Effect of Rat Strain Stereotactic Coordinates on Infarct Volume

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Effect of Rat Strain and Stereotactic Coordinates on Infarct Volume

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

By

SAAGAR KAMLESH SANGHVI
B.S.E., University of Michigan, 2010

2013
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Saagar Sanghvi ENTITLED Effect of Rat Strain and Stereotactic Coordinates on Infarct Volume BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Ischemic stroke makes up 87% of all hospital-admitted stroke cases annually; the primary treatment for these cases is intravenous administration of tPA within a 3.5 hour window from stroke onset. A long-term delayed ischemic stroke treatment proposed by this study was a combination of the pharmaceuticals Fluoxetine (SSRI), Simvastatin (statin), and ascorbic acid (Vitamin C). 51 adult rat subjects (10-12 months of age; 44 Sprague Dawley, 7 Long Evans) were given a combination of the drugs for 31 days. Drugs were given through voluntary oral administration via sugar cookie-dough balls to reduce inhibition of neurogenesis through stress-related glucocorticoid production. Drug combinations were as follows: FSA - 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin and 20 mg/kg ascorbic acid; FS - 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin; and the vehicle control. Endothelin-induced cortical stroke was administered using 2 different set of coordinates relative to bregma: Group 1 - (AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm); Group 2 - (AP: 0.0 mm, ML: -2.5mm) and (AP +2.3; ML -2.5 mm). To analyze functional deficit, rats were subject to Montoya Staircase functional test once pre-stroke and twice post-stroke, and the Forelimb Asymmetry functional test once pre-stroke and thrice post-stroke. Results showed that Long Evans rats sustain a significantly larger infarct volume compared to Sprague Dawley rats using Group 2 coordinates; Group 1 cortical injection coordinates produced a larger infarct than Group 2 coordinates.
in FSA Sprague Dawley rats; drug treatment showed no effect on total infarct volume, however, this may be attributed to use of generic fluoxetine in Group 2 Sprague Dawley rats.
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INTRODUCTION

Problem: Stroke

As the 2nd leading cause of death in the world surpassed only by ischemic heart disease, Cerebrovascular disease in the US claims 133,000 lives every year (NSA, 2012). Almost 800,000 strokes will occur nationwide this year alone – once every 40 seconds, causing a fatality approximately every 4 minutes (NSA, 2012). Stroke is defined as an acute condition: blood supply to the brain is altered in affected areas due to a vascular blockage or hemorrhaging vessel, and the resulting tissue ischemia can cause cell death within minutes of stroke onset. However, with 7,000,000 adult stroke survivors – two-thirds of who endure some type of stroke-related disability everyday – the effects of stroke are chronic (VL, 2012). Visual impairments, paralysis, aphasia, and other sensory and motor deficits can result from brain tissue damage. American patients' average direct cost of care for 90 days post-stroke is greater than $15,000, with additional costs from rehabilitation and preventative medications that can last years. The US currently spends $43 billion on stroke a year (Di Carlo, 2009). Considering three-fourths of all stroke patients are older than 65 years, a population expected to grow 200% worldwide over the next 30 years, the healthcare system itself faces a long-term economic problem in addition to the growing human cost (AHA, 2012).

What is a stroke and how is it treated?

There are two defined types of stroke– ischemic and hemorrhagic. Ischemic strokes account for the vast majority (87%) of all stroke cases (AHA, 2012). This
condition results from gradual atherosclerotic plaque and fatty-acid deposit accumulation in a brain blood vessel. These blockages either originate at the site (thrombotic stroke) or are transported from another location in the body vasculature (embolic stroke). Risk factors for an ischemic attack include hypertension, high cholesterol, diabetes, obesity, alcoholism, and conditions such fibromuscular dysplasia and atrial fibrillation (NSA, 2012). Less common hemorrhagic strokes result from a blood vessel rupture in or on the surface of the brain; treatments associated with this condition promote blood coagulation and reducing brain pressure.

The primary treatment for an ischemic stroke attack is administration of recombinant Tissue Plasminogen Activator (rt-PA) within a 3.5-hour window of stroke onset. tPA, a natural human enzyme found in endothelial cells lining blood vessels, activates plasminogen in order to dissolve blood clots. rt-PA is most commonly injected through a vein in the arm, but can also be delivered directly to the infarct site via catheter. Up to 70% of hospital stroke admissions are for ischemic stroke; however, only 3.5 to 8% of these patients are able to receive the rt-PA clot-buster within the 3.5-hour administration window. Most admitted ischemic stroke patients must seek an alternate method of treatment (Mayo Clinic, 2010).

The vast majority of diagnosed ischemic stroke patients are prescribed aspirin for for its blood-thinning effect, primarily to prevent another stroke. Other blood-thinning drugs such as warfarin (Coumadin), heparin and clopidogrel (Plavix) are also options for preventative stroke treatment, but the presence of aneurysms or other risk factors for hemorrhagic stroke must be taken heavily into consideration (Mayo Clinic, 2010). Invasive surgical procedures such as stent angioplasties and carotid endarterectomies are
useful only for a small percentage of stroke patients. Most stroke patients have numerous surgical complications and therefore increased probabilities of triggering another ischemic attack, weak vessels hemorrhaging, or recurrent vessel stenosis. Considering that 25% of victims will have a repeat incidence within 5 years, and more than 50% of all ischemic stroke victims will die in 8 years, a need for an effective long-term stroke treatment aside from prescribing aspirin remains (ISC, 2012).

A Drug-Cocktail for Delayed Treatment of Ischemic Stroke

A treatment model that increases the window of time available to administer rt-PA or aides in developing a long-term treatment solution needs to either diminish the onset of ischemic stroke or curb the effects of stroke after onset. Treatment strategies examining various elements of the ischemic stroke cascade, including calcium channels and glutamate receptors, have either been unsuccessful or are currently undergoing trials (Shabitz, 2006). A probable strategy for treating neural sensory and motor deficits caused by stroke, however, can be related to curing the damaged brain tissue itself. Growth of neurons and glial cells have been linked to various growth factors that may also play a role in repairing damaged tissue (Allen, 2006). Up-regulating factors involved in neurogenesis or growth factor production may play a role in treating brain deficits related to stroke.

Two germinal niches have been identified as regions of neurogenesis: the subventricular zone (SVZ) and the subgranular zone (SGZ). The SVZ is found adjacent to the lateral ventricles of the forebrain (see Figure 1), and the SGZ is found within the dentate gyrus of the hippocampus. Self-renewing, multipotent cells that have been shown to create various neural cells are found in both regions; however, the SVZ is the largest
shown to be the largest neurogenic niche in the adult brain (Quinoneshinojosa, 2007).

