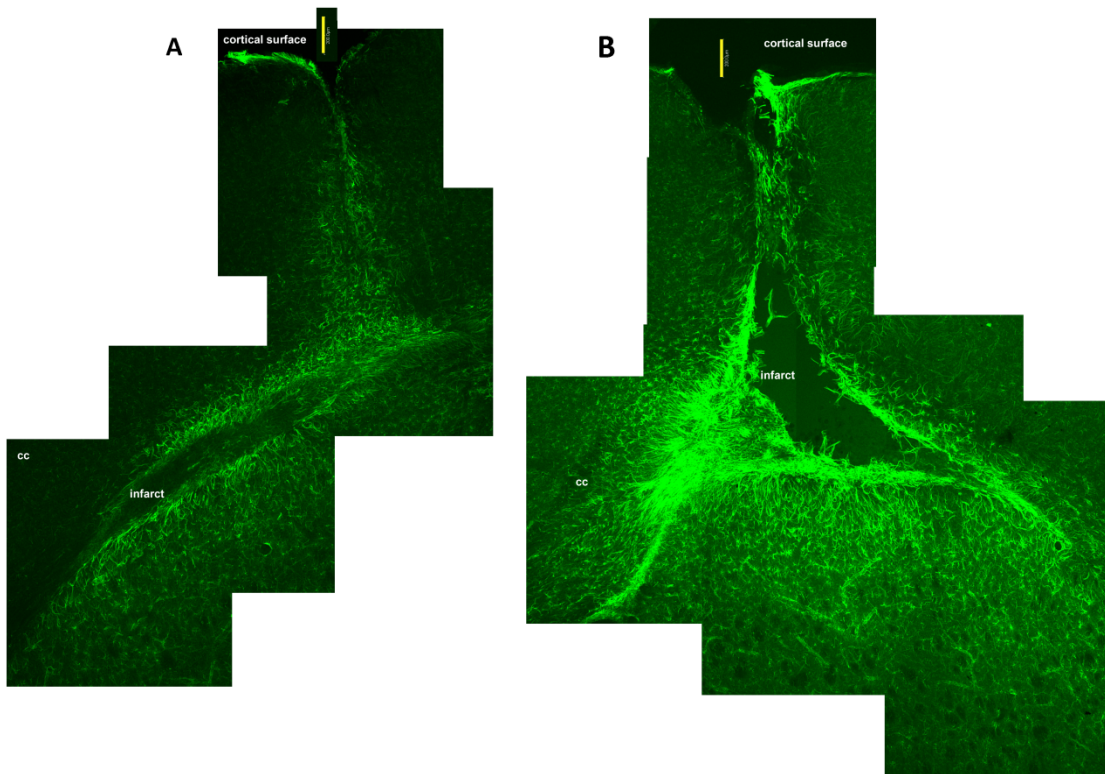


Figure 29 eNOS Montages. **A.** This is a sample montage from control Rat 371. The intensity of the eNOS staining is lower when compared to that of the montage on the right. Cortical surface is at the top. **B.** This is a sample montage from an FAA treated animal, Rat 381. There is very robust eNOS staining surrounding the infarct. Cortical surface is at the top. Scale bar (yellow) is 200 μ m. Black area above tissue was not counted in tissue area measurements.

Glial Fibrillary Acidic Protein Expression

Glial fibrillary acidic protein (GFAP, stains for stem cells and glia) was used to observe changes in glial scarring between control and FAA drug treated animals. Table 5 shows results for specific animals that went into producing the following figures (n=7 for control, n=7 for FAA). The tissue area average is a measure of the total tissue area in the montages of each rat. These results were gathered to be able to account for total area when observing fluorescence. The GFAP average is the average of GFAP fluorescence in montages for each individual rat. The final column, GFAP staining/mm was created by dividing the GFAP average value by the total tissue area average. This is the GFAP density per rat within this montage. While all of the rats included in eNOS analysis had ten brain sections with infarcts to be analyzed, one animal analyzed for GFAP had only six brain sections with infarcts to analyze. Because of this, instead of using the total tissue area sum and the GFAP sum (as we did with the total tissue area sum and the eNOS sum) we used the total tissue area average and the GFAP average to account for the different number of infarcts analyzed for each animal. Figure 30 shows average tissue area per brain slice found in the montages for GFAP fluorescence for control (yellow bar) and FAA treated (green bar) animals (P= 0.671, t test). The mean tissue area average for control animals is 3.418 mm while the mean tissue area average for FAA treated animals is 3.198 mm. As the statistics show, there was not a statistical difference between the

