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The Use of Forelimb Asymmetry Functional Tests to Determine Motor Recovery With Various Drug Treatments Following Endothelin-Induced Stroke

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THE USE OF FORELIMB ASYMMETRY FUNCTIONAL TESTS TO DETERMINE
MOTOR RECOVERY WITH VARIOUS DRUG TREATMENTS FOLLOWING
ENDOTHELIN-INDUCED STROKE

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

By

KELLY REBECCA LEACH

B.S., Wright State University, 2010

2012

Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

July 27th, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Kelly Rebecca Leach ENTITLED The Use of Forelimb Asymmetry Functional Tests to Determine Motor Recovery With Various Drug Treatments Following Endothelin-Induced Stroke BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Our laboratory has found increased functional recovery post-stroke with Long Evans rats that received a combination of fluoxetine, simvastatin, and vitamin C. The objectives of this study were to test that treatment and other drug combinations on Sprague Dawley rats, to determine whether Forelimb Asymmetry is a reliable behavioral measure for stroke studies, and to determine the most optimal surgical procedure on Sprague Dawley rats. There were more significant increases in functional recovery observed on the Forelimb Asymmetry than on Montoya Staircase behavioral tests, but there were no significant differences between treatment groups and control. The largest number of rats that had at least a 20% deficit was in the group that underwent stroke induction through injecting 1.5 μ l endothelin into the stereotactic sites AP (0), ML (-2.5), DV (2.0) and AP (1.5), ML (-2.5), DV (2.0) from bregma, which should be utilized in future stroke studies with Sprague Dawley rats.

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I. INTRODUCTION

Stroke is the number one cause of long-term, serious disability in the United States (1). The Framingham Heart Study (FHS) by the National, Heart, Lung, and Blood Institute (NHLBI) found these disabilities six months after stroke in ischemic stroke survivors at least 65 years old: 50% exhibited some hemiparesis, 30% were unable to walk without assistance, 26% needed help with daily living, 19% exhibited aphasia, 35% exhibited symptoms of depression, and 26% had to live in a nursing home (1). When logistic regression analyses were performed on the data from the FHS, it was found that women had higher instances of post-stroke disability than men, but it was due to the correlation of greater disability with older age at stroke onset, not gender (2). Six million four hundred thousand Americans at least 20 years old have had a stroke (1). About 795,000 people have a stroke each year, which means on average, every 4 minutes someone dies from a stroke (1). The average cost of healthcare for stroke victims in 2010 was \$73.7 billion (1), so there needs to be studies on the long-term healthcare costs for ischemic stroke (3). This is a major nationwide issue. Part of the problem is due to the fact that the median time between stroke onset and arrival at the emergency room is 3 to 6 hours; the clot buster drug must be given within 3.5 hours of stroke onset in order to be effective, and there is risk of hemorrhage with that treatment (1). Therefore, the only treatment option most stroke victims have is rehabilitation, which can be helpful; rats in an enriched environment display an increase in progenitor cell proliferation and neurogenesis in the subventricular zone (SVZ) and exhibit improved function on the

beam-traversing and Montoya Staircase tests (4-6), and rodents that run on the wheel on a regular basis show an increase of BDNF receptors, CREB mRNA, and growth-associated protein (GAP-43) (7). However, the high instances of disability and death show that rehabilitation alone does not completely help a stroke victim regain function.

NEUROGENESIS POST-ISCHEMIA

After stroke, studies have shown that neurogenesis occurs to compensate for and fix the damage caused by the ischemia. In mice that have undergone an ischemic injury of the central nervous system (CNS), there is an increase in neurogenesis from GFAP-expressing progenitor cells in the SVZ, and new cells migrate to a niche in the peri-infarct cortex (8). New neurons have been found near the infarct in even human postmortem stroke victims (9). There is evidence that brain derived neurotrophic factor (BDNF) works as a chemoattractant for immature neurons migrating from the lateral ventricles (10-16) After ischemic stroke in rats, BDNF was found in both hemispheres; more was found in the hemisphere with the lesion than in the undamaged hemisphere (17). At both four and 24 hours after stroke, there was an increase in BDNF activity in neurons and ependymal cells in the undamaged hemisphere, whereas an increase in BDNF activity was found in microglial cells, endothelial cells of cerebral arterioles, and astrocytes in the damaged hemisphere was found during those times (17). At 2 hours post-stroke in rats that have undergone middle coronary artery occlusion (MCAO), but not at later time points, there was a several-fold increase of the number of BDNF-immunoreactive cells in the ipsilateral, cingulate, and frontal cortices outside the damaged area from the MCAO (18). Those animals in that study with cortical injury showed loss of BDNF-immunoreactive fibers in the striatum at 2-24 hours post-stroke,

whereas rats with cell damage confined to the striatum exhibited no such change (18). At 2-16 hours post-stroke, strongly BDNF-immunoreactive fibers were observed along the myelinated fascicles medially in the striatum, in the anterior commissure, and in the corpus callosum ipsilateral to the MCAO (18). BDNF protein levels were increased by 133-213% at 2 hours post-stroke in the cingulate and frontal cortices and decreased by 40% at 24 hours post-stroke in the striatum (18). Enhancing this phenomenon with a pharmacological treatment could possibly lower the instances of disability and death after ischemic stroke. The question is: what sorts of pharmacological drugs and drug combinations would be successful in treating ischemic stroke?

POSSIBLE THERAPEUTIC EFFECTS OF FLUOXETINE

Antidepressants may be a valid option; they have been shown to increase the number of cells in the hippocampus, promoting neurogenesis, which could help with functional recovery (19). Many stroke patients develop depression, and there are many studies that indicate that SSRIs may have other benefits, especially fluoxetine. In one study, patients were given 20 mg fluoxetine or a placebo daily for 90 days starting 5-10 days after an ischemic stroke (20). The group that received fluoxetine had more improved Fugl-Meyer Motor Scale (FMMS) scores than the group that received placebo; the treatment group's score increased by an average of 14.5 points (95% CI [7.3-21.6]), whereas the placebo group's score increased by an average of 9.8 points (95% CI [3.4-16.1]) (20). However, digestive disorders may be a side effect of fluoxetine (20). Still, the ability to regain function may outweigh the side effects. One 20 mg dose of fluoxetine can increase the activation of the muscles recorded by EMG of the forearm by 21.5% on average in stroke patients (21).

One way fluoxetine may promote neurogenesis is through BDNF. Daily doses of 10 mg/kg fluoxetine or desipramine increase the gene expression of BDNF in the dentate gyrus (DG) of transgenic mice (15). However, mature BDNF protein is increased more rapidly than BDNF mRNA after treatment with 10 mg/kg fluoxetine daily (15). Fluoxetine and paroxetine upregulate BDNF, vascular endothelial growth factor (VEGF), and VGF mRNA expression, decrease glycogen production, and increase glucose utilization and lactate production in Swiss albino mice astrocytes, and these effects are not mediated by serotonin (22).

Chronic treatment with fluoxetine is more effective than acute treatment. For example, in one study, rats were given 10 mg/kg fluoxetine once daily for 1 day, 4 days, 7 days, 14 days, and 21 days; after 4 days of treatment, BDNF mRNA had decreased in the hippocampus (by 27% in CA1 and by 15% in CA3) and in the medial habenular (by 38%) and periventricular thalamic nuclei (by 30%), while after 14 days of treatment, BDNF-immunoreactive cells were increased in the hippocampus (by 47% in CA1, by 61% in CA3, by 53% in the DG, and by 40% in the parietal cortex) and in the medial habenular (by 101%) and periventricular thalamic nuclei (by 157%) (13). Also, fluoxetine does not promote cell proliferation when it is not a chronic treatment; rats were given 5 mg/kg fluoxetine daily for 1, 5, 14, or 28 days, and the number of BrdU-labeled cells in the DG was significantly greater in rats that were treated for 14 or 28 days than in the rats only treated for 1 or 5 days by about 1000 cells (19). Another way that fluoxetine promotes neurogenesis is that it increases cell proliferation, the incidence of cell clusters, and number of cells per cell cluster in the DG of stressed rats (10). These positive effects of fluoxetine are not always observed; rats treated with fluoxetine as well as

rehabilitation exhibited no significant functional recovery (23). However, to administer the treatment, 2 surgeries to implant an osmotic pump were performed on these rats (23); this could have caused stress in these rats, and stress reduces neurogenesis.

POSSIBLE THERAPEUTIC EFFECTS OF PAROXETINE

Another antidepressant that may have therapeutic effects for ischemic stroke patients is paroxetine; one study found that 20-40 mg paroxetine taken daily for 6 months restores the abnormal N-acetyl aspartate/creatine and choline/creatine ratios in the hippocampus and thalamus that are present in stroke survivors with depression (24). However, this study had no placebo group, so more research needs to be done to elucidate the possible therapeutic effects. Many patients who develop depression after a stroke take paroxetine, fluoxetine, or other SSRIs because they are safe to use; tricyclic antidepressants and serotonin-norepinephrine reuptake inhibitors can increase blood pressure and worsen cardiovascular disease (25). Also, paroxetine taken along with aspirin does not inhibit blood clotting (26). Therefore, antidepressants, especially fluoxetine and paroxetine, may be a safe and effective therapeutic agent for victims of ischemic stroke.