The subventricular zone (SVZ) contains multipotent neural stem cells (type B cells) that have been identified as the neural progenitor cell (Gonzales-Perez, 2012). This self-renewing astrocytic type B cell can then generate self-renewing “transit-amplifying” progenitor cells (type C cells) (see Figure 2). These type C

Figure 1. (A) Rat brain: Neurogenic Centers, Rostral Migratory Stream leading to olfactory bulb Image from Scientopia.org
(B) Human Brain: Subventricular Zone (SVZ), adjacent to the lateral ventricles Image from Oscar Arias-Carrión, 2008

Figure 2. Self-renewing multipotent neural stem cells (type B) can differentiate into transit-amplifying cells (type C). Self-renewing type C cells can differentiate into migrating neuroblasts (type A). Image from Gonzales-Perez, 2012
cells may then differentiate into various migrating neuroblasts (type A cells), that develop into end cells such as neurons, oligodendrocytes, and astrocytes. Type B cells are made up of Type B1 and Type B2 astrocytes. Type B1 cells associate closely with the ependymal layer through adherens and gap junctions, and the brain parenchymal blood vessels and basal lamina. Their apical processes extend to the ventricular surface (Gonzales-Perez, 2012). These associations have suggested that type B cells may play a role in neurogenesis through direct interaction and supporting growth. The newly formed type A neuroblasts migrate to the olfactory bulb along the rostral migratory pathway. Type B1 cells express molecular markers associated with their astrocytic morphology, a few of which are glial fibrillary acidic protein (GFAP), nestin, and vimentin. Type C cells can be identified using epidermal growth factor receptors (EGFR). Type A-cell neuroblasts can be identified with doublecortin (Zhang, 2004).

Growth factors associated with neurogenesis include epidermal growth factor (EGF) and the family of neurotrophins, specifically brain-derived neurotrophin (BDNF). EGF has been shown to support proliferation of SVZ neural progenitors such as type B1 cells (Gonzales-Perez, 2009); BDNF has been shown to support existing neurons while promoting neurogenesis and synaptogenesis (Mattson, 2008).

The primary difference between the human and rodent subventricular zone is that in humans, the 2nd SVZ layer (hypocellular gap) is devoid of cell bodies, and 3rd layer astrocytes are organized as a ribbon of GFAP+ cells that do not contact the ependymal layer (see Figure 3). In both species, the SVZ 1st layer is made of multi-ciliated ependymal cells that regulate substance such as cerebrospinal fluid at the ventricular surface. The 4th layer has myelin sheaths that act as a transition zone into the brain
A drug cocktail developed to repair damaged brain tissue can work through promoting neurogenesis or the production of growth factors shown to support neurogenesis. Statins have been commonly used for reducing cholesterol levels in preventing various artery diseases; studies have also established pleiotropic effects of statins which include inducing neurogenesis and synaptogenesis in the hippocampal dentate gyrus (Lu et al., 2007; Chen et al., 2003) up-regulation of endothelial nitric oxide synthase (eNOS) and tPA activity, and increased levels of brain-derived neurotrophic factor (BDNF) in the ischemic boundary zone after stroke. After phosphorylation and subsequent activation of eNOS, statins have been shown to increase the amount of NO, though the exact mechanism is not fully understood (Datar et al., 2010). eNOS has also been shown to up-regulate BDNF (Wu et al., 2006).

Selective-serotonin re-uptake inhibitors (SSRIs) have been used to treat depression for years, but have recently been associated with promoting neurogenesis and BDNF production. Studies have shown a marked reduction in neurogenesis when cells are damaged within the dentate gyrus in rodents. Chronic treatment with Fluoxetine
specifically has been proven to up-regulate neurogenesis in the hippocampus and the production of BDNF in rodent studies (Ploughman et al, 2009). Upregulation of BDNF is linked to serotonin binding to 5-HT subtype receptors in both the SVZ and SGZ (Benasr et al, 2003). Chollet's recent FLAME study (2011) has shown enhanced motor recovery in ischemic stroke patients after chronic fluoxetine treatment. Patient subjects were given 20 mg fluoxetine to take orally for 90 days. The treatment was started 5-10 days after stroke and was coupled with physiotherapy.

The drug cocktail in this study developed for delayed post-stroke treatment included Simvastatin (statin) and Fluoxetine (SSRI). The final component added was Ascorbic acid (Vitamin C), an antioxidant (see Figure 4). Ascorbic acid has been shown to enhance the effects of SSRIs that are otherwise very sensitive oxidative degradation (Idayu et al, 2010). Furthermore, ascorbic acid increases the effectiveness of statins by supporting the endothelial eNOS system that up-regulates BDNF (Chen et al, 2005). The majority of neurogenesis studies conducted have focused on the SGZ hippocampal niche. Therefore this study will concentrate on the more active SVZ neurogenic niche in rats in post-stroke studies.

**Stroke and Animal Model**

The Middle Cerebral Artery Occlusion (MCAo) is the most common focal
ischemia model used in young rodents (Windle et al, 2006). A modern approach to create this occlusion is by inserting an intraluminal suture through the external carotid artery, through the internal carotid artery, and up to the point of MCA opening. Another method involves a craniectomy, electrocoagulation of the MCA, and a final cut of the artery to insure occlusion (Macrae et al, 2006). The use of aneurysm clips allows for occlusion followed by immediate vascular reperfusion upon removal. The injury caused by immediate reperfusion is not completely analogous to reperfusion injury in post-stroke patients, which occurs over a timescale of hours. Other disadvantages of MCAo include: relatively variability in the amount of injury caused, the ischemic injury sometimes does not affect the motor cortex, or other arteries become inadvertently blocked with or without MCAo therefore not representing a true MCA infarct (Liu, 2011). Inadvertently blocked MCA adjacent arteries can result in feeding difficulties that will affect food and drug intake, as well as deterioration of long-term health (Windle et al, 2006). These factors can heavily influence outcomes of motor deficit studies.

**Figure 5.** A schematic representation of the various stroke models presented, induced by endothelin-1. *Image from Windle et al, 2006*
A less invasive method of inducing stroke are various ways of applying Endothelin-1 (ET-1), a potent vasoconstrctor that can produce ischemic injury when injected directly into brain tissue. ET-1 injection has shown a more consistent injury profile (Soleman et al, 2010) as well as more consistent MCA infarcts (Windle et al, 2006). ET-1 also allows for reperfusion injury to occur over several hours as opposed to clips and sutures that allow for reperfusion almost instantly after removal, thus better mimicking a human stroke (Windle et al, 2006). The amount of stroke injury can adjusted comparison to MCA infarcts (see Figure 5). Though the topical injection produces more concentrated lesions than the intracortical injection, the intracortical method creates lesions large enough for viable motor deficits. The “success rate” in this study was gauged by the number of animals that survived stroke induction surgery, coupled with a minimum of 20% forelimb function deficit in the Montoya Staircase behavioral test. The success rate was highest for intracortical injection (up to 91%)

![Figure 6.](image)

**Figure 6.** Cortical stroke induced by ET-1 resulted in the highest Montoya Staircase deficit both 1 week (A) and 1 month (B) post-surgery.

![Figure 7.](image)

**Figure 7.** Cortical stroke induced by ET-1 resulted in the highest % of ipsilateral function 1 week post-surgery (A), and remained high 1 month post-surgery (B)
depending on dosage) in comparison to both MCAo (50%) and topical injection (75%). The Intracortical injection method is also shown to maintain the highest percentage of ipsilateral forelimb function 1-week post-surgery and remains significantly higher than the contralateral limb 1-month post-surgery. Also, the percentage of Montoya Staircase deficit is highest in the intracortical injection subjects than other methods 1-month post surgery. (see Figures 6 & 7). Therefore, intracortical injection using Endothelin-1 causing a cortical stroke (Windle et al, 2006) was the developed stroke induction model modified for use by our laboratory.

