The Use of Doublecortin to Quantify the Effects of Pharmacological Treatment on Neurogenesis and Functional Recovery after Stroke

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THE USE OF DOUBLECORTIN TO QUANTIFY THE EFFECTS OF PHARMACOLOGICAL TREATMENT ON NEUROGENESIS AND FUNCTIONAL RECOVERY AFTER STROKE

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

By

AMBER LEE HENSLEY
B.S., Wright State University, 2011

2016
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amber Lee Hensley ENTITLED The Use of Doublecortin to Visualize the Effects of Pharmacological Treatment on Neurogenesis and Functional Recovery after Stroke BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Hensley, Amber Lee. M.S. Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2016. The Use of Doublecortin to Visualize the Effects of Pharmacological Treatment on Neurogenesis and Functional Recovery after Stroke.

Ischemic strokes account for 87% of all strokes and can have debilitating effects on language, sensory, and motor skills. Currently, tPA is the only medication approved by the FDA for the treatment of ischemic stroke, but the window of time to administer the drug is very small. In this thesis, we investigate the use of a simvastatin and fluoxetine drug combination (FS) as a possible alternative treatment for ischemic stroke victims. To analyze the effects of FS on neurogenesis and functional recovery, we utilize the Montoya Staircase and quantify the amount of neurogenesis using doublecortin.

Although the results of this study show that the drug treatment did not produce the significant increase in neurogenesis when comparing the control with the drug-treated animals as we had hoped for, nor did it translate to an increase in functional recovery, there was significantly more neurogenesis in the right hemisphere in the anterior and middle region of the subventricular zone (SVZ) of the FS rats than the left. This tells us that the drug treatment did produce a significant amount of neurogenesis in the SVZ, but further work needs to be done to better understand how the FS drug treatment effects neurogenesis and functional recovery. There may be an optimal window of time and dosage that will lead to a greater significance in recovery and higher levels of neurogenesis. This could open the door for a better treatment option to increase the quality of life for ischemic stroke patients.
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I. INTRODUCTION

STROKE RESEARCH

Stroke is the fifth leading cause of death for Americans, and each year approximately 130,000 Americans are killed by stroke. That is one out of every 20 deaths, with 66% of those deaths being adults over the age of 65. The cost of health care, treatment medications, and missed days of work amount to an estimated 34 billion dollars each year. (CDC, 2015).

Types of Stroke

There are two main different types of stroke, hemorrhagic and ischemic. There is also a transient stroke, which is a form of ischemic stroke. They are discussed in greater detail below.

Hemorrhagic

Hemorrhagic stroke results from the rupturing of weak or damaged blood vessels that bleed into the brain. There are two types of vessels that can lead to hemorrhagic bleeding, an aneurysm and arteriovenous malformation. Aneurysms are vessels that balloon out due to fragile vessel walls, which break when they become too thin.
Arteriovenous malformation are bundles of blood vessels that are formed abnormally, which are more delicate and prone to rupturing (The American Heart Association, 2015a).

Ischemic

Ischemic strokes account for 87% of all stroke victims. Ischemic strokes occur when the vessels in the brain become blocked or occluded. The primary cause for the occlusion is atherosclerosis, which is when fatty deposits line the blood vessel walls and impedes the flow of blood. This impedance of flow can be caused by either a cerebral thrombosis or cerebral embolism. Cerebral thrombosis occurs when a blood clot develops in the narrowed walls of the blood vessels of the brain. Cerebral embolism is when a blood clot from another location in the circulatory system travels to the brain and then becomes lodged in the narrowed vessel (The American Heart Association, 2015b).

Transient Ischemic Attack (TIA)

Lastly, another form of stroke is the transient ischemic attack or TIA. This is very similar to an ischemic stroke, except the clot that blocks the blood vessel in the brain is temporary or transient. This ‘attack’ differs from a stroke, because the blood clot is broken up before it causes permanent damage in the brain. This typically only lasts roughly five minutes, but it is a precursor to a larger or full-fledged stroke and should be treated as such (The American Heart Association, 2012).
NEUROGENESIS

Neurogenesis is the birth of new neurons in the brain. It was long believed that this only occurred in the prenatal and developing brain, and the mature brains of adults could not actively give birth to new neurons until Joseph Altman’s work in the 1960’s lead to the discovery of adult neurogenesis (Altman, 1963; Saaltink & Vreugdenhil, 2014). Neurogenesis after ischemic stroke aids in the repair of damaged tissue from the lack of blood flow. In turn, if the tissue can be repaired, the function coordinating to that tissue may also be restored.

Neurogenesis is commonly localized in two locations in the adult brain, the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ). The SVZ will be the focus of this study because it is the location corresponding with post-stroke neurogenesis. This can be deduced from the deficits that result from the majority of acute strokes; language, sensory, and motor skills. These functions are largely affected by a stroke and do not originate from the hippocampus (Carmichael, 2010; R. L. Zhang, Zhang, & Chopp, 2008). Some stroke victims do see damage to their hippocampus, but the forelimb motor cortex was targeted for this study, so a deficit in motor skills was analyzed. When visualizing neurogenesis in the SVZ, we look at levels in the anterior, middle, and posterior regions of each hemisphere. The three different regions can be visualized in Figure 1.
Figure 1: From top to bottom, a series of images from one hemisphere of the anterior, middle, and posterior subventricular zone from the rat brain atlas.
Stress Effects on Neurogenesis

Adult neurogenesis is greatly affected by hormones released during stress. The release of glucocorticoids during stress stimulates a decrease in the rate of stem or progenitor cell proliferation and can even cause tissue atrophy in the adult hippocampus (Mirescu & Gould, 2006; Warner-Schmidt & Duman, 2006). Due to our interest in neurogenesis, it is of great importance that stress be reduced to a minimum so the effect of the drug combination can be accurately quantified. To achieve a low-stress environment during drug administration we used a voluntary oral method used in previous work. This voluntary oral method of drug delivery involved placing the powered drug in a ball of sugar cookie dough. If the animal was in the control group, they received the dough by itself. This method resulted in significantly lower stress levels and higher results of consistent ingestion (A. Corbett, McGowin, Sieber, Flannery, & Sibbitt, 2012).

