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Effects of Delayed Pharmacological Treatment and Limb Rehabilitation on Infarct Size and Functional Recovery After Stroke

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EFFECTS OF DELAYED PHARMACOLOGICAL TREATMENT AND LIMB REHABILITATION ON INFARCT SIZE AND FUNCTIONAL RECOVERY AFTER STROKE

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Maria Helen Harley Balch ENTITLED Effects of Delayed Pharmacological Treatment and Limb Rehabilitation on Infarct Size and Functional Recovery After Stroke BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Previous studies show a fluoxetine/simvastatin drug combination successfully reduced infarct size and increased functional recovery when administered 20-26 hours post-stroke. This project tested the hypothesis that earlier drug delivery at 6-12 hours post-stroke will improve functional recovery and decrease infarct size, and that limb rehabilitation will improve functional recovery.

Pre-stroke function was determined in rats, a stroke was induced, and daily treatment began 6-12 hours post-stroke. Baseline functional deficit was established, and additional testing over 90 days monitored functional recovery. Limb rehabilitation was provided for designated animals, and brain analysis measured infarct size.

In animals with no rehabilitation, drug treatment provided better recovery; with rehabilitation, recovery was similar whether animals received drug treatment or not, suggesting a drug treatment could replace rehabilitation if needed. Larger infarcts were also present with earlier drug delivery, implying the possibility of hemorrhagic transformation due to earlier induction of VEGF expression by our drug combination.

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For my loving husband

I. INTRODUCTION

STROKE

Every forty seconds, someone in the United States has a stroke (Centers for Disease Control and Prevention (CDC): National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP), Division for Heart Disease and Stroke Prevention (DHDSP), 2013). Strokes affect nearly 795,000 people each year, and 610,000 of those cases occur in patients who have not previously had a stroke (CDC: NCCDPHP, DHDSP, 2013). Census data analysis suggests that incidence of stroke each year will double by 2050 (Howard and Goff, 2012). Stroke is also a leading cause of serious long-term disability in the United States (CDC: NCCDPHP, DHDSP. 2013; Go *et al*, 2014). When accounting for healthcare expenses, cost of medications, and lost productivity, studies estimate the national annual cost of stroke at $38.6 billion (CDC: NCCDPHP, DHDSP, 2013).

The fourth leading cause of death (Towfighi and Saver, 2011; Murphy *et al*, 2013), strokes kill almost 130,000 Americans each year (CDC: NCCDPHP, DHDSP, 2013). Approximately one out of every 19 deaths is caused by a stroke (Go *et al*, 2014), with someone dying of a stroke every four minutes (CDC: NCCDPHP, DHDSP, 2013). In addition, the Acute Cerebrovascular Care in Emergency Stroke Systems (ACCESS)

study reported that only half of the American population has timely access to a primary stroke center (PSC) (Albright *et al*, 2010).

Stroke clearly stands as one of the most significant health issues in the United States (Hall *et al*, 2012). Injury following a stroke develops via a multifaceted sequence of pathophysiological events (Dirnagl *et al*, 1999). In order to comprehend these events and develop treatment options, the immediate and post-stroke conditions within the brain and the associated mechanisms must be understood (Hossmann, 2006). Stroke research provides a means to reach this understanding and is thus essential to the health of the country.

Types of Stroke

There are two major types of stroke: ischemic and hemorrhagic. Both types of stroke, whether caused by a clot or bleeding, can result in damage and death of cells in the brain.

Ischemic strokes account for 87 percent of stroke cases (American Heart Association (AHA)/American Stroke Association (ASA), 2012) and occur when a vessel that supplies blood to the brain is blocked by a clot. Ischemic strokes can be either thrombolic – where a clot forms within a vessel in the brain – or embolic – where a clot forms in a vessel elsewhere in the body and moves to the brain (National Stroke Association, 2014b).

Unlike ischemic strokes, hemorrhagic strokes do not involve a blood clot. Instead, a vessel bursts or leaks, causing damage and increased pressure on brain tissue. Aneurysms are ballooning regions of weak-walled blood vessels, while arteriovenous

malformations (AVMs) are clusters of abnormally-formed vessels (AHA/ASA, 2012). Both aneurysms and AVMs are capable of rupturing and causing hemorrhagic strokes. When a ruptured vessel bleeds into the brain, the event is called an intracerebral hemorrhagic stroke. Conversely, when a ruptured vessel bleeds outside of the brain into the subarachnoid space, the event is called a subarachnoid hemorrhagic stroke (National Stroke Association, 2013).

In addition to ischemic and hemorrhagic strokes, transient ischemic strokes (TIAs), also known as mini-strokes, can occur. TIAs exhibit similar symptoms as ischemic strokes, but the effects are brief because the clot only temporarily blocks the vessel (AHA/ASA, 2012).

Current Pharmacological Treatment for Stroke

Recombinant tissue plasminogen activator (tPA) was approved for use treating ischemic stroke in 1996 (Zivin, 2009) and remains the only Food and Drug Administration (FDA)-approved treatment for stroke (AHA/ASA, 2013). Blood clots form when activated platelets aggregate onto fibrin threads. Plasmin is a protease capable of breaking down the fibrin meshes via fibrinolysis. The inactive form, plasminogen, must be activated by a plasminogen activator (PA). One such PA is tPA. Because timely reperfusion is imperative to save the ischemic brain tissue (Zhang *et al*, 2012), recombinant tPA was developed as a pharmacological treatment for ischemic stroke (National Institute of Neurological Disorders and Stroke (NINDS), 1995), acting by breaking down the clot and resulting in recanalization of the previously-occluded blood vessel (Gravanis and Tsirka, 2008).

A precise window of administration exists when using tPA, as a patient must present to the hospital for treatment within three hours post-stroke (Clark *et al*, 1999; Hacke *et al*, 2004; NINDS, 1995) (up to four-and-one-half hours in certain qualifying patients) (Stemer and Lyden, 2010). If the three-hour post-stroke treatment window has passed, a patient is considered ineligible to receive the drug. Unfortunately, this is often the case with patients because they are unable to recognize the symptoms or arrive at a hospital in time (Y. Li *et al*, 2014). Though previously-reported national averages for patients receiving tPA treatment vary (AHA, 2009; Reeves *et al*, 2005), it is estimated that less than five percent of ischemic stroke patients are able to be treated with tPA (California Pacific Medical Center, 2013).

tPA administration is time-sensitive because of the possibility of hemorrhagic transformation, or secondary bleeding after an initial ischemic stroke has occurred (Jickling *et al*, 2014; Chapman *et al*, 2014; National Stroke Association, 2014a). Some studies suggest harmful effects of tPA arise beyond the approximate three-hour post-stroke mark because of its ability to activate matrix metallo-proteinase-9 (MMP-9), which is an enzyme that degrades extracellular matrix (ECM) components (Zhao *et al*, 2004) and contributes to blood-brain barrier (BBB) breakdown (Chaturvedi and Kaczmarek, 2013). Other studies suggest involvement of low-density lipoprotein (LDL) receptor-related protein (LRP) (Yepes *et al*, 2003) or that both LRP and MMP-9 mediators contribute to hemorrhagic transformation with tPA (Ortolano and Spuch, 2013). Mast cell degranulation and caspase-8 have also been investigated as mediators of tPA toxicity (Ishrat *et al*, 2012). Many studies speak to the importance of understanding the deleterious effects of tPA on cytotoxicity, cerebral edema, neurovascular unit

permeability, and intracranial bleeding (Armstead *et al*, 2006; Sappino *et al*, 1993; Yepes *et al*, 2009). Regardless of the mechanism, it is clear that the undeniable thrombolytic benefits of tPA are compromised due to administration restrictions and possible brain hemorrhage, thus making it available to only a small minority of patients.

It has been stated that the argument whether tPA should be considered neuroprotective or neurotoxic could continue indefinitely (Gravanis and Tsirka, 2008). Evidence is certainly shown for both cases. Research is aimed at the goal of treating more ischemic stroke patients with tPA. Combination therapies coupling tPA with other neuroprotective agents are being tested and explore mediators such as MMP inhibitors, LRP antagonists, mast cell stabilizers, platelet inhibition, free radical scavengers, neuroserpin, and estrogen therapy. These studies have shown a reduction in vascular damage or hemorrhagic transformation and, therefore, suggest tPA use can continue, more patients can benefit, and negative side effects can be avoided (Ishrat *et al*, 2012; Zhang *et al*, 2012). Studies are also attempting to identify ways to accurately predict the risk of hemorrhagic transformation in ischemic stroke patients (Shinoyama *et al*, 2013).

Endogenous tPA

While recombinant tPA was developed as a pharmacological treatment for ischemic stroke (NINDS, 1995), tPA itself is produced naturally in the human body. It is expressed in both developing and mature brain tissue in neurons and microglia. Besides its role in fibrinolysis and preservation of nonthrombogenic vascular surfaces (Bednar and Gross, 1999; Lucking *et al*, 2013), it is suggested that endogenous tPA mediates

neuronal death and microglial activation following excitotoxic injury as well (Ishrat *et al*, 2012).

Research has shown that cerebral ischemia increases levels of endogenous tPA (Copin *et al*, 2011), and this increase induces opening of the BBB (Yepes *et al*, 2003). While normal tPA production may be a function of all endothelium (Ishrat *et al*, 2012), the origin of tPA in brain tissue after injury to the brain is not well described (Zhao *et al*, 2004).

As previously discussed, tPA administration presents with the risk of hemorrhagic transformation. Some publications propose endogenous tPA contributes to hemorrhagic transformation by enhancing MMP-9 expression or proteolytic activation (Tang *et al*, 2010; Umemura, 2005). However, the precise involvement of endogenous tPA in hemorrhagic transformation is unclear.

Rehabilitation and Stroke

Because stroke is the leading cause of adult disability in the United States, rehabilitation therapy for stroke patients is of utmost importance. Occupational therapy assists with relearning everyday activities, and speech language pathology helps patients rebuild speaking skills. Because depression, frustration, and anxiety are commonly observed in stroke patients, psychological treatment may also be involved. Of all available therapies, physical therapy is considered the cornerstone of the rehabilitation process and aims to restore movement, balance, and coordination (NINDS, National Institute of Health (NIH), 2014).

Brain-Derived Neurotrophic Factor (BDNF)

Neurotrophins are a family of growth factors associated with neuronal growth and survival (Miller and Kaplan, 2001). One such neurotrophin, BDNF, is widely distributed in the central nervous system (Binder and Scharfman, 2004). In addition to stimulating neurogenesis (Schäbitz *et al*, 2007), BDNF promotes neuroplasticity (Madinier *et al*, 2013) and assists with learning and memory (Bekinschtein *et al*, 2008; Binder and Scharfman 2004; Ploughman *et al*, 2009). Aside from its function in supporting existing neurons, BDNF also appears to play a crucial part in brain injury and recovery.

A gradual increase in astrocytic expression of neurotrophic factors has been observed in the brain after ischemic injury. Studies suggest this increase may protect these brain regions from further damage and facilitate brain repair (Kleim *et al*, 2003). Specifically, BDNF initiates anti-apoptotic mechanisms, reduces infarct size, and decreases instance of secondary neuronal cell death (Schäbitz *et al*, 2007).

After an ischemic event, rehabilitation is necessary to support brain plasticity (Kleim *et al*, 2003). While neuroplasticity engages multiple signaling pathways, BDNF has been shown to play a critical role in motor learning and recovery through rehabilitation post-stroke (Mang *et al*, 2013; Ploughman *et al*, 2009). Following stroke, forced use and rehabilitation of an impaired limb improves functional recovery (Livingston-Thomas *et al*, 2014), and, in turn, stimulates increases of BDNF in the brain (MacLellan *et al*, 2011). Interestingly, exercise independent of ischemic injury also results in increased levels of BDNF as well as other defenses against cell death (MacLellan *et al*, 2011; Ploughman *et al*, 2009).

Adult Neurogenesis

The term neurogenesis refers to the growth and development of new neurons. This process is most active during embryonic development, and until 1962, it was believed the adult nervous system was fixed, incapable of regeneration. However, it is now known that neurogenesis can occur at two locations in the adult brain: the subventricular zone (SVZ), lining the lateral ventricles, and the subgranular zone, forming part of the dentate gyrus of the hippocampus (Mandal, 2014; Nogueira *et al*, 2014). A resident population of neural progenitor cells provides the source of new neurons (Elder *et al*, 2006).

A microtubule-associated protein (MAP) known as doublecortin (DCX) assists with the young neuronal migration into cortical layers of the brain during embryological development. DCX expression in areas with extended neuronal recruitment allows this endogenous protein to be used as a marker for adult neurogenesis (Vellema *et al*, 2014).

Stroke has been shown to trigger neurogenesis, not only in the SVZ for example, but also in areas of the brain that do not exhibit neurogenesis under normal conditions. It is possible that radial microglia are involved with guiding migration of neuroblasts to the ischemic brain tissue. There is also evidence that neurogenesis is linked to angiogenesis (Seki *et al*, 2011), which is the process that forms new blood vessel branches from existing vessels (Mayes, 2006).

The hope of assisting cell replacement after an ischemic event has directed some stroke research to focus on drug therapy that may stimulate neurogenesis.

STROKE RESEARCH

Because stroke persists as one of the most substantial health issues today (Hall *et al*, 2012), much research centers on developing pharmacological treatments for stroke. With ischemic stroke accounting for almost 90 percent of strokes (AHA/ASA, 2012), and with a narrow – and many times unreachable – window for tPA administration (Clark *et al*, 1999; Hacke *et al*, 2004; NINDS, 1995), a treatment option available after three hours post-stroke is ideal.

Animal Model

When developing an animal model for ischemic stroke research, features similar to those in humans must be considered so the outcomes can be addressed with the least possible limitations. Biological factors, physiological reactions, and pathological implications must be reviewed (Casals *et al*, 2011).

The most relevant animal models for stroke research are rodents or lagomorphs, such as rabbits (Casals *et al*, 2011). Rats and mice are most often used (Howells *et al*, 2010), as their cerebrovasculature closely matches that of higher organisms (Ginsberg and Busto, 1989). Rodents also present similar Circle of Willis collateralization and variation to that found in humans (Howells *et al*, 2010; Mergenthaler and Meisel, 2012). This is an important consideration when understanding how the brain responds to stroke induction, depending on the method used, and how the brain responds to treatment.

Previous studies in our laboratory found that Sprague Dawley rats had better blood vessel collateralization than the Long Evans strain. When the same amount of endothelin was injected into both rat strains, a much smaller infarct was produced in the

Sprague Dawley strain, suggesting there is better blood vessel collateralization in the cortex.

Earlier studies in our laboratory used Long Evans rats. It was noted, however, that their blood vessel collateralization does not closely match that in humans. In Long-Evans rats, endothelin was injected to either side of the forelimb motor cortex, causing injury but not complete infarction of this important center. With our drug treatment, fast recovery of forelimb motor function was observed within 30 days, most likely because the tissue was only injured rather than killed. In addition to these noted concerns, data suggest that Long Evans rats may be more susceptible to damage in the hemisphere contralateral to the injury site than are Sprague Dawley rats (Tan *et al*, 2009).

Sprague Dawley rats are now used in our laboratory instead because they much more closely match human brain collateralization. To address the recovery matter, the location of stroke induction was changed to destroy the forelimb motor cortex itself rather than affect the tissue surrounding it. A lasting deficit is now attainable in an animal model that more closely relates to humans; this should provide a better translational approach.

Our rat subjects are all female because the male rats are >60 g heavier than the females (Casals *et al*, 2011) and will not fit in the functional test apparatus (Montoya Staircase, Lafayette Industries; 400 g limit). The female rats used in this study, at 10 to 12 months of age, weighed approximately 320-385 g.

Though no single animal model can fully represent the variables affecting human ischemic stroke patients (Bacigaluppi *et al*, 2010; Howells *et al*, 2010), studies suggest the lack of translation lies not completely with the choice of animal model but in how

such models and their resultant data are applied (Howells *et al*, 2010). Most often, stroke research is conducted using young animals. However, human stroke patients are typically elderly and may present other health concerns (Bacigaluppi *et al*, 2010; Casals *et al*, 2011). In addition, young brains react differently to stroke (Casals *et al*, 2011). Our study sought to eliminate this limitation by using older animals, aged 10 to 12 months, which is approximately middle-aged for a rodent.

Induction Model

Several induction models have been introduced in stroke research. Many of the models are successful in following the condition found with human stroke patients in relation to large artery occlusion. Still, other models result in extremely large infarcts that may not imitate instances of the most common, treatable human strokes (Carmichael, 2005). With human ischemic strokes typically only affecting approximately 5% of total brain volume (Brott *et al*, 1989), it is important to find a model that best matches human ischemic stroke.

Most frequently used is mechanical occlusion of the proximal middle cerebral artery (MCA) (pMCAo). This large-vessel occlusion produces a large infarct and is usually achieved by inserting a silicon-coated nylon suture into the internal carotid artery to the circle of Willis to occlude the MCA at its source. For a more restricted occlusion of superficial vessels, mechanical occlusion of the distal MCA (dMCAo) can be performed. This small-vessel occlusion is accomplished by exposing and altering superficial MCA branches via a craniotomy (Bacigaluppi *et al*, 2010). One study showed most MCAo models produce infarcts between 21-45% of the affected cerebral

hemisphere, and the dMCAo model specifically produces smaller infarcts than the pMCAo model, with infarct sizes of approximately 14-25% of the affected hemisphere. Still, these models more closely mimic large-volume or hemorrhagic strokes in humans rather than typical ischemic stroke when reviewing infarct size (Carmichael, 2005).