In developing a viable animal model, adult rats were chosen for the study. This is because studies in younger rats have shown a near-linear increase in motor recovery in control models – adults rats do not display such rapid recovery rates (see Figure 8) (Weissner et al, 2003). Cultivating this rate of improvement in an adult rat could be correlated to an increase in neurogenesis. Additionally, human stroke occurs most often in those above age 55, and adult rat subjects around 1

**Figure 8.** Control models show rapid initial recovery in motor function in young rats

*Graphs taken from Weissner et al, 2003*
year old (2 year life-span) would have a greater correlation to the human stroke model (Cameron et al, 1982). This is another reason MCAo could not be used; adult rats do not sustain a neurogenic growth rate large enough to recover from such a large injury.

Sprague Dawley and Long-Evans rats have traditionally been used as Montoya Staircase subjects. Sprague Dawley rats were proven to be most successful in achieving pre-stroke training criteria when compared to Wistar and Long-Evans rats. Female rats were used in the study because they fulfill weight limit criteria for commercial Montoya Staircase models – 400 grams). Female weight range is 250-300 grams while males range between 450-520 grams.

**Drug Delivery Method**

An administration method had to be developed to effectively deliver the Fluoxetine, Simvastatin, and Ascorbic acid drug cocktail to rats. Because drugs were to be delivered into the bloodstream daily, an administration method that minimized stress was also essential. The physiological effects of stress include anxiety, hypertension and other cardiovascular effects, increased susceptibility to infection and other immunological effects, and increased levels of glucocorticoids released in the bloodstream (NIMH, 2012). Glucocorticoids, especially corticosterone, have been shown to inhibit neurogenesis among other neurophysical effects (Cameron et al, 1999).

A common method for daily administration of drugs is through subcutaneous injection. A recent study compared the effects on corticosteroid levels of subcutaneous injection versus oral administration of buprenorphine (Goldkuhl et al, 2008). The results showed that oral administration of buprenorphine significantly reduced corticosteroid levels while subcutaneous injection subjects showed no reduction in these hormone
levels.

Oral administration through oral gavage is a commonly used method and has been shown to be an effective method of drug delivery. This method, however, has been shown to produce esophageal injury as well as restraint related stress during administration (Atcha et al, 2010). Studies have shown that voluntary oral administration causes less distress and decreased glucocorticoid levels in comparison to using an oral gavage. This is especially pertinent for administering the stroke drug cocktail for this study because medications must be delivered into the bloodstream daily.

Oral administration through syringe or jello (Flecknell et al, 1999) are useful for medication that dissolve easily in an aqueous solution or are liquid to begin with. The drug cocktail of Fluoxetine, Simvastatin, and Ascorbic acid, however, is lipid soluble solid. Therefore, an effective vehicle for administration was found in dissolving the powder substance into Pillsbury sugar cookie-dough. Sugar cookie-dough also chosen because it was shown to completely absorb lipid-soluble drugs, it could be handled easily, and be reliably digested by rodent subjects. Alternative drug-vehicles, such as food wafers (Ferguson et al, 2009) were not as reliable throughout the process, from handling to drug delivery. Sprague Dawley rats were shown to especially digest the cookie-dough with the least failure when compared to other rat strains. In this study, high performance liquid chromatography (HPLC) analysis showed serum levels of norfluoxetine and fluoxetine 2-4 hours after drug administration (Corbett et al, 2012).
MATERIALS AND METHODS

Experimental Design

A total of 51 rat subjects – 44 Sprague Dawley, 7 Long-Evans – were used for stroke treatment testing. These two adult rat stocks (10-12 months of age) were chosen because of their efficacy in completing Montoya Staircase training (see below).

2 drug combination vehicles and 1 vehicle control, making 3 total drug treatment groups, were tested over a 1-month period; Drug combination 1: 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin and 20 mg/kg ascorbic acid (FSA); Drug combination 2: 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin (FS); Vehicle control (C).

During the endothelin-induced stroke (described below), 2 different sets of cortical injection coordinates were used with two injection site in each set; Group 1: (AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm); Group 2: (AP: 0.0 mm, ML: -2.5 mm) and (AP +2.3; ML -2.5 mm).

Animal subjects completed Forelimb Asymmetry (described below) and Montoya Staircase training within a maximum time of 2 weeks. 20 to 26 hours after stroke induction, the first drug vehicle was given through voluntary oral administration. Treatment continued daily until 31 days post-stroke, at which time the animal was euthanized. Rats were subject to the Forelimb Asymmetry functional test at 3, 16, and 28 days post-stroke induction. Rats were subject to the Montoya Staircase functional test on post-stroke days 8-10 and 29-31.

Daily conditions: Rats had ad libitum access to food and water except during
Montoya Staircase training and functional testing post-stroke. Subjects were housed in 12-hour light/12-hour dark conditions, which were approved, along with all other animal procedures, by the IACUC at Wright State University.

**Endothelin-Induced Cortical Stroke**

*Procedure prior to Endothelin Injection*

Anesthesia was induced by inhalation through rodent facial mask of 5% isoflurane; concentration was maintained at 2-2.5% throughout the stroke-induction surgery. Isoflurane was selected because animal subjects reach the surgical plane for anesthesia and recover the quickest from this inhalant in comparison to injectable anesthetics such as ketamine. Concentration levels are also more easily changed using the inhalant anesthetic versus injectable.

The rat's head was mounted in a stereotactic apparatus that utilizes non-traumatic ear bars to reduce stress. Puralube ointment was applied to the eyes to maintain moisture and the head was shaved. The surgical site at the top of skull was shaved and then cleaned in a 3-step process using proviodine, then 70% ethanal, and finally proviodine again to insure a clean incision and surgical procedure (as according to Wright State Animal-Care Protocol). The analgesic Bupivicaine (0.25%) was applied drop-wise to the midline incision in the skin. Unlike most alternative analgesics,
Bupivicaine acts locally without pervading the systemic circulation. To drill 2 holes in the skull, a 0.9 mm burr micro-drill (Fine Science Tools) was used. The drilling coordinates for two holes in the skull relative to bregma for Group 1 were (AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm), and coordinates for Group 2 were (AP: 0.0 mm, ML: -2.5 mm) and (AP +2.3; ML -2.5 mm) (see Figure 9).

Endothelin Injection and Post-Injection Procedure

Using a 10 microliter Hamilton syringe, 1 microliter of endothelin solution was injected at a depth of 2.3 mm (Long Evans) or 2.0 mm (Sprague Dawley) into the drilled holes. The endothelin solution (Human and Porcine ET-1, EMD Chemicals) was maintained at a concentration of 400 pmoles/microliter and the injection took place over a 2-minute time period (modified from procedures described in Windle et al, 2006).

Vicryl resorbable sutures resealed the incision, and the surgical area was again coated with provoidine. Analgesics were not administered post-surgery as they have been demonstrated to promote or inhibit growth of neural progenitors (Hoehn et al, 2005; Sasaki et al, 2003; Sargeant et al, 2008).

Voluntary Oral Drug Administration

Purchased pharmaceuticals of Simvastatin (80 mg tablet) and Fluoxetine HCl (20 mg capsule) were used as the primary components for stroke drug treatment. The amount of active ingredients within each pharmaceutical correlates to the total drug weight by a...
specific correction factor; this factor accounts for the amount of “fill” compounds present that are inert. For examples, the correction factor for Simvastatin is 10X, and for Fluoxetine it is 7.5X using generic drugs – correction factor was recalculated upon every new drug purchase. Ascorbic acid (Vitamin C, Fluka) was purchased and administered in its pure form. Encapsulated drugs were dumped directly onto the weighing dish; pills were first pulverized before weighing.