average tissue area for control and FAA treated animals. Figure 31 shows the average GFAP staining (P= 0.654). The yellow bar represents control animals and the green bar represents FAA treated animals. The mean GFAP staining for control animals is 0.386 mm and the mean GFAP staining for FAA treated animals is 0.429 mm. Again, there is no statistical difference in the average GFAP staining between control and FAA treated animals. Figure 32 shows the GFAP staining normalized for differences in tissue area, as density of GFAP staining/mm², with yellow bar representing control animals and green bar representing FAA treated animals (P= 0.183, t-test). The mean GFAP staining/mm² for control animals is 0.109 while the mean for FAA treated animals is 0.136. Although not statistically significant, there is a trend that shows increased GFAP expression in animals that received the drug combination which is opposite of what was expected. Figure 33 shows montages for representative glial fibrillary acidic protein expression for an infarct in an animal that received the control vehicle (left panel) and an infarct in an animal that received the FAA drug treatment (right panel). Table 15 shows the results for the GFAP morphology data. Next to rat ID is information on whether or not there was GFAP expression near the infarct or spread out in the tissue away from the infarct. When this information was analyzed using a Fischer's Exact Test it was shown to be significant with a P value of 0.0291.

Rat	Tissue Area Average	Average GFAP Area/Slice	Average GFAP staining/mm ²
364	3.563	0.231	0.064
368	3.793	0.317	0.083

371	2.234	0.152	0.068
380	5.698	0.847	0.148
387	2.446	0.283	0.115
391	2.785	0.417	0.150
393	3.409	0.456	0.133
366	3.568	0.539	0.151
369	2.663	0.557	0.209
372	2.587	0.336	0.129
378	2.603	0.299	0.114
381	4.374	0.492	0.112
388	3.441	0.348	0.101
392	3.155	0.437	0.138

Table 14 Various GFAP results for individual animals, control animals highlighted in yellow at top of table. Column 1 shows rat ID number. Column 2 shows the average tissue area for all montages from each rat. Column 3 shows the average GFAP sum for all montages for each rat. Column 4 shows the GFAP density which was gathered by dividing the GFAP average for each animal by the average tissue area for each animal. All values in mm.

GFAP Stain Montaged Tissue Area Average (per slice)

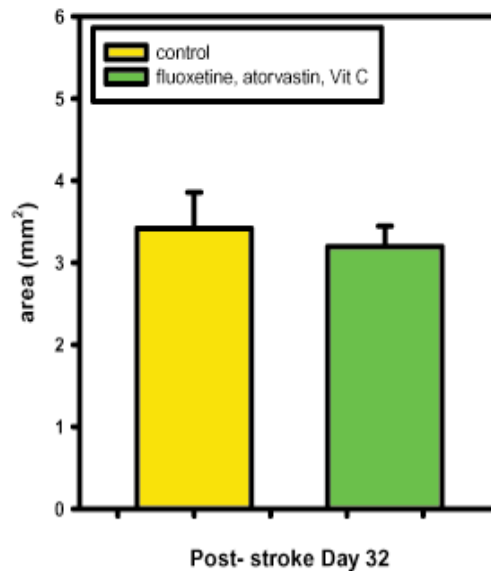


Figure 30 GFAP Average tissue area. This graph shows that there was not a statistical different between the average tissue area for control and FAA treated animals. It was thought that because the control animals generally had larger infarcts that the average tissue area in montages would be larger as well. With a P value of 0.671, this is not the case. n=7 for control, n=7 for FAA