Another model for stroke induction involves intravenous injection of a photosensitive dye. Once injected, photothrombosis is induced when the brain is illuminated trans-cranially and the irradiated tissue coagulates. While this model can produce precise small strokes (Bacigaluppi *et al*, 2010), the dye itself can damage the endothelium (Carmichael, 2005). In the case where a drug that targets endothelial cells is being studied for use in stroke treatment, this model would not be appropriate.

Cerebral veins and sinus thrombosis (CVT) is a disorder affecting younger adults or children more often than arterial stroke. A model for inducing CVT involves occluding the superior sagittal sinus, mechanically or thermally damaging the superior sagittal sinus, or photothrombosis of the dorsal cerebral veins (Bacigaluppi *et al*, 2010). Because this study seeks to research the most common instance of ischemic stroke in humans, this model is not fitting.

Some models have been designed to specifically mimic the effects of an arterial blockage by a blood clot. Thrombotic occlusion is obtained by injecting blood clots, thrombin, or synthetic macrospheres into the MCA or internal carotid artery to produce a large infarct like that of permanent pMCAo. To produce smaller, multifocal infarcts, synthetic microspheres can be used. However, the high variability in infarct size and results associated with this model have caused a lack of translation to human stroke (Bacigaluppi *et al*, 2010).

Other approaches have been developed in order to gain more control over the size of the infarct produced. Injecting the potent vasoconstrictor endothelin intracerebrally produces a focal ischemic infarct by severely reducing blood flow to the affected area. Though it can be more difficult to precisely localize the lesion, the endothelin model for producing focal ischemia is easier, faster, and technically simpler than other rat stroke models (Bacigaluppi *et al*, 2010).

Our study employs the endothelin model for stroke induction. Endothelin's vasocontrictive mechanism induces a stroke in approximately 30 minutes, with lasting effects for 24 hours before the vasoconstriction releases (Windle *et al*, 2006). A sure consequence of stroke is neurodegeneration, which appears early in this model (Wolinski and Glabinski, 2013). This method has also proven to be easily performed and reproducible in rats (Bacigaluppi *et al*, 2010). Because of the ability to produce smaller, more controlled infarcts, in addition to its ease of use, confirmed repeatability, and accuracy in generating ischemic stroke, the endothelin model was chosen for our study.

PHARMACOLOGICAL TREATMENT DESIGN

In order to propose a delayed pharmacological treatment for stroke, several standards must be established. The individual drugs involved, the proper dosage of each drug, and the ideal timeframe for drug delivery post-stroke must all be defined.

Previous studies in our laboratory have reviewed combinations of fluoxetine, simvastatin, and ascorbic acid in hopes of optimizing the dose of essential constituents (Corbett *et al*, 2013). Combination therapies have proven to be more effective than a single drug in treating many health concerns, from depression (Blier *et al*, 2010) to cancer (F. Li *et al*, 2014), and from diabetes (Tosi *et al*, 2003) to human

immunodeficiency virus (HIV) (Pakker *et al*, 1998). There was already work in the literature showing that fluoxetine alone (Schäbitz *et al*, 2007) and simvastatin alone (Rodríguez-Yáñez *et al*, 2008) increase neurogenesis, but no one had ever tested the combination. Information on each drug, its class, and the final drug combination is presented below.

Selective Serotonin Reuptake Inhibitors (SSRIs) and Stroke

Fluoxetine belongs to a group of drugs known as SSRIs. SSRIs act to treat depression by inhibiting presynaptic reuptake of serotonin. This inhibition increases the availability of serotonin at the synaptic cleft (Costagliola *et al*, 2008).

In addition to its ability to help manage depression, SSRIs may improve recovery after stroke. SSRIs appear to improve neurological impairment and disability; this finding holds true even in patients who are not depressed (Mead *et al*, 2012). Additionally, SSRIs inhibit platelet aggregation, decrease ischemic risk, protect against inflammatory neurotoxicity, and stimulate neuroplasticity, neurogenesis, and angiogenesis (Rao and Andrade, 2011).

Many antidepressants have been reviewed for their ability to treat depression and, separately, to assist with stroke recovery. For the treatment of depression alone, studies compared the use of fluoxetine to that of other SSRIs, tricyclic antidepressants (TCAs), and heterocyclic and related antidepressants. Significant differences surfaced regarding the efficacy and tolerability of the drugs reviewed for depression treatment, but the study concluded uncertainties in the application, and no clinical implications could be drawn (Cipriani *et al*, 2005; Magni *et al*, 2013). However, another study compared the use of SSRIs to TCAs for stroke treatment and found that SSRIs significantly reduced the risk

of stroke as compared with TCAs (Lee *et al*, 2013). While certain antidepressants did not stand out against others in terms of treatment for depression, SSRIs presented obvious benefits for stroke recovery.

Fluoxetine

When evaluating SSRIs for stroke treatment, studies have used fluoxetine, sertraline, paroxetine, citalopram, or escitalopram (Mead *et al*, 2013b). Although it seems acceptable that all SSRIs would have similar effects, most of the stroke research using SSRIs has been conducted using fluoxetine (Rao and Andrade, 2011).

Fluoxetine crosses the BBB (Warren, 2012) and increases expression of BDNF, which plays a role in cognitive recovery (Liu *et al*, 2013) and neurogenesis (Schäbitz *et al*, 2007). Fluoxetine also greatly improves motor recovery with ischemic stroke patients (Chollet *et al*, 2011; Chollet *et al*, 2013; Marquez-Romero *et al*, 2013) and results in less disability (Cramer, 2011). Studies have followed patients through their recovery and have seen enhanced motor improvement after three months post-stroke (Chollet *et al*, 2011; Cramer, 2011). Such mechanisms are believed to involve modulation of brain plasticity (Chollet *et al*, 2011).

Furthermore, neuroprotective effects accompanied the advances in motor recovery. Fluoxetine's neuroprotection may be associated with its anti-inflammatory effects. Microglial activation, expression of proinflammatory markers, and neutrophil infiltration were all suppressed in brain tissue after being treated with fluoxetine post-stroke, suggesting that fluoxetine protects the brain against ischemic injury (Lim *et al*, 2009).

In addition to neurogenesis promotion via BDNF, anti-inflammatory effects, and reduced motor cortex excitability, fluoxetine may act by inducing expression of vascular endothelial growth factor (VEGF). VEGF is a mediator of neurogenesis, neuroprotection, and angiogenesis after ischemia. By upregulating VEGF – an action specific to SSRIs, as TCAs do not affect the VEGF pathway – fluoxetine facilitates other avenues of brain recovery after stroke (Gaillard and Mir, 2001).

Some debate exists regarding the efficacy of fluoxetine at different doses (Zhu BG. *et al*, 2012) as well as possible risks and interactions. SSRIs block serotonin uptake by platelets, which impairs the platelet hemostatic response. Some have expressed concern about a possible increase in bleeding risk when used as stroke treatment. However, only epidemiologic studies of gastrointestinal bleeding have been performed, and the study concluded that the data are too limited to permit interpretations about influences on stroke (Andrade *et al*, 2010). Additionally, there are postulated interactions of fluoxetine with other drugs. Studies suggest fluoxetine interacts with the anticoagulant warfarin, which is prescribed to prevent blood clot formation (Dent and Orrock, 1997); the anticonvulsant diazepam, which is prescribed for patients who experience seizures after a stroke (Dent and Orrock, 1997); and the antiplatelet drug clopidogrel, which is often prescribed after a stroke for secondary prevention (Gonzenbach *et al*, 2011), or efforts used to prevent future stroke in a patient who has already had a stroke. Unfortunately, most of these interactions are not well defined or established (Dent and Orrock, 1997; Gonzenbach *et al*, 2011).

Studies suggest larger trials will help determine whether post-stroke SSRI administration should be routine (Mead *et al*, 2012; Mead *et al*, 2013a). Current clinical

trials using fluoxetine to study the implications of SSRI use in stroke recovery include Fluoxetine or Control Under Supervision (FOCUS) in the United Kingdom, Assessment oF FluoxetINe In sTroke recoverY (AFFINITY) in Australia, and Efficacy oF Fluoxetine - a randomisEd Controlled Trial in Stroke (EFFECTS) in Sweden (Mead *et al*, 2013a).

Statins and Stroke

Simvastatin belongs to a group of drugs called statins. Statins help lower cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes a rate-limiting step in the biosynthesis of cholesterol. By reducing levels of LDL cholesterol in plasma, statins serve to decrease cardiovascular risk (Hernández-Perera *et al*, 1998).

In addition to their role in cholesterol metabolism, studies suggest statins possess cholesterol-independent pleiotropic effects that improve endothelial function, inhibit thrombogenic response, stabilize atherosclerotic plaques, and decrease inflammation and oxidative stress (Liao and Laufs, 2005). Statins also provide neuroprotection against ischemic injury, making them an important candidate in stroke treatment (Hernández-Perera *et al*, 1998; Miedema *et al*, 2010; Stepień *et al*, 2005).

Though the neuroprotective mechanisms of statins are only partially understood (Sierra *et al*, 2011), it is believed that changes in inflammation, platelet aggregation, immune response, glutamate metabolism, and apoptosis are involved (Rodríguez-Yáñez *et al*, 2008). Statins provide protection against ischemic injury by upregulating endogenous tPA (Asahi *et al*, 2005). Statins have also been shown to protect against vascular remodeling, an adaptive process that can negatively contribute to vascular

pathophysiology (Dzau and Gibbons, 1993), by suppressing the Rho/Rho-kinase pathway (Ma *et al*, 2012). Inhibition of isoprenoids, which serve as lipid attachments for intracellular signaling molecules, may also mediate some of the pleiotropic effects (Liao and Laufs, 2005).

Moreover, statins promote angiogenesis, formation of new synapses, and neurogenesis (Rodríguez-Yáñez *et al*, 2008). One avenue through which statins initiate neurogenesis and protect against ischemic injury is by upregulating endothelial nitric oxide synthase (eNOS) (Asahi *et al*, 2005; Mital *et al*, 2000). Nitric oxide (NO) is normally produced in the brain by the neuronal isoform of NOS and negatively regulates precursor cell proliferation. However, in the injured brain, NO overproduction actually promotes neurogenesis (Estrada and Murillo-Carretero, 2005).

Recent studies show statins consistently reduce the risk of ischemic stroke in patients (Tuttolomondo *et al*, 2014) and are recommended to lower the risk of stroke recurrence or other vascular events after an ischemic stroke (X. Li *et al*, 2012). Statins have also been shown to support neurovascular recovery and improve neurological outcome after intracerebral hemorrhage (Yang *et al*, 2012).

Simvastatin

Studies have reviewed all statins in order to determine which statin works best to protect the brain. The nine statins can be divided into three categories: natural or fungus-derived, semi-synthetic, and synthetic. Data show the natural and semi-synthetic statins, also known as monacolin J derivatives, are the best contenders for preventing neurodegeneration. Reasons for this conclusion include a higher potential for BBB

penetration, the safe manner in which they lower cholesterol in neurons, and observations regarding protection against cell death in culture. Of the nine total statins, simvastatin, also a monacolin J derivative, presented as the best option for preventing neurodegenerative conditions (Sierra *et al*, 2011).

Because simvastatin is lipophilic (Thelen *et al*, 2006), it can easily cross the BBB (Wood *et al*, 2010) and is more likely to passively diffuse into endothelial cells than hydrophilic statins (Zhu M. *et al*, 2012). These properties explain why use of simvastatin has resulted in improving the function of ischemia-disturbed BBBs (Jiang *et al*, 2012). In addition, simvastatin increases BDNF expression (Yang *et al*, 2012), which, as previously discussed, works in neuroprotection and reduction of infarct size (Kleim *et al*, 2003; Schäbitz *et al*, 2007). Simvastatin also upregulates VEGF, which initiates angiogenesis (Wu *et al*, 2008).

In ischemic stroke, high extracellular levels of glutamate overstimulate glutamate receptors. This overstimulation causes calcium entry into neurons through N-methyl-D-aspartate receptors (NMDARs), which is a central influence of neuronal excitotoxicity. Simvastatin has been shown to significantly decrease infarct area after ischemia and increase expression of protein phosphatase 2A (PP2A). PP2A is a family of serine-threonine phosphatases controlling multiple cellular processes through negative signal regulation, including suppression of NMDARs, thus aiding in protection against neuronal excitotoxicity (Zhu M. *et al*, 2012).

Simvastatin is believed to influence vascular tone by moderating endothelial vasoactive factor expression (Hernández-Perera *et al*, 1998). Simvastatin also increases

eNOS activity (McGirt *et al*, 2002), and, as noted earlier, NO overproduction promotes neurogenesis (Estrada and Murillo-Carretero, 2005).

Simvastatin and tPA

Clinical benefits are observed when administering simvastatin alone or when coupling simvastatin with tPA. When used together, simvastatin increases the therapeutic window for tPA and encourages neuroprotection (Lapchak and Han, 2010).

Though some benefits exist, combining simvastatin with tPA is a controversial topic. Some studies suggest a possible increase in the risk of hemorrhagic transformation when combining simvastatin with tPA (Laloux, 2014; Scheitz *et al*, 2014), while others state that simvastatin reduces tPA-induced hemorrhage (Lapchak and Han, 2010), decreases neurovascular unit dysfunction (Zhang *et al*, 2012), and produces no increase in hemorrhagic transformation risk (Campos *et al*, 2013). The proper dose of simvastatin with tPA and window of administration has not yet been determined (Lapchak and Han, 2010).

Pre-Stroke Exposure to Statins

Pre-stroke statin exposure trends toward improved results in ischemic stroke patients. While patients taking statins did have less severe strokes, the effects were not significantly different. Nevertheless, results suggest a tendency toward lower stroke severity and improved outcomes short-term post-stroke (Phipps *et al*, 2013).

Ideal Drug Combination

Because of its pleiotropic actions that improve the disturbed BBB (Jiang *et al*, 2012), increase BDNF expression (Yang *et al*, 2012), upregulate eNOS activity (McGirt *et al*, 2002), promote neurogenesis (Estrada and Murillo-Carretero, 2005), and aid in neuroprotection and infarct size reduction (Kleim *et al*, 2003; Schäbitz *et al*, 2007), simvastatin was chosen as a component in this study's drug combination.

Similarly, fluoxetine was chosen for use in our study due to its ability to stimulate neurogenesis through BDNF expression (Liu *et al*, 2013; Schäbitz *et al*, 2007), improve motor recovery (Chollet *et al*, 2011; Chollet *et al*, 2013; Marquez-Romero *et al*, 2013), promote anti-inflammatory effects (Lim *et al*, 2009), and enhance neuroprotection and angiogenesis through VEGF expression (Gaillard and Mir, 2001).

Because simvastatin and the eNOS enzyme are both easily oxidized, and because ascorbic acid is an antioxidant that should help retain normal function in both substances, asborbic acid was originally reviewed as a possible component of the combination. However, it was later discovered that the major filler in simvastatin is already ascorbic acid, so simvastatin and fluoxetine together proved to be the desired combination.

The drug combination used in this study was 5 mg/kg fluoxetine, 1 mg/kg simvastatin. In the "fluoxetine for motor recovery after acute ischaemic stroke (FLAME)" clinical trial, a dose of 20 mg fluoxetine was administered post-stroke (Chollet *et al*, 2011), which corresponds to an approximate dose in rats of 2.5 mg/kg (Reagan-Shaw *et al*, 2007). In humans, a patient is typically started at a dose of 20 mg fluoxetine; the dose is later increased as needed. Our laboratory's rat dose of 5 mg/kg fluoxetine correlates to a human dose of approximately 40 mg/kg, which is well within

the normal human dose range of 20-80 mg for this drug (Rx List, 2014a). Two rat doses of simvastatin have been used in our laboratory's studies: 0.5 mg/kg and 1 mg/kg. These rat doses correlate to approximate human doses of 5 mg or 10 mg respectively. These doses are well within the normal human dose range of 5-40 mg for this drug (Rx List, 2014b).

With the individual drugs comprising the combination outlined and the proper dosage of each drug defined, the ideal timeframe for drug delivery post-stroke must be established.

HYPOTHESIS

A previous study noted success with the fluoxetine/simvastatin drug combination in reducing infarct size and increasing functional recovery when delivered 20-26 hours post-stroke (Corbett *et al*, 2013). This study seeks to test the hypothesis that earlier drug delivery will improve functional recovery and decrease infarct size by determining the effects of the fluoxetine/simvastatin drug combination when administered at 6-12 hours post-stroke. This study also seeks to test the hypothesis that limb rehabilitation will improve functional recovery.

SPECIFIC AIMS

The focus of this study is to determine the effects of the earlier fluoxetine/simvastatin drug combination delivery on functional recovery and infarct size as well as define the effects of limb rehabilitation on functional recovery. This work aims to help identify the prime window of drug delivery for optimum effects on increasing functional recovery and reducing infarct volume in the ischemic brain.

Two experiments were preformed to test the hypotheses. Experiment 1 reviewed earlier drug delivery at 6-12 hours post-stroke. No rehabilitation was included in Experiment 1. Two groups of animals were defined in this portion of the study: drug-treated and control.

Experiment 2 reviewed earlier drug delivery at 6-12 hours post-stroke as well, using the same drug combination and dosage as Experiment 1. Additionally, Experiment 2 included and reviewed a rehabilitation component. Four groups of animals were defined in this portion of the study: drug-treated with rehabilitation, drug-treated without rehabilitation, control with rehabilitation, and control without rehabilitation.

To test the hypothesis that earlier drug delivery will improve functional recovery, pre-stroke function can be established for all animals using the Montoya staircase functional grasping test. After stroke induction, the animals can be retested to establish a baseline functional deficit. Following treatment for 90 days, the functional assessment data will quantify functional recovery, which will be compared between the drug-treated and control animals to determine whether earlier drug delivery improves functional recovery. After euthanasia and brain tissue analysis, infarct volume can be calculated for each animal. Comparison of infarct size in drug-treated animals to control animals will allow for testing of the hypothesis that earlier drug delivery will decrease infarct size. This process is followed in Experiment 1.