All drugs were encapsulated within a 4 gram Pillsbury sugar cookie-dough “ball”, the drug vehicle for voluntary oral administration. A drug-free vehicle was given to the rats starting 2 days before surgery, and again after stroke-induction surgery to acclimate them to this novel food. Preparation for the cookie-dough “ball” went as follows: 1) the 4-gram balls were prepared with gloved hands (see Figure 10); 2) a depression within each ball was made; 3) the appropriate weight of drugs relative to animal's body mass was placed within the depression (Step 3 was skipped for control rat subjects); 4) the cookie-dough balls are wrapped and re-rolled up, ensuring a homogenous solid containing all medication is created. These cookie-dough balls are placed in each rat cage at approximately noon each day of the 31-day stroke treatment regimen. Most often, the drug-vehicle was consumed within 10 minutes of presentation. On occasion, consumption time would last longer; therefore, the petri-dish in which the drug-vehicle is presented on was allowed to sit for 1 full day before being replaced. Remaining drug vehicle that goes unconsumed is counted against the total medication consumed; 3 or more days of failing to fully consume the drug vehicle was criteria for exclusion from the study.

**Montoya Staircase Functional Test**

The training period for the Montoya Staircase can last a maximum for 2 weeks.
The apparatus was set up as shown (see Figure 11) with three 50 mg sucrose pellets on each well for 7 levels of the staircase (both on the right and left sides). Sucrose pellets were painted with maple extract and allowed to dry prior to placement within the apparatus. Training sessions were for a 15-minute period, once a day, in the 12-hour dark phase. The final 3 days of training were used to create the pre-stroke baseline; of these 3 days, the day in which the most pellets were retrieved is used. If multiple days showed the same maximum number of pellets received but varied between forelimbs, the results were averaged. During this time, animal subjects receive a restricted chow diet of 85% of their ad lib feed per day, and their weights were not allowed to drop below 90% of their ad lib feeding weight. Any animal unable to retrieve 9 pellets total in each forepaw by training termination was excluded from functional analysis.

Montoya staircase functional testing post-stroke took place for 3 days in the dark-phase, and was always preceded by an overnight fast. Specifically, testing took place on days 8-10 and 29-31 post-stroke. The testing subjects' diets were restricted to 10 grams total between the drug vehicle and rat chow. Any animal unable to retrieve a single pellet during
post-stroke tests was excluded from functional analysis.

**Forelimb Asymmetry Functional Test**

This test examines motor deficits within each forepaw by counting the number of wall-contacts the rat subject makes with each paw individually over a 5-minute time period. The animal was placed into a clear, 9 inch ID acrylic tube, and the subject was attracted to the maple extract painted 14 inches from the inside-bottom of the cylinder (see Figure 12). To ensure results were accurate, wall contacts were counted from a video recording made of each test played back in slow-motion.

**Euthanization and Cardioperfusion**

Animals subjects were euthanized 32 days post-stroke induction. The anesthetic used was 100 mg/kg pentobarbital (Euthasol), administered via intraperitoneal injection. Euthasol was chosen because it puts animals in the surgical plane relatively quickly, maintains cardiac function to prevent clotting during perfusion, and enables subjects to display the “cardioperfusion dance” (described below). Once the anesthesia was appropriately set, cardioperfusion was established with a minimum of 150 mLs PBS solution. The PBS solution was then stopped and almost instantly the neural fixative solution was perfused. The fixative used was 4% paraformaldehyde in PBS, and a minimum of 150 mLs was circulated. A successful cardioperfusion is initially gauged by a pale discoloration of the liver as well as a “cardioperfusion dance” in which the rat's limbs move around in a swimming motion caused by fixative induced skeletal muscle contractions.

**Immunohistochemistry**

Following cardioperfusion, all collected rat brains are immediately placed in 4%
paraformaldehyde in PBS blocking solution at 5°C. The brains are postfixed for 24 hours in this solution before being transferred into 30% sucrose in PBS at 5°C for 3 days.

50 micrometer thick sections were sliced from each rat brain ensuring the entire infarct was present somewhere in between the first and last section taken. 1 out of every 3 sections was retained in PBS solution for estimation of infarct volume.

Group 2 Long Evans and Group 1 Sprague Dawley rat infarct section were first dried on slides, and then labeled using Nissl stain. This stain labels nissl bodies found primarily in the nucleus in the cortex, allowing damaged cells due to stroke to be shown in contrast to the remainder of the undamaged section. Stained sections then underwent a destaining and dehydration process. Slides were dipped in these solutions sequentially: Cresyl Violet (Nissl) stain (15 min), Distilled water (5 min), 70% ethanol (5 min), 95% ethanol (5 min), 100% ethanol (5 min), 100% ethanol (5 min), Xylene (5 min), Xylene (5 min). Nissl stain was created using 2.5 grams of Cresyl Violet dissolved in a 500 mL solution made of water (300mL), 1M Na acetate (30 mL), and 1M acetic acid (170 mL). The permanent mordant DPX was used to cover-slip the slides.

The retained sections from Group 2 Sprague Dawley rats were transferred from the PBS solution into PBS containing 1% Triton and 3% donkey serum (serum from host of secondary antibody). These sections are reacted with 8-hydroxyguanosine primary antibody (1:500 dilution) overnight for about 16 hours (antibody purchased from Abcam® company). 8-hydroxyguanosine is a marker of oxidative stress in DNA; it is a modified DNA base damaged from hydroxyl radicals produced by oxidative stress (Abcam, 2008). The secondary antibody used was a fluorescence label: Donkey Anti-mouse IgG Rhodamine-X-Red (1:100 dilution), which was interacted with washed
sections for 2.5 hours. After a final set of washes, the stained sections were mounted onto gel-subbed slides and cover-slipped using Vectashield® to prevent photo-bleaching.

**Image Capture and Infarct Volume Calculation**

![Image](image.png)

**Figure 13.** Infarct tracing using ImageJ to calculate infarct area of a section. Spot Scope image, Nissl stain (left); Confocal image, 8-hydroxyguanosine stain (right)

The cortical stroke infarct was located and imaged using both the Olympus Epi Fluorescence Spot Scope (for Nissl stained sections) and Olympus FV300 Confocal Microscope (for 8-hydroxyguanosine stained sections). In cases of large infarcts, multiple images were captured and montaged using Adobe Photoshop.

Individual infarct areas for each section were calculated using the NIH ImageJ Program. To calculate an infarct volume, the following steps were followed: 1) Trace the infarct area using ImageJ (*see Figure 13*); 2) Calculate the Infarct Area (mm²); 3) Multiply: Infarct Area (mm²) X 0.05 (mm) Section Width = Section Volume; 4) Multiply each Section Volume X 3 = Total Section Volume; 5) Sum: ΣTotal Section Volumes = Infarct Volume.

The student t-test was used to analyze the infarct volumes.
Statistical Analysis

Two Way Repeated Measures ANOVA was used to analyze the functional data for both Forelimb and Montoya Staircase tests. Post-hoc test varied. To analyze differences in infarct volume, t-test was used for two groups or ANOVA for three groups.