Neurogenesis Labeling

Neurogenesis begins with progenitor cells located outside the hippocampus, in the SGZ of the dentate gyrus. Here, they proliferate, differentiate, and then become new neurons. Each phase of neurogenesis expresses different markers, so it is important to select the proper marker to monitor the stage of neurogenesis we wish to see (von Bohlen und Halbach, 2007).
Use of Doublecortin

Doublecortin, or DCX, is a microtubule-associated protein (MAP) that is expressed in both the cell body and processes of migrating neurons. Particularly, it is present in developing as well as mature rodent brains (Vellema, Hertel, Urbanus, Van der Linden, & Gahr, 2014) DCX expression is specific for newly formed neurons, because the doublecortin-positive cells express antigens for early neurons (von Bohlen und Halbach, 2007).

Why not Bromodeoxyuridine?

Bromodeoxyuridine (BrdU) is an analog of thymidine that integrates DNA of dividing cells during the synthesis, or S-phase, of the cell cycle. Because of this, BrdU is traditionally used for birth dating and monitoring cell proliferation, but it has been used more recently as an in-situ marker for neurogenesis (Taupin, 2007). BrdU only labels cells during DNA replication and for approximately 24 hours after injection, while DCX labels new neurons from a two-week timeframe (Balthazart & Ball, 2014). The main problem with this characteristic is that BrdU can give false positives when looking at tissue after trauma. There is natural DNA repair after stroke induction that will be labeled by BdrU, which is not neurogenesis, whereas doublecortin tags only the migrating neurons of repair (Nowakowski & Hayes, 2000).
STROKE INDUCTION

Middle Cerebral Arterial Occlusion Model

The middle cerebral arterial occlusion or MCAO model was not used in this study because we utilized older rats. Occlusion of the middle cerebral artery is a very common method of inducing stroke in younger rats, but it creates more damage than desired for this research. This method typically involved surgical insertion and removal of clips and sutures and the older rats in this study would not have been able to survive a larger stroke. To create less damage, endothelin-1 was used to induce stroke. Endothelin-1 is a powerful vasoconstrictor that allows the blood flow to be cut off from the targeted area for a length of approximately 12 hours before it wears off and blood flow continues. This is very similar to how ischemic strokes present in the human brain; the clot breaks up naturally, but the blood flow is restricted long enough to damage the surrounding tissue. This caused a smaller and more targeted restriction of blood flow to supply greater control over the damage. This also allowed for a more targeted stroke area with a less invasive surgery (Nikolova et al., 2009).

SELECTION OF TREATMENT DRUGS

Standard Treatment

The standard treatment for ischemic stroke patients is the administration of the drug tissue plasminogen activator or tPA. This is the only drug approved by the FDA for the treatment of ischemic stroke and the window of time to administer the drug is very small (Rivera-Bou, Wanda; Cavanas, Jose; Villanueva, 2015). Research shows that stroke
victims have a window of three hours after the onset of their symptoms to receive tPA if they hope to see improvement over the next three months. If the drug is administered after the three-hour window, the risk of causing secondary bleeding in the brain increases (Hacke et al., 2004). There are successful treatments that reestablish brain perfusion and minimize the probability of a recurrent stroke, but there is no treatment proven to promote neurological repair from the damage induced by stroke (Mead et al., 2013).

**Fluoxetine**

Fluoxetine is a selective serotonin reuptake inhibitor or SSRI, which is highly prescribed to treat depression. More recently, fluoxetine is being investigated as a possible treatment drug for ischemic stroke patients, because it has been found to “improve motor recovery and reduce dependency after stroke when given to people without depression” (Chollet et al., 2011). This is correlated with the connection between stress and neurogenesis discussed earlier, in which administration of a chronic antidepressant treatment could up-regulate neurogenesis, and possibly block or reverse the atrophy and damage caused by stress or the damage from a stroke (Warner-Schmidt & Duman, 2006). Fluoxetine given on a daily basis one week after stroke can increase the number of proliferating neurons in the SVZ (Xiaoyu Sun et al., 2015, 2016).

In a similar study, hippocampal neurogenesis was disrupted after the onset of ischemic stroke to analyze the ability of fluoxetine to stimulate neurogenesis in the brain. This disruption ceased the beneficial effect of fluoxetine on cognitive impairment post-stroke. This suggests that fluoxetine treatment after an ischemic event can improve
spatial cognitive function recovery via neurogenesis in the subgranular zone as well (Li et al., 2009).

**Simvastatin**

Simvastatin is a statin, also known as an HMGR inhibitor. HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase, or HMGR, catalyzes the committed step in cholesterol biosynthesis. Due to the inhibition of this synthesis, simvastatin effectively lowers blood cholesterol levels and is commonly prescribed in the treatment of hypercholesterolemia and stroke prevention. In addition to lowering cholesterol, simvastatin promotes the nitric oxide mediated stimulation of new blood vessel growth (Istvan & Deisenhofer, 2001). When administered after stroke, it has been shown to augment functional outcome and stimulate neurogenesis (Chen et al., 2003).