POSSIBLE THERAPEUTIC EFFECTS OF SIMVASTATIN

Statins may also be a good option for treatment of the effects of an ischemic stroke, especially simvastatin. Statins increase cerebral blood flow, decrease infarct size, improve neurological function, and upregulate endothelial nitric oxide synthase (eNOS) in mice after an ischemic stroke (27). Simvastatin has much potential as a possible therapeutic agent for treating ischemic stroke. For example, one study found that

simvastatin increases Notch signaling activity and Presenilin 1 expression, which induces arteriogenesis, which could help the healing process of the infarct from an ischemic stroke (28). Another study found that simvastatin directly induces angiogenesis by increasing the expression of VEGF (16). Simvastatin also increases the expression of BDNF, which induces cell proliferation and differentiation in the DG, and simvastatin enhances the recovery of spatial learning in rats after a traumatic brain injury (16). Simvastatin also lowers oxidative stress by lowering serum 8-isoprostane levels, an oxidant (29).

POSSIBLE THERAPEUTIC EFFECTS OF ATORVASTATIN

Atorvastatin has some potential as a therapeutic agent for treating or preventing ischemic stroke. For example, in one study, patients that had experienced an ischemic stroke were given 80 mg atorvastatin or placebo daily for 7 days; more of the patients in the atorvastatin group than the placebo group resulted in a disability score of less than 2 on the modified Rankin scale after treatment (odds ratio, atorvastatin vs placebo, 0.74; 95% CI [0.26-2.17]) (30). However, this effect was only found with patients that had experienced a mild stroke, and there was no difference in infarct size between the treatment and placebo groups (30). This lack of effect may be due to the patients only being treated for 7 days. One study found that atorvastatin improves function and plasticity by increasing levels of VEGF, increasing levels of cyclic guanosine monophosphate, angiogenesis, cell proliferation, neurogenesis, and levels of synaptophysin in rats after stroke (12). Also, fewer carotid artery plaques are observed after subjects are treated with atorvastatin, which could lower their risk factor for stroke (31). Atorvastatin also increases latency to seizures and is neuroprotective after stroke,

brain injury, and excitotoxic amino acid exposure in rats (32). Two 15mg/kg doses of atorvastatin given 12 hours apart lower the risk of hemorrhage by lowering excess hemoglobin by the infarct in type 2 diabetic Goto-Kakizaki rats that had undergone ischemic stroke through MCAO (33). Also, atorvastatin in combination with ezetimibe reduces inflammation and thrombosis in patients with atrial fibrillation, which could help prevent stroke (34). Even combining simvastatin with atorvastatin could help the healing process; with rats that were treated with both simvastatin and atorvastatin after a traumatic brain injury, spatial learning was improved, neurological loss in the CA3 region of the hippocampus was reduced, neurogenesis in the DG was observed, and an increased rate of angiogenesis was observed (35).

POSSIBLE PROBLEMS AND SIDE EFFECTS OF STATINS

However, some studies show a lack of sufficient evidence that statins could be helpful in treating survivors of ischemic stroke (36-37). In one study, patients that had experienced an acute, ischemic stroke within the previous 96 hours were given either 80 mg of atorvastatin or placebo daily for 30 days, and the infarct size was measured by perfusion computed tomography 3 and 30 days after the start of treatment; the infarct size was not significantly altered by atorvastatin: day 3: 454 mm² (IQR: 107, 1765), day 30: 462 mm² (IQR: 43, 1399) (36). A meta-analysis review found that statins do not significantly lower mortality in patients that had experienced ischemic stroke or transient ischemic attack compared to placebo: pooled odds ratio: 1.51, 95% CI [.6, 3.81] (37). Unfortunately, the safety risks of treating with statins are not completely clear. On the one hand, it may actually be dangerous to stop treatment with statins after a stroke; statin withdrawal in ischemic stroke victims increases the likelihood of death, dependency for

daily living, early neurological deterioration, and infarct volume (38-39). In one study, ischemic stroke patients who had been treated with statins chronically before stroke were given 20 mg atorvastatin daily for 3 months either within 24 hours or 3 days after stroke; patients that had to undergo statin withdrawal showed a higher instance of disability (modified Rankin score > 2): 60.0% vs 39.0% ($p = 0.043$), early neurologic deterioration (END): 65.2% vs 20.9% ($p < 0.0001$), and greater infarct volume: 74 vs 26 mL ($p = 0.002$) compared with the patients who were treated immediately (38). Statin withdrawal was also correlated with 4.66X the risk of death or dependency and 8.67X the risk of END after adjusting for age and stroke severity (38). On the other hand, taking statins could be dangerous; one major risk of using statins as a chronic treatment is that the patient could develop rhabdomyolysis, a disorder where the muscle fibers are broken down and released into the bloodstream (37, 40-42). Another risk is that some statins can interact with some antidepressants, namely nefazodone, and can cause rhabdomyolysis and other severe side effects (41). If used with some caution, simvastatin or atorvastatin could help stroke victims regain function.

POSSIBLE THERAPEUTIC EFFECTS OF VITAMIN C

High concentrations of vitamin C are found in the brain and other neuroendocrine organs (43). Vitamin C works as an antioxidant in the brain (43). It is utilized in many enzyme reactions, including catecholamine synthesis, collagen production, and regulation of antibodies (43). Vitamin C also works as an antioxidant along with vitamin E by improving endothelial function, decreasing levels of superoxide and NAD(P)H oxidase in the blood, and increasing levels of the NOS and nitric oxide in the blood (44). Since a drop in the level of vitamin C in the brain is observed after reperfusion after ischemic

stroke (45), the antioxidants would need to be replenished in the brain. The brain does this by enhancing expression and activity of sodium-dependent vitamin C transporter 2 (46). Vitamin C could therefore have therapeutic effects for patients with ischemic stroke, Alzheimer's, Parkinson's, and Huntington's (43). However, there is not much evidence of therapeutic effects of vitamin C for ischemic stroke patients. In one study, in stroke patients that were treated with vitamin C, their blood level of antioxidants increased, but their neurological function was not improved (47). Also, vitamin C can be broken down into ascorbate radicals, which is not conducive to healing (48-49). Still, vitamin C may have therapeutic effects on ischemic stroke patients when taken in combination with other medications, namely antidepressants and statins.

REASONS FOR THIS STUDY

Our laboratory has found increased functional recovery post-stroke with Long Evans rats that received a combination of fluoxetine, simvastatin, and vitamin C (50), so we performed some experiments to elucidate whether these or other medications would improve motor function in Sprague Dawley rats that have had an ischemic stroke from the injection of endothelin, to test whether Forelimb Asymmetry is a good measure of neurological recovery after stroke compared to Montoya Staircase, and to determine the most optimal surgical procedure on Sprague Dawley rats to produce a similar infarct size and contralateral deficit as the Long Evans rats in the aforementioned study. Forelimb Asymmetry is a measure of exploratory behavior in rats and has been used in many studies as an indicator of motor function, and Montoya Staircase measures a rat's ability to reach for and grasp a sugar pellet and has been used in many studies to measure fine motor skills in rats (4, 23, 51-64). In the previous experiment (50) and the experiments

detailed in this thesis, we injected endothelin into the cortex instead of performing MCAO because the cortical endothelin injection causes as much functional deficit, but MCAO has a higher mortality rate (51). The injection also results in a smaller infarct, which makes it a more authentic stroke model. Most stroke studies use Long Evans rats instead of Sprague Dawley rats because it is easier to produce a long-lasting reduction in blood flow in Long Evans rats because Sprague Dawley rats have more collateral blood supply in the brain (65). However, it is important to develop a method for studying stroke in Sprague Dawley rats because the vasculature of their brain is more analogous to the anatomy of humans than the brain anatomy of Long Evans rats, so different stereotactic coordinates and amounts of endothelin were tested. The animals were tested on Forelimb Asymmetry before and after stroke and were given either vehicle, fluoxetine in combination with simvastatin and vitamin C, fluoxetine with simvastatin, fluoxetine in combination with atorvastatin and vitamin C, or paroxetine in combination with simvastatin and vitamin C in generic or brand name form. Some animals were also tested on Montoya Staircase before and after stroke. Here, we show evidence of recovery over time but no differences in neurological function between treatment groups on Forelimb Asymmetry and develop a reliable method for inducing stroke in Sprague Dawley rats.