Like Experiment 1, Experiment 2 analyzes functional recovery and infarct volume. Unlike Experiment 1, Experiment 2 also tests the hypothesis that limb rehabilitation will increase functional recovery. In Experiment 2, certain control animals and certain drug-treated animals are given rehabilitation using reaching shelves outside

their cages. The functional recovery of drug-treated animals with rehabilitation can then be compared to that of drug-treated animals without rehabilitation; the functional recovery of control animals with rehabilitation can be compared to that of control animals without rehabilitation; and all animals receiving rehabilitation (regardless of drug treatment) can be compared to all animals not receiving rehabilitation. These comparisons will allow for the determination of whether rehabilitation increases functional recovery.

Infarct size and functional recovery data from Experiments 1 and 2 can also be analyzed to determine if a correlation exists between the two factors. In addition, the data from this study (Experiments 1 and 2), in which drug delivery is at 6-12 hours post-stroke, can be compared to the data from the previous study, in which drug delivery was at 20-26 hours post-stroke. This will provide valuable insight as to the optimum window for drug delivery and contribute to the current research aim of developing a treatment for stroke.

II. MATERIALS AND METHODS

All studies described in this paper were performed in accordance with protocols approved by the Wright State University Institutional Animal Care and Use Committee (IACUC).

PRE-STROKE TRAINING

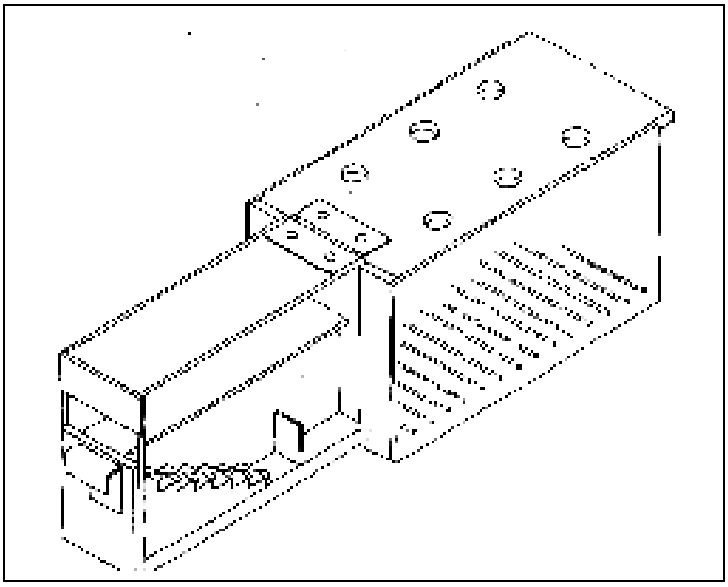
Female Sprague Dawley outbred retired breeder rats aged 10 to 12 months were trained on Montoya Staircases. The Montoya Staircase (Figure 1) is a functional test that presents food pellets on a bilateral set of stairs, requiring the rat to crawl onto a raised platform, extend its forelimbs at increasing distances to reach each stair, and grasp pellets to retrieve them (Montoya *et al*, 1991). Three sucrose pellets – some painted with maple extract – were placed in each of the wells (seven on each side) to total 21 pellets per side. Each animal was trained for 15 minutes on the Montoya Staircase and returned to its cage. Training occurred daily for one-and-one-half weeks during the dark cycle to best accommodate the animals' nocturnal circadian rhythm (Dauchy *et al*, 2010). Training data were collected to determine pre-stroke function. The number of pellets retrieved by each forepaw was measured, and the highest number recovered was noted as the pre-stroke function. By the end of training, animals must have been able to retrieve at least nine pellets with each forepaw to be included in the post-stroke analysis.

Figure 1.

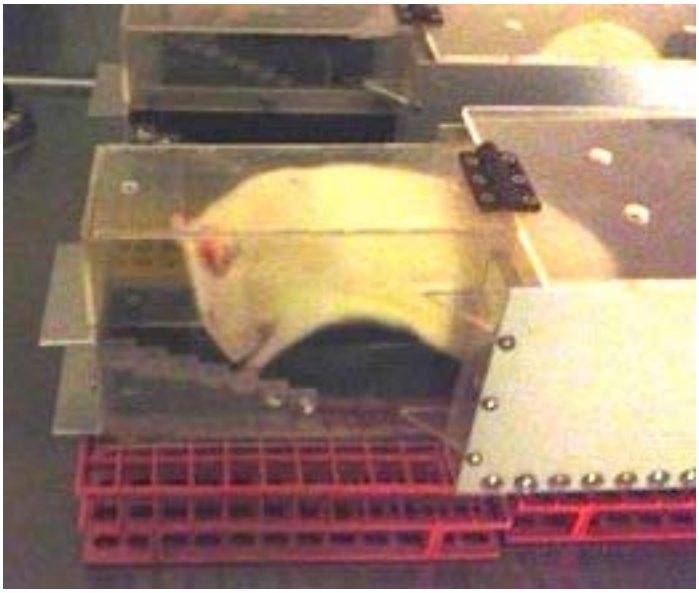
The Montoya Staircase. (A) This schematic shows the Montoya Staircase, a functional forelimb reaching and grasping test for rats. The rat is placed inside a Plexiglas box with access to a raised platform that sits in the middle of a removable double staircase. The double staircase offers progressive levels of reaching difficulty and allows for the assessment of individual forelimb function (Montoya *et al*, 1991). (B) The photograph shows a rat performing the Montoya Staircase test.

Figure 1.

(A)



(B)



Diet Restriction

During training, the animals were placed on a restricted diet to optimize Montoya Staircase results. To measure the average amount of food eaten ad libitum by the rats, the food given to the animals the day after their arrival was weighed, and the food remaining after two days was weighed again. The animals were then fasted overnight and began Montoya Staircase training the next morning during the dark cycle. Their diet was restricted to 85% of their normal intake. To monitor their weight loss, the rats were weighed immediately prior to food restriction as well as every third day until the training period was complete. If the animals neared their goal weight loss of 15 percent, their daily food was increased to stabilize their weight. In general, most animals only lost five to 10 percent of their weight during the training period.

Sucrose Pellets

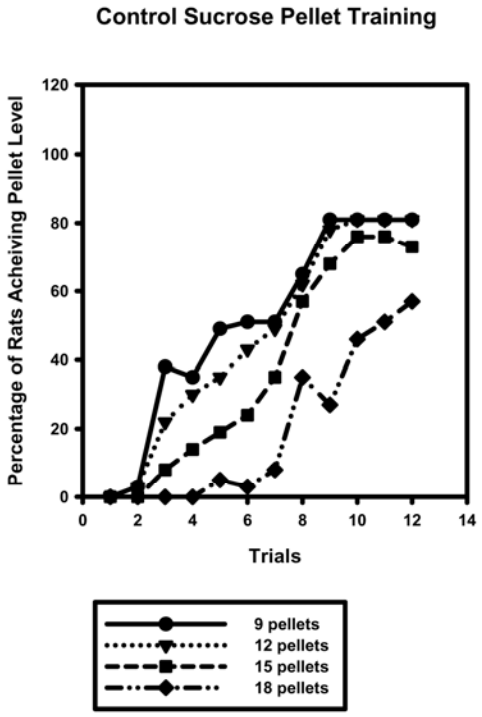
Sucrose pellets were used to encourage training (Figure 2A). Some of the sucrose pellets were painted with maple extract. A previous study in our laboratory found that painting all sucrose pellets with maple extract encouraged the rats to retrieve them (Figure 2B). However, this study determined that even if only some of the pellets are painted with maple extract, the rats are still encouraged to retrieve the pellets (Figure 2C).

Figure 2.

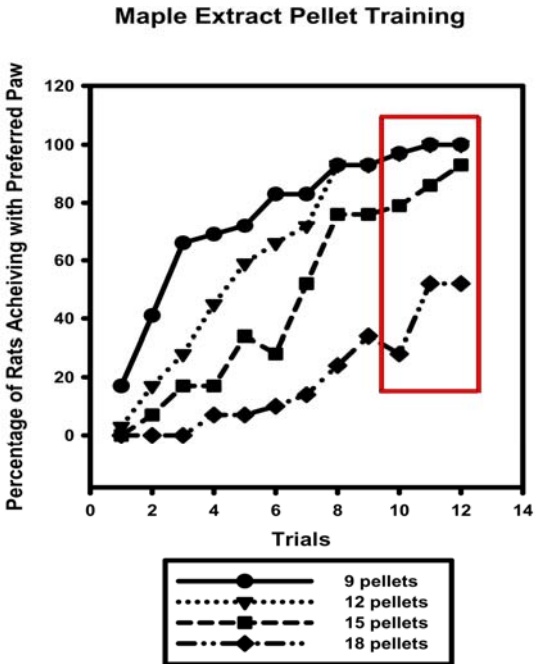
Figures showing data regarding how rats retrieved sucrose pellets painted with different degrees of maple extract. Thirteen trials were performed on the Montoya Staircase where pellet retrieval was measured. The x-axis represents the trial number. The y-axis denotes percentage of pellets retrieved with the animal's preferred paw, meaning if an animal retrieved 20 pellets with the left forelimb and 21 pellets with the right forelimb, the right forelimb is considered the preferred paw and this number was used in our calculations. Data are presented according to number of pellets retrieved. The minimum number of pellets an animal had to retrieve with each forelimb to be contained in our study was nine of the 21 total pellets. Circles represent animals that retrieved 9-11 pellets. Triangles represent animals that retrieved 12-14 pellets. Squares represent animals that retrieved 15-17 pellets. Diamonds represent animals that retrieved 18-21 pellets. (A) This figure displays data from a previous study in the laboratory showing the control, where plain sucrose pellets were used and none were painted with maple extract. (B) This figure displays data from a previous study in the laboratory showing that animals retrieved more pellets if all of the sucrose pellets were painted with maple extract. (C) This figure displays data from our study showing that, even if only some of the sucrose pellets were painted with maple extract, animals were still encouraged to retrieve a higher number of pellets compared to the control.

Figure 2.

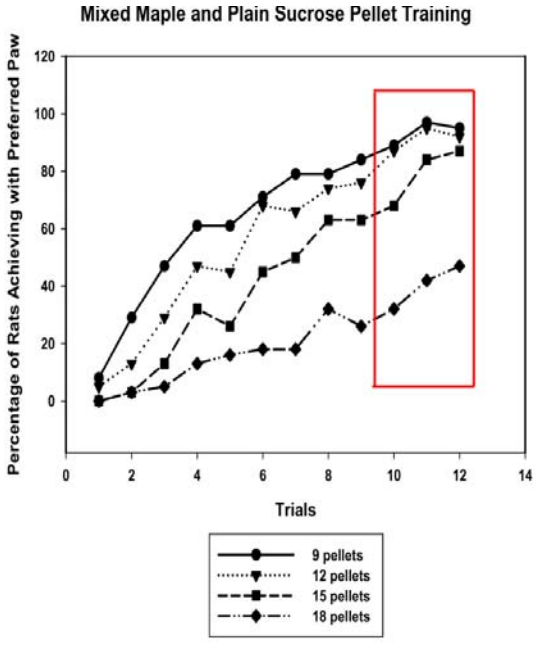
(A)


Control Sucrose Pellet Training
Percentage of Rats Acheiving Pellet Level
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Trials
9 pellets
12 pellets
15 pellets
18 pellets

(B)


Maple Extract Pellet Training
Percentage of Rats Acheiving with Preferred Paw
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Trials
9 pellets
12 pellets
15 pellets
18 pellets

(C)


Mixed Maple and Plain Sucrose Pellet Training
Percentage of Rats Achieving with Preferred Paw
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Trials
9 pellets
12 pellets
15 pellets
18 pellets

STROKE INDUCTION

All animals in the study had stroke surgery. Strokes were induced under anesthesia using two injections of endothelin, a potent vasodilator, into the forelimb motor cortex of the right hemisphere. All animals had a stroke in the right hemisphere, which would affect the left forelimb, regardless of handedness or preferred paw consideration. Coordinates measured from bregma facilitated calculation of the forelimb motor cortex location.

A list of the substances administered during the course of the stroke induction has been compiled (Table 1).

Pre-operative Care

The animal was placed in an induction chamber with 5% isoflurane. Once under anesthesia, the animal was removed from the chamber, and its head was shaved. The animal was then placed on paper overlying a heating pad and positioned in a stereotactic device to stabilize its head. A gas mask supplied a constant flow of 2-3% isoflurane during the procedure. A foot-pinch withdrawal reflex was performed on the animal to ensure full anesthetization, and Puralube eye ointment was applied to keep the animal's eyes moist.

Table 1.

Table listing substances administered during the course of the stroke induction surgical procedure.

Table 1.

Substance	***Concentration***	***Vehicle***	***Volume***	***Route***	***Notes***
Isoflurane	5%	Oxygen		Inhalation	Surgical anesthetic induction
Isoflurane	2-3%	Oxygen		Inhalation	Surgical anesthetic maintenance
Puralube eye ointment				Topical	Preoperative; applied to eyes
Povidone-iodine				Topical	Preoperative: once to clean incision site, once to scrub incision site Postoperative: once to treat sutured incision site
Ethanol				Topical	Preoperative; once to clean incision site, performed between cleaning and scrubbing with povidone-iodine
Bupivacaine	0.25%	Water	0.15 ml		Analgesic
Endothelin	400 pmoles/μl	Water	1.5 μl/site	Cortical injection (2 mm depth)	Injected into each of two cortical sites during surgery to induce stroke
Sterile saline	Isotonic		2 ml	Subcutaneous injection	Postoperative

Surgical Procedure

The incision site was cleaned with povidone-iodine, cleaned with ethanol, and then scrubbed with povidone-iodine. A midline incision was made on the animal's head, and the edges of the incision were dabbed with bupivacaine. Bregma was then located on the animal's skull and marked with a fine-tip marker.

A small burr bit in a microdrill was aligned with bregma. Coordinate measurements were then used to determine the location of the forelimb motor cortex in the right hemisphere. The drill was moved anterior-posterior (AP) a distance of 0 mm and medial-lateral (ML) a distance of -2.5 mm from bregma to drill the first of two holes. To drill the second hole, the drill was moved AP +1.5 mm and ML -2.5 mm from bregma. The drill was removed and replaced with a Hamilton syringe containing endothelin, and 1.5 μl of 400 pmoles/μl endothelin was injected over the course of several minutes into each site. The injection depth was 2.0 mm.

The incision was closed with resorbable sutures and treated again with povidone-iodine.

Post-Operative Care

A subcutaneous injection of 2 ml sterile saline was administered before placing the animal in its cage on a heated pad to recover from anesthesia. The animal was monitored until awake and moving, at which point it was returned, in its cage, to the animal rack. When the animal was moving around well, it was given moist chow on the floor of its cage. The animal was switched back to normal chow by post-stroke day 2.

POST-STROKE TREATMENT

Treatment after stroke induction involved both a pharmacological component and a rehabilitation component.

Pharmacological Treatment

Pharmacological treatment was provided in both Experiments 1 and 2. Treatment began 6-12 hours after stroke induction. Animals received either the vehicle only or the drug combination fully incorporated into the vehicle.

Because stress can influence neurogenesis among other physiological processes, it was crucial that stress associated with pharmacological treatment be reduced (Corbett *et al*, 2012). The vehicle used in this study was a 3-4 g ball of Pillsbury sugar cookie dough. This method provided a voluntary oral means for drug delivery and thus helped decrease stress on the animal (Corbett *et al*, 2012).

If an animal failed to ingest the cookie dough on three or more occasions, the animal was removed from the study.

No additional substances were administered to assist with post-operative pain control or recovery because non-steroidal anti-inflammatory drugs (NSAIDs) and analgesic opioids can both influence neurogenesis (Goncalves *et al*, 2010; Hoehn *et al*, 2005; Sargeant *et al*, 2008). The infarct analysis aspect in this study depended on the effects of our drug combination on neurogenesis; therefore, it was imperative that neurogenesis not be modified by other drugs.

Treatment: Drug-Treated Rats

The drug combination consisted of 5 mg/kg fluoxetine and 1 mg/kg simvastatin. The powdered drug combination was fully incorporated into the cookie dough vehicle and placed in the animals' cages. Animals received the drug treatment once daily for 90 days following the stroke.

Treatment: Control Rats

Control rats received only the cookie dough vehicle beginning 6-12 hours post-stroke. They continued receiving the vehicle once daily for 90 days.

Limb Rehabilitation

Limb rehabilitation was provided in Experiment 2 only. The control rats were divided into two groups: control with rehabilitation and control without rehabilitation (Table 2). The drug-treated animals were also divided into two groups: drug-treated with rehabilitation and drug-treated without rehabilitation (Table 2).

Beginning on post-stroke day 8, a rehabilitation shelf containing peanut butter was hung every other night outside the rehabilitation animals' cages, and this was continued for five and one half weeks. The shelves were positioned in such a way so the animals could only use the contralateral forelimb (stroke-affected limb) to reach the peanut butter (Figure 3).

Table 2.

Table displaying how animals were grouped. Experiment 1: drug-treated and control. Experiment 2: drug-treated with rehabilitation, drug-treated without rehabilitation, control with rehabilitation, and control without rehabilitation.

Table 2.

Experiment 1: Rats 530-566		*Experiment 2: Rats 600-637*			
Group	**Total Animals***	**Group**	**Total Animals**	**Rehabilitation**	**No Rehabilitation**
FS	18	FS	18	15	3
Control	18	Control	17	14	3
Died	1	Died	3		

*No animals in Experiment 1 received rehabilitation.

Figure 3.