**Drug combination**

We wanted to further investigate the possibility of using fluoxetine and simvastatin as a drug combination that can be used as a treatment to stimulate neurogenesis and repair functional deficits after an ischemic stroke. We used a combination of 5 mg/kg of fluoxetine and 1 mg/kg of simvastatin; in which the dosage was chosen based on similar dosage recommendations given to humans and scaled-down to the rat model.
HYPOTHESIS

We believe the simvastatin and fluoxetine drug combination will significantly increase functional recovery by stimulating neurogenesis when administered 6-12 hours after ischemic stroke.
II. MATERIALS AND METHODS

All research discussed in this document was performed in accordance with protocols approved by the Wright State University Institutional Animal Care and Use Committee (IACUC).

EXPERIMENTAL OVERVIEW

Research Animals

Sprague Dawley outbred retired breeder rats were chosen for this experiment. The female Sprague Dawley was selected due to the size needed to fit into the Montoya Staircase training apparatus; the males were too large to accommodate. The rats ranged from 10 to 12 months in age and were ordered from Harlan Laboratories in Indianapolis, Indiana. The adults were selected to mirror the physiological development of a typical human stroke patient. Rat pups were not of the correct neurological maturity to make accurate comparisons (Sengupta, 2013).

The rats were housed in separate, numbered cages to aid in accurate data collection. The cages were 952cm² hanging shoeboxes with wire lids and ¼ - ½ inch depth of Harlan Teklad Sani-chip bedding. All rats were kept in a room on a light schedule of 12 hours on and 12 hours off with a room temperature of 23-24°C. The food given throughout the entire experiment was Harlan Teklad rodent chow 8640. The rats
were placed on a restrictive diet during the training to encourage retrieval of the reward pellets. To determine the amount of food to be considered ‘restricted’ the amount of ad lib food eaten over a two-day period was calculated. They were given a weighed amount of food upon arrival and it was left in their cages for two days. After the two days, the remaining food was weighed, and subtracted from the initial weight to determine the ad lib food eaten. The restricted amount of food was calculated to 85% of the weighed amount of food that was eaten the first two days. The rats’ weight loss was closely monitored to maintain a healthy size. They were weighed prior to administration of the restricted diet and then subsequently weighed every three days until the completion of training. The rats then fasted overnight before the training began and were then fed their restricted diet after training session was completed each day. If the loss of weight rose to 10% of the original, the diet was increased to counterbalance that loss.

Montoya Staircase

The Montoya Staircase is a test developed by Montoya to assess the reaching capability of each forelimb. The ‘staircase’, as seen in Figure 2, is a clear Plexiglas box with a removable raised platform with seven bilateral graded steps. Each step has a well in which food pellets may be placed. This device allows the rat to step into the box on the platform and reach with the forelimbs into the wells to grasp the food. The steps allow for a gradual increase in difficulty of reaching the items in the wells due to an increase in the forelimb extension and grasping that takes place as the steps reach the bottom of the box (Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991). Plain and maple extract
painted sucrose pellets (Bioserv 45 mg dustless precision pellets) were used in this study as an incentive to train in the staircase. Using three pellets per well, a total of 21 pellets were placed on each side of the platform.

Each rat was trained in the Montoya Staircase on a daily basis during the dark cycle for 1.5 weeks prior to surgery. Three staircases were placed side-by-side and three rats (one in each staircase) were placed into them to allow for more rats to be tested in a shorter amount of time. They were placed in the staircase for 15 minutes and then returned to their cages. The number of pellets taken from the wells was recorded and the highest values were noted as the pre-stroke number of pellets taken. Any rat that did not take more than nine out of the 21 pellets was excluded from the analysis. These rats were considered to have not taken to the training or did not like the sucrose pellets as a reward and could not be reliable to collect data from post-surgery because their deficit could not be reliably quantified. After surgery was performed the animals were retested on the Staircase to determine their initial functional deficit (post-stroke days 3-5) and then their functional recovery (tested every thirty days).
Figure 2: A rat performing the functional testing in the Montoya Staircase.
INDUCTION OF STROKE

Summary

Stroke was induced to the right hemisphere of all rats in this study. This induction caused impairment to the left forelimb, or the contralateral limb. The stroke was induced via two injections of a vasoconstrictor, endothelin-1, while under anesthesia.

Prior to Stroke Induction

The rats’ cages were taken out of their room individually and brought into the operation room during surgery. Once taken out of their cage, the empty cage was placed onto a heating pad so it was warm for the rat during recovery. The rat was placed into a chamber that administered 5% isoflurane gas. Once the rat was sedated, it was taken out of the chamber and the top of the head was shaved. Puralube eye ointment was applied to the eyes to keep them from drying out and the head was sterilized with povidone-iodine, ethanol, and then povidone-iodine again. They were then laid on top of a heating pad to keep their body temperature at a safe level during the procedure and placed in the stereotactict apparatus to stabilize their head. A specialized gas mask was attached to the rat’s nose to continually administer a 2-3% isoflurane gas during surgery. The rate of respiration, observation of color of the flesh, as well as a foot-pinche withdrawal reflex method was used to monitor the effectiveness of the anesthesia and the health of the rats during surgery.
**Stroke Induction Operation**