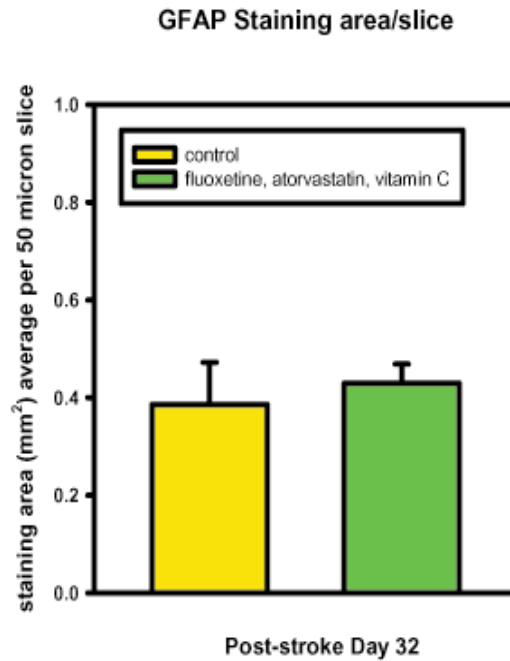


Figure 31 GFAP Average. This graph shows that there was not a statistical different between the GFAP average from all montages for control and FAA treated animals. There is not yet a statistical different shown between GFAP expression between the two groups ($P=0.654$). $n=7$ for control, $n=7$ for FAA

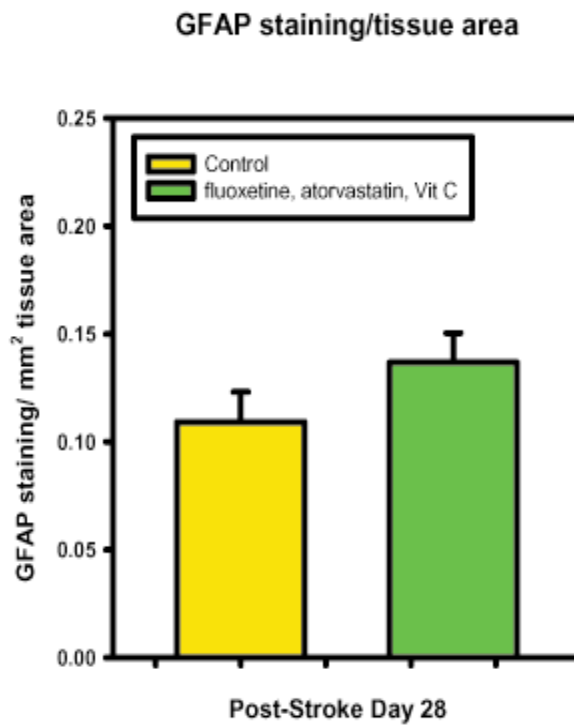


Figure 32 GFAP density (staining/mm²). This graph shows a trend towards increased glial scarring in animals that received the drug combination, which was not expected (P=0.183). This graph accounts for the average tissue area from each montage because the GFAP average was divided by the average tissue area. This is the most accurate representation of the GFAP staining for each group (control, FAA). n=7 for control, n=7 for FAA