II. METHODS

ENDOTHELIN-INDUCED STROKE

Endothelin has been successfully used in many studies to induce an ischemic stroke in rats (23, 52-53, 66-68). Anesthesia was induced by inhalation of 5% isoflurane. The head was shaved, Puralube™ ointment administered to the rodent's eyes, and the animal mounted in a stereotactic apparatus using non-traumatic ear bars. Anesthesia was maintained with 2-2.5% isoflurane inhalation during surgery. The surgical site was cleaned first with povidone iodine, then 70% ethanol, and finally povidone iodine and a midline incision was made on the top of the head. Bupivacaine (0.25%) was administered to the incision in several drops. A micro-drill (Fine Science Tools), with a 0.9 mm burr drill, was used to drill two holes in the skull at the stereotactic sites indicated for each experimental group in each experiment. Endothelin (Human and Porcine, EMD Chemicals) at a concentration of 400 pmoles/ μ l (1 μ l unless otherwise indicated) was injected into each site (modified from the procedure of Windle et al. (51)) over the time-course of 3 minutes. The incision was sutured with Vicryl resorbable sutures and painted with povidone iodine. No post-operative analgesia (besides bupivacaine) was used, as all have been shown to modulate neurogenesis (67-68). All animals were given the drug vehicle (4 grams of purchased sugar cookie dough) following surgery to help accustom them to the sugar cookie dough. These methods have been modified from the procedure of Corbett et al. (50).

VOLUNTARY ORAL DRUG ADMINISTRATION

Pharmaceutical drugs (simvastatin, 80 mg tablet; fluoxetine HCl, 20 mg capsule; atorvastatin, 80 mg tablet; paroxetine, 20 mg capsule) were used and weights of active ingredients were compared to total dry drug weights, to account for any fill compounds. The correction factors for fill compounds (10X for simvastatin and atorvastatin and 7.5X for fluoxetine HCl and paroxetine) were used to measure out the correct amount of active ingredients for each animal. Ascorbic acid (Vitamin C, Fluka) was used in pure form.

The drug vehicle was 4 grams of purchased sugar cookie dough (Pillsbury). The cookie dough was weighed, rounded into a ball and then a depression was made into the ball with a push of a finger. The individually weighed drugs were put into the depression in the dough ball and rim edges of the depression were brought together and sealed so that all the dry chemicals were enclosed in the dough ball. The dough ball was thoroughly mixed manually to incorporate all of the chemicals into the dough, and reformed into a ball. Each ball either contained no drugs (vehicle) or the designated dosages of drugs for each animal assigned to the different drug groups. The dough balls were presented to individually housed rats in a glass petri dish around noon each day and left in the cage until the next day. Any remaining sugar cookie dough found the next day was counted against the complete ingestion of the drug or vehicle (reliability of the voluntary oral administration). Any animal that failed to completely eat their vehicle or medicine for a total of 3 days or more was excluded from the study. These methods were perfected by a previous study (69).

FORELIMB ASYMMETRY TEST

For this test, animals were placed in a translucent, acrylic cylinder (diameter: 9 inches) with maple extract painted 14 inches from the bottom for 5 minutes. A video camera recorded the animals' movements from a location adjacent and perpendicular to the vertical axis of the cylinder, and mirrors were placed on the rear lateral sides of the cylinder at an angle to ease identification of forepaws when the animal is turned away from the video camera. The animals were tested before the stroke and during various times after the stroke. The number and timing of the post-stroke trials will be indicated for each experimental group in each experiment. The videos were later viewed on QuickTime software in slow motion and scored by an observer blind to treatment conditions. The number of times the ipsilateral (right) forepaw and contralateral (left) forepaw touched the side of the cylinder as the animal was exploring were counted. A touch was counted if any part of the forepaw (even just the tips of the digits) touched the side of the cylinder. A paw sliding on the side of the cylinder only counted as one touch; the paw had to be lifted in order for another touch to be counted. Simultaneous touching of both forepaws was counted as one ipsilateral and one contralateral touch. Proportion of ipsilateral ($\text{ipsilateral touches}/(\text{ipsilateral touches} + \text{contralateral touches})$) and contralateral ($\text{contralateral touches}/(\text{ipsilateral touches} + \text{contralateral touches})$) touches were calculated for each trial. For the post-stroke trials, deficits for each forepaw were calculated by dividing the proportion of touches for the forepaw of interest over the proportion of touches for said forepaw in the pre-stroke trial. Therefore, if the quotient for an animal approaches 1, it is back to normal pre-stroke performance. If the quotient for an animal is less than 1, then it has a functional deficit in that forepaw (e.g. a value of

0.6 indicates the animal has 60% of its pre-stroke function, or a functional deficit of 40%).

MONTOYA STAIRCASE

Prior to beginning training on the Montoya Staircase, animals were fasted overnight. During the training animals received restricted rat chow, equivalent to 85% of their ad lib feed/day. Training took place during the dark phase, with one 15 minute test each day for each rat for a maximum of two weeks. Each well of the staircase contained three 50 mg sucrose pellets, which had been painted with maple extract and allowed to completely dry. The final three days of training were used to establish the pre-stroke baseline, with the best performance (total pellets retrieved) used. If more than one trial had the same number of total pellets retrieved for the best performance, but different performance for the forelimbs, then the results were averaged. Animal weight was not allowed to fall below 90% of their ad lib feeding weight during training. The number and timing of the post-stroke tests will be indicated for each experimental group in each experiment. Post-stroke tests followed an initial overnight fast and testing was for three days in the dark phase. Animal rat chow and sugar cookie dough ball for this time period totaled 10 grams per day. Any pellets the animal retrieved were not counted against their total food in either the training or post-stroke testing. Only animals retrieving at least 9 pellets in each forepaw by the end of training were included in this part of the functional analysis. Animals failing to retrieve any pellets during post-stroke tests were excluded from this part of the functional analysis.

STATISTICAL ANALYSIS

All statistical analysis used repeated measures ANOVA with Tukey post-hoc analysis unless the data did not pass the Shapiro-Wilk normality test or the equal variance test, in which case the Mann-Whitney rank sum test was used. A student's t-test was used for comparing 2 sets of data. $\alpha < .05$ was considered significant.

ANIMALS AND EXPERIMENTAL DESIGN

Female Sprague Dawley rats (10-12 months) were housed under 12-hour light/12-hour dark conditions with *ad libitum* access to food and water outside of training or testing for Montoya Staircase, where food was restricted. All animal procedures were approved by the IACUC at Wright State University. To perform the different experiments, the rats were designated into five different groups. The experimental conditions for each group are summarized in Table 1.

The first group of rats (group 1) were tested in one Forelimb Asymmetry trial to test their baseline motor function and then subjected to endothelin-induced cortical stroke; 1 μ l endothelin was injected in these two stereotactic sites: AP (0), ML (-2.3), DV (2.3) and AP (2.3), ML (-2.3), DV (2.3). Following the stroke, animal groups were given vehicle ($n = 14$) or one of two drug combinations in generic form (5 mg/kg fluoxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C ($n = 15$), or 5 mg/kg paroxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C ($n = 15$)) daily starting 20-26 hours after stroke throughout behavioral testing to compare the therapeutic effects of fluoxetine versus paroxetine. The animals were tested on Forelimb Asymmetry on post-stroke days 3, 16, and 31, and these results were compared to the results of the pre-stroke trial as indicated previously. One third of the rats from group 1 were euthanized at post-stroke

day 10, one third of the rats from group 1 were euthanized on post-stroke day 20, and the remaining third from group 1 were euthanized on post-stroke day 32 in order to perform Brain Histology (data not shown). Therefore, all of the rats from group 1 were observed in the vertical column pre-stroke and post-stroke day 3, two thirds of those rats were observed in the vertical column post-stroke day 16, and only one third of those rats were observed in the vertical column post-stroke day 31.

The second group of rats (group 2) were trained for Montoya Staircase retrieval of sucrose pellets for two weeks, a trial during the final three days of training was chosen to serve as a baseline, and the post-stroke Montoya trials were post-stroke days 8-10 and 29-31 (data not shown). The animals were then tested in one Forelimb Asymmetry trial to test their baseline motor function, and then they were subjected to endothelin-induced cortical stroke; 1 μ l endothelin was injected in these two stereotactic sites: AP (0), ML (-2.5), DV (2.0) and AP (2.3), ML (-2.5), DV (2.0). Following the stroke, animal groups were given vehicle ($n = 11$) or the drug combination 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C in brand name form ($n = 12$), daily starting 20-26 hours after stroke throughout behavioral testing. The decision to switch to brand name drugs was made because it has been found that the enantiomers of fluoxetine have been changed in the generic form of the drug, resulting in more of a non-active metabolite. In brand names fluoxetine, there is a larger amount of an active metabolite, which lasts for long periods of time. The animals were tested on Forelimb Asymmetry on post-stroke days 3, 16, and 28, and these results were compared to the results of the pre-stroke trial as indicated previously.