A straight line was cut down the midline of the skull using scissors and bupivacaine was applied to the lacerated skin. The location of bregma was found and identified with a fine point marker and aligned with a burr bit in a microdrill. The forelimb motor cortex was located with an atlas and coordinates were used from Bregma to find the appropriate location. To drill the first hole in the skull, the drill was moved -2.5mm medial-lateral and 0mm anterior-posterior from bregma. The second hole was drilled 2.5mm medial-lateral and +1.5mm anterior-posterior from bregma. The microdrill was removed and a Hamilton syringe was inserted into the stereotactic apparatus. The syringe contained 3µl of endothelin (400pmoles/µl). The endothelin (1.5µl per hole) was slowly injected into the drill sites at a depth of 2.0mm. After the injections were administered, the syringe was removed and the skin was sutured. Resorbable sutures were used and after closure, the site was sterilized again with povidone-iodine.

**Post-Stroke Care**

A 2ml injection of saline was administered subcutaneously prior to placing the rat back in its warm cage. After being placed in the cage, the rat was monitored until it woke up from the anesthesia and began moving normally again. After normal activity was observed, the rat and its cage were taken back into the room where they were housed, and the next rat was brought out. All of the rats received moist food on the floor of their cages for two days after surgery to make sure food was accessible and easy to consume.
TREATMENT AND TRAINING

Drug Administration

Treatment for the stroke started 6-12 hours post-procedure and lasted for 90 days. The rats received either the drug/vehicle combination or they just received the vehicle. The rats that received the vehicle only were in the control group. The drug combination used in this study contained 1mg/kg of Simvastatin and 5mg/kg of Fluoxetine, also referred to as FS throughout this paper. This drug combination was selected based on a previous study which found a 19-fold increase in neurogenesis in ischemic rats treated with this drug combination versus controls (A. M. Corbett et al., 2015). This drug combo was in a powder form and placed into the vehicle. To reduce stress, a 3-4g ball of Pillsbury sugar cookie dough was the chosen vehicle used to administer the drugs. This was selected specifically because the rats ate the dough voluntarily and in entirety in a previous study (A. Corbett et al., 2012). If a rat did happen to refuse the cookie dough 3 or more times, they were removed from the analysis.

There was no additional administration of pain medication after the stroke. This includes non-steroidal anti-inflammatory drugs as well as analgesic opioids because there has been research performed that has found that these medications influence neurogenesis (Ajmone-Cat & Minghetti, 2008).

Post-Operative Montoya Staircase Training

The rats were given three to five days to heal before the functional testing began, and then the post-operative testing was done after this recovery period to gauge the level
of deficit caused by the stroke. The number of pellets taken from the wells during this time was recorded, then divided by their performance with the same paw pre-stroke, to give us a normalized contralateral function and ipsilateral function. If this quotient was less than one, then the animal had a functional deficit, which could be determined by subtracting this value from one. For example, if a rat retrieved 6 pellet post-stroke with its left paw, but it had retrieved 18 pellets pre-stroke, then its contralateral function would be 0.33, and the rat would have a 67% deficit in that limb. The rats were again placed in the staircase at 30, 60, and 90 days post-surgery, to monitor functional recovery. The exact days on which the rats were tested are as follows: post-stroke days 3 to 5, post-stroke days 29 to 31, post-stroke days 59 to 61, and post-stroke days 89 to 91.

EUTHANASIA AND HISTOLOGY

Euthanasia

After 91 days of post-operative study, the rats were euthanized. Each rat was taken into a room for anesthesia and euthanization separately to minimize trauma and stress. An overdose of sodium pentobarbital (100mg/kg Euthasol) was given and then exsanguination was initiated by clamping a catheter allowing phosphate buffered saline (PBS) flow into the apex of the heart (left ventricle), while cutting the right atria. Saline was pumped into the left ventricle of the heart while the blood drained from the right atria. Following approximately 100 ml of PBS infused through the heart, the solution was switched to 4% paraformaldehyde in PBS and the animal was kept on this solution until
at least 150 ml had been pumped through the heart. Dissection and removal of the brain followed and the left hemisphere was marked.

**Preservation and Preparation of Brain Slides**

Each brain was placed immediately into a solution of four percent paraformaldehyde in PBS overnight after blocking the brain (cutting it into coronal sections) and retaining the section, which included the infarct. The following day the brains were moved into a solution of 30 percent sucrose for a duration of three days. The brains were taken from the sucrose solution and placed into Optimal Cutting Temperature compound to a cutting post. A Peltier cooling device on the cryostat machine was used to freeze the brain and Optimal Cutting Temperature combination. This freezing process lasted approximately 30 minutes, because the tissue and compound had to reach the same temperature of the cryostat before the tissue could be sectioned safely. If the sectioning was completed too quickly, the tissue could be damaged and lost.

The brain was then sectioned into coronal slices measuring 50 µm in thickness and placed into four labeled vials of solution. The solution in the vials was a phosphate buffered solution. The tissue was separated into four vials because other experiments needed to utilize the same tissue from the experiment for a separate analysis. This means that after the sections are placed in their separate vials, they will each be stained or marked with a different solution for their specific experiment. The vial of sections used in this experiment were marked with doublecortin.
Sectioning

The sectioning was done throughout the entire ventricle area of the brain. Once the ends of the ventricles were reached, the sections could be prepared for mounting on slides. The vials were emptied into petri dishes of fresh phosphate buffered solution and carefully placed on a glass slide with a small paintbrush to minimize any tearing of the tissue. Up to five sections could be placed onto one slide for mounting. Once the sections were strategically placed on the slides they were set aside to dry and placed in a slide holder.