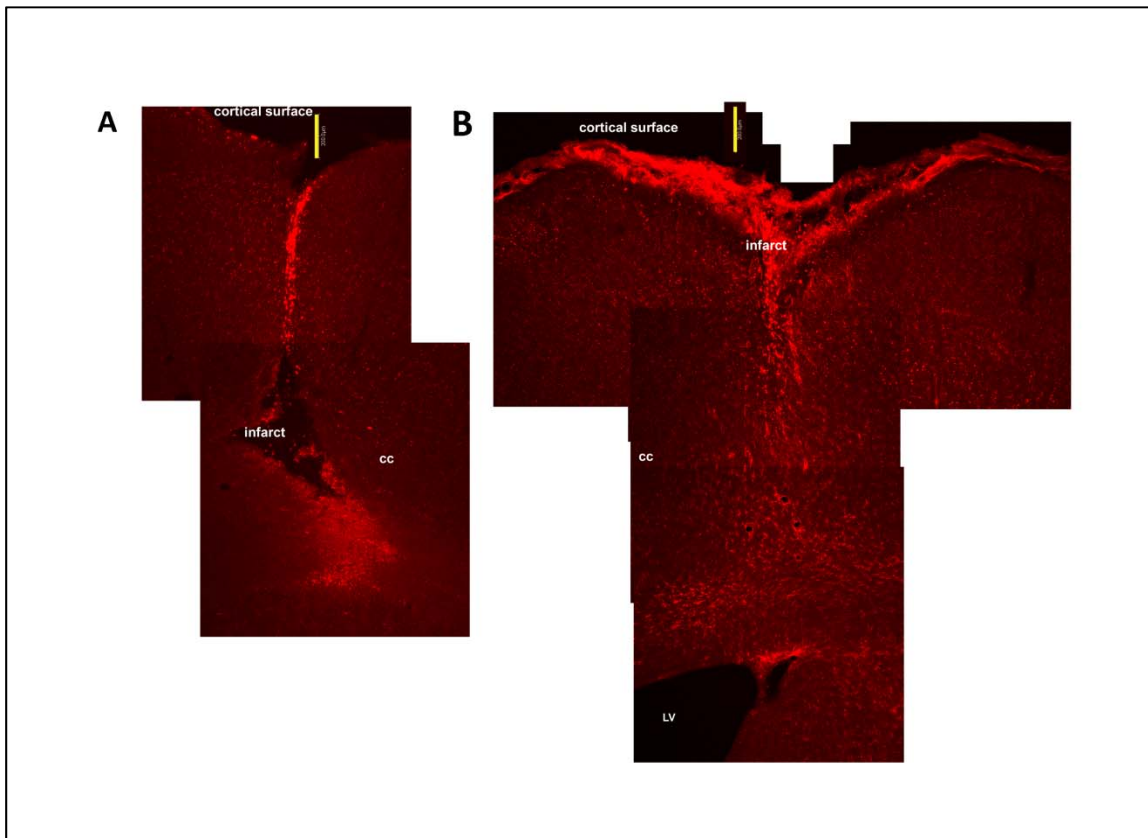


Figure 33 GFAP Montages. **A.** This is a sample montage from a control animal, Rat 371. There is GFAP expression seen around the infarct, but not as much as is seen in the FAA treated animals. Cortical surface is at the top. **B.** This is a sample montage from an FAA treated animal, Rat 369. There is slightly increased GFAP expression when compared to control. The staining near the ventricle represents progenitor cells, not glia. Cortical surface is at the top. Lateral Ventricle shown at bottom. Scale bar (yellow) is 200µm. Black area above tissue was not counted in tissue area measurements

Rat ID#	GFAP Expression Near Infarct	Diffuse GFAP Expression
364	Yes	No
368	Yes	No
371	Yes	No
380	Yes	No
387	Yes	No
391	No	Yes
393	Yes	No
366	No	Yes
369	No	Yes
372	No	Yes
378	No	Yes
381	Yes	No
388	No	Yes
392	No	Yes

Table 15 GFAP Morphology. Control animals shown in yellow. This table shows results for each individual rat analyzed for GFAP and whether or not they showed the most intense GFAP staining near the site of the infarct or the most intense GFAP staining in the tissue surrounding the infarct (not at the site of injury). P=0.0291