Pictures of rehabilitation shelves. (A) This series of pictures shows varying degrees of peanut butter retrieval. Because of the shelf design, it was easy to measure whether an animal took no peanut butter, some peanut butter, or all of the peanut butter. (B) This picture shows a rat taking peanut butter while using the rehabilitation shelf.

Figure 3.

(A)

(B)


Hanging Shelf for Rehabilitation
of Contralateral Forelimb

POST-STROKE FUNCTIONAL TESTING

After stroke induction, animals underwent a series of Montoya Staircase testing. Each set of testing days allowed for calculation of an initial baseline deficit and ultimately the measurement of functional recovery. Figure 4 displays a timeline of all events included in this study.

Calculation of Baseline Functional Deficit

The first set of post-stroke Montoya Staircase testing occurred daily on post-stroke days 3, 4, and 5. The number of pellets retrieved during this initial post-stroke functional evaluation allowed for the establishment of a baseline functional deficit. Both contralateral (affected by stroke) and ipsilateral retrieval was measured.

An animal's best performance during post-stroke days 3, 4, and 5 was divided by the animal's best performance from the pre-stroke training days; multiplying this figure by 100% produced the percentage of pre-stroke function the animal retained; subtracting this number from 100% produced the baseline percentage of functional deficit resulting from the stroke. The formulas and sample calculations are presented in Table 4.

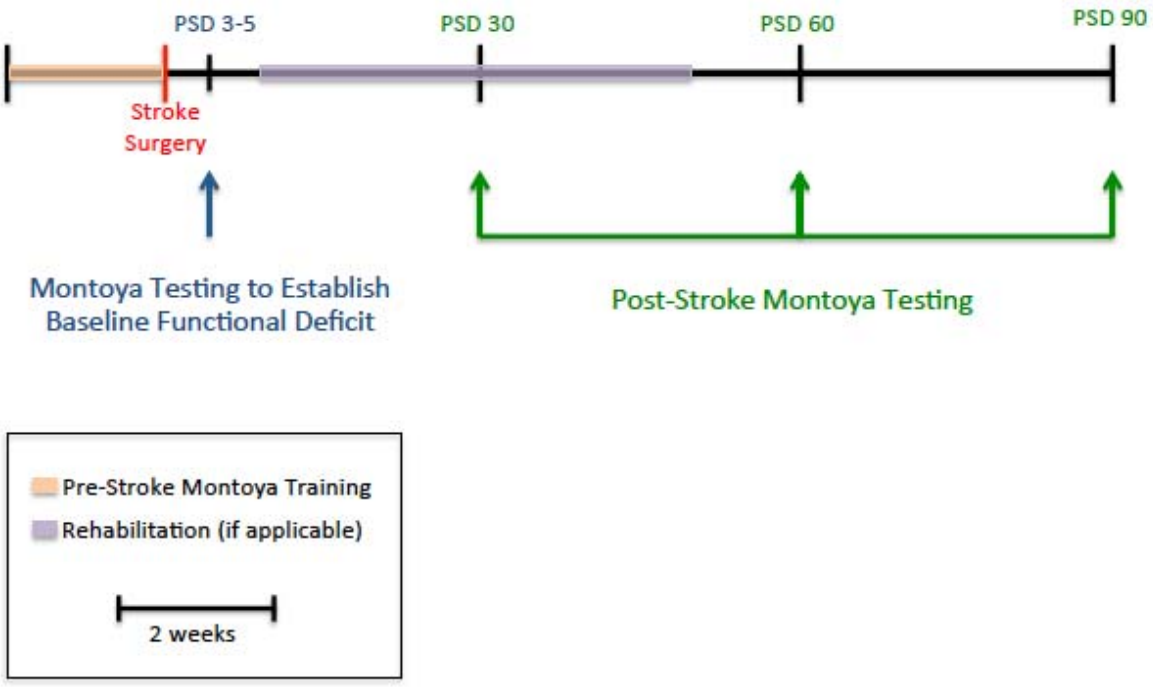
Charting Functional Recovery

Additional series of Montoya Staircase testing charted functional recovery over time. A total of four testing series took place on post-stroke days 3-5, 29-31, 59-61, and 89-91.

Figure 4.

Timeline showing timing of details of this study. Events included two weeks of pre-stroke Montoya training, the stroke surgery, Montoya testing to establish baseline functional deficits at post-stroke days 3-5, and additional Montoya testing periods to chart functional recovery at post-stroke days 29-31, 59-61, and 89-91. If assigned to a rehabilitation group, animals received rehabilitation beginning on post-stroke 8 for five-and-one-half weeks.

Figure 4.


PSD 3-5
PSD 30
PSD 60
PSD 90
Stroke
Surgery
Montoya Testing to Establish
Baseline Functional Deficit
Post-Stroke Montoya Testing
Pre-Stroke Montoya Training
Rehabilitation (if applicable)
2 weeks

After 91 days, the best performance from post-stroke days 89, 90, and 91 can be divided by the best pre-stroke testing figure to give the final functional ability; multiplying this number by 100% gives the percent of functional ability; subtracting this number from 100% gives a final figure for functional deficit. Subtracting the resulting functional deficit from the original baseline functional deficit gives the percentage of pre-stroke function recovery.

All functional recovery calculations were performed for both contralateral (affected by stroke) and ipsilateral forelimbs.

INFARCT ANALYSIS

Euthanization

Animals were injected intraperitoneally with Euthasol (100 mg/kg sodium pentobarbital). Full anesthetization was confirmed with a foot-pinch withdrawal reflex test.

The animals were then exsanguinated. An incision was made to expose the heart. The apex of the heart was gently clamped with a hemostat. The apex was cut with scissors, and a blunt-edged cannula was inserted through the apex incision into the left ventricle at a depth deeper than the location of the hemostat. An incision was also made in the right atrium. The tissue was perfused first with 100 ml of phosphate buffered solution (PBS) and then fixed using a 4% paraformaldehyde in PBS.

After euthanization, the brain was removed and placed in 4% paraformaldehyde overnight followed by a 30% sucrose solution for three days.

Cryostat Sectioning of Brain Tissue

The tissue was set in Optimal Cutting Temperature (OCT) compound and frozen using a Peltier device on the cryostat. In approximately 30 minutes, equilibrium was reached between the tissue temperature and the chamber temperature. The frozen tissue was then placed in the cryostat and sliced at a thickness of 50 μm. Each slice was placed sequentially into one of four vials containing PBS, and the vials were labeled with the corresponding rat's identification number. The infarct analysis vial was used for this study; the remaining three vials were used for other work studying evidence of neurogenesis using expression of DCX and MAP 2.

Slide Preparation

In order to analyze the brain tissue, each slice was mounted on gel-subbed glass slides and stained with Cresyl Violet stain in anticipation of microscope examination.

Tissue Mounting

The vial containing an animal's brain slices for infarct analysis was dumped into a Petri dish and filled half-way with additional PBS. A glass slide was labeled with the corresponding rat's identification number, placed at an angle in the dish, and wetted with PBS. Using a small-tip paintbrush, tissue slices were mounted onto the slide. Once the slide was full, it was set aside to dry.

As the brain slices were mounted, care was taken to ensure the tissue was not folded or damaged.

Staining

After the tissue was dry, the slides were placed into slide racks. The tissue was stained using a Cresyl Violet (Nissl) stain composed of 2.5 g Cresyl Violet, 300 ml water, 30 ml 1 M sodium acetate, and 170 ml 1 M acetic acid. The stain and wash solutions were poured into a series of staining dishes in a chemical hood. The slices were then stained using a series of timed washes: Cresyl Violet stain for 15 minutes, distilled water for 5 minutes, 70% ethanol for 5 minutes, 95% ethanol for 5 minutes, two washes of 100% ethanol for 5 minutes each, and two washes of Xylene for 5 minutes each.

The slides were left in the slide racks in the hood to dry. After the stained tissue was dry, cover slips were placed over the tissue using DPX, a permanent mordant.

Image Capture Using SPOT Scope

The slides were analyzed using Wright State University's Microscopy Core Facility's Olympus Epi Fluorescence SPOT Scope with RT color camera. Slides were examined under brightfield using the 4x scan objective lens. SPOT software provided means to add calibration marks (200 μm) and capture images of the damaged brain tissue in each brain slice.

Creating Image Montages

In cases where brain slices contained large areas of damage, multiple overlapping images were captured to record all damage. These images were merged into one large image using Adobe Photoshop.

Measuring Infarct Volume

Images were opened using ImageJ software. In ImageJ, the measuring tool was calibrated and used to measure infarct volume by tracing the area of damage and recording the listed measurement. This measurement gave infarct volume in mm^3.

Infarct Volume Calculations

Because of the fact that every fourth slice (rather than every slice) of tissue was used for capturing infarct data, calculations were required to determine total infarct volume for each animal.

First, the infarct area for each individual tissue slice provided by ImageJ was multiplied by 0.05 because the tissue sections were 50 μm (0.05 mm) thick; multiplying the area by the 0.05 mm thickness provided the total infarct volume for that slice. The slice volumes for each rat were then added together, and the sum was multiplied by 4 (because every fourth slice was used) to provide the infarct volume for a single rat.

III. RESULTS

PRE- AND POST-STROKE ANALYSIS

Data for this study were obtained from two separate experiments. Both experiments used the same drug combination, dosage, pre-stroke analysis, and post-stroke analysis.

Pre-Stroke Analysis

All animals received pre-stroke training using the Montoya Staircase daily for one-and-one-half weeks. Each animal was trained for 15 minutes and returned to its cage.

By counting the number of pellets retrieved by each animal during training period, pre-stroke function was established. This was important because it allowed for calculation of functional deficit and retained pre-stroke function after a stroke was induced. Animals that did not retrieve at least nine pellets with each forepaw did not meet training criteria and were therefore not used to obtain functional recovery data.

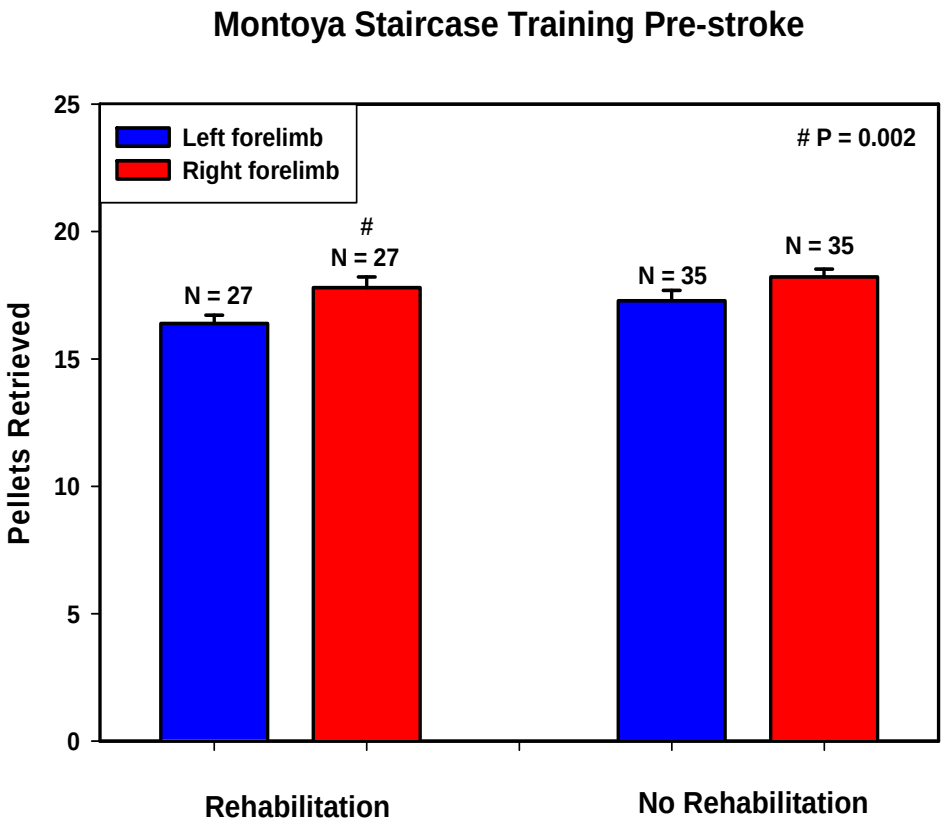
Pre-stroke Montoya training data were reviewed for animals that would receive rehabilitation (rehabilitation animals in Experiment 2) and animals that would not receive rehabilitation (non-rehabilitation animals for Experiment 2 plus all Experiment 1 animals). Figure 5 shows that the mean number of pellets retrieved out of 21 total pellets

with each forelimb ranged from 16-18 pellets. Even though animals did trend toward retrieving more pellets with their right forelimb, handedness was not an apparent issue because the numbers retrieved with each forelimb were very similar. There was a small significant difference between pre-stroke function of the right and left forelimb for those animals that eventually underwent post-stroke rehabilitation, but there was no significant difference in right and left forepaw performance in the animals that would receive no post-stroke rehabilitation.

Figure 5.

Figure displaying the performance by right and left forepaws for animals that were assigned into either the post-stroke rehabilitation group (Experiment 2) or the post-stroke non-rehabilitation group (Experiments 1 and 2). The maximum number of pellets that could be retrieved by either forepaw was 21 pellets. The right forepaw retrieved significantly more pellets in the group that eventually underwent post-stroke rehabilitation (ANOVA, $p= 0.002$; Tukey post-hoc test). No significant difference existed between right and left forepaw retrieval of the animals that did not eventually receive rehabilitation.

Figure 5.


Montoya Staircase Training Pre-stroke
Left forelimb
Right forelimb
P = 0.002
Pellets Retrieved
25
20
15
10
5
0
N = 27
#
N = 27
N = 35
N = 35
Rehabilitation
No Rehabilitation

Post-Stroke Analysis

After stroke induction, animals in the drug-treated group received 5 mg/kg fluoxetine and 1 mg/kg simvastatin via a sugar cookie dough vehicle. Animals in the control group received the vehicle only. Drug/vehicle administration took place once daily for 90 days post-stroke.

A Montoya Staircase functional test was performed at post-stroke days 3-5. This data allowed us to establish a baseline deficit for each animal that met pre-stroke training criteria. This also allowed us to determine if the animal exhibited a contralateral forelimb deficit, a bilateral deficit, or no deficit.

Additional Montoya Staircase functional tests were performed at post-stroke days 29-31 (simplified as "post-stroke day 30"), post-stroke days 59-61 (simplified as "post-stroke day 60"), and post-stroke days 89-91 (simplified as "post-stroke day 90"). This data allowed us to chart functional recovery.

After euthanization and tissue preparation, the brain tissue was analyzed. Based on the endothelin injection site, evidence of ipsilateral infarcts (caused by the endothelin injections), contralateral infarcts, corpus callosum damage, and bilateral infarcts were reviewed. Infarct volume was also measured.

EXPERIMENT 1

In 2012, Experiment 1 reviewed earlier administration of the defined drug combination – 5 mg/kg of fluoxetine and 1 mg/kg of simvastatin – with delivery at 6-12 hours post-stroke. No rehabilitation component was involved. The animals were divided into one of two groups: drug-treated or control.

Presented Deficits

Both animal groups were reviewed for deficit type. Three control animals presented less than a 20% deficit after stroke surgery. Animals are considered to have no deficit if the baseline functional deficit measured less than 20%, and such animals were not included in the study of functional recovery. In regard to functional deficit, thirteen animals (nine drug-treated and four control animals) presented bilateral deficits. Bilateral deficits can be explained by analyzing the brain tissue for the presence of either contralateral cortical damage in addition to the ipsilateral infarct (caused by the endothelin injections) or corpus callosum damage. These data are presented in Table 3.

Of the nine drug-treated animals with bilateral deficits, four showed evidence of contralateral cortical damage in addition to ipsilateral infarcts. Three animals did not have contralateral cortical damage but did show corpus callosum damage; this corpus callosum damage could potentially cause the bilateral deficit. The source of the bilateral deficit could not be determined in two animals: Rat 566 and Rat 544. Rat 566 was used for a gene expression study, so brain tissue sections were not available for analysis; therefore, it cannot be determined whether this animal had contralateral or corpus callosum damage. Rat 544 showed bilateral deficits but did not show contralateral damage or corpus callosum damage; perhaps the bilateral deficit was due to damage not visible on the tissue slices we reviewed, since we only analyzed every fourth slice.

Of the four control animals with bilateral deficits, three animals showed evidence of contralateral cortical damage in addition to ipsilateral infarcts. The one animal that did not show contralateral cortical damage did demonstrate ipsilateral corpus callosum damage, which could have caused the bilateral deficit.

Table 3.

A table showing how animals were grouped in Experiment 1 and the types of deficits observed. None of the animals in this experiment received rehabilitation.

Table 3.

Experiment 1: Rats 530-566						
Group	**Total Animals**	**Montoya Criteria Not Met**	**No Deficit (< 20%)**	**Bilateral Deficits**	**Contralateral Cortical Damage**	**Ipsilateral Corpus Callosum Damage**
FS	18	4	0	9	4	3
Control	18	4	3	4	3	1
Died	1					

Functional Recovery

Functional deficits and recovery were charted over time. The percent of pre-stroke function retained was calculated by dividing an animal's best performance at a given time (e.g., best performance from post-stroke days 29-31) by the animal's best performance from the pre-stroke training days and multiplying that figure by 100%. Subtracting this value from 100 provided the percent of functional deficit. Percent functional recovery was calculated by subtracting the function retained at post-stroke days 3-5 (when the baseline deficit was determined) from the function retained at a given time (e.g., function retained at post-stroke days 29-31). Sample calculations are presented in Table 4.

Bilateral Deficits

Seven animals with bilateral deficits displayed evidence of contralateral cortical damage in addition to the ipsilateral infarcts (caused by the endothelin injections). Contralateral recovery and total recovery of these animals over time was analyzed (Table 5).