Staining

Once the slices of tissue were dry, they could then be tagged. In this study, a marker was used instead of a stain. The binding protein doublecortin, DCX, was used to visualize new neurons at the site of damage. This protein binds to new neurons to mark or tag the site of neurogenesis. The tissue was blocked with PBS-Tween containing the serum from the host of the secondary antibody for one hour. The doublecortin antibody was then added and the vials were left to shake overnight in the cold. The antibody solution was then removed and the vials were washed with 5 ml of PBS-Tween two times before putting on the secondary antibody in blocking solution for two hours at room temperature.
Microscopy and Image Collection

The slides were analyzed via brightfield microscopy using a 4X objective lens on Wright State University’s Olympus Epi Fluorescence SPOT Scope with RT color camera. This equipment is available to Wright State University students through the Microscopy Core Facility in the Biological Sciences building. The microscopes can be checked out for chunks of time using the iLab Solutions website.

Once the images were collected via the SPOT microscope, they were pieced together, or montaged, using Adobe Photoshop. This method was only used when the images of the stained brain slice were too large to fit into one screen for saving purposes. When this occurred, multiple images were captured and then overlapped into one large picture with Adobe Photoshop.

ImageJ Capture for Quantification

The amount of neurogenesis had to be quantified in each section of tissue. To accomplish this, the software program ImageJ was utilized. The software allows the user to select the desired area (in this case it is the area of doublecortin marked tissue as seen in Figure 3) and calculate that area. The program was calibrated to utilize the same scale as the microscope, 150µm. It then produced an area in mm² that could then be stored in Excel to later analyze.
Calculation of Area from ImageJ

The area of neurogenesis was then calculated using some simple math to represent the average amount of neurogenesis taking place in each rat. The doublecortin-labeled tissue was outlined and the threshold was adjusted. Once this was done, the program created a mask of the original image to include only the labeled tissue. When this mask was produced, values for the area of this mask were simultaneously placed in a table. Each value produced from ImageJ per rat was first added together and then averaged to give an average area of neurogenesis. This number signified the amount of neurogenesis for one rat.
Figure 3: Brightfield microscope images of right ventricles from both the control group and FS-treated group used for DCX analysis in ImageJ. These images were chosen because their average DCX levels were representative of their respective groups.
III. RESULTS

ANALYSIS SOFTWARE

Both unpaired t-tests and two-way ANOVAs were performed using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com.

MONTOYA STAIRCASE TRAINING ANALYSIS

Weight Analysis

Weight was monitored during the experimental process and documented, as seen in Table 1. A two-way ANOVA with repeated measures was done on these weights and the control group was significantly heavier in grams than the drug-treated rats. In Figure 4, the significance between the two groups is seen at the beginning of the experiment and remains constant throughout the experiment. This means, although there was a significant difference in weight between the two groups, it happened by chance and was not related to the administration of the treatment drug.
Table 1: This table lists the weights in grams of each rat in both groups throughout the duration of the experiment. Along the left hand column are the numbers assigned to each rat.

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Figure 4: Graph shows the average rat weights in grams for both the control and FS groups throughout the duration of the experiment. The control animals were significantly heavier than the drug-treated group, but this was not related to treatment.
**Functional Analysis**

Every rat was trained on the Montoya Staircase for one and a half weeks prior to stroke induction. The training occurred every day for a duration of 15 minutes per rat during the nighttime cycle. This training established a baseline for pre-stroke forelimb function. The sucrose pellets taken by each rat were counted and documented to later determine the deficit induced by the stroke, as well as to gauge the level of recovery and possible neurogenesis. If a rat failed to collect at least nine pellets during the initial pre-stroke training, they were excluded from this study.

After stroke induction, Montoya testing was continued after a three to five day recovery period to heal. The testing was then repeated at 30-day intervals to observe functional deficit and recovery. There were some rats that did not show a contralateral deficit, which means the number of pellets taken after surgery did not change from their baseline value. If they showed a decrease in both the contralateral and ipsilateral sides post-stroke, their deficits were bilateral. This information is listed in Table 2.
Table 2: This table summarizes the groups of rats by numbers in each group and the types of deficits that occurred.

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<th>Bilateral Deficit</th>
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<tr>
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<td>0</td>
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To establish a post-stroke deficit, the number of sucrose pellets taken from the wells were divided by their pre-stroke value for both the contralateral and ipsilateral side; the contralateral side being the left limb and the ipsilateral side being the right limb. This number was multiplied by 100 to give the percentages seen in the two graphs below which represent functional recovery based on the pre-stoke functional baseline values. See Figure 5.

A two-way ANOVA with repeated measures was performed on the contralateral side. The control group showed a significantly higher percent recovery from the FS group, p-value of 0.0005, F (3,57) = 6.89 and as time progressed, each training session showed a significant increase in function, p-value of 0.021, F (1,19) = 6.298. The same analysis was conducted for the ipsilateral side with no significance seen.
Montoya Forelimb Mobility Results

Figure 5: Results from the Montoya training for the contralateral and ipsilateral forelimb function between control and FS throughout the experiment based on the pre-stroke function.
To further visualize the final functional recovery between the contralateral and ipsilateral limbs, the daily recovery was calculated by subtracting the percentage of contralateral and ipsilateral recovery on the current day of training from the percentages calculated on the first day of training after surgery (Figure 6). This would mean that if a rat took fewer pellets from the wells on one side during day 60 of Montoya testing than it did on day 3, a negative value would be calculated for the recovery on that day. A two-way ANOVA with repeated measures resulted in a significant increase in the recovery over time for the contralateral limb (p-value = 0.029, F (2, 38) = 3.89), but there was no significance for the ipsilateral recovery.