Exclusions

As mentioned previously, any rat subject that failed to fully ingest the drug vehicle for 3 days was removed from analysis. Also, subjects that failed to successfully retrieve 9 pellets with each paw during Montoya Staircase training were removed. Deficit Criteria: Any subject that does not display a minimum 15% functional deficit in Forelimb Asymmetry and 15% functional deficit in Montoya Staircase was excluded from the study.
RESULTS

Experimental Design Summary

Three different post-stroke medicated treatments were tested; **Drug combination 1**: 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin and 20 mg/kg ascorbic acid (FSA); **Drug combination 2**: 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin (FS); **Vehicle control** (C). In addition to testing drug combinations, two different sets of stereotactic coordinates were used during endothelin injection. Rats were divided into two groups; **Group 1**: (AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm); **Group 2**: (AP: 0.0 mm, ML: -2.5 mm) and (AP +2.3; ML -2.5 mm). The two stocks tested were Sprague Dawley (SD) and Long Evans (LE). The infarct volume of each subject was measured after the 31-day treatment regimen post-stroke and subsequent euthanization on day 32.

Forelimb Motor Functional Testing

Data was collected for all rats in the study using both the Forelimb Asymmetry and Montoya Staircase functional tests (see Tables 1 and 2). Rats were subject to Montoya Staircase testing two times during the post-stroke study, aside from pre-stroke training. Forelimb Asymmetry testing occurred three times during the post-stroke study.

After analyzing post-stroke data, I used Montoya Staircase testing data to study deficit correlations with infarct volume because Forelimb Asymmetry data showed spontaneous recovery in control rats, with significant improvement in % of contralateral limb use 16 and 28 days post-stroke (PS) (see Figure 14). Rapid recovery could be due to the small infarct volumes induced, but this was only seen in Forelimb Asymmetry data.
Figure 14. Forelimb Asymmetry Test Results. Error bars represent SEM. # indicated $P<0.05$ using Repeated Measures ANOVA and post hoc test.

Results analyzed from a previous study conducted in our lab by Kailee Haggerty.
Figure 15. Infarct Volume Comparison: Group 2 LE rats. Treatment groups: FSA (Fluoxetine, Simvastatin, Vit. C), Control. Error bars - SEM
**Effect of Post-stroke Drug-treatment**

Post-stroke medical treatments were compared by infarct volume in Group 2 Long Evans rats. The treatment groups tested were FSA and the vehicle control (*see Figure 15*). No significant difference was found between an FSA mean infarct volume of 5.4696 m$^3$ (SEM = 1.6762) and Control mean infarct volume of 9.6310 m$^3$ (SEM = 2.6527) ($p = 0.221$; T-test).

Group 2 LE rat treatment groups were separated by rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) and rats that failed Montoya training criteria. FSA and Control groups were separately evaluated, and then these two groups were further divided by criteria fulfilled. In Group 2 LE, FSA rats, all rats that met training criteria also met deficit criteria (*see Figure 16*). The Met All Criteria mean infarct volume (6.7380 mm$^3$) was found to be relatively similar to the Training Criteria Not Met mean infarct volume (4.2013 mm$^3$). Statistical analysis could not be conducted with sample sizes smaller than N = 3. Also, because rats that did not meet training criteria were excluded from the study and therefore functional testing, the degree of functional deficit caused by the infarcts could not be determined.

The Group 2 LE, Control group was divided by criteria fulfilled (*see Figure 17*). Again, all rats that met training criteria in this group also met the deficit criteria. The Met All Criteria mean infarct volume (7.5866 mm$^3$) was relatively similar to the Training Criteria Not Met mean infarct volume (13.7198 mm$^3$). Statistical analysis could not be conducted because of small sample size; functional deficit caused by infarcts in the Training Criteria Not Met group could not be determined.

Overall findings suggest that infarct volume does not correlate with post-stroke
Figure 16. Infarct Volume Comparison: Group 2 LE, FSA rats.
Figure 17. Infarct Volume Comparison: Group 2 LE, Control rats.
drug treatment. Data showed that mean infarct volumes in rat treatment groups that met and did not meet criteria were relatively similar. Conclusions could not be drawn regarding a correlation between infarct size and functional deficit attained because there were no rats that met training criteria but failed to meet deficit criteria.

A few problems were encountered with the LE rat stock used in these experiments. These rats were observed to have a high mortality rate of 25-30% during endothelin-induced stroke surgery coupled with a problem in reliably ingesting the drug vehicle (significantly different from other rats stocks or strains tested; Chi squared test). Furthermore, LE adult rats at 10-12 months were relatively unavailable commercially. For these reasons, Sprague Dawley rats were used for the remainder of testing. Post-stroke medical treatments were compared by infarct volume in Group 2 SD rats. The treatment groups tested were FSA and the vehicle control (see Figure 18). No significant difference was found between an FSA mean infarct volume of 0.8835 \( \text{mm}^3 \) (SEM = 0.2165) and Control mean infarct volume of 0.9275 \( \text{mm}^3 \) (SEM = 0.1864) (\( p = 0.373; \) T-test).

The Group 2 SD rat treatment group was separated by rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) and rats that failed Montoya training criteria. Each treatment group was looked at individually and each group based on criteria fulfilled was compared (see Figure 19). When examining Group 2 SD, FSA rats, no significant difference was found between the Met All Criteria mean infarct volume (1.0630 \( \text{mm}^3 \); SEM = 0.6876), Deficit Criteria Not Met (0.6566 \( \text{mm}^3 \); SEM = 0.2576), and Training Criteria Not Met (1.1580 \( \text{mm}^3 \); SEM = 0.3477) (\( p = 0.390; \) One-way ANOVA). Group 2 SD, Control rat treatment group was separated by rats that
Figure 18. Infarct Volume Comparison: Group 2 SD rats. Treatment groups: FSA (Fluoxetine, Simvastatin, Vit. C), Control. Error bars - SEM
Figure 19. Infarct Volume Comparison: Group 2 SD, FSA rats. Error bars - SEM.
Figure 20. Infarct Volume Comparison: Group 2 SD, Control rats. Error bars - SEM. Middle bar (Deficit Criteria Not Met has no error bar).
met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) and rats that failed Montoya training criteria (see Figure 20). No significant difference was found between the Met All Criteria mean infarct volume (1.0425 mm$^3$; SEM = 0.4791), Deficit Criteria Not Met (0.6110 mm$^3$), and Training Criteria Not Met (0.9708 mm$^3$; SEM = 0.0864 mm$^3$) (p = 0.281; One-way ANOVA).

Overall findings for Group 2 SD rats suggest that infarct volume does not correlate with post-stroke drug treatment. Results show that mean infarct volumes in rat groups Met All Criteria versus rats were relatively similar to rat groups Deficit Criteria Not Met. This implies that a lack of forelimb motor deficit in some rats was not caused by a faulty endothelin injection, but was rather due to a mislocation of the infarct relative to the forelimb motor cortex.

**Effect of Rat Stock on Infarct Volume**

Group 2 SD and LE rats treatment groups were also compared against one another, according to infarct volume, to test for a correlation in the infarct induced with rat stock used (see Figure 21). As previously described, the two treatment groups tested in Group 2 LE and SD rats were FSA and the vehicle control. These findings find a significant difference in mean infarct volume between rats stocks in both the FSA (LE = 5.4696, SEM = 1.6762; SD = 0.8835, SEM = .2165; p = 0.025, T-test) and Control groups (LE = 9.6310, SEM = 2.6527; SD = 0.9275, SEM = 0.1864; p = 0.014, T-test). SD rats were found to have smaller infarcts induced within both treatment groups.