The third group of rats (group 3) were trained for Montoya Staircase retrieval of sucrose pellets for two weeks, a trial during the final three days of training was chosen to serve as a baseline, and the post-stroke Montoya trials were post-stroke days 8-10 and 29-31 (data not shown). The animals were tested in one Forelimb Asymmetry trial pre-stroke to test their baseline motor function, and then they were subjected to endothelin-induced cortical stroke; 1 μ l endothelin was injected in these two stereotactic sites: AP (0), ML (-2.5), DV (2.0) and AP (1.5), ML (-2.5), DV (2.0). Following the stroke, animal groups were given vehicle ($n = 11$) or one of four drug combinations in generic form (5 mg/kg fluoxetine, 0.5 mg/kg atorvastatin, and 20 mg/kg vitamin C ($n = 11$), 5 mg/kg paroxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C ($n = 11$), (5 mg/kg fluoxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C ($n = 12$), or 5 mg/kg fluoxetine and 0.5 mg/kg simvastatin ($n = 11$)) daily starting 20-26 hours after stroke throughout behavioral testing to compare the therapeutic effects of fluoxetine versus paroxetine and simvastatin versus atorvastatin and to determine whether vitamin C is necessary for functional recovery. The animals were tested on Forelimb Asymmetry on post-stroke days 3, 16, and 28, and these results were compared to the results of the pre-stroke trial as indicated previously.

The fourth group of rats (group 4) were trained for Montoya Staircase retrieval of sucrose pellets for two weeks, a trial during the final three days of training was chosen to serve as a baseline, and the only post-stroke Montoya trial was post-stroke days 3-5 (data not shown). The animals were tested in one Forelimb Asymmetry trial pre-stroke to test their baseline motor function, and then they were subjected to endothelin-induced cortical stroke; 1.5 μ l endothelin was injected in these two stereotactic sites: AP (0), ML (-2.5), DV (2.0) and AP (1.5), ML (-2.5), DV (2.0). Long Evans rats may be more sensitive to

the endothelin than Sprague Dawley rats, so the amount of endothelin injected was increased in order to produce a larger infarct size. Following the stroke, animal groups were given vehicle ($n = 5$) or the drug combination 5 mg/kg fluoxetine, 0.5 mg/kg simvastatin, and 20 mg/kg vitamin C in brand name form ($n = 5$), daily starting 20-26 hours after stroke throughout behavioral testing. The animals in group 4 were only tested pre-stroke and 3 days post-stroke since they were euthanized at post-stroke day 3 or post-stroke day 10 for RNA preparation from the peri-infarct region (not shown).

Rat Group	Stereotactic Coordinates	Endothelin	Treatments	Forelimb Trials
1	AP (0 & 2.3), ML (-2.3), DV (2.3)	1 μ l	FSA, PSA	pre-stroke, PSD 3, 16, 31
2	AP (0 & 2.3), ML (-2.5), DV (2.0)	1 μ l	FSA	pre-stroke, PSD 3, 16, 28
3	AP (0 & 1.5), ML (-2.5), DV (2.0)	1 μ l	FAA, PSA, FSA, FS	pre-stroke, PSD 3, 16, 28
4	AP (0 & 1.5), ML (-2.5), DV (2.0)	1.5 μ l	FSA	pre-stroke, PSD 3

Table 1. Experimental conditions of all animal groups. Under treatments, FSA stands for 5 mg/kg fluoxetine/0.5 mg/kg simvastatin/20 mg/kg vitamin C, PSA stands for 5 mg/kg paroxetine/0.5 mg/kg simvastatin/20 mg/kg vitamin C, FAA stands for 5 mg/kg fluoxetine/0.5 mg/kg atorvastatin/20 mg/kg vitamin C, and FS stands for 5 mg/kg fluoxetine/0.5 mg/kg simvastatin. Under forelimb trials, PSD stands for post-stroke day. Groups 4 and 5 were given brand name drugs; the rest were given generic drugs.

EXCLUSIONS

Any animal failing to completely ingest post-stroke daily medicine or vehicle for a total of 3 or more days was completely excluded from the study. Animals failing to display at least a 20% functional deficit compared to pre-stroke values in both functional tests (post-stroke day 3 in Forelimb Asymmetry; post-stroke days 8-10 in Montoya Staircase) were excluded from the study. Any animal failing to retrieve at least 9 pellets in each forepaw by the end of training for the Montoya Staircase was excluded from this

part of the study only. Animals failing to retrieve any pellets (either paw) in any of the post-stroke Montoya tests were excluded from this part of the study only.

Total	Just Forelimb Asymmetry	Just Montoya Staircase	Both Functional Tests	Excluded
86	16 (18.6%)	14 (16.27%)	35 (40.69%)	21 (24.41%)

Table 2. Animals analyzed in each functional test. This table shows the number of rats that were statistically analyzed in each behavioral test (percentage of total are in the parentheses). Only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke day 3 on forelimb asymmetry was analyzed for that test and only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke days 8-10 on Montoya Staircase was analyzed for that test. Any animal failing to retrieve at least 9 pellets in each forepaw by the end of training or to retrieve any pellets (either paw) in any of the post-stroke Montoya tests was excluded from this part of the study only.

III. RESULTS

According to Table 3, using the stereotactic coordinates AP (0), ML (-2.3), DV (2.3) and AP (2.3), ML (-2.3), DV (2.3) and rat strain Sprague Dawley, 52.27% of animals in group 1 had greater than 20% deficit on post-stroke day 3. The average deficit for the control animals in group 1 that made the deficit criteria was 35.9%.

	Beginning Total	< 20% Deficit	Post-Stroke Day 3	Post-Stroke Day 16	Post-Stroke Day 31
Control	14	6 (42.86%)	8 (57.14%)	6 (42.86%)	3 (21.43%)
Fluoxetine/Simvastatin/ Vit. C	15	9 (60%)	6 (40%)	2 (13.33%)	2 (13.33%)
Paroxetine/Simvastatin/ Vit. C	15	6 (40%)	9 (60%)	4 (26.67%)	2 (13.33%)
Total	44	21 (47.73%)	23 (52.27%)	12 (27.27%)	7 (15.9%)

Table 3. Forelimb Asymmetry analysis for group 1. This table shows in the first column the number of rats in each treatment group, in the second column the number of rats that exhibited a deficit smaller than 20% on post-stroke day 3 and were removed from analysis (percentage of total are in the parentheses), and in the subsequent columns the number of rats from in each treatment group that provided data from each post-stroke measurement that was statistically analyzed (percentage of total are in the parentheses). All rats were observed in the vertical column pre-stroke and post-stroke day 3, two thirds of those rats were observed in the vertical column post-stroke day 16, and only one third of those rats were observed in the vertical column post-stroke day 31. Only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke day 3 was analyzed.