The final day of Montoya testing occurred on days 89-90. The number of pellets taken on these days was compared to the number taken prior to stroke induction. The same values calculated from the first days post-stroke were then subtracted from the number calculated on the final day as described above to give a total recovery value. These values were analyzed with a two-way ANOVA, p-value = 0.0099, F (1, 38) = 7.36) with the contralateral recovery being significantly higher than the ipsilateral recovery. There was no significance between control and FS groups. See Figure 7.
Figure 6: Contralateral and ipsilateral recovery values based on the deficit 3-5 days after stroke induction.
Figure 7: Overall functional recovery analysis from Montoya training on contralateral and ipsilateral sides.
ANALYSIS OF DOUBLECORTIN LEVELS

Doublecortin levels were analyzed in ImageJ using the quantitative tool to find the area of doublecortin-stained tissue in the SVZ. The anterior, middle, and posterior ventricles of both the left and right hemispheres were all documented. T-tests with Welch’s correction were run on the subventricular zone to compare the control with the FS rats, no significance was found between the two groups. When looking at the left and right hemispheres within both the control and FS-treated rats, there was significantly more doublecortin in the right hemisphere of the anterior (p = 0.047) and middle (p = 0.034) regions of the FS-treated rats but not the control. To compare the variances, the F-test was completed and showed the anterior region to be non significant, while the middle region showed significance with a p-value of 0.0007. There was no significance between the two hemispheres in the posterior region. See Figures 8-10.
Doublecortin Levels in the Anterior SVZ

Figure 8: On the top an analysis of doublecortin levels in the anterior SVZ for control and FS groups, no significance, and on the bottom an analysis of doublecortin levels in the right and left anterior hemispheres for the FS group, $p = 0.047$. 
Figure 9: On the top an analysis of doublecortin levels in the middle SVZ for control and FS groups, no significance, and on the bottom an analysis of doublecortin levels in the right and left middle hemispheres for the FS group, $p = 0.034$. 
Doublecortin Levels in the Posterior SVZ

Figure 10: On the top an analysis of doublecortin levels in the posterior SVZ for control and FS groups and on the bottom an analysis of doublecortin levels in the right and left posterior hemispheres for the FS group with no significance in either posterior region.
The average areas (in mm$^2$) for each rat are shown in Tables 3 and 4. The control animals are listed first, followed by the FS-treated animals. The number of coronal slices per region and hemisphere are listed beside their respective position. The areas of these slices were averaged to get the value for each location in the brain per rat. The numbers in the colored boxes are the outlier values that were excluded from the graph in Figure 12, labeled “WITHOUT outliers”. These outlier values (8 values total) were excluded based on the results from the ROUT method outlier test in Prism. This exclusion of outliers was done to assume a normalized sample size out of our data points.

The right and left hemispheres were separately analyzed in both the control and FS-treated group by regions in the SVZ; anterior, middle, and posterior, respectively. See Figure 11. Only the left hemisphere showed any significance. Using a two-way ANOVA, the control showed a significantly higher average area of doublecortin levels than the FS-treated group in the left hemisphere, p-value $0.048$, $F (1, 58) = 4.09$.

The same analysis was completed for the values minus the outliers. A total of 8 values were excluded when plotting without the outliers. In this analysis, the left hemisphere showed no significance while the right hemisphere showed a significance between the regions, p-value $0.0016$, $F (2,51) = 7.360$. This can be seen in Figure 12.
Table 3: This table shows all the average measurements of doublecortin levels in the control rats. The number of coronal slices included in each average is listed beside the average. The colored values were the outlier values determined in Prism.
Table 4: This table shows all the average measurements of doublecortin levels in the FS-treated rats. The number of coronal slices included in each average is listed beside the average. The colored values were the outlier values determined in Prism.
Doublecortin Levels of Right and Left Hemispheres

Figure 11: The average area of doublecortin was analyzed by group and region in the left and right hemispheres, respectively. A significantly higher area of DCX was found in the left hemisphere of the control group than the FS group $p = 0.048$. 
Figure 12: The average area of doublecortin was analyzed after excluding the outlying areas seen in tables by group and region in the left and right hemispheres, respectively. A significance between the regions was found $p = 0.0016$. 

Doublecortin Levels of Right and Left Hemispheres 
*WITHOUT Outliers*
IV. DISCUSSION

FINDINGS

This study found no significance in the functional recovery between the control and drug-treated animals, though both groups did show a significant increase in contralateral limb function as the experiment progressed. Neurogenesis was not found to be significantly higher in the fluoxetine and simvastatin-treated group than the control animals. There was a significantly higher area of neurogenesis in the anterior and middle subventricular zone of the stroke-damaged right hemisphere than the left hemisphere when the drug-treated group was analyzed separately from the control group, which did not show this same significance.

Functional Recovery

Both the control group and the FS-treated rats showed a consistent and significant increase in functional recovery of their contralateral forelimb every 30-days during Montoya testing. This means that both groups showed a significant improvement in the stroke-damaged forelimb throughout the duration of the experiment, but they were not significantly different from each other (see Figure 6). This could be because it has been shown that small infarcts induced via endothelin-1 have enhanced neurogenesis in the
SVZ in adult mice without pharmacological intervention (Wang, Jin, & Greenberg, 2007). It is possible that the effects of the drugs were not high enough for us to tell the difference between the natural recovery and the treatment-induced recovery.