The same comparison as seen in Figure 21 was made, but only using rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) (see Figure 22). Again, the mean infarct volume of FSA-LE (6.7380 mm$^3$; SEM = 1.0571) was found
Figure 21. Infarct Volume Comparison: Group 2 LE vs SD rats; Treatment groups: FSA (Fluoxetine, Simvastatin, Vit.C), Control. Error bars - SEM.
Figure 22. Infarct Volume Comparison: Group 2 LE vs SD rats. Treatment groups: FSA (Fluoxetine, Simvastatin, Vit.C), Control. Error bars - SEM. Excluded - Subjects that failed to meet forelimb motor deficit criteria.

LE bars with N = 2 have no error bars.
to be significantly less than that of FSA-SD (1.0630 mm\(^3\); SEM = 0.6876) (p = 0.017, T-test). Though the mean infarct volume of Control-LE (7.5866 mm\(^3\); SEM = 2.9278) seems clearly smaller than that of Control-SD (1.0425 mm\(^3\); SEM = 0.4791), this difference was not found to be significant (p = 0.133, T-test), most likely due to a small sample size and large variability in the infarct volumes.

Overall, these findings suggest that endothelin-induced cortical strokes produce a significantly larger infarct volume in LE rats in comparison to SD rats. The difference seen here can be attributed to the greater number of cerebral collateral vessels found in SD rats versus LE rats (Ginsberg, 1989). Collateral vessels allow blood flow to circumvent an infarct, thus reducing the total amount of tissue damage caused by the ischemic attack.

In order to create “LE-sized” infarct volumes within SD rats, different cortical injection coordinates must be used in SD rats. Therefore, AP coordinates for one injection site were moved, relative to bregma, to +1.5 (Group 1) instead of +2.3 (Group 2). Thus the two endothelin injection sites were closer to each other for this final group of subjects – Group 1 SD rats.

*Effect of Post-stroke Drug Treatment*

Post-stroke medical treatments were compared by infarct volume in Group 1 SD rats. The treatment groups tested were FSA, FS, and the vehicle control (*see Figure 23*). No significant difference was found between any of the groups – an FSA mean infarct
volume of 3.4561 mm$^3$ (SEM = 0.5463), an FS mean infarct volume of 2.6579 mm$^3$ (SEM = 0.5361), and a Control mean infarct volume of 2.8176 mm$^3$ (SEM = 0.8531) ($p = 0.612$; One-way ANOVA). The Group 1 SD Control group infarct volumes were

![Figure 23](image)  

**Figure 23.** Infarct Volume Comparison: Group 1 SD rats. Treatment groups: **FSA** (*Fluoxetine, Simvastatin, Vit.C*), **FS** (*Fluoxetine, Simvastatin*), **Control**. Error bars - SEM.
Figure 24. Infarct Volume Comparison: Group 1 SD, FSA rats. Error bars - SEM. Middle and right bars with N=2 have no error bars.
Figure 25. Infarct Volume Comparison: Group 1 SD, FS rats. Error bars - SEM. Right bar, with N=1, has no error bar.
measured outside of this study by Kailee Haggerty, M.S. using 8-hydroxyguanosine instead of Nissl stain.

Group 1 SD rat treatment group was separated by rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) and rats that failed Montoya training criteria. Each treatment group was looked at separately, and then further divided by criteria fulfilled. When examining Group 1 SD, FSA rats, groups were found to be relatively similar between the Met All Criteria mean infarct volume (3.7943 mm$^3$; SEM = 0.7061), Deficit Criteria Not Met (1.6319 mm$^3$), and Training Criteria Not Met (3.9277 mm$^3$) (see Figure 24).

In Group 1 SD, FS rats, mean infarct volumes were again found to be relatively similar along all groups (see Figure 25). Mean infarct volume for Met All Criteria was 2.4364 mm$^3$ (SEM = 0.9031), mean infarct volume for Deficit Criteria Not Met was 2.5809 mm$^3$ (SEM = 0.7790), and the mean infarct volume for Training Criteria Not Met was 4.0733 mm$^3$. Comparing the Met All Criteria and Deficit Criteria groups directly, these groups were found to have no significant difference (p = 0.439, T-test).

Overall, findings suggest that infarct volume does not correlate with post-stroke drug treatment in Group 1 SD rats. Furthermore, results show that mean infarct volumes in rat groups Met All Criteria were relatively similar to rat groups Deficit Criteria Not Met.

Effect of Stereotactic Coordinates on Infarct Volume

Stereotactic coordinates between Group 1 and Group 2 SD rats were varied at one injection site in AP coordinates, +1.5 and +2.3 respectively. The FSA drug group was used in both groups in this study; therefore, these groups were used to analyze the effect
Figure 26. Infarct Volume Comparison: Group 1 and 2, SD, FSA rats
Group 1 – AP: 0.0 and +1.5 mm; Group 2 – AP: 0.0 and +2.3.
Error bars - SEM.
Figure 27. Infarct Volume Comparison: Group 1 and 2, SD, FSA rats
Group 1 – AP: 0.0 and +1.5 mm; Group 2 – AP: 0.0 and +2.3
Excluded – Subjects that failed to meet forelimb motor deficit criteria
Error bars - SEM.
of stereotactic coordinates on infarct volume (see Figure 26).

The Group 1, SD, FSA mean infarct volume (3.4561 mm$^3$; SEM = 0.5463) was found to be significantly larger than the Group 2, SD, FSA mean infarct volume (0.8835 mm$^3$; SEM = 0.2165) ($p < 0.001$).

Group 1 and Group 2, SD, FSA rats were compared again, except only using rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) (see Figure 27). Group 1, SD, FSA rats were found to have a nearly significantly larger mean infarct volume (3.7943 mm$^3$; SEM = 0.7061) than that of Group 2, SD, FSA rats (1.0630 mm$^3$; SEM = 0.6876) ($p = 0.057$).

These results suggest that Group 1 coordinates [(AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm)] create larger infarcts than Group 2 coordinates [(AP: 0.0 mm, ML: -2.5mm) and (AP +2.3; ML -2.5 mm)] in SD rats. Larger infarcts can be attributed to the fact that Group 1 coordinates are closer to each other than Group 2 coordinates. Closer injection coordinates may allow the initial growing endothelin-induced infarcts to coalesce more readily and therefore disrupt a greater number of brain vessels, thus creating a larger infarct.

Montoya Deficit vs. Infarct Volume

Finally, Montoya Deficit (1 - % Prestroke performance) was plotted against infarct volume in all SD rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) (see Figure 28) using data from post-stroke day 9. A linear regression was calculated using these data points ($r^2 = 0.632137$).

Montoya Deficit was again plotted against infarct volume in all SD rats that met a post-stroke/pre-stroke deficit criteria of 0.85 (15% forelimb motor deficit) (see Figure
using data from post-stroke day 30. A linear regression was calculated using these data points ($r^2 = 0.708356$).

These linear correlations, though only moderately strong, suggest that rat subjects who sustained an adequate forelimb motor cortex deficit to meet criteria had infarcts that hit at least a portion of the motor cortex. Those animals with larger infarcts that correlated with larger deficits could be inferred to have hit more of the motor cortex. Those animals with smaller infarcts could be inferred to have hit a smaller portion of the motor cortex for forelimb.
Figure 28. Montoya Deficit vs Infarct Volume: Group 1 and 2 SD rats
PSD ~ 9; Linear regression shown: $r^2 = 0.632137$
Excluded – Subjects that failed to meet forelimb motor deficit criteria
Figure 29. Montoya Deficit vs Infarct Volume: Group 1 and 2 SD rats
PSD ~ 30; Linear regression shown: $r^2 = 0.708146$
Excluded – Subjects that failed to meet forelimb motor deficit criteria
### Table 1.