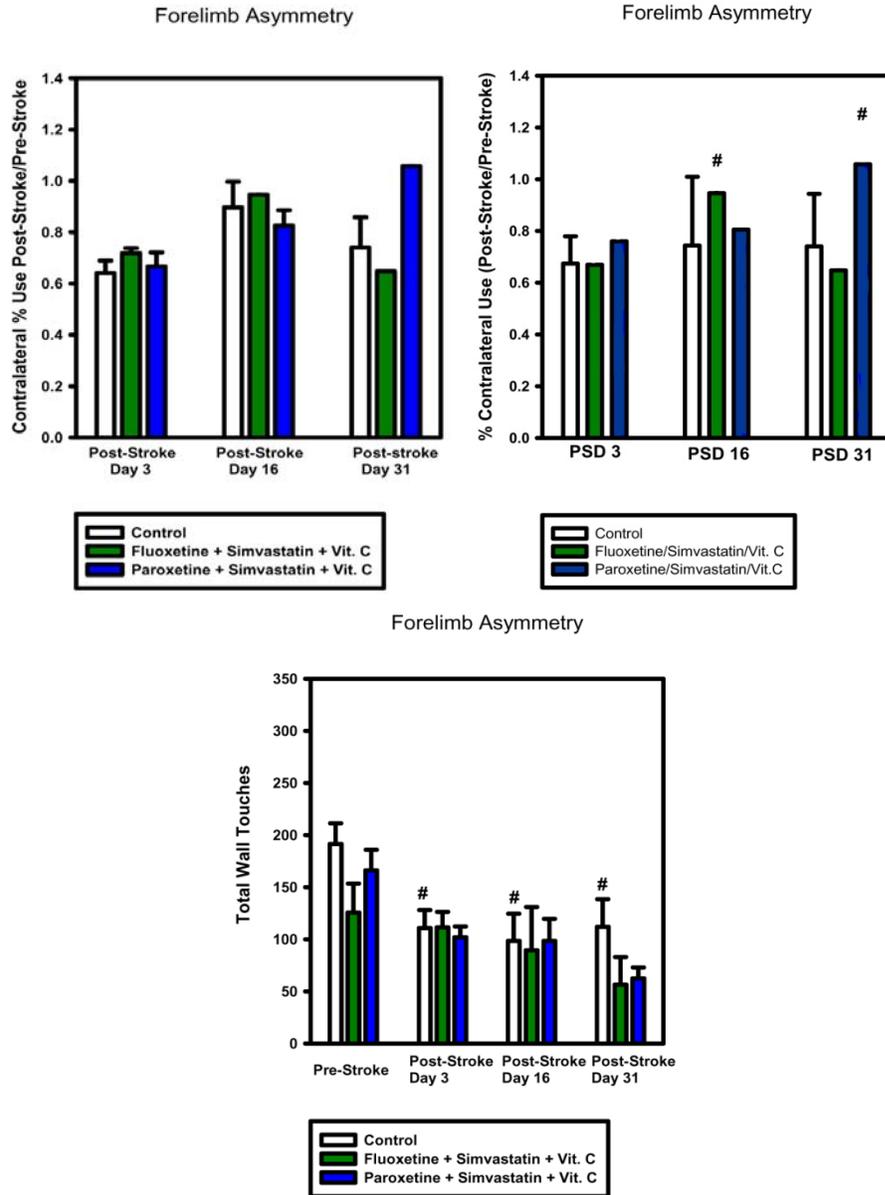


Figure 1. Forelimb Asymmetry analysis for group 1. The top graphs depict the functional recovery of the contralateral limb ((contralateral touches post-stroke/total touches post-stroke)/(contralateral touches pre-stroke/total touches pre-stroke)) during the Forelimb Asymmetry trials post-stroke days 3, 16, and 31. The graph on the left includes all rats in group 1. The graph on the right includes only the rats that were euthanized post-stroke day 32 and were therefore analyzed for all trials. The values on the y-axis are proportional, so 1 = 100% recovery, e.g. 0.8 = 80% recovery (20% deficit). When all rats were analyzed, there was an overall significant increase in functional recovery at post-stroke day 16 compared to post-stroke day 3, but not at post-stroke day 31 (top left), which is not shown. When only rats that were euthanized post-stroke day 32 were analyzed, there was a significant interaction between treatment group and day of trial in functional recovery (top right), which is not shown. The bottom graph depicts the total number of wall touches (ipsilateral touches + contralateral touches) for every rat in group 1 during each Forelimb Asymmetry trial. # indicates significant difference from post-stroke day 3 (top) or pre-stroke (bottom).

In the top panels of Figure 1, data on contralateral paw use and recovery following ischemic stroke for group 1 is shown. Again, because the data is showing post-stroke function divided by pre-stroke function, if the numbers are less than 1, they indicate a functional deficit and as the numbers reach 1, they indicate recovery to pre-stroke values. The data from group 1 for the Forelimb Asymmetry trials passed the Shapiro-Wilk normality test and the equal variance test. Two way repeated measures ANOVA along with Tukey post hoc testing show that with group 1, when all rats were analyzed, there was an overall significant increase in functional recovery at post-stroke day 16 compared to post-stroke day 3 ($p = .023$), but not at post-stroke day 31 ($p = .191$), and when only rats that were euthanized post-stroke day 32 were analyzed, there was a significant interaction between treatment group and day of trial in functional recovery ($p = .043$). Also, when only rats that were euthanized post-stroke day 32 were analyzed, there was a significant increase in functional recovery in the fluoxetine/simvastatin/vitamin C (FSA) group from post-stroke day 3 to post-stroke day 16 ($p = .04$), and there was a significant increase in functional recovery in the paroxetine/simvastatin/vitamin C (PSA) group from post-stroke day 3 to post-stroke day 31 ($p = .041$).

In the bottom panel of Figure 1, the total number of wall contacts for group 1 in the Forelimb Asymmetry test is analyzed to determine whether or not we can have confidence in these results. The control group displayed significantly fewer wall touches at every trial post-stroke compared to pre-stroke (post-stroke day (PSD) 3: $p = .003$, PSD 16: $p = .001$, PSD 31: $p = .009$). The reason the decreases in total wall touches of the 2 treatment groups over the course of the post-stroke testing do not reach significance may

be because the data of more rats were statistically analyzed in the control group than in each of the 2 treatment groups for post-stroke days 16 and 31. Another explanation may be that the treatments may enhance the rats' ability to smell the maple extract, so the rats in the treatment groups do not habituate as quickly as the control rats. The lowest number of wall contacts is at least 50, which gives confidence in these values.

According to Table 4, using the stereotactic coordinates AP (0), ML (-2.5), DV (2.0) and AP (2.3), ML (-2.5), DV (2.0) and rat strain Sprague Dawley, 43.48% of animals had greater than 20% deficit. The average deficit for the control animals in group 2 that made the deficit criteria was 32.7%.

	Beginning Total	< 20% Deficit	Analyzed
Control	11	5 (45.45%)	6 (54.54%)
Fluoxetine/Simvastatin/Vit. C	12	8 (66.67%)	4 (33.33%)
Total	23	13 (56.52%)	10 (43.48%)

Table 4. Forelimb Asymmetry analysis for group 2. This table shows in the first column the number of rats in each treatment group, in the second column the number of rats from in each treatment group that exhibited a less than 20% deficit on post-stroke day 3 and were removed from analysis (percentage of total are in the parentheses), and in the third column the number of rats in each treatment group that provided data that was statistically analyzed (percentage of total are in the parentheses). Every rat was observed in the vertical column pre-stroke and on post-stroke days 3, 16, and 28, but only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke day 3 was analyzed.

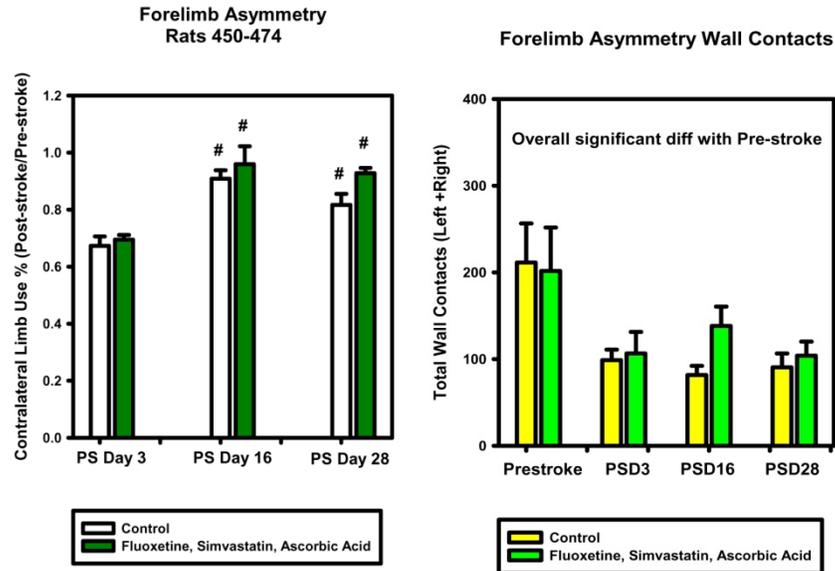


Figure 2. Forelimb Asymmetry analysis for group 2. The graph on the left depicts the functional recovery of the contralateral limb ((contralateral touches post-stroke/total touches post-stroke)/(contralateral touches pre-stroke/total touches pre-stroke)) during the Forelimb Asymmetry trials post-stroke days 3, 16, and 28. The values on the y-axis are proportional, so 1 = 100% recovery, e.g. 0.8 = 80% recovery (20% deficit). The graph on the right depicts the total number of wall touches (ipsilateral touches + contralateral touches) during each Forelimb Asymmetry trial. # indicates statistically significant difference from post-stroke day 3. There was also an overall significant decrease in wall touches at post-stroke days 3, 16, and 28 compared to pre-stroke.

In the left panel of Figure 2, data on contralateral paw use and recovery following ischemic stroke for group 2 is shown. Again, because the data is showing post-stroke function divided by pre-stroke function, if the numbers are less than 1, they indicate a functional deficit and as the numbers reach 1, they indicate recovery to pre-stroke values. The data from group 2 for the Forelimb Asymmetry trials passed the Shapiro-Wilk normality test and the equal variance test. Two way repeated measures ANOVA along with Tukey post hoc testing show that with group 4, there was a significant increase in functional recovery in both the control group and the FSA treatment group at post-stroke days 16 and 28 compared to post-stroke day 3 (PSD 16: $p < .001$, PSD 28: $p < .001$).

In the right hand panel of Figure 2, the total number of wall contacts for group 2 in the Forelimb Asymmetry test is analyzed. The data from group 2 that measured wall touches for the Forelimb Asymmetry trials failed Mauchly's Test of Sphericity ($p < .002$), so the Greenhouse-Geisser value was used as an adjusted value for Epsilon during the ANOVA test. Two way repeated measures ANOVA along with Tukey post hoc testing show that there was an overall significant decrease in wall touches at post-stroke days 3, 16, and 28 compared to pre-stroke (PSD 3: $p = .01$, PSD 16: $p = .008$, PSD 28: $p = .002$). This finding was not unexpected, since it is very typical of rats to get habituated to the environment and therefore explore the environment less over time (52-53). The lowest number of wall contacts is close to 100, which gives confidence in these values.