The drug-treated rats showed a significantly lower contralateral forelimb mobility than the control group (see Figure 5). Although the FS group displayed significantly lower contralateral forelimb functional recovery, they did show a higher recovery rate from their initial post-stroke deficit, though not significant. This means that the early administration of the drugs may have caused more damage after the stroke, but it did result in a greater, though insignificant, recovery than the control group. In fact, in a parallel study, it was found that earlier administration of the drug combination (6-12 hours post-stroke) resulted in not only a larger infarct size than had previously been seen when this drug treatment was delivered beginning 20-26 hours after stroke induction, but in some cases, secondary hemorrhagic stroke was observed. Images of the secondary hemorrhagic stroke from this same study can be visualized in Figure 13 (Balch et al., 2015; Ragas et al., 2015).
Figure 13: Image A shows a control animal from the 6-12 hour administration time study with the infarct area outlined. Image B shows an FS-treated animal from the 6-12 hour administration time study with infarct and secondary hemorrhagic damage outlined. Image C is a control animal from the 20-26 hour drug administration study with infarct outlined. Image D is the FS-treated animal from the 20-26 hour administration time study with infarct outlined. Figure from work by Maria Balch et al.
Possible Explanations for Increase in Functional Recovery

We saw an increase in contralateral functional recovery in all of our research animals at each 30-day Montoya testing for the duration of our study, with the 90-day analysis being the highest. We looked specifically at neurogenesis in this study, but there are other factors that contribute to functional recovery after ischemic stroke.

Angiogenesis and neuroplasticity also play major roles in increasing functional recovery. Neuroplasticity is the ability of the brain to alter pathways, which bypass damage, and has been studied as a factor in functional recovery after an ischemic stroke. There are multiple ways that the brain responds to the injury. One way it compensates for the damaged tissue is to utilize pathways in the brain that are only activated after the damage-inducing stroke occurs. There are also new synapses that sprout from fibers of surviving neurons and create new pathways. Neural routes also exist in the central nervous system which are typically redundant or parallel in function, and they can replace the role of some of the broken circuitry and retain the function of the lesioned tissue (Font, Arboix, & Krupinski, 2010; Lee & van Donkelaar, 1995).

Angiogenesis is the formation of new blood vessels. Small microvessels form after MCAO-induced stroke, and although they prove to be temporary and fragile, they may be crucial for the removal of necrotic tissue and begin the process of angiogenesis. Within hours after stroke induction, growth factors that influence angiogenesis (FGF-2, PDGF, VEGF, and their receptors) are seen in the brain and up-regulated to begin the growth of new blood vessels. Within five to seven days, fully functioning vessels are
present (Font et al., 2010; Xiong, Mahmood, & Chopp, 2010). These new blood vessels also aid in neurogenesis because they deliver the nutrients and support for their survival.

This same increase in angiogenesis may have also led to the secondary hemorrhagic stroke in the drug-treated animals discussed previously. Vascular endothelial growth factor (VEGF) is a growth factor associated with angiogenesis and the increased permeability of blood vessels. This is very important during repair and neurogenesis because it allows for the delivery of essential restoration components after ischemic stroke (Duffy, Bouchier-Hayes, & Harmey, 2000; Z. G. Zhang et al., 2000). It has been found in previous studies that fluoxetine and simvastatin increase levels of VEGF (Chen et al., 2003, 2005; Warner-Schmidt & Duman, 2007), but in some cases this is not always beneficial.

A study conducted in 2000, found that early administration of VEGF did not result in the typical positive result of angiogenesis to the repair of damaged tissue. When administered 1-hour after stroke, it caused excess permeability to the blood vessels in the surrounding area and led to collapse of the vessels and hemorrhaging (Z. G. Zhang et al., 2000). This is the most probable reason for our secondary hemorrhagic damage in our FS-treated rats. The VEGF levels were so high, the newly formed blood vessels became too permeable, and their structures collapsed, leading to blood leaking into the brain and creating additional damaged tissue.
Rehabilitation

It should also be noted that as part of another study, the same rats in this paper were also given rehabilitation to study its effects on recovery. It has been studied previously that the voluntary forced rehabilitation after stroke has yielded a significant increase in functional recovery (Livingston-Thomas, McGuire, Doucette, & Tasker, 2014), but rehabilitation was shown to not have a significant effect on functional recovery results when comparing all the control animals to all the drug-treated animals in our particular study. Only rats not given any treatment at all (neither rehabilitation nor the simvastatin and fluoxetine drug combination) displayed a significantly lower functional recovery. This means that rehabilitation did not increase functional recovery (Ragas et al., 2015). We had hoped to see increased functional recovery correlated with the increased production of new neurons; however, we saw no correlation between the two. Drug treatment did not produce significant increases in neurogenesis nor did it translate to an increase in functional recovery.