Four animals with bilateral deficits had corpus callosum damage in addition to the ipsilateral infarcts. Contralateral recovery and total recovery of these animals over time was analyzed as well (Table 6).

Total Recovery

Total recovery was charted over time and involved measuring contralateral recovery (recovery of the stroke-affected limb) together with ipsilateral recovery (Figure 6). While recovery at post-stroke days 30 and 60 was not statistically different between groups, total recovery at post-stroke day 90 was statistically higher for drug-treated animals over controls.

Table 4.

Tables showing sample calculations of baseline functional deficit at post-stroke days 3-5 and functional deficit/recovery at post-stroke days 29-31, 59-61, and 89-91. This sample used data for Rat 605 from the drug-treated with rehabilitation group in Experiment 2.

Baseline functional deficit was calculated as follows:

post-stroke # pellets / pre-stroke # pellets = baseline function retained

(1 – baseline function retained) x 100 = % baseline functional deficit

Functional Deficit and Functional Recovery were calculated as follows (calculations were performed for PSD 60 and 90 as well; calculations were also performed for both contralateral and ipsilateral data):

PSD30 # pellets / pre-stroke # pellets = PSD 30 function retained

(1 – PSD30 function retained) x 100 = % deficit

(PSD 30 function retained – baseline function retained) x 100 = % recovery

Table 4.

Rat 605, Pre-stroke Function

Best Performance Pre-stroke
20 L, 21 R

Rat 605, Baseline Functional Deficit

Best Performance PSD 3, 4, 5	**Contralateral Deficit**	**Ipsilateral Deficit**
11 L, 20 R	0.45 (45% deficit)	0.05 (5% deficit)

Rat 605, Deficit/Recovery at 30 Days Post-Stroke

Best Performance PSD 29, 30, 31	**Contralateral Deficit**	**Ipsilateral Deficit**	**Contralateral Recovery**	**Ispilateral Recovery**
13.5 L, 20.5 R	0.33 (33% deficit)	0.02 (2% deficit)	0.13 (13% recovery)	0.02 (2% recovery)

Rat 605, Deficit/Recovery at 60 Days Post-Stroke

Best Performance PSD 59, 60, 61	**Contralateral Deficit**	**Ipsilateral Deficit**	**Contralateral Recovery**	**Ispilateral Recovery**
15 L, 19 R	0.25 (25% deficit)	0.095 (9.5% deficit)	0.20 (20% recovery)	-0.05 (-5% recovery)

Rat 605, Deficit/Recovery at 90 Days Post-Stroke

Best Performance PSD 89, 90, 91	**Contralateral Deficit**	**Ipsilateral Deficit**	**Contralateral Recovery**	**Ispilateral Recovery**
17 L, 20 R	0.15 (15% deficit)	0.048 (4.8% deficit)	0.30 (30% recovery)	0.00 (0% recovery)

Table 5.

Review of contralateral recovery (recovery of the stroke-affected limb) and total recovery for animals that functionally presented bilateral deficits, specifically those animals with contralateral cortical damage in addition to the ipsilateral infarcts. Values for contralateral recovery and total recovery over time from post-stroke days 29-31, 59-61, and 89-91 are displayed.

Table 5.

Experiment 1	*PSD 29-31*		*PSD 59-61*		*PSD 89-91*	
Group	**Contralateral Recovery**	**Total Recovery**	**Contralateral Recovery**	**Total Recovery**	**Contralateral Recovery**	**Total Recovery**
FS (N=4)*	29.02%	55.66%	33.68%	70.93%	34.71%	73.00%
C (N=3)**	31.22%	52.24%	22.22%	45.74%	18.52%	50.60%

*FS rats: 540, 542, 546, 553

**Control rats: 539, 547, 562

Table 6.

Review of contralateral recovery (recovery of the stroke-affected limb) and total recovery for animals that functionally presented bilateral deficits, specifically those animals with corpus callosum damage (not contralateral cortical damage) in addition to the ipsilateral infarcts. Values for contralateral recovery and total recovery over time from post-stroke days 29-31, 59-61, and 89-91 are displayed.

Table 6.

Experiment 1	*PSD 29-31*		*PSD 59-61*		*PSD 89-91*	
Group	**Contralateral Recovery**	**Total Recovery**	**Contralateral Recovery**	**Total Recovery**	**Contralateral Recovery**	**Total Recovery**
FS (N=3)*	1.76%	26.06%	22.31%	46.79%	20.74%	57.76%
C (N=1)**	47.06%	62.85%	44.12%	67.80%	50.00%	73.68%

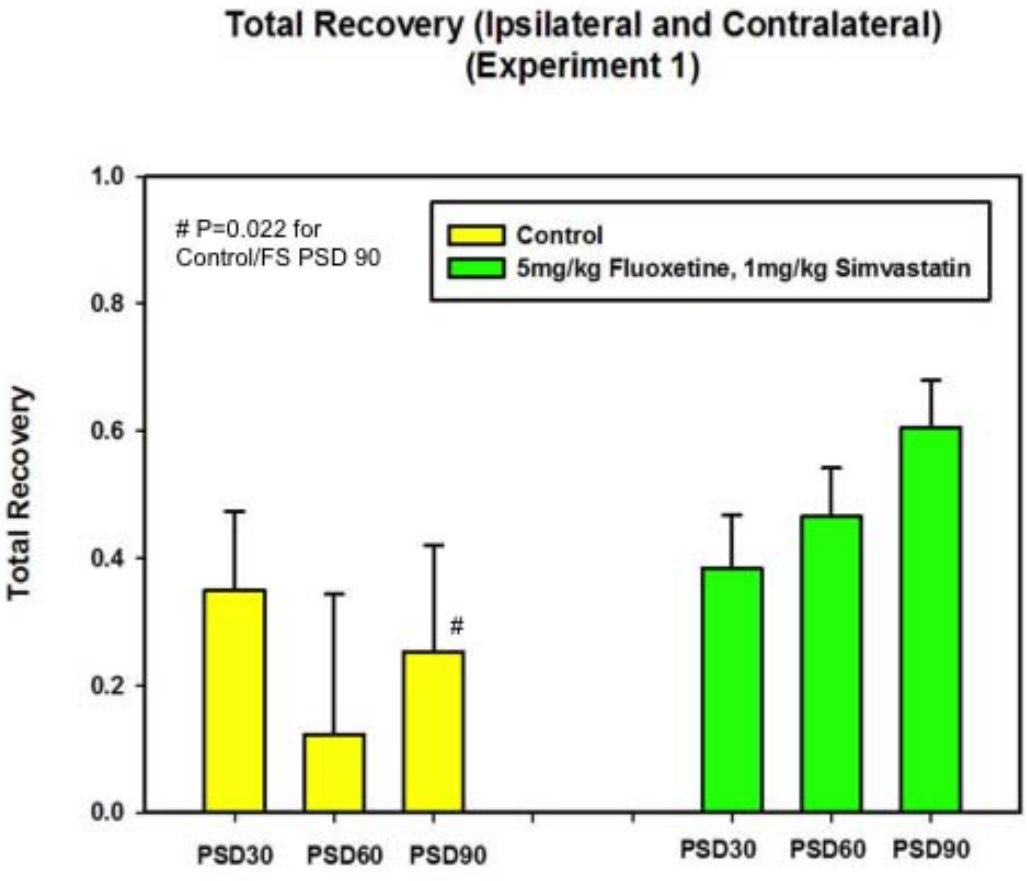
*FS rats: 549, 557, 563

**Control rats: 545

Figure 6.

Comparison of total recovery (ipsilateral and contralateral combined) in control animals versus drug-treated animals at post-stroke days 30, 60, and 90. The x-axis shows time at post-stroke days 30, 60, and 90 for the control animals in yellow on the left and the drug-treated animals in green on the right. The y-axis denotes total recovery; multiplying the y-axis values by 100% gives the total recovery in a percentage. While no significant difference is shown at post-stroke days 30 or 60, there is a significant difference (T test, $p=0.022$) between the recovery of drug-treated animals and control animals at post-stroke day 90.

Figure 6.


Total Recovery (Ipsilateral and Contralateral)
(Experiment 1)
P=0.022 for
Control/FS PSD 90
Control
5mg/kg Fluoxetine, 1mg/kg Simvastatin
Total Recovery
1.0
0.8
0.6
0.4
0.2
0.0
#
PSD30
PSD60
PSD90
PSD30
PSD60
PSD90

Infarct Volume

In contrast to the previous study in our laboratory where drugs were administered at 20-26 hours post-stroke, larger infarcts were presented in this study (6-12 hour post-stroke delivery). Control animals, showed similar infarct sizes to those in the previous study. However, the drug-treated animals had much larger infarcts than previously observed.

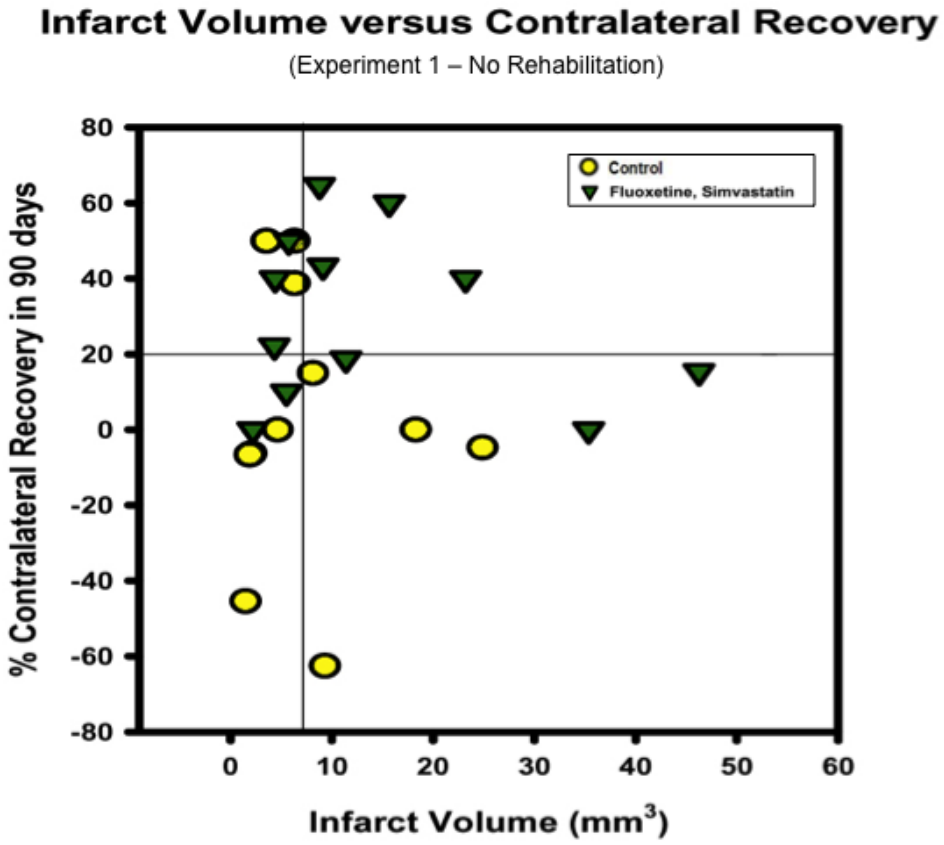
The previous study in our laboratory with drug administration at 20-26 hours post-stroke also exhibited functional recovery along with smaller infarct sizes; there appeared to be a correlation between the two factors. Yet, a correlation did not appear to be evident in this study (6-12 hour post-stroke delivery).

A comparison of infarct size to contralateral functional recovery is presented in Figure 7. The best recovery was observed in animals with the smallest infarcts around 8 mm^3. A trend was observed between animals with recovery less than 20% and animals with recovery greater than 20%, as more drug-treated animals had recovery above 20% while more control animals had recovery below 20%. In addition, at smaller infarct sizes, there was not much separation between recovery in drug-treated animals and recovery in control animals. However, at larger infarct sizes, more separation between recovery in drug-treated animals and recovery in control animals is observed.

Figure 7.

Figure plotting individual animal data comparing infarct volume to contralateral functional recovery (recovery of stroke-affected limb) in animals from Experiment 1. Limb rehabilitation was not a factor in this experiment. The yellow circles represent the 10 control animals. The green triangles represent the 12 drug-treated animals. The x-axis denotes infarct volume in mm^3 (measured at the end of the study). The y-axis shows percent contralateral recovery seen after 90 days. The horizontal line is placed at 20% contralateral recovery for reference. The vertical line is placed at 8 mm^3 infarct size for reference. A trend (Fisher's Exact Test, $p=0.23$) was present between drug-treated and control animals with recovery less than 20% and drug-treated and control animals with recovery greater than 20%. The drug-treated animals appeared to show a higher percentage of animals with recovery greater than 20%. In order to achieve sufficient power for this statistical set, the sample size for each group would need to be doubled at the very least.

Figure 7.


Infarct Volume versus Contralateral Recovery
(Experiment 1 – No Rehabilitation)
Control
Fluoxetine, Simvastatin
% Contralateral Recovery in 90 days
80
60
40
20
0
-20
-40
-60
-80
0
10
20
30
40
50
60
Infarct Volume (mm3)

EXPERIMENT 2

In 2013, Experiment 2 reviewed earlier administration of the defined drug combination – 5 mg/kg of fluoxetine and 1 mg/kg of simvastatin – with delivery at 6-12 hours post-stroke; this is the same as Experiment 1. However, in Experiment 2, a rehabilitation component was added. The animals were divided into one of four groups: drug-treated with rehabilitation, drug-treated without rehabilitation, control with rehabilitation, or control without rehabilitation.

Presented Deficits

All animal groups were reviewed for deficit type. Seven animals (two drug-treated animals with rehabilitation and five control animals with rehabilitation) presented less than a 20% deficit after stroke surgery. Animals are considered to have no deficit if the baseline functional deficit measured less than 20%, and such animals were not included in the study of functional recovery. Four animals (one drug-treated animal with rehabilitation, two control animals with rehabilitation, and one control animal without rehabilitation) presented bilateral deficits when analyzing their post-stroke functional deficit. Bilateral deficits can be explained by the presence of contralateral cortical damage in addition to the ipsilateral infarct (caused by the endothelin injections). No animals in Experiment 2 displayed corpus callosum damage as a sole explanation for bilateral deficits. These data are presented in Table 7.

The one drug-treated animal with a bilateral deficit received rehabilitation and showed evidence of contralateral cortical damage in addition to ipsilateral infarcts; the contralateral damage explains the bilateral deficit.

Of the three control animals with bilateral deficits, two received rehabilitation and showed evidence of contralateral cortical damage in addition to ipsilateral infarcts; this contralateral damage explains the bilateral deficits. The third control animal with bilateral deficits did not receive rehabilitation. This animal (Rat 617) was used for a gene expression study, so brain tissue sections were not available for analysis. Therefore, it cannot be determined whether this animal had contralateral cortical damage.

Contralateral and ipsilateral deficits across the four groups were examined. These data are presented in Figure 8. Contralateral deficit (deficit in stroke-affected limb) seemed to increase across groups, with rehabilitation groups showing less deficit than non-rehabilitation groups, regardless of whether they were control or drug-treated animals. The control animals with rehabilitation showed the lowest contralateral deficits. The drug-treated animals with rehabilitation had larger contralateral deficits. The control animals without rehabilitation had larger contralateral deficits still. Finally, the largest contralateral deficits were seen in the drug-treated animals without rehabilitation. Ipsilaterally, the control animals with rehabilitation showed the smallest deficits. The drug-treated animals without rehabilitation had larger ipsilateral deficits, followed only with a slight increase by drug-treated animals with rehabilitation. Finally, the largest ipsilateral deficits were seen in the control animal without rehabilitation.

Table 7.

A table showing how animals were grouped in Experiment 2 and the types of deficits observed.

Table 7.

Experiment 2: Rats 600-637							
Group	**Total Animals**	**Rehab.**	**No Rehab.**	**Montoya Criteria Not Met**	**No Deficit (< 20%)**	**Bilateral Deficits**	**Contralateral Cortical Damage**
FS	18	15	3	2 R	2 R	1R	1R
Control	17	14	3	1 NR	5 R	2R, 1NR	2R
Died	3						

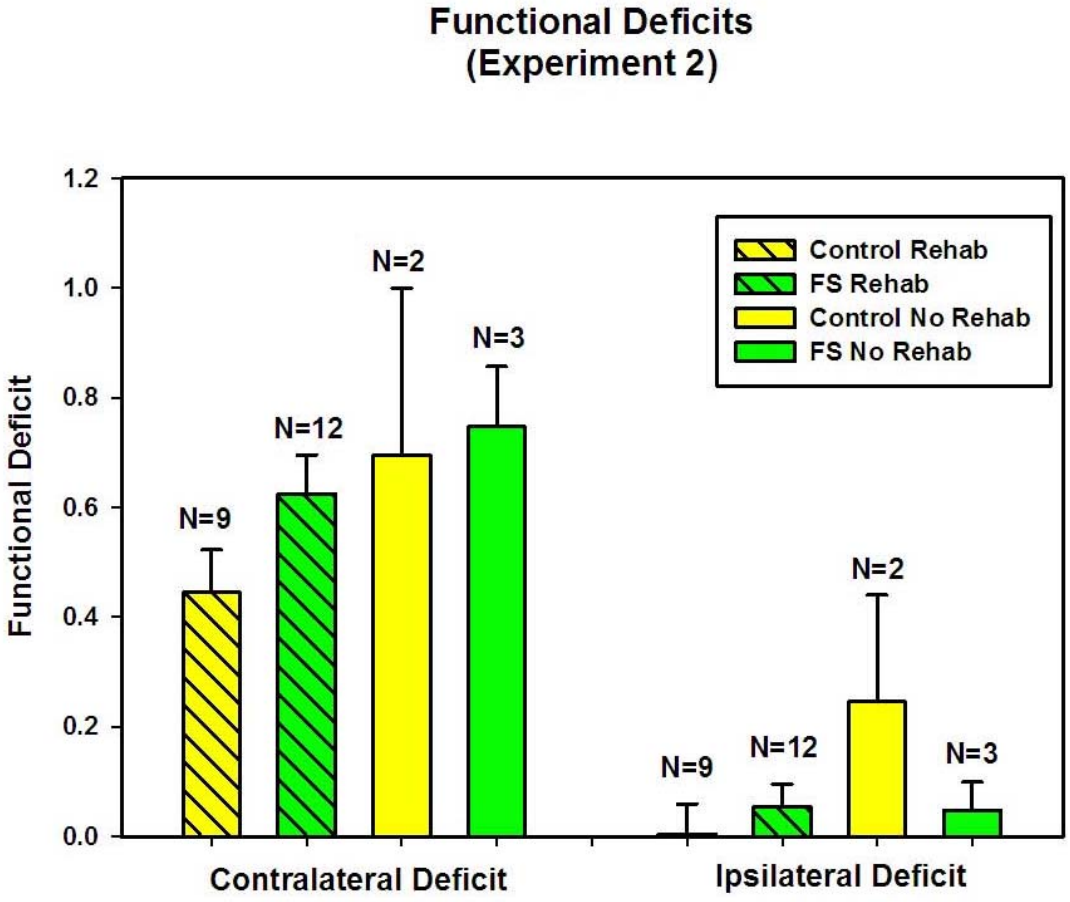
R=Animal received rehabilitation

NR=Animal received no rehabilitation

Figure 8.