According to Table 5, using the stereotactic coordinates AP (0), ML (-2.5), DV (2.0) and AP (1.5), ML (-2.5), DV (2.0) and rat strain Sprague Dawley, 58.93% of animals had greater than 20% deficit. The average deficit for the control animals in group 3 that made the deficit criteria was 29.1%.

	Beginning Total	< 20% Deficit	Analyzed
Control	11	3 (27.27%)	8 (72.73%)
Fluoxetine/Atorvastatin/Vit. C	11	5 (45.45%)	6 (54.54%)
Paroxetine/Simvastatin/Vit. C	11	7 (63.64%)	4 (36.36%)
Fluoxetine/Simvastatin/Vit. C	12	4 (33.33%)	8 (66.67%)
Fluoxetine/Simvastatin	11	4 (36.36%)	7 (63.63%)
Total	56	23 (41.07%)	33 (58.93%)

Table 5. Forelimb Asymmetry analysis for group 3. This table shows in the first column the number of rats in each treatment group, in the second column the number of rats from in each treatment group that exhibited a less than 20% deficit on post-stroke day 3 and were removed from analysis (percentage of total are in the parentheses), and in the third column the number of rats from in each treatment group that provided data that was statistically analyzed (percentage of total are in the parentheses). Every rat was observed in the vertical column pre-stroke and on post-stroke days 3, 16, and 28, but only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke day 3 was analyzed.

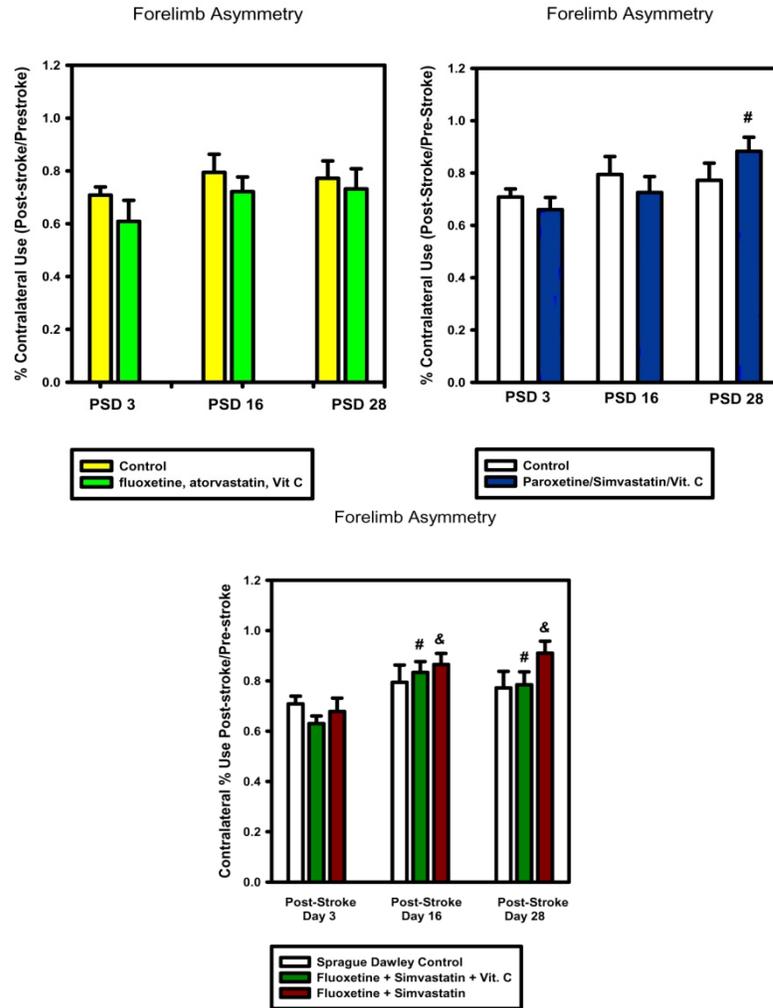


Figure 3. Forelimb Asymmetry analysis for group 3. These graphs depict the functional recovery of the contralateral limb ((contralateral touches post-stroke/total touches post-stroke)/(contralateral touches pre-stroke/total touches pre-stroke)) during the Forelimb Asymmetry trials post-stroke days 3, 16, and 28. The values on the y-axis are proportional, so 1 = 100% recovery, e.g. 0.8 = 80% recovery (20% deficit). # indicates statistically significant difference from paroxetine/simvastatin/vit. C on post-stroke day 3 (top) and fluoxetine/simvastatin/vit. C on post-stroke day 3 (bottom). & indicates statistically significant difference from fluoxetine/simvastatin on post-stroke day 3.

In Figure 3, data on contralateral paw use and recovery following ischemic stroke for group 3 is shown. Again, because the data is showing post-stroke function divided by pre-stroke function, if the numbers are less than 1, they indicate a functional deficit and as the numbers reach 1, they indicate recovery to pre-stroke values. The data from group 3 for the Forelimb Asymmetry trials passed the Shapiro-Wilk normality test and the equal variance test. Two way repeated measures ANOVA along with Tukey post hoc testing

show that there was a significant increase in functional recovery in the FSA and fluoxetine/simvastatin (FS) treatment groups at post-stroke day 16 compared to post-stroke day 3 (FSA: $p = .001$; FS: $p = .015$), and there was a significant increase in functional recovery in the PSA, FSA, and FS treatment groups at post-stroke day 28 compared to post-stroke day 3 (PSA: $p = .025$; FSA: $p = .005$; FS: $p < .001$). However, there were no statistical differences from the control group. In previous tests with Long Evans rats, the FSA treatment group had shown significant differences compared to the control group (50).

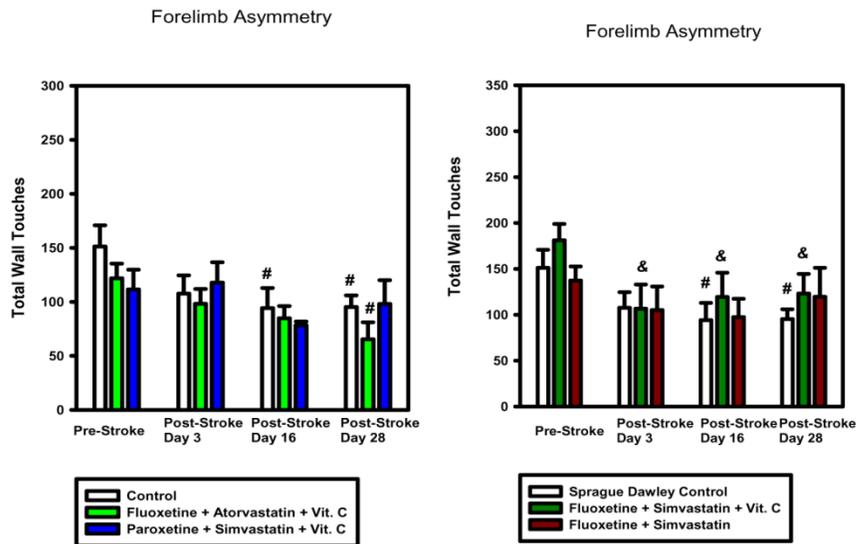


Figure 4. Forelimb Asymmetry analysis for group 3. This graph depicts the total number of wall touches (ipsilateral touches + contralateral touches) during each Forelimb Asymmetry trial. # and & indicate statistical significance from pre-stroke.

In Figure 4, the total number of wall contacts for group 3 in the Forelimb Asymmetry test is analyzed. The control group displayed significantly fewer wall touches at post-stroke days 16 and 28 compared to pre-stroke (PSD 16: $p = .014$, PSD 28: $p = .016$). The fluoxetine/atorvastatin/vitamin C (FAA) treatment group displayed significantly fewer wall touches at post-stroke day 28 compared to pre-stroke ($p = .043$).

The FSA treatment group displayed significantly fewer wall touches at all post-stroke days compared to pre-stroke (PSD 3: $p = .003$, PSD 16: $p = .018$, PSD 28: $p = .029$). The lowest number of wall contacts is at least 50, which gives confidence in these values.

According to Table 6, using the stereotactic coordinates AP (0), ML (-2.5), DV (2.0) and AP (1.5), ML (-2.5), DV (2.0) and rat strain Sprague Dawley while injecting 1.5 μ l of endothelin instead of 1 μ l into each stereotactic site, 80% of animals had greater than 20% deficit. The average deficit for the control animals in group 4 that made the deficit criteria was 32.1%.