Stroke-Induced Neurogenesis

While there was no correlation between an functional recovery and neurogenesis, we found a significantly higher amount of neurogenesis in the right hemisphere of the anterior and middle subventricular zone in the drug treated animals when we analyzed them separately from the control group. These findings suggest that the increase in neurogenesis was not just from the stroke induction itself, which can cause an increase in production of neurons at the site of trauma (Lichtenwalner & Parent, 2006).
Another possibility could be that the drug dosage was insufficient to create a quantifiable difference in neurogenesis when compared to the control group. Previous work showed that fluoxetine treatment increased neurogenesis but it did not translate to functional recovery (X. Sun et al., 2015; Windle & Corbett, 2005). This may have been what happened in our study as well, but it is difficult to compare the two studies due to the administration of the drug treatment. We administered our drug combination via cookie dough to lower stress while Sun, et al. used an implanted pump, which may have caused additional stress to the animals. To look further into this aspect, the experiment needs to be repeated, to see if the drug dosage needs to be altered.

_Prolonged Fluoxetine_

There is another possible explanation for the lack of evidence of a significant increase in neurogenesis between the drug-treated group and the control group. It has been shown that prolonged administration of fluoxetine may decrease the growth of new neurons. A study in 2011 found that chronic treatment with fluoxetine for more than 6 weeks decreased neurogenesis in the subventricular zone of adult mice (Ohira & Miyakawa, 2011). Our study lasted a duration of 90 days, which exceeds the length of time they found to show a decrease in neurogenesis. They had some distinct differences in their study; the use of mice instead of rats and the dosage of fluoxetine. Their dosage of fluoxetine was 15mg/kg, while we chose a lower dosage of 5mg/kg.
FUTURE WORK

The research conducted for this thesis provided us with some confounding information, and it has raised some questions on how to proceed from this point. There is promise for some answers to these questions with more experimentation.

Repeat Study

There were multiple issues that could be resolved if these experiments were repeated. The elements of the drug treated group, rehabilitation, increasing the number of animals in the study, and monitoring neurogenesis are all variables that could give clearer results, if altered.

Changes to the Drug Treatment

A previous study was conducted in our lab which showed that the drug combination chosen for this study resulted in the highest increase in functional recovery after ischemic stroke (A. M. Corbett et al., 2015). Repeated research would yield more information on the best time, dosage, and duration of the FS drug combination.

Time of Drug Delivery

We saw a larger functional deficit in the drug-treated animals and this is most likely due to the early delivery (between 6-12 hours after stroke induction) of the drug combination causing a secondary hemorrhagic stroke which was observed in a parallel study and seen in Figure 13 (Balch et al., 2015). A later administration of the drug treatment of 20-26 hours was tested previously without the observation of additional
damage to the animal (A. M. Corbett et al., 2015). This is the most crucial change to the methods of this research when repeating because of the additional damage to the neural tissue and the resulting functional deficits it invoked.

*Alter the Drug Dosages*

There have not been additional experiments conducted that alter the dosage amounts for each drug in the combination. It would be extremely beneficial to repeat this study and try multiple variations of dosage combinations to see which one yields the highest increase in not only functional recovery but also neurogenesis.

*Duration of Drug Treatment*

Once the drug combination factor is determined, it would be beneficial to reproduce this work with different duration times to see which produces the optimal results. Based on the findings from a study which found that chronic fluoxetine treatment of 6 weeks decreased neurogenesis (Ohira & Miyakawa, 2011), it would be interesting to see how the duration of drug administration would affect functional recovery as well as neurogenesis. This is a key element to resolve for the treatment of human patients, especially if chronic treatment after a certain amount of time proves to be detrimental to the patient as opposed to being beneficial.

*Removal of Rehabilitation Component*

It would also be advantageous to take out the rehabilitation factor when repeating this work. It added a component to the experiment that did not show any benefit when
conducted with a drug. It may have also confounded the results by adding a variable, which was hard to account for when trying to quantify the effect of a drug treatment on neurogenesis.

*Increase Number of Animals*

We had a total of 22 rats (11 control and 11 FS-treated) in this study and some of our results yielded a high amount of error due to a couple of outlying data points. When your sample size is this low, a couple of outliers increase the error drastically and decrease the chance of getting data with a significant p-value. A clearer picture of the significance of drug treatment on neurogenesis would be seen with an increase in sample size.

*Monitor Neurogenesis*

There are limited ways to quantify neurogenesis at the moment. There is no neuroimaging software or functional test to calculate the amount of neurogenesis without euthanizing the rat. The best way to monitor neurogenesis would be to repeat the research conducted in this study and euthanize at different intervals. This will tell us when neurogenesis is at its highest. It will also help us find the best window of time for drug administration to give us peak neurogenesis and functional recovery results.
CONCLUSIONS

This study did not find a correlation between functional recovery and increased neurogenesis. Though we saw no significance increase in the contralateral functional recovery of drug-treated animals, there was significantly more neurogenesis in the right hemisphere in the anterior and middle region of the SVZ of the FS rats. This tells us that the drug treatment did produce a significant amount of neurogenesis at the site of damage, but further work needs to be done to see if an alteration of the drugs administered can produce a significance from the control group as well.

The secondary hemorrhagic stroke caused by the early administration of the drugs may have confounded some of the findings of this study. The secondary stroke most likely led to the contralateral functional recovery being significantly lower for the drug-treated group than the control. The drug treated group did show a higher total recovery, which means that without the initial damage of the secondary stroke to repair; they may have displayed significantly higher contralateral functional recovery from the beginning of the study.

Further research needs to be conducted to better understand how the fluoxetine and simvastatin drug treatment affects neurogenesis and functional recovery. There may be an optimal window of time and dosage that will lead to a greater significance in recovery and higher levels of neurogenesis. This is key to helping human patients not only recuperate after experiencing a stroke, but to increase their quality of life after losing functional capabilities.
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