Comparison of functional deficits at the end of the study in control with rehabilitation (hashed yellow), control without rehabilitation (solid yellow), drug-treated with rehabilitation (hashed green), and drug-treated without rehabilitation (solid green). Contralateral (stroke-affected limb) and ipsilateral deficits were plotted separately. The x-axis distinguishes the contralateral deficits as compared to the ipsilateral deficits. The y-axis shows functional deficit; multiplying the y-axis values by 100% gives the functional deficit in a percentage. The control group without rehabilitation had a sample size of 2, so the bars on this group are standard deviation bars, while the bars on all other groups are standard error bars. Due to the low sample sizes of the control and drug-treated animal groups without rehabilitation, statistics were not run.

Figure 8.


Functional Deficits
(Experiment 2)
Functional Deficit
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control Rehab
FS Rehab
Control No Rehab
FS No Rehab
N=9
N=12
N=2
N=3
N=9
N=12
N=2
N=3
Contralateral Deficit
Ipsilateral Deficit

Functional Recovery

Functional deficits and recovery were charted over time. The percent of pre-stroke function retained was calculated by dividing an animal's best performance at a given time (e.g., best performance from post-stroke days 29-31) by the animal's best performance from the pre-stroke training days and multiplying that figure by 100%. Subtracting this value from 100 provided the percent of functional deficit. Percent functional recovery was calculated by subtracting the function retained at post-stroke days 3-5 (when the baseline deficit was determined) from the function retained at a given time (e.g., function retained at post-stroke days 29-31).

Contralateral Recovery

Contralateral recovery (recovery of the stroke-affected limb) of the control and drug-treated animals was reviewed over time at post-stroke days 30, 60, and 90. Data were analyzed separately with rehabilitation animals and non-rehabilitation animals.

For the rehabilitation animals (Figure 9), no significant differences existed in contralateral recovery between drug-treated and control animals at any point in time. Because no significant difference existed, it could be stated that drug treatment does not appear to add any benefit when rehabilitation is received because recovery is similar between drug-treated and control groups across all time points. However, one notable observation was that drug-treated animals at post-stroke day 30 showed recovery above 30% while control animals showed recovery below 20%; this suggests it is possible that adding drug treatment to rehabilitation facilitates a quicker initial increase in recovery early on.

For the non-rehabilitation animals (Figure 10), control animals appeared to show higher contralateral recovery at post-stroke day 30 over drug-treated animals. However, recovery appeared to drop dramatically in control animals at post-stroke days 60 and 90 while the drug-treated animals showed increases in contralateral recovery consistently over time. Drug-treated animals appeared to show higher contralateral recovery in the end. Both control and drug-treated animals displayed functional recovery around 20% at 30 days post-stroke. However, while control animals lost recovery over time and end the study with recovery around 10%, drug treatment allowed the animals to gain recovery over time and end the study with recovery around 30%, thus better maintaining the initial recovery. It could be stated that trends are evident as described above, but despite these observations, the sample size of non-rehabilitated animals in Experiment 2 was extremely small. To best analyze data pertaining to non-rehabilitated animals, Figure 13 should be referenced because it combines all non-rehabilitation animal data from Experiments 1 and 2, resulting in a larger sample size, and does in fact present significant differences.

Figure 9.

Figure comparing contralateral recovery (recovery of stroke-affected limb) over time between control animals that received rehabilitation and drug-treated animals that received rehabilitation. The x-axis denotes time, reviewing post-stroke days 30, 60, and 90 for control animals on the left in yellow and drug-treated animals on the right in green. The y-axis exhibits contralateral recovery; multiplying the y-axis value by 100% gives the contralateral recovery in a percentage.

Figure 9.

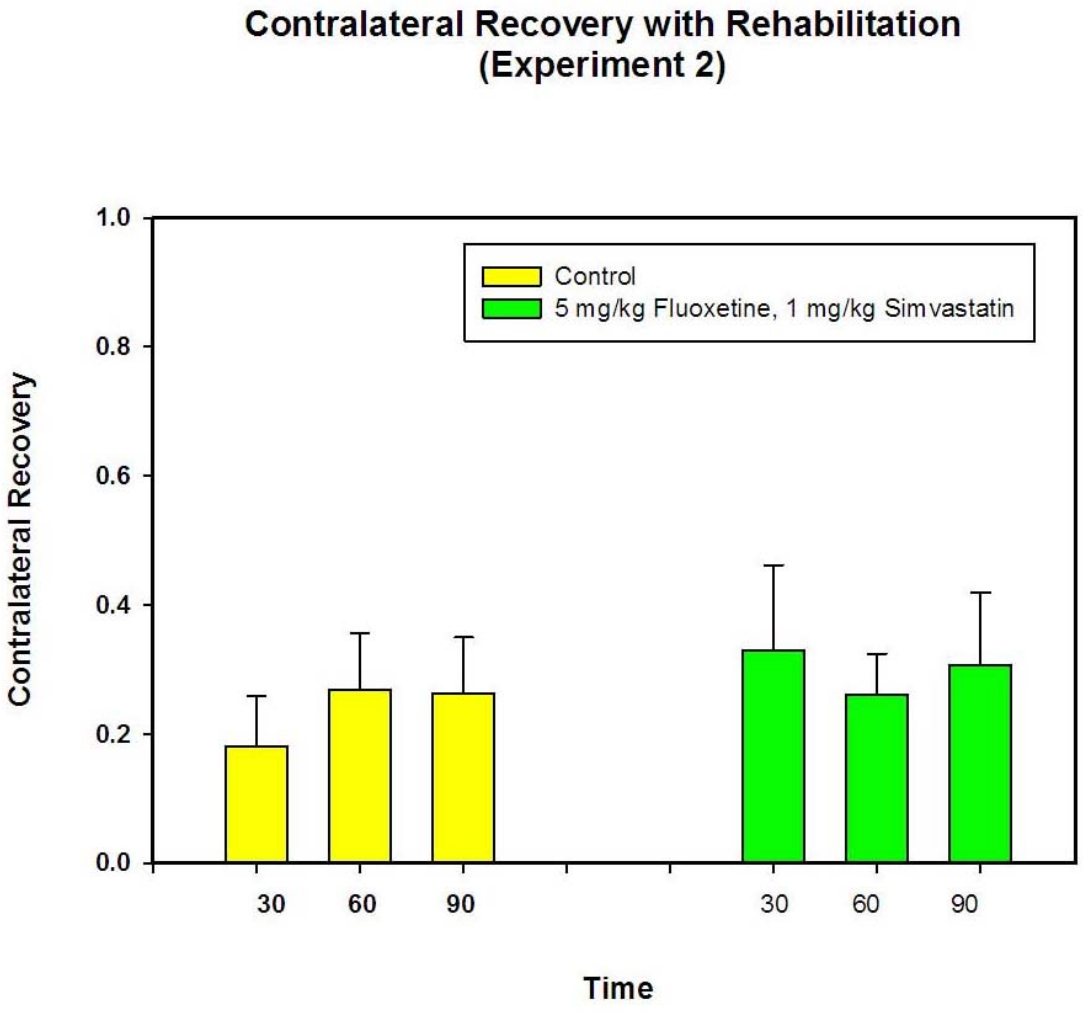
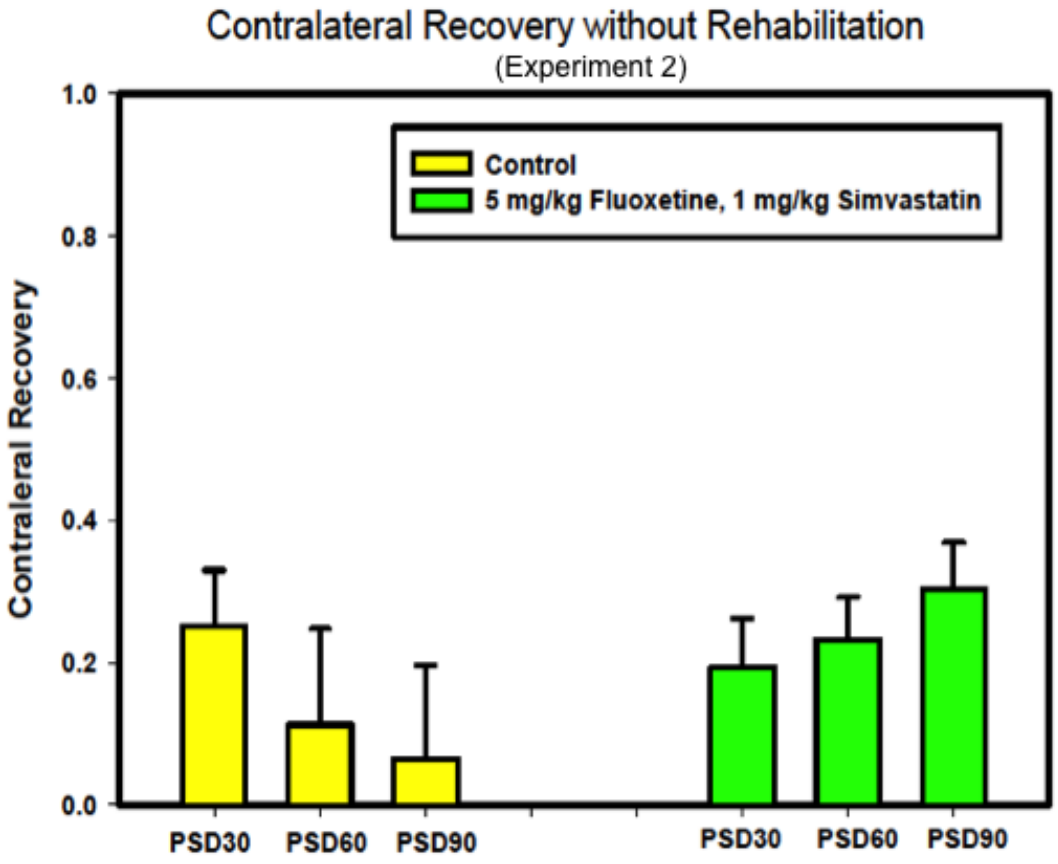

Contralateral Recovery with Rehabilitation
(Experiment 2)
Contralateral Recovery
1.0
0.8
0.6
0.4
0.2
0.0
Control
5 mg/kg Fluoxetine, 1 mg/kg Simvastatin
30
60
90
30
60
90
Time

Figure 10.

Figure comparing contralateral recovery (recovery of stroke-affected limb) over time between control animals that did not receive rehabilitation and drug-treated animals that did not receive rehabilitation. The x-axis denotes time, reviewing post-stroke days 30, 60, and 90 for control animals on the left in yellow and drug-treated animals on the right in green. The y-axis exhibits contralateral recovery; multiplying the y-axis value by 100% gives the contralateral recovery in a percentage. It is important to note only 6 animals composed the sample size for this figure (3 drug-treated animals and 2 control animals).

Figure 10.


Contralateral Recovery without Rehabilitation
(Experiment 2)
Control
5 mg/kg Fluoxetine, 1 mg/kg Simvastatin
Contraleral Recovery
1.0
0.8
0.6
0.4
0.2
0.0
PSD30
PSD60
PSD90
PSD30
PSD60
PSD90

Infarct Volume

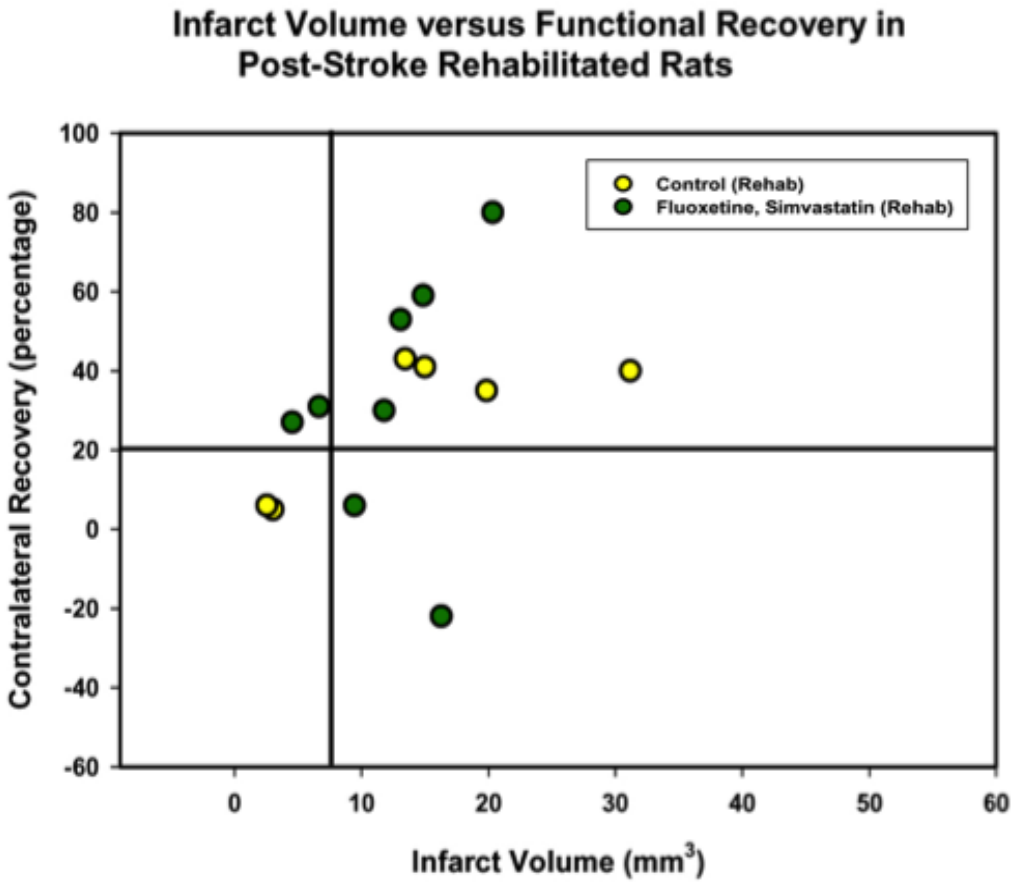
Like Experiment 1, larger infarcts were presented with Experiment 2 when compared to our previous study with drug administration at 20-26 hours post-stroke, and a correlation between infarct size and functional recovery was not observed.

A comparison between infarct size and contralateral functional recovery is presented in Figure 11 for all animals in Experiment 2 that received rehabilitation. The best recovery appeared to be observed in animals with larger infarcts greater than 8 mm^3 in size. No significant difference was observed between the control and drug-treated groups with recovery greater than 20%. The majority of drug-treated animals with rehabilitation had recovery above 20%; the majority of the control animals with rehabilitation also had recovery above 20%. In addition, at smaller infarct sizes, there was not much separation between recovery in drug-treated animals and recovery in control animals. However, at larger infarct sizes, more separation between recovery in drug-treated animals and recovery in control animals was detected.

Figure 11.

Figure plotting individual animal data comparing infarct volume to contralateral functional recovery (recovery of stroke-affected limb) in animals from Experiment 2, which involved a limb rehabilitation component. The yellow circles represent six control animals that received rehabilitation. The green circles represent eight drug-treated animals that received rehabilitation. Animals that did not receive rehabilitation are not represented in this figure. The x-axis denotes infarct volume in mm^3 (measured at the end of the study). The y-axis shows percent contralateral recovery seen after 90 days. The horizontal line is placed at 20% contralateral recovery for reference. The vertical line is placed at 8 mm^3 infarct size for reference. No significant difference (Fisher's Exact Test) was present between the control and drug-treated groups with recovery greater than 20%.

Figure 11.


Infarct Volume versus Functional Recovery in Post-Stroke Rehabilitated Rats
Control (Rehab)
Fluoxetine, Simvastatin (Rehab)
Contralateral Recovery (percentage)
100
80
60
40
20
0
-20
-40
-60
0
10
20
30
40
50
60
Infarct Volume (mm3)

COMBINED DATA

Data from Experiments 1 and 2 were combined to analyze the full scope of early drug administration at 6-12 hours post-stroke. All animals from Experiment 1 (no rehabilitation) were grouped with the non-rehabilitation animals from Experiment 2.