IV. DISCUSSION

Functional Analysis

Two different strains of rats were used to produce the results in this thesis. Not only did the strain differ between these animals, the stereotactic coordinates for the stroke survival surgery and the functional deficit requirements were also different. For Long Evans, a 30% deficit was required to be included in the functional analysis and the coordinates used were farther apart (AP: 0.0 and 2.3 mm). When Sprague Dawley animals underwent stroke surgeries, the coordinates were changed (AP: 0.0 and 1.5) to shorter distances away from each other. Smaller infarcts were seen in these Sprague Dawley animals which led to smaller deficits than the Long Evans animals. That is why the deficit requirement for Sprague Dawley animals was reduced from 30% to 20%. In the Long Evans rats, the mean control infarct volume was 9.631 (+/-4.59 SD) mm³ and the drug combo mean infarct volume was 5.469 mm³ (+/-3.35 SD). These were not significantly different from each other. In the Sprague Dawley rats the mean control infarct volume was 2.818 (+/-2.41 SD) mm³ and the drug combo mean infarct volume was 1.556 mm³ (+/-1.04 SD). The difference in infarct volume speaks directly to the difference in deficit seen between the two strains of animals. The difference in infarct volume could be caused by different anatomy in the two strains. The Long Evans rats had larger infarcts which could mean that their arteries have less collateralization than Sprague Dawley rats. If the main arteries branch in such a way that blood flow is maintained proximal to the occlusion, areas that are not receiving blood flow because of the clot (endothelin induced in this case) are saved. It would make sense that Long Evans rats don't have this branching which leads to greater damage (infarct volume). Whatever

the reason, the endothelin induced strokes affected the strains differently. Possibly because of this, there was also a difference in the functional recovery that was seen. The Long Evans rats, which received the FSA treatment, did show significant recovery of function. The Sprague Dawley rats, which received the FAA treatment, did not. The difference in infarct volume, and thus deficit, could have changed the way the drug recovered the function in both the Montoya Staircase and Forelimb Asymmetry.

Another difference between the two sets of rats was the chemical make-up of the drugs used to treat them. Drugs are made up of enantiomers. Enantiomers are stereoisomers that are mirror images of each other. There are R and S enantiomers which specify the orientation of the isomer. It is possible that only one enantiomer is responsible for causing the physiological effects on the body that is intended by a drug. The other enantiomer can either be less active or completely inactive and in some cases can even result in unfavorable effects. Pharmaceutical drugs can have differing percentages of enantiomers based on whether or not they are of brand name or generic origin. Fluoxetine is made up of R-fluoxetine and S-fluoxetine, both of which are active enantiomers. However, the metabolites of these enantiomers, R-norfluoxetine and S-norfluoxetine respectively, differ in activity (only S-norfluoxetine is active) and have a much longer half-life than the original fluoxetine enantiomers. Brand name Prozac™ is made with equal amounts of R-fluoxetine and S-fluoxetine. Generic drugs are not regulated on the amount of the specific enantiomers and through HPLC analysis with a chiral column, Dr. Audrey McGowin (Chemistry Dept., Wright State University) discovered that the generic drugs we used in this series of experiments had a 15% larger amount of the R-fluoxetine compared to the S-fluoxetine, which would produce more of

the long-lasting inactive metabolite. Both the Long Evans and Sprague Dawley animals were treated with generic fluoxetine from two different suppliers. For generic drugs, the chemical make-up can change from batch to batch without the FDA being notified. If the fluoxetine used in the Long Evans animals was made up of the more active enantiomer which is metabolized to a long-lasting active metabolite then it would make sense that these animals showed functional recovery while the Sprague Dawley animals did not. Simvastatin is a semi-synthetic statin (containing the monacolin J molecule structure) and atorvastatin is a synthetic statin and they have severely different structures. Although there is not much information on how these structures could be leading to differing physiological effects, one study did compare nine different statins (including simvastatin and atorvastatin) in the hopes of finding differences between them when it comes to neuroprotective capabilities. It was shown that natural and semi-synthetic (simvastatin) statins work best because they have increased penetration of the BBB and prevention of cell death through okadaic acid. Additionally, the paper came to the conclusion that simvastatin is the best option when treating cerebral diseases (43).