	Beginning Total	< 20% Deficit	Analyzed
Control	5	0 (0%)	5 (100%)
Fluoxetine/Simvastatin/Vit. C	5	2 (40%)	3 (60%)
Total	10	2 (20%)	8 (80%)

Table 6. Forelimb Asymmetry analysis for group 4. This table shows in the first column the number of rats in each treatment group, in the second column the number of rats from in each treatment group that exhibited a less than 20% deficit on post-stroke day 3 and were removed from analysis (percentage of total are in the parentheses), and in the third column the number of rats in each treatment group that provided data that was statistically analyzed (percentage of total are in the parentheses). Every rat was observed in the vertical column pre-stroke and on post-stroke day 3, but only data from rats that exhibited a contralateral deficit of at least 20% on post-stroke day 3 was analyzed.

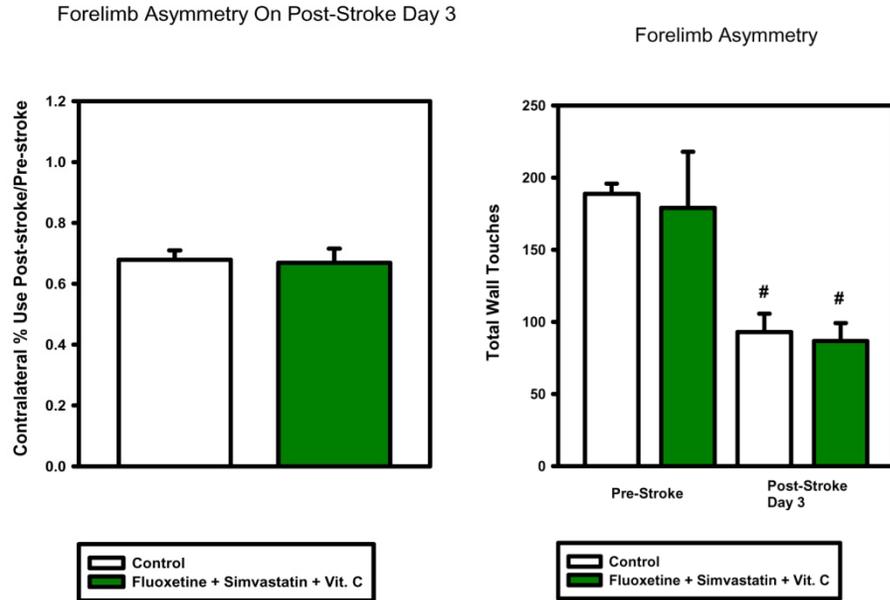


Figure 5. Forelimb Asymmetry analysis for group 4. The graph on the left depicts the functional recovery of the contralateral limb ((contralateral touches post-stroke/total touches post-stroke)/(contralateral touches pre-stroke/total touches pre-stroke)) during the Forelimb Asymmetry trials post-stroke day 3. The values on the y-axis are proportional, so 1 = 100% recovery, e.g. 0.8 = 80% recovery (20% deficit). The graph on the right depicts the total number of wall touches (ipsilateral touches + contralateral touches) during each Forelimb Asymmetry trial. # indicates statistical significance.

In the left panel of Figure 5, data on contralateral paw use and recovery following ischemic stroke for group 4 is shown. Again, because the data is showing post-stroke function divided by pre-stroke function, if the numbers are less than 1, they indicate a functional deficit and as the numbers reach 1, they indicate recovery to pre-stroke values. The data from group 4 for the Forelimb Asymmetry trials passed the Shapiro-Wilk normality test and the equal variance test. A student's t-test shows that with group 5, there was no significant difference between the control and FSA groups in function at post-stroke day 3 ($t(6) = .188, p = .857$).

In the right hand panel of Figure 5, the total number of wall contacts for group 4 in the Forelimb Asymmetry test is analyzed. Both groups displayed significantly fewer

wall touches at post-stroke day 3 compared to pre-stroke (control: $p = .002$, FSA: $p = .007$). The lowest number of wall contacts is close to 100, which gives confidence in these values.

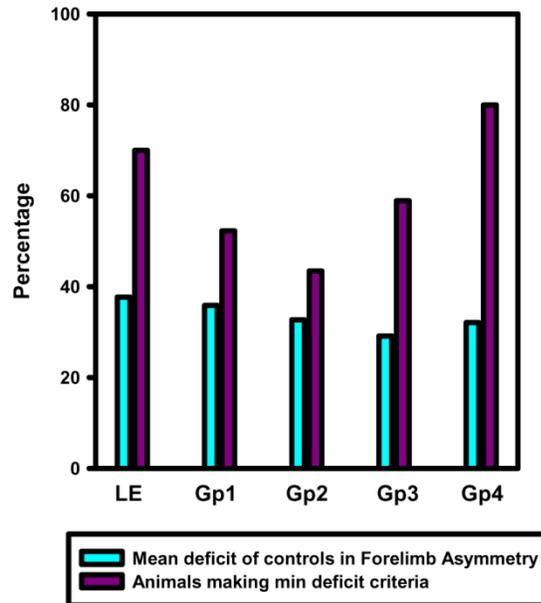


Figure 6. Forelimb Asymmetry analysis for all groups. This graph depicts the mean deficit of controls and percentage of animals making the minimum deficit criteria of 20% on post-stroke day 3 for every group in this study and the Long Evans rats in our previous study (50). LE stands for the Long Evans rats, Gp1 stands for group 1, Gp2 stands for group 2, Gp3 stands for group 3, and Gp4 stands for group 4.

In figure 6, the mean deficit of controls and percentage of animals making the minimum deficit criteria of 20% on post-stroke day 3 for every group in this study and the Long Evans rats in our previous study (50) are shown. In our previous study, 70% of the Long Evans rats made the deficit criteria, and the average deficit for those control animals that made the deficit criteria was 37.7% (50). The average deficit of group 1 was closest to what was previously found with Long Evans rats at 35.9%. The percentage of animals that made deficit criteria in group 4 was larger than what was previously found

with Long Evans rats at 80%. The average deficit of group 3 was the smallest at 29.1%.

Group 2 had the lowest percentage of animals make minimum deficit criteria at 43.48%.

IV. DISCUSSION

The FSA treatment did not work for group 1 (stereotactic coordinates AP (0), ML (-2.3), DV (2.3), and AP (2.3), ML (-2.3), DV (2.3)) to allow lasting functional recovery compared to the control group. There was a significant increase in functional recovery in the FSA treatment group from post-stroke day 3 to post-stroke day 16 which had subsided by post-stroke day 31, and there was a significant increase in functional recovery in the PSA treatment group from post-stroke day 3 to post-stroke day 31. There are many possible reasons for this result. One possible reason could be that paroxetine is a better treatment than fluoxetine. However, there were no significant differences between treatment groups, particularly from control. Another reason could be that the chemical composition of the generic form of fluoxetine may not be reliably effective; the enantiomers of fluoxetine have changed in the generic form of the drug, such that it has produced more of a non-active metabolite. This study needs to be replicated, utilizing brand-name pharmaceuticals instead of generic forms. Another possibility is that the fluoxetine is working more quickly; 60% in the FSA group did not make the minimum deficit criteria of 20%, whereas only 40% in the PSA group and 42.85% of the control group did not make the criteria. There was also an overall significant increase in functional recovery at post-stroke day 16 compared to post-stroke day 3, but not at post-stroke day 31. That is problematic because this is not a typical pattern; the increase in function was not long-lasting for the FSA group. This may be due to human error while scoring the videos for Forelimb Asymmetry for this group; the videos may need to be

scored by a separate party. The average deficit for the control animals in group 1 that made the deficit criteria was 35.9%. In our previous study with Long Evans rats, the average deficit for the Long Evan control animals that made the deficit criteria was 37.7% (50). It is a good sign that the percentage of Sprague Dawley rats that made this criteria is so close to the percentage of Long Evans rats because it is typically more difficult to create a deficit in Sprague Dawley than in Long Evans rats (65). However, only 52.27% of animals in group 1 made the minimum deficit criteria whereas 70% of the Long Evans rats made the deficit criteria (50), so there is probably another stroke-inducing method that may induce a larger deficit in Sprague Dawley rats.