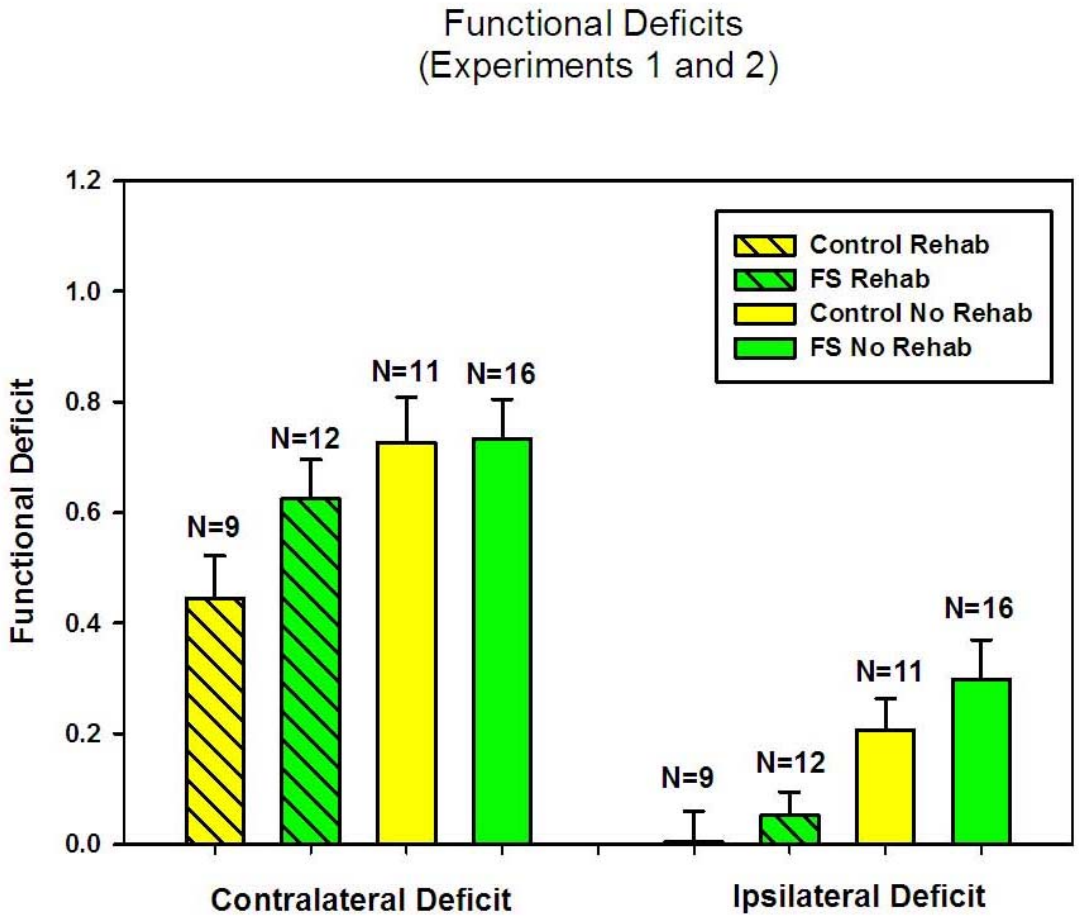
Presented Deficits

Contralateral (in stroke-affected limb) and ipsilateral deficits across the combined groups from Experiments 1 and 2 were reviewed: control animals with rehabilitation, drug-treated animals with rehabilitation, control animals without rehabilitation, and drug-treated animals without rehabilitation. These data are presented in Figure 12. Functional deficit seemed to increase across groups, with rehabilitation groups showing less deficit than non-rehabilitation groups, regardless of whether they were control or drug-treated animals. The control animals with rehabilitation showed the lowest functional deficits, both contralateral and ipsilateral. The drug-treated animals with rehabilitation had larger deficits, both contralateral and ipsilateral. The control animals without rehabilitation had larger deficits still, both contralateral and ipsilateral. Finally, the largest deficits were seen in the drug-treated animals without rehabilitation, both contralateral and ipsilateral. Additionally, while the drug-treated animals without rehabilitation showed larger deficits than the control animals without rehabilitation, the difference was very small when looking at contralateral deficits between the two groups. Statistical analysis showed the ipsilateral deficits were significantly smaller than the contralateral deficits. However, there was no significant difference between any drug-treated group when compared to its corresponding control group.

Figure 12.

Comparison of functional deficits at the end of the study in all animals from Experiments 1 and 2 combined: control with rehabilitation (hashed yellow), control without rehabilitation (solid yellow), drug-treated with rehabilitation (hashed green), and drug-treated without rehabilitation (solid green). Contralateral (stroke-affected limb) and ipsilateral deficits were plotted separately. The x-axis distinguishes the contralateral deficits as compared to the ipsilateral deficits. The y-axis shows functional deficit; multiplying the y-axis values by 100% gives the functional deficit in a percentage. Statistical analysis showed the ipsilateral deficits are significantly different from the contralateral deficits. However, there is no significant difference between any drug-treated group when compared to its corresponding control group. This figure is similar to the data shown in Figure 8; however, Figure 12 represents Experiment 1 data added to the Experiment 2 data from Figure 8.

Figure 12.


Functional Deficits
(Experiments 1 and 2)
Functional Deficit
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control Rehab
FS Rehab
Control No Rehab
FS No Rehab
N=9
N=12
N=11
N=16
N=9
N=12
N=11
N=16
Contralateral Deficit
Ipsilateral Deficit

Functional Recovery

Functional deficits and recovery were charted over time. The percent of pre-stroke function retained was calculated by dividing an animal's best performance at a given time (e.g., best performance from post-stroke days 29-31) by the animal's best performance from the pre-stroke training days and multiplying that figure by 100%. Subtracting this value from 100 provided the percent of functional deficit. Percent functional recovery was calculated by subtracting the function retained at post-stroke days 3-5 (when the baseline deficit was determined) from the function retained at a given time (e.g., function retained at post-stroke days 29-31).

Contralateral Recovery

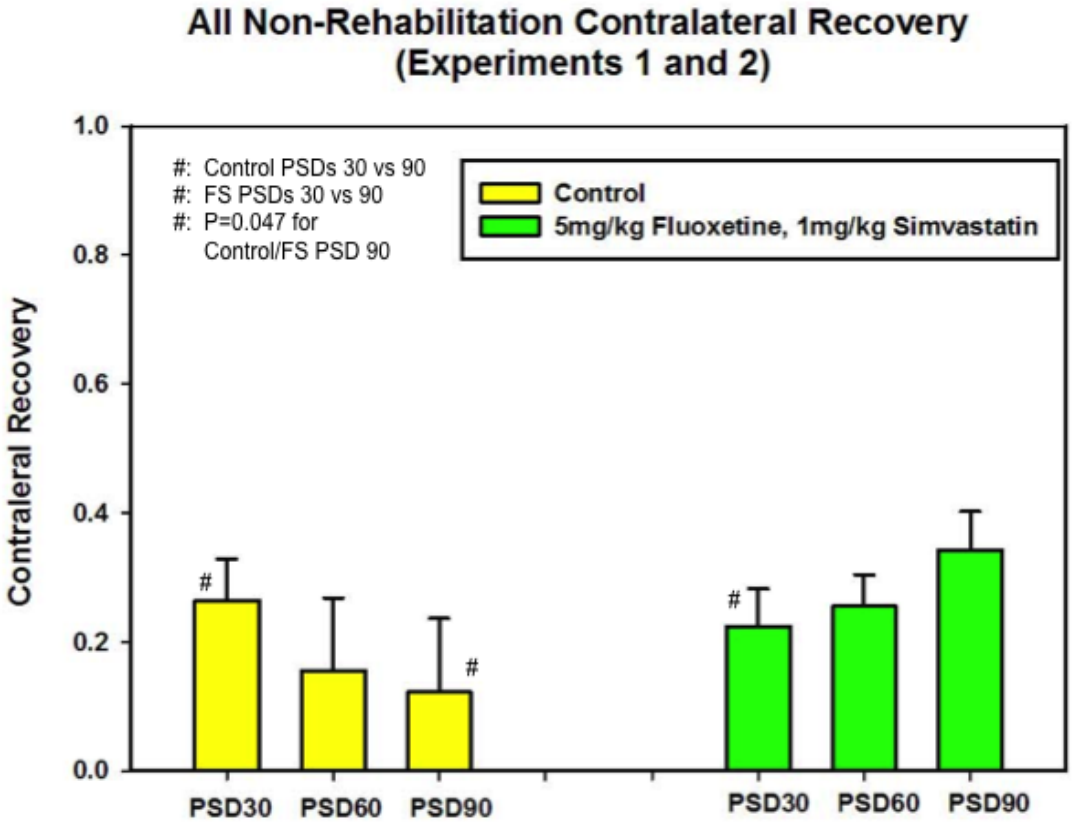
Contralateral recovery (recovery of stroke-affected limb) of all non-rehabilitation control and drug-treated animals from Experiments 1 and 2 combined was reviewed over time at post-stroke days 30, 60, and 90 (Figure 13).

A significant difference existed between drug-treated animals at post-stroke days 30 and 90, showing recovery improved significantly over time with drug treatment. There was a significant difference between the control animals at post-stroke days 30 and 90 as well, showing recovery decreased significantly over time without drug treatment. Finally, there was a significant difference between drug-treated animals and control animals at post-stroke day 90, which demonstrates drug-treated animals recovered significantly better than the controls.

Figure 13.

Figure comparing contralateral recovery (recovery of stroke-affected limb) over time between all non-rehabilitation control and drug-treated animals from Experiments 1 and 2 combined. The x-axis denotes time, reviewing post-stroke days 30, 60, and 90 for control animals on the left in yellow and drug-treated animals on the right in green. The y-axis exhibits contralateral recovery; multiplying the y-axis value by 100% gives the contralateral recovery in a percentage. A significant difference exists between drug-treated animals at post-stroke days 30 and 90. A significant difference also exists between the control animals at post-stroke days 30 and 90. Lastly, a significant difference (Two-Way Repeated Measures ANOVA, Holm-Sidak post-hoc, $p=0.047$) exists between drug-treated animals and control animals at post-stroke day 90.

Figure 13.

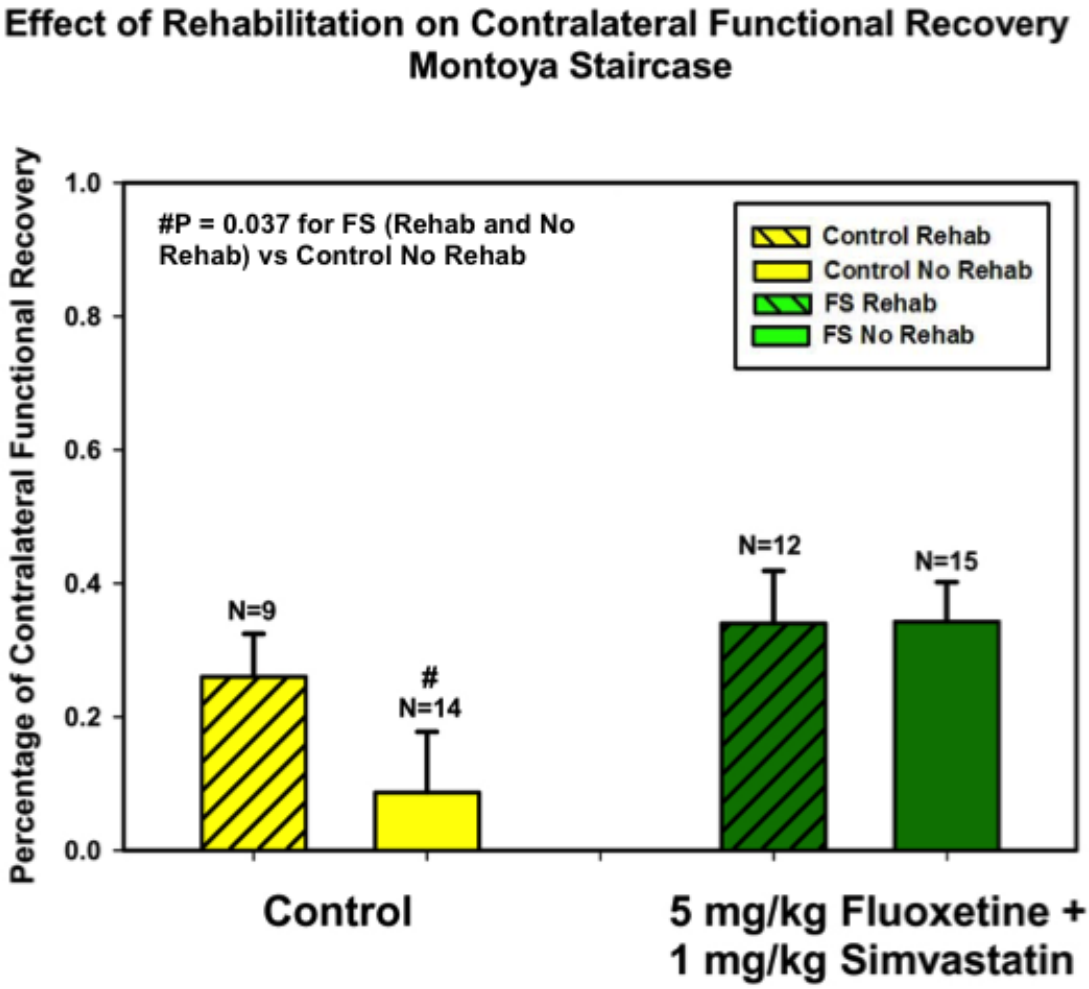

All Non-Rehabilitation Contralateral Recovery
(Experiments 1 and 2)
#: Control PSDs 30 vs 90
#: FS PSDs 30 vs 90
#: P=0.047 for
Control/FS PSD 90
Control
5mg/kg Fluoxetine, 1mg/kg Simvastatin
Contraleral Recovery
1.0
0.8
0.6
0.4
0.2
0.0
#
#
#
PSD30
PSD60
PSD90
PSD30
PSD60
PSD90

Contralateral recovery (recovery in stroke-affected limb) at the end of the study from Experiments 1 and 2 was reviewed. The data are presented in Figure 14. No significant difference was seen in the drug-treated animals between those that received rehabilitation and those that did not; recovery was virtually the same. The control animals appeared to show more functional recovery if they had rehabilitation, but no significant difference was seen between control animals that received rehabilitation and those that did not. No significant difference was seen between control animals with rehabilitation and drug-treated animals with rehabilitation, although drug-treated animals with rehabilitation appeared to show slightly higher recovery than the controls with rehabilitation. One significant difference did present, though, showing drug-treated groups (both rehabilitation and no rehabilitation) produced significantly higher recovery than the control group without rehabilitation.

Figure 14.

Figure showing the effects of rehabilitation on contralateral recovery (recovery of stroke-affected limb) at the end of the study across Experiments 1 and 2. The four groups reviewed are control with rehabilitation (hashed yellow), control without rehabilitation (solid yellow), drug-treated with rehabilitation (hashed green), and drug-treated without rehabilitation (solid green). The x-axis denotes the control groups as compared to the drug-treated groups. The y-axis shows contralateral functional recovery as a decimal; multiplying this number by 100% provides the percentage of contralateral functional recovery. A significant difference is observed for Factor A (control versus drug) in the ANOVA. Tukey Post Hoc analysis showed a statistical difference between both drug-treated groups (rehabilitation and no rehabilitation) and control animals in the no rehabilitation group. No significant difference exists between the control animals with rehabilitation and the control animals without rehabilitation. There was, however, a trend, which would need increased sample sizes to resolve. No significant difference exists between the control animals with rehabilitation and the drug-treated animals with rehabilitation.

Figure 14.


Effect of Rehabilitation on Contralateral Functional Recovery
Montoya Staircase
Percentage of Contralateral Functional Recovery
1.0
0.8
0.6
0.4
0.2
0.0
#P = 0.037 for FS (Rehab and No Rehab) vs Control No Rehab
Control Rehab
Control No Rehab
FS Rehab
FS No Rehab
N=9
#
N=14
N=12
N=15
Control
5 mg/kg Fluoxetine +
1 mg/kg Simvastatin

Infarct Volume

While our laboratory believed drug-treated animals would exhibit smaller infarct sizes than controls, this was not observed in this study. Figure 7 compared infarct size to contralateral functional recovery with Experiment 1 (no rehabilitation); Figure 11 compared infarct size to contralateral functional recovery with all rehabilitated animals in Experiment 2. Figure 15 compares Figure 7 (Figure 15A) to Figure 11 (Figure 15B).

In Figure 15A (no rehabilitation), the best recovery developed in animals with smaller infarcts around 8 mm^3. No significant difference was presented between drug-treated/control animals with recovery less than 20% and animals with recovery greater than 20%, although a trend was observed toward drug-treated animals having greater recovery.

While Figure 15A showed smaller infarcts produced better recovery in non-rehabilitated animals, Figure 15B reviewed only rehabilitated animals and shows the best recovery with larger infarcts greater than 8 mm^3. No significant difference was presented in Figure 15B between drug-treated/control animals with recovery less than 20% and animals with recovery greater than 20%, and in contrast to Figure 15A, a trend was not observed, suggesting drug-treated animals did not have greater recovery over controls.

Animals in the right upper quadrant of the figures (infarcts larger than 8 mm^3, recovery greater than 20%) were compared to animals elsewhere in the figures. When the data from Figure 15A data (no rehabilitation) was compared to the data from Figure 15B (rehabilitation), a statistical difference was shown, implying control animals recover better if they receive rehabilitation. Without rehabilitation, no control animals

showed recovery above 20% unless their infarct size was smaller than 8 mm^3. However, with rehabilitation, recovery was seen above 20%, even with larger infarcts.

Figure 15.