The other possibility that could explain the failure of the Sprague Dawley animals to recover is that because of the differences in the strain, coordinates, and drug combination make-up, these animals required longer treatment. There was a clinical trial conducted using fluoxetine following cerebral brain ischemia. The drugs were given 5-10 days following stroke and the treatment continued for 90 days. Using the Fugl-Meyer motor scale, motor function was analyzed pre- and post-treatment with fluoxetine. The fluoxetine was shown to significantly improve the FMMS ($P=0.003$) at 90 days but not at 30 days (44). Longer treatment using the drug combination could have resulted in

significant increases in the eNOS expression and reduction of the infarct volume which could have eventually led to functional recovery.

Angiogenesis

The strong trend towards increased eNOS production in Sprague Dawley rats treated with the FAA drug combination was encouraging ($P=0.070$). Thus, we look to explain why we saw an increase in eNOS and try to explain why it wasn't significant. As mentioned in the Introduction, statins and fluoxetine have the ability to increase vascular endothelial growth factor (VEGF) which has many downstream effects. Most importantly, VEGF leads to phosphorylation and activation of the Akt pathway. In turn, we see increased levels of eNOS. If both drugs had been shown to be responsible for this increase, VEGF could be the reason there is a trend that shows increased eNOS in brains where rats had been treated with the FAA drug combination. The question now is whether or not different statins lead to changes in upregulation of VEGF. Although data is not available at this time, it is thought that perhaps the FSA drug combination would show a significant difference in eNOS fluorescence between control and drug treated animals because the FSA combination worked to significantly recover motor function. It makes sense that a drug combination that had more integrity in recovering motor function would also produce greater results in other areas. Finally, are the effects of the fluoxetine and the atorvastatin additive in respect to the eNOS expression (as they were with the doublecortin expression) or synergistic?

Neurogenesis

Upregulation of VEGF also helps the proliferation of progenitor cells. Together with BDNF, it could be responsible for increased neurogenesis (as indicated by a trend towards smaller infarct volumes in FAA treated rats). Similar to VEGF, increased BDNF has been shown with statins and fluoxetine. Statins act to increase tissue plasminogen activator in endothelial cells, which has been shown to cleave pro-BDNF into BDNF. If we have increased VEGF, which helps neural stem cells progress through the growth phase, and increased BDNF, which helps aid in the survival of newborn neurons, we would expect to see increased neurogenesis. The reason for this assumption stems from the fact that it has been proven that neurogenesis is aided by many different growth factors (45). If we have this increase in growth factors resulting from the drug combination, we would expect an increase in neurogenesis and thus smaller infarct volumes in the FAA treated animals. Although the results were not significant ($P=0.072$), there was a strong trend showing just that. It has been shown that neurogenesis increases, even in adult animals, following injury, but it is short lived. Thus, it is to be assumed that the neurogenesis in the FAA treated animals was heightened and prolonged, leading to less cortical damage. The initial goal of this drug treatment was to increase neurogenesis in adult animals, which it did. The next attempt was to increase it over a longer period of time in adult animals following ischemic stroke, and the trend showing reduced infarct volume at the end of the 32 days study moves to suggest that that goal was met. Again it should be mentioned that, for various reasons already discussed, the FSA combination may have led to significant results in regards to infarct volume.