In group 2 (stereotactic coordinates AP (0), ML (-2.5), DV (2.0), and AP (2.3), ML (-2.5), DV (2.0)), there was a significant increase in functional recovery in both the control group and the FSA treatment group observed during the Forelimb Asymmetry trials at post-stroke days 16 and 28 compared to post-stroke day 3, but there was no significant difference in contralateral deficit between the control and the FSA group. These animals were also tested on the Montoya Staircase, which was administered by Nicholas Wyatt in our laboratory. There was no significant difference in contralateral deficit observed during the Montoya Staircase trials between the two groups. This may be due to the small sample of rats that were observed in each test, which could have lowered the statistical power of these tests, so the results may have just not reached significance. This may be a sufficient explanation for the lack of significant results from the Montoya trials, but not for the Forelimb Asymmetry data since there were significant results, just not between the groups. This may be due to the fine motor skills involved in grasping the sugar pellets, which requires more control of movement than exploring the column

during the Forelimb Asymmetry trials. Another possibility is that the animals analyzed on the Montoya Staircase may be different animals observed on Forelimb Asymmetry; only 21.74% of the animals in group 2 made the criteria for both tests, 21.74% of the animals only made the criteria for Forelimb Asymmetry, 13.04% of the animals only made the criteria for Montoya Staircase, and 43.48% did not make criteria for either test. Why would both the control and the treatment groups show functional recovery over time and not just the treatment group? This could be due to spontaneous recovery. Studies have shown that a brief period of functional recovery occurs in the days following ischemia, but this is a false positive result because the neuroprotection is only temporary and is reversible (70). It often occurs in stroke-induced animals with small infarct sizes; in Sprague Dawley rats that have endured cortical damage from MCAO stroke, functional recovery observed in Forelimb Asymmetry is correlated with a smaller infarct size, but infarct size is not correlated with scores on reaching tasks (71-72), which could explain the difference in results between the two behavioral tests. The average infarct size for the treatment group was $.8835 \text{ mm}^3$, and the average infarct size of the control group was $.9577 \text{ mm}^3$, so we may need to increase the size of the infarct to induce a larger deficit. Also, the similar infarct sizes between the control and treatment groups in group 3 may be the reason both groups exhibited similar results. Only 43.48% of animals in group 2 made the minimum deficit criteria, and the average deficit for the control animals in group 2 that made the deficit criteria was 32.7%, five percentage points lower than the Long Evans rats. There is probably another stroke-inducing method that may induce a larger deficit in Sprague Dawley rats. Again, this experiment needs to be replicated with

a different surgical procedure, either changing the stereotactic coordinates or increasing the dose of endothelin to induce a larger infarct size and therefore a larger deficit.

The PSA treatment group from group 3 (stereotactic coordinates AP (0), ML (-2.5), DV (2.0), and AP (1.5), ML (-2.5), DV (2.0)) exhibited a significant increase in functional recovery on Forelimb Asymmetry at post-stroke day 28 compared to post-stroke day 3, but there were no significant differences between treatment groups, particularly with the control group. There was a significant increase in functional recovery on Forelimb Asymmetry in both the FS and FSA treatment groups in group 3 at post-stroke days 16 and 28 compared to post-stroke day 3. Does that mean vitamin C is not necessary for functional recovery? It is possible; in one study, in stroke patients that were treated with vitamin C, their blood level of antioxidants increased, but their neurological function was not improved (47). However, it could be because the average infarct sizes (determined by Saagar Sanghvi) of the two treatment groups were not significantly different (FSA = 4.5651 mm³; FS = 2.7686 mm³), and both groups exhibited spontaneous recovery. All of the treatment groups had lower percentages of animals making the deficit criteria than the control group, which could be a sign that three days of treatment are actually causing recovery. The results of the Montoya Staircase show no significant effect of treatment group or day of trial on function of contralateral limb, so functional recovery was observed on Forelimb Asymmetry, but not on the Montoya Staircase. This could be because Forelimb Asymmetry is less objective and more prone to human error; judging whether a rat managed to retrieve a pellet out of a container is easier than defining what is considered a wall contact. Therefore, these results could be false positives. 58.93% of animals in group 3 made the minimum deficit criteria, but the

average deficit for the control animals in group 3 that made the deficit criteria was only 29.1%. There is probably another stroke-inducing method that may induce a larger deficit in Sprague Dawley rats. Again, this experiment needs to be replicated with a different surgical procedure, either changing the stereotactic coordinates or increasing the dose of endothelin.

In group 4 (stereotactic coordinates AP (0), ML (-2.5), DV (2.0), and AP (1.5), ML (-2.5), DV (2.0), 1.5 μ l of endothelin instead of 1 μ l), no significant difference between the control and FSA treatment groups in function was observed during the Forelimb Asymmetry trials at post-stroke day 3, so the switch to brand name drugs may not have been successful. That is not certain, however, because only one post-stroke behavioral trial was done, so the brand name drugs need to be tested on rats that go through all post-stroke trials of Forelimb Asymmetry and possibly Montoya Staircase. More studies will need to be done to elucidate the specific benefits of the drug combinations outlined in these experiments and to determine whether brand name medications are more effective than generic medications. The FSA group had 60% of animals making the deficit criteria, but all of the animals from the control group made the deficit criteria, which could be a sign that three days of treatment are actually causing recovery. Even though there was no significant difference between the control and FSA groups in function, 80% of animals in group 4 made the minimum deficit criteria, and the average deficit for the control animals in group 4 that made the deficit criteria was 32.1%. Even though group 1 exhibited the highest average deficit, percentage of rats making the minimum deficit criteria in group 4 was even larger than the Long Evans rats, so future experiments with Sprague Dawley rats should utilize the stereotactic coordinates used for

group 4 while increasing the dosage of endothelin to 1.5 μ l to increase the size of the infarct.

The most consistent results from all of the experiments is that the number of wall touches significantly decreased over time. This finding was not unexpected since it is very typical of rats to get habituated to the environment and therefore explore the environment less over time (52-53). Also, there were more significant increases in functional recovery observed on the Forelimb Asymmetry than on Montoya Staircase behavioral tests. However, while about the same proportion of rats exhibited the 20% or larger deficit required for analysis on Forelimb Asymmetry as on the Montoya Staircase, the largest deficit seen on the Montoya Staircase was 100%, whereas the largest deficit seen on Forelimb Asymmetry was 71.7%. Why, then, were there more significant results observed on Forelimb Asymmetry than on Montoya Staircase? This may be due to the fine motor skills involved in grasping the sugar pellets, which requires more control of movement than exploring the column during the Forelimb Asymmetry trials. Forelimb Asymmetry may also be a less sensitive test than the Montoya Staircase and is more likely to produce falsely positive results. Therefore, Montoya Staircase may be a more reliable method for measuring contralateral deficit than Forelimb Asymmetry. However, instead of falsely positive results from spontaneous recovery, it could be that some of the drug combinations are working within the first three days post-stroke, particularly the fluoxetine/simvastatin/vitamin C combination treatment, so in future studies, we may have to begin behavioral observations earlier (around post-stroke day 1) or not start the treatment until after the first trial 3 days post-stroke. One problem with these solutions is that altering the timing of behavioral observation and treatment could in itself affect the

therapeutic effects of the drugs; beginning behavioral trials that soon after surgery could cause stress in the rats, which would in turn prevent neurogenesis, and starting the treatments later could be detrimental to the mechanism of the drugs. For example, after ischemic stroke in rats, there is an increase in BDNF activity in neurons and ependymal cells in the undamaged hemisphere and an increase in BDNF activity in microglial cells, endothelial cells of cerebral arterioles, and astrocytes in the damaged hemisphere at both four and 24 hours after stroke (17), and at 2 hours post-stroke, but not at later time points, there is a large increase of the number of BDNF-immunoreactive cells in the ipsilateral, cingulate, and frontal cortices outside the infarct (18), so the lack of BDNF later on could prevent the treatment from promoting neurogenesis. We could possibly do an experiment where we test whether these changes affect the functional recovery of the animals.

It is difficult to determine from these studies whether the drug combinations tested would be effective at improving neurological function in ischemic stroke patients. The low number of animals in each test lowered the statistical power, making absolute conclusions about functional recovery problematic. The results for the drug cocktail fluoxetine/simvastatin/vitamin C were especially inconsistent; while we have demonstrated good evidence of the therapeutic effects of that combination in our previous study (50), in the present study, when the FSA combination did effect functional recovery over time, there was never any significant difference from control, and, in one instance, the effect did not last throughout the testing period. The paroxetine/simvastatin/vitamin C and fluoxetine/simvastatin treatment groups did exhibit functional recovery over time; it may be tempting to conclude that paroxetine is a more effective treatment and that vitamin C is not necessary for functional recovery, but these effects were not significantly

different from the control groups, so we cannot make any conclusions about the therapeutic effects of these drugs. No recovery was seen with the fluoxetine/atorvastatin/vitamin C treatment group, so that drug cocktail may not be an effective treatment.

Overall, these results raise more questions than answer them. Do brand name drugs improve recovery from stroke more than generic drugs? Are these drug combinations really improving motor function within three days, or are we just observing the effects of temporary and reversible spontaneous recovery? Which drug combination is most effective? Does Forelimb Asymmetry give too many false positive results? However, an effective surgical procedure to induce an ischemic stroke in Sprague Dawley rats has been successfully developed, and these questions can be answered in future studies utilizing the stereotactic coordinates AP (0), ML (-2.5), DV (2.0), and AP (1.5), ML (-2.5), DV (2.0) while injecting 1.5 μ l of endothelin in Sprague Dawley rats. These studies will hopefully lead to an effective delayed pharmacological treatment that will increase neurological function in ischemic stroke victims.

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