**Group 1 Functional Test Data and Deficit Results**

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Infarct Volume (mm³)</th>
<th>Group</th>
<th>Rat Stock</th>
<th>Montoya Staircase ^PSD 9</th>
<th>Montoya Staircase PSD 30</th>
<th>Forelimb Asymmetry PSD 3</th>
<th>Forelimb Asymmetry PSD 28</th>
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Group 1 Cortical Injection Coordinates: (AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm)

**PSD:** Post-stroke Day

**PTC:** Pre-stroke Testing Criteria. Rats unable to retrieve a minimum of 9 pellets with each forepaw during pre-stroke testing were excluded from the study

**FSA:** Fluoxetine, Simvastatin, Ascorbic Acid; **FS:** Fluoxetine, Simvastatin

* All Criteria Met

^ Training Criteria Not Met

& Deficit Criteria Not Met
### Table 2.

**Group 2 Functional Test Data and Deficit Results**

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<th>Rat #</th>
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<th>Rat Stock</th>
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Group 2 Cortical Injection Coordinates: (AP: 0.0 mm, ML: -2.5 mm) and (AP +2.3 mm; ML: -2.5 mm)

C: Control

* All Criteria Met

^ Training Criteria Not Met

& Deficit Criteria Not Met
DISCUSSION

_Montoya Staircase vs. Forelimb Asymmetry_

Animal subjects were gauged on whether or not an adequate contralateral forepaw motor deficit was attained based on Montoya Staircase functional testing. As mentioned, Forelimb Asymmetry functional testing was also conducted for all rats. Forelimb Asymmetry data could not be used for this study, however, because of the spontaneous recovery observed on post-stroke days 16 and 24 (see Figure 14) with some of the infarcts in Sprague Dawley rats.

I attributed this spontaneous recovery of both control and drug treatment groups to the very small infarcts seen post-euthanization in rat subjects, especially Group 2 Sprague Dawley rats. Smaller infarcts can potentially have a greater chance of preserving the specific forelimb function tested in the Forelimb Asymmetry apparatus. The Forelimb Asymmetry test is not as rigorous a functional test as the Montoya Staircase – it involves less complex motor function to complete the task. Therefore, a valid hypothesis could be that Montoya function (grasping and holding) can be more easily altered and less easily preserved than Forelimb Asymmetry function. Further testing using a greater number of Sprague Dawley rats with larger endothelin-induced infarcts must be conducted to see if this theory explains the spontaneous recovery.

_Contralateral Forepaw Deficit Criteria_
Rats had to sustain a 15% contralateral forepaw deficit in Montoya Staircase functional testing to fulfill deficit criteria. This deficit criteria was lowered from an original 20% deficit because many animals did not sustain an adequate deficit to meet this higher criteria. This could again be attributed to the smaller infarcts induced in Group 2 Sprague Dawley rats.

As the drug-treatment data showed, infarct volumes were relatively similar in groups that met and did not meet deficit criteria (see Figures 19, 20, 24, 25). This led to the conclusion that an adequate deficit was not attained not because of a faulty endothelin injection (too little endothelin injected), but rather a mislocation of the injection site relative to the forelimb motor cortex. A faulty endothelin injection would result in significantly smaller induced infarcts in those animals not meeting deficit criteria. A mislocation of the infarct could occur for a number of reasons: Bregma, the reference point for all coordinates, was not easily identified or was indistinct in some rats; the brain underneath the rat skulls (and therefore, the reference marks) may have been slightly misaligned relative to each animal; or the injection needle did not penetrate tissue in a perfectly vertical plane and thus may have affected a nearby location to the motor cortex.

A small trend may seem to appear in Figures 19, 20, and 24 suggesting that rats not meeting deficit criteria have smaller infarcts. However, in Figure 19, a p-value of 0.390 clearly indicates no significant difference between any group; in Figures 20 and 24, the sample size of N < 3 was too small to conduct statistical analysis or draw any conclusions. Figures 19 and 25 have Deficit Criteria Not Met groups that had an adequate sample size (N > 2), and in both cases, Deficit Criteria Not Met was found to be clearly similar to the Met All Criteria group (p = 0.390 and 0.439, respectively).
Group 2 Long Evans rats had no cases that resulted in rats meeting training criteria but failing to meet deficit criteria. This could be due to a small sample size (N = 7), which would imply that there was simply not enough of a chance to have an animal that met training but failed deficit criteria. Another theory, however, could be that more accurate infarcts hitting the motor cortex, thus inducing an adequate deficit, could be attained in Long Evans rats. This could be attributed to a potentially less variable brain-to-skull alignment, a more distinct bregma reference point, or simply that Long Evans rats sustained larger infarcts than Sprague Dawley rats. Further testing using a greater number of Long Evans rats is needed to determine the cause.

Functional tests determining whether or not rats met deficit criteria were conducted on days 8, 9, and 10 post-stroke. Perhaps conducting this test closer to the day of stroke induction would show more accurately the amount of deficit induced by stroke infarct, and would lessen the potential effects of small recovery that may be attained during the first ten days post-stroke.

*Effect of Rat Stock on Infarct Volume*

Group 2 coordinates (AP: 0 & +2.3) were initially used for endothelin stroke induction surgery in Long Evans rats. These coordinates produced adequately large infarcts in this rat stock. However, our laboratory switched to the Sprague Dawley rat stock because of the reasons previously mentioned: 1) Sprague Dawley were much easier to attain commercially, 2) Long Evans rats had a large mortality rate during stroke surgery while Sprague Dawley had only about a 3% mortality, and 3) Sprague Dawley rats were shown to ingest the drug vehicle with significantly greater reliability compared
to Long Evans and Wistar rat stocks in a study conducted by this lab previously (Corbett et al, Laboratory Animals *in press*).

Infarcts sustained in Sprague Dawley rats using Group 2 coordinates were significantly smaller than infarcts sustained in Long Evans rats using Group 2 coordinates (*see Figures 21 & 22*). This difference was seen in both FSA and Control groups (*p* = 0.014 and 0.025 respectively) and was attributed to the lesser number of collateral vessels found in Long Evans versus Sprague Dawley rats (Ginsberg, 1989; Prieto, 2005). An occluded vessel may re-route blood flow to smaller surrounding vessels due to increased pressure from the blockage. If blood has more avenues through which it can continue flowing to the next major vessel, more tissue volume surrounding the occlusion can be retained. Greater tissue damage results when blood cannot circumvent an occlusion, thus creating a larger sustained infarct.

*Stereotactic Coordinates for Endothelin Cortical Injection*

As mentioned, Group 2 coordinates were first used in Long Evans rats, and then in Sprague Dawley rats. When it was observed that Sprague Dawley rats sustain much smaller infarcts using these cortical injection coordinates, our laboratory changed the AP coordinates so that the two injection sites were closer for the Group 1 Sprague Dawley rats (Group 1 – AP: 0 & +1.5). The aim was to develop Sprague Dawley infarcts that mimicked the Long Evans-sized infarcts seen initially. These closer injection coordinates proved to induce significantly larger infarcts than the farther Group 2 coordinates (*see Figures 26 & 27*). An AP coordinate of +1.5 was chosen because this had been previously shown to be the center of the forelimb motor cortex (Kleim et al., 1996).
A larger infarct may be induced in coordinates that are closer to one another because of the greater additive effect of concentration gradients from closer endothelin injections. As endothelin perfuses away from the injection site through brain tissue, its total concentration decreases with distance, creating a concentration gradient. When the injection sites are farther apart, there is a lower total additive concentration when these gradients intersect, thus fewer vessels are occluded.