Glial Scar Morphology

The decreased infarct volume and increased eNOS expression (not significant) in response to the FAA drug treatment were hoped for. Another thing hypothesized was decreased GFAP expression in FAA treated animals. However, this was not seen. In fact, there was a trend ($P=0.183$) showing the opposite, increased GFAP expression in FAA treated animals. When montages were analyzed to try to understand the morphology of the GFAP it was seen that there was a significance in the expression separating the control animals from the FAA treated animals ($P=0.0291$). Six out of the seven control animals analyzed were shown to exhibit GFAP expression near the infarct which is indicative of a glial scar. Six out of the seven FAA treated animals analyzed were shown to exhibit GFAP expression in tissue surrounding the infarct. GFAP was included in analysis because it has been shown that intense GFAP expression can be associated with the glial scar (46). The same paper that reported that also stated that the presence of GFAP decreased in the areas away from the injury, which is opposite of what is seen in FAA treated animals because they have labeling in the surrounding tissue (46). Glial scars form largely from an accumulation of astrocytes which are responsible for producing high levels of GFAP which forms a dense network within the scar (47). A glial scar is not favorable for axon sprouting or angiogenesis and it has been shown that aged rats develop glial scars earlier and more robustly following MCAO when compared to young rats (48). However, this paper did not move to explore the ideas behind the formation of the glial scar so little information is known about how the FAA drug combination could be acting to decrease the glial scars presence in the brains of the drug treated rats while the control animals still show labeling that is indicative of scars. One possibility that explains the GFAP expression in surrounding tissue in the FAA treated

animals stems from the fact that astrocytes have been shown to have processes that wrap around blood vessels in the cortex to maintain their stability (49). The FAA drug combination did show a trend towards increased angiogenesis so maybe the creation of new blood vessels was causing astrocytes to migrate to these areas more robustly than in control animals. As stated, additional research needs to be conducted to understand the reason for the increased glial scar seen in the control animals and increased GFAP expression in the tissue surrounding infarcts of the FAA treated animals. Again, it could also be due to the fact that the fluoxetine used in the FAA combination was of generic origin which has 15% more of the inactive enantiomer, R-fluoxetine.

Conclusion

Strong trends indicating reduced infarct size (increased neurogenesis) and increased eNOS expression (increased angiogenesis) in FAA treated rats suggests there is some promise in this drug combination as a delayed pharmacological treatment for ischemic stroke. Significant differences in morphology of GFAP fluorescence (which showed less glial scarring following treatment) could be helpful in reaching functional recovery in the future. Further research is needed to fully explain the lack of functional recovery. The differences in the results seen for the two drug combinations could be due to the differences in the brand name fluoxetine and generic fluoxetine and further research needs to be done using strictly brand name fluoxetine to decide if replacing simvastatin with atorvastatin is a viable option.

Future Studies

The future of these studies has important clinical relevance and the current results can move to shape what happens in the future. We see trends for increased eNOS expression and decreased infarct areas without functional recovery. All three of these need to be compared so that a connection can be drawn between the three. We were able to produce reliable deficits and see functional recovery in the Long Evans animals of this study but not in Sprague Dawley animals using 400 pmoles of endothelin per injection site at the given stereotactic coordinates. We think the change in rat strain proposed a problem because of different blood vessel collateralization seen within the brains of the two strains: the same amount of endothelin produced much larger deficits in the Long Evans strain. It is thought that Sprague Dawley animals, which showed signs of having higher collateralization, are closer to what is seen in humans. Moving forward, Sprague Dawley animals need to be held as the optimum animal model for studying endothelin-induced strokes as long as the surgery can be altered in order to produce deficits like those seen in Long Evans; current work in the laboratory has achieved this with 600 pmoles endothelin injection per site. The other thing that needs to be modified is the supply of fluoxetine used. The FDA requires any changes made to brand name drugs be reported so either the fluoxetine needs to always be brand name or the chemical make-up (racemic enantiomers) of the generic drugs needs to be recorded. The time the animals are on the drugs could also be increased especially since more time on the drugs could mean more functional recovery. The only way a true comparison between simvastatin and atorvastatin can be made is by using the same rat strain, same drug supplier, and same stroke surgery coordinates. Once all of these things are held stable, the differences

in statins but also the connections between eNOS, infarct volume, and possibly even GFAP morphology can begin to be understood more clearly. In making correlations with changes in infarct size, angiogenesis and glial scarring, it is important to separate animals based on whether they were included in the functional analyses or not. Separating animals by whether they met deficit criteria or not and then correlating any changes in these immunological findings would enable one to better understand the changes necessary for functional recovery.

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