A future study may look at this overlapping concentration gradient theory by moving these closer injection coordinates relative to this “dead-on” forelimb motor cortex spot of AP +1.5. Group 1 coordinates used AP coordinates of 0 and +1.5, with the +1.5 being right on the forelimb motor cortex (Kleim et al., 1996). Therefore, the two perfusing concentration gradients would coalesce next to but not on this “dead-on” spot.

A future study could test AP coordinates of +0.75 and +2.25 (“Group 3” coordinates), which would contain the dead-on spot right in between and allow the gradients to intersect at this spot, making it the center of the large “combined” infarct. If more rats are able to meet deficit criteria using these potential Group 3 coordinates versus the Group 1 coordinate subjects in this study, this may show that coalescing endothelin concentration gradients produce one greater infarct that occludes many vessels. If fewer rats are able to meet deficit criteria, this result would suggest coalescing endothelin concentration gradients still have a smaller additive effect than a single infarct that is placed directly on the motor cortex.

The ability of intersecting concentration gradients to occlude vessels depends on the total endothelin concentration at the edge of the perfusing concentration gradient. Suppose the edge has half of the total endothelin concentration – this would suggest that
two additive “edges” intersecting would create a fully concentrated endothelin induced infarct. Such an infarct would theoretically occlude as many vessels as if there was a direct endothelin injection into this “edge - intersecting” area. Suppose the perfusing edge has one-fourth the total endothelin concentration – this would suggest the two additive “edges” intersecting would sum to only half of the total endothelin concentration. This infarct would theoretically occlude fewer vessels and maintain more tissue integrity than if each edge made up half of the total concentration. Essentially, the smaller the perfusing gradient (or the greater the total concentration on each “edge”), the greater probability of occluding more vasculature, thus creating a larger infarct.

Furthermore, an observation in this study is that Long Evans rats showed trend toward recovery in the FSA group versus Control. Group 2 coordinates used here surrounded the forelimb motor cortex, but were not directly on the “dead-on” forelimb motor cortex spot of AP +1.5 (Kleim et al., 1996). A theory for why recovery was seen here could be the fact that the two coalescing endothelin concentration gradients may damage the tissue in between (forelimb motor cortex), but not completely destroy and kill it. Images taken to measure the infarct show a clear area beneath the injection site of destroyed tissue. Damaged tissue can potentially show a higher propensity for recovery in comparison to tissue that is completely dead.

*Montoya Staircase Functional Deficit vs. Infarct Volume*

Montoya Deficit (1 – Prestroke function %) was plotted against Infarct Volume for all Group 1 Sprague Dawley rats that met deficit criteria. Linear trends observed in Figures 25 and 26 ($r^2 = 0.632137$ and $0.708146$ respectively) suggest that greater infarct volumes result in greater Montoya Deficits in rats that met deficit criteria. Specific points
to note are rat 430 in Figure 25, and rats 417 and 430 in Figure 26. All three of these data points show an infarct volume sustained around 6 mm$^3$ and Montoya Deficit of around 0.75 or greater. These infarcts can be determined as “hitting the spot” of the forelimb motor cortex. Though rat 432 sustained the largest infarct volume of all Group 1 Sprague Dawley rats that met deficit criteria, it did not sustain the greatest Montoya Deficit. This difference implies that the rat 432 infarct was not completely within the forelimb motor cortex. Infarcts such as the one seen with rat 432 may explain why these charts in Figure 25 and 26 do not show a tight correlation ($r^2 > 0.90$) – part of the infarct was not within the motor cortex, thus creating a large infarct with a relatively smaller Montoya deficit.

**Drug Treatment Effects on Infarct Volume**

Results showed that the drug combinations FSA (fluoxetine, simvastatin, and ascorbic acid) and FS (fluoxetine, simvastatin) had no effect on infarct volume when compared to Control subjects (see Figures 15, 18, and 23). Infarct volumes for all drug and control groups showed no significant difference in all situations where statistical analysis could be conducted (N > 2).

One explanation may the difference in active metabolite enantiomers in the brand name fluoxetine (Prozac) versus the generic brand used in Group 2 Sprague Dawley rats. Prozac is made up of an approximately 50:50 ratio of R:S fluoxetine enantiomers (Kim, 2004). The active metabolite of these enantiomers is S-norfluoxetine is shown to be at least 20 times more active than its counterpart R-norfluoxetine (McDonagh et al., 2011; Kim, 2004). Both R and S-norfluoxetine have been shown to have a half-life of 7 to 15 days, while R and S-fluoxetine have a half-life of 2 to 4 days (Gury et al., 1999). HPLC analysis conducted after this study showed that generic brand fluoxetine had a 15%
higher amount of R-fluoxetine and a 15% lower amount of S-fluoxetine in comparison to brand name Prozac™ (study was conducted by Audrey McGowin – Chemistry Dept. at Wright State University). This would imply that there is a lower amount of the active metabolite S-norfluoxetine in the generic fluoxetine used - this may influence the ability of generic brand fluoxetine to affect motor deficits in Group 2 Sprague Dawley rats because there is less long-lasting active metabolite build-up in the brain.

Another explanation for the lack of drug effect on infarct volume may simply be due to the fact that drugs are not offered for voluntary oral administration until 20-26 hours after stroke induction. This time lapse may be too great to influence infarct volume – perhaps administering drugs sooner could have an effect on the infarct. Further testing using a shorter time lapse between stroke induction and voluntary oral drug administration would have to be conducted to see if this hypothesis is valid or if the drugs were ineffective.

The ability of statins to improve motor deficits in past studies may also be due to upregulation of VEGF along with the previously described upregulation of BDNF. VEGF promotes angiogenesis - new vasculature can theoretically help regain function in a damaged brain. Studies are currently being conducted in Dr. Corbett’s lab to analyze the influence of angiogenesis on motor recovery in rats.

**Conclusion**

When endothelin-induced cortical strokes are induced in Long Evans and Sprague Dawley rats using Group 2 coordinates [(AP: 0.0 mm, ML: -2.5mm) and (AP +2.3; ML -
2.5 mm)], Long Evans rats are shown to sustain a significantly larger infarct volume in comparison to the Sprague Dawley rat stock.

Comparing infarct volumes induced by the closer Group 1 cortical injection coordinates [(AP: 0.0 mm, ML: -2.5 mm) and (AP +1.5 mm; ML: -2.5 mm)] in comparison to Group 2 coordinates (see above), the closer Group 1 coordinates were shown to produce a larger infarct volume in Sprague Dawley rats.

Drug treatment groups FSA (fluoxetine, simvastatin, ascorbic acid) and FS (fluoxetine and simvastatin) were shown to have no effect on infarct volumes when compared, respectively, to the vehicle control.
References


Lu, Dunyue, Changsheng Qu, Anton Goussev, Hao Jiang, Chang Lu, Timothy Schallert, Asim Mahmood, Jieli Chen, Yi Li, and Michael Chopp. "Statins Increase