Comparison of infarct volume to contralateral functional recovery (of stroke-affected limb) between Experiments 1 and 2. The x-axes denote infarct volume (at end of study) in mm^3, and the y-axes show percent contralateral recovery after 90 days. The horizontal lines at 20% recovery and the vertical lines at 8 mm^3 infarct size are for reference.

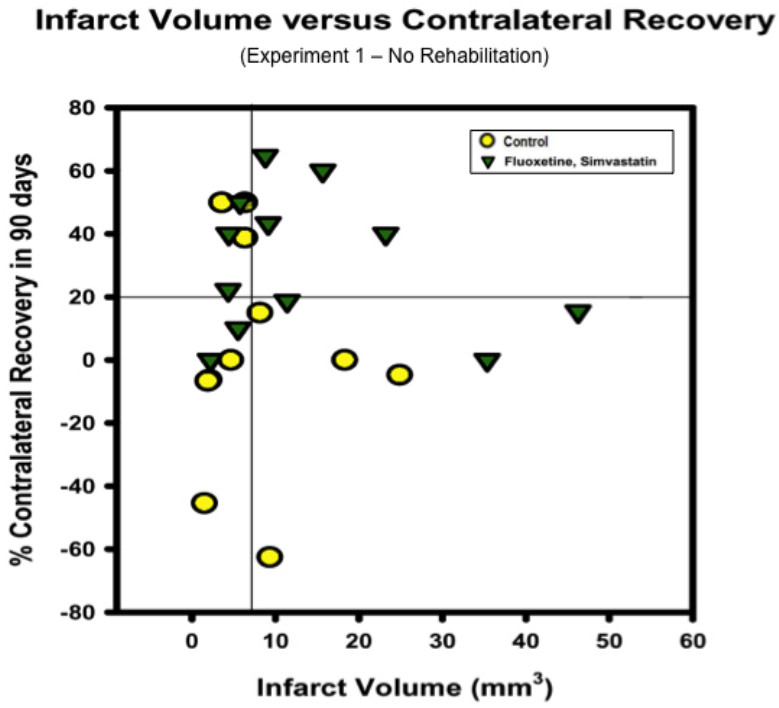
(A) Infarct volume and contralateral recovery from Experiment 1, where limb rehabilitation was not involved, are compared. The yellow circles represent 10 control animals. The green triangles represent 12 drug-treated animals. No significant difference existed between drug-treated and control animals with recovery less than 20% and animals with recovery greater than 20%; however, a trend (Fisher's Exact Test, $p=0.23$) was evident, suggesting a 77% chance that drug-treated animals would show better recovery if sample sizes were larger.

(B) Infarct volume and contralateral recovery from Experiment 2 animals that received rehabilitation are compared. The yellow circles represent six control animals that received rehabilitation. The green circles represent the eight drug-treated animals that received rehabilitation. Animals that did not receive rehabilitation are not represented. No significant difference was presented between drug-treated and control animals with recovery less than 20% and animals with recovery greater than 20%.

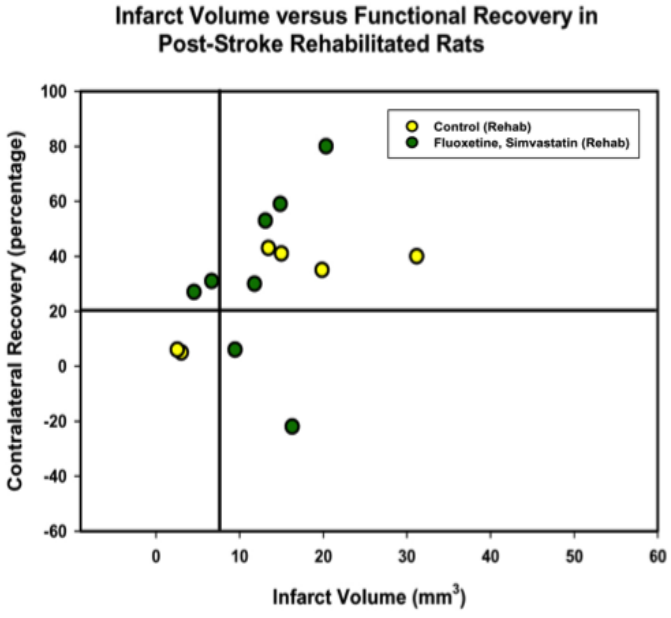
A Fisher's Exact Test was run to compare the two figures. Animals in the right upper quadrant (infarcts larger than 8 mm^3, recovery greater than 20%) were compared to animals falling elsewhere in the figures. A statistical difference existed between these data from Figure 15A compared to these data from Figure 15B ($p=0.008$), suggesting that control animals recovered better, even with larger infarcts, if they received rehabilitation.

Figure 15.

(A) Experiment 1 (No Rehabilitation)



(B) Experiment 2 (All Rehabilitated Animals)



IV. DISCUSSION

RESULTS SUMMARY

The results of this thesis present three main discoveries. Variability in functional recovery with drug treatment and limb rehabilitation as factors was observed. A lack of correlation between functional recovery and infarct size was also perceived. Lastly, larger (and sometimes bilateral) infarcts were observed in this study (6-12 hours post-stroke drug delivery) when compared with previous studies from our laboratory (20-26 hours post-stroke drug delivery), which may directly relate to our earlier drug administration.

Effects of Drug Combination and Limb Rehabilitation on Functional Recovery

The observations pertaining to functional recovery when considering drug treatment and limb rehabilitation are discussed below.

Functional Recovery: Without Rehabilitation

Without rehabilitation where drug treatment was the only variable, the drug-treated animals showed significantly higher contralateral functional recovery (of stroke-affected limb) over the control animals. Control animals showed a significant

decrease in recovery from post-stroke day 30 to post-stroke day 90 (Figure 13). Conversely, drug-treated animals showed a significant increase in recovery from post-stroke day 30 to post-stroke day 90 (Figure 13). Total recovery at post-stroke days 30 and 60 was not much different between the control and drug-treated animals, but total recovery at post-stroke day 90 was significantly higher in the drug-treated animals (Figure 6, Figure 13). Because the goal of the stroke surgery was to heavily impair the forelimb motor cortex rather than simply damage it, it took the full course of the study to see improvement in recovery. The data showed that, in the absence of rehabilitation, drug-treatment does in fact provide for increased functional recovery compared to the control. The control animals actually displayed a decrease in recovery and an increase in deficit over time. This finding is important because some patients may not be able to perform physical therapy due to other health problems or restrictions; if drug treatment alone is an option, such patients could obtain a level of increased recovery without having to take part in rehabilitation.

Functional Recovery: With Rehabilitation

With voluntary rehabilitation, no statistical difference was presented between recovery in the drug-treated animals and the control animals (Figure 9). The control animals appeared to recover just as well as drug-treated animals in the presence of rehabilitation. This suggests that rehabilitation in-and-of itself may present an opportunity for recovery, which is an important consideration for patients who may have access to rehabilitation but cannot afford medication or may not be able to take certain medications.

Though drug treatment may not add any benefit when rehabilitation is received, it is possible that drug treatment allows for a quicker initial recovery when rehabilitation is involved. Recovery over time was about equal between drug-treated and control animals with rehabilitation, but the drug-treated animals appeared to show faster improvement initially at post-stroke day 30 over the controls (Figure 9); this suggests that drug treatment in combination with rehabilitation could possibly be beneficial for patients by improving functional recovery sooner after stroke.

Functional Recovery: Comparison of All Experimental Groups

Contralateral recovery across the four groups (control with rehabilitation, control without rehabilitation, drug-treated with rehabilitation, drug-treated without rehabilitation) (Figure 14) showed the drug-treated groups (both rehabilitation and no rehabilitation) produced significantly higher recovery than the control group without rehabilitation. This confirms that without rehabilitation, drug treatment provides for better recovery. There was no significant difference between drug-treated animals that received rehabilitation and those that did not, suggesting rehabilitation does not add anything to the drug treatment. No difference was noted between drug-treated animals with rehabilitation and control animals with rehabilitation; this implies that rehabilitation alone may be effective as well.

No difference presented between control animals that received rehabilitation and those that did not, but there did appear to be a trend insinuating that control animals show more functional recovery if they have rehabilitation. A statistical difference was observed when animals with larger infarcts (greater than 8 mm^3) and recovery above 20%

were compared to the remaining animals in Experiments 1 and 2 (Figure 15), implying control animals recovered better, even with larger infarcts, if they received rehabilitation. Without rehabilitation, no control animals showed recovery above 20% unless their infarct size is smaller than 8 mm^3. This suggests rehabilitation may be a promising avenue for stroke patients presenting larger infarcts, such as those who have experienced hemorrhagic stroke.

Many possible implications for stroke treatment in humans have surfaced regarding the effects of drug treatment or rehabilitation on recovery. This study proposes drug treatment alone, rehabilitation alone, and a combination of both could be beneficial in different cases. It is important to perform additional research in these areas.

Functional Recovery: Gray Matter versus White Matter Recovery

Differences were not observed in functional recovery of gray matter (cortical damage) versus white matter (corpus callosum damage) in the few cases that showed bilateral deficits. Recovery was typically noted on both contralateral (stroke-affected limb) and ipsilateral sides with the drug treatment.

Lack of Correlation Between Infarct Size and Functional Recovery

In contrast to the previous study with drug delivery at 20-26 hours post-stroke, functional recovery did not seem to correlate well with infarct size in this study. Several reasons could explain this lack of connection. A large infarct could be detected with very little consequence to the animal's function if the infarct encompassed a large somatosensory area rather than a large motor area. Conversely, a small infarct could be

detected but with great detriment to the animal's function if the infarct encompassed a small but important motor area. This heterogeneity in damage raises questions about how to classify and analyze damage as well as how to best compare the damage with the animal's recovery.

Figure 15 can be referenced for data associated with the issue of heterogeneity in damage. It is possible that a large infarct could have little effect on an animal's motor function if the damage involved mostly the somatosensory area; on the other hand, a small infarct impairing a sensitive portion of the forelimb motor cortex could greatly decrease the animal's motor function despite the small size of the infarct. In Figure 15A, the two yellow circles representing control animals seen in the lower left of the figure show very small infarct volumes but largely negative recovery values; it is possible these animals had severe damage, though small foci, in the forelimb motor cortex.

Without rehabilitation, drug-treated animals and control animals showed more separation in recovery with larger infarcts, while the recovery between the two groups was similar if the infarcts were smaller (Figure 15A). The question has been raised regarding the effectiveness of fluoxetine at different levels of severity, with one study suggesting fluoxetine only works if the level of damage is severe (Kirsch, 2008). However, another study suggests it is not that severely depressed patients respond better to the drug but that they respond less to the placebo (Kirsch *et al*, 2008). Again, further investigation is necessary.

Measuring Off of Bregma

To locate the two endothelin injection sites for stroke induction, coordinates were measured off of bregma to take out the forelimb motor cortex. An additional consideration regarding the issue of broader damage is whether measuring off of bregma is reliable. If the injection site was misplaced, it is possible that a different area of the cortex was damaged.

Possible Explanations of Larger Infarcts

Larger infarcts were seen in this study with drug administration at 6-12 hours post-stroke when compared to our laboratory's previous study with drug administration at 20-26 hours post-stroke. The data are shown in Figure 16. Both the control and drug-treated groups from Experiment 1 (6-12 hour post-stroke delivery) appeared to exhibit larger infarct volumes than those from the previous study (20-26 hour post-stroke delivery), with a significant difference present between the two drug-treated groups. Additionally, the previous study (20-26 hour post-stroke delivery) showed smaller infarct volumes in the drug-treated animals over the controls. Conversely, Experiment 1 of this study (6-12 hour post-stroke delivery) showed larger infarct volumes in the drug-treated animals over the controls.

While larger infarcts are of course undesirable, drug-treated animals with rehabilitation showed more functional recovery even with larger infarcts (Figure 15B). Future studies on this observation could potentially be important with recovery in hemorrhagic stroke patients, which exhibit larger infarcts.

Endothelin Injection Issue

One possible explanation for larger infarcts seen in this study is an issue with the endothelin injection. If some of the endothelin came back up out of the injection hole and spread across the surface of the cortex, less than the normal amount of endothelin would have entered the injection site. The endothelin that spread across the cortex could have constricted surface arteries beyond the targeted injection site, resulting in larger damage. This could have been caused by differences in the speed of injection.

Secondary Hemorrhagic Stroke

Another possible explanation of larger infarct sizes seen with the drug-treated animals is the occurrence of a secondary hemorrhagic stroke. Several different infarct configurations were observed in this study. Figures 17-20 show examples of each and explain the different configurations of damage.

Figure 16.

Figure comparing infarct volumes from Experiment 1 from this study (with drug administration at 6-12 hours post-stroke) to infarct volumes from our laboratory's previous study (with drug administration at 20-26 hours post-stroke). The solid yellow represents control animals from this study; the diagonally-hashed yellow represents control animals from the previous study. The solid green represents drug-treated animals from this study; the diagonally-hashed green represents drug-treated animals from the previous study. The x-axis denotes control groups compared to drug-treated groups. The y-axis shows infarct volume measured in mm^3. A significant difference is present between the two drug-treated groups. A significant difference ($p=0.016$) is apparent between the infarct sizes of drug-treated animals from this study (delivery at 6-12 hours post-stroke) and drug-treated animals from the previous study (delivery at 20-26 hours post-stroke).

Figure 16.

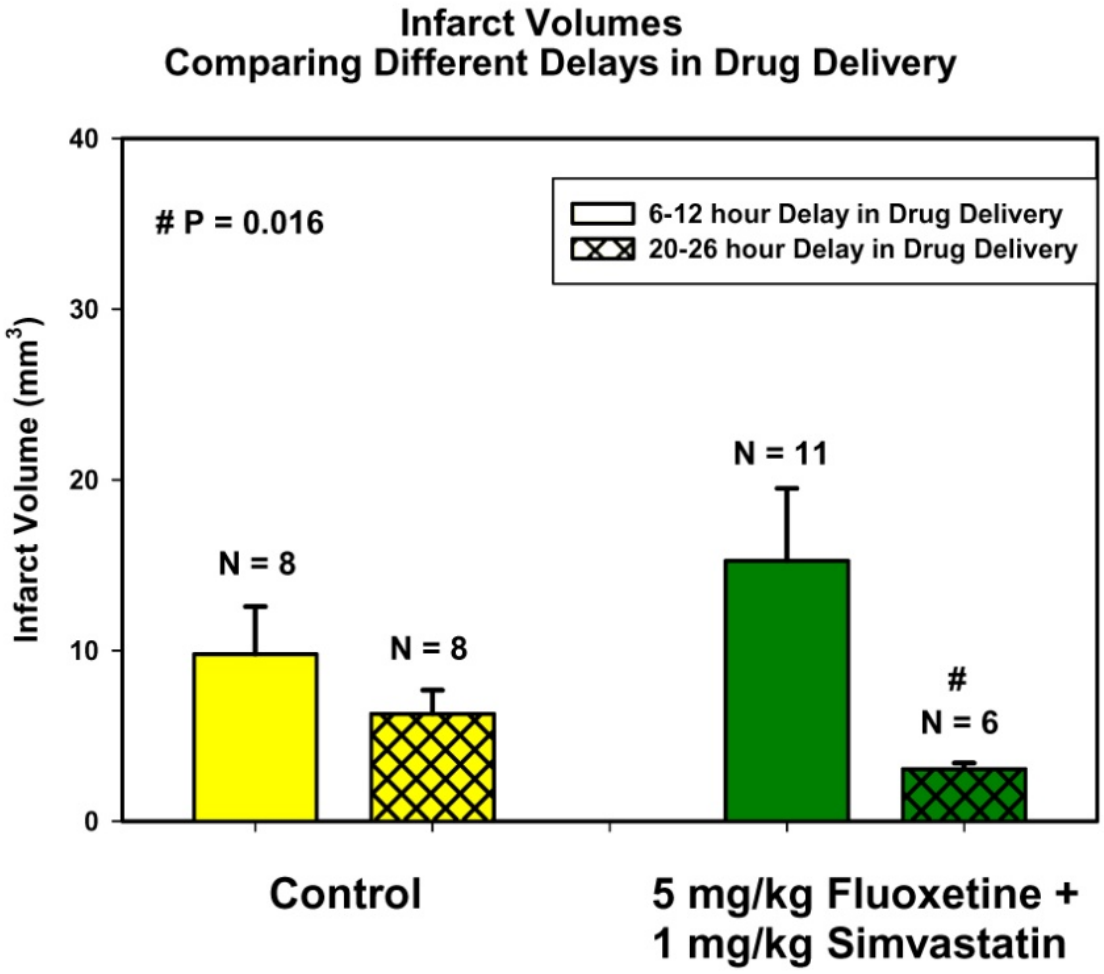
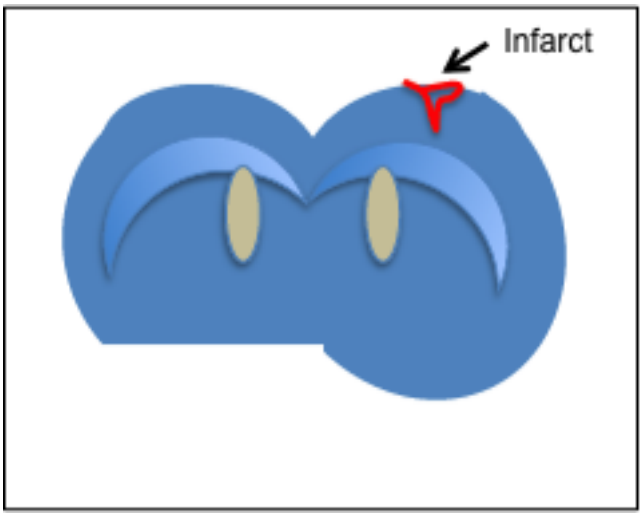

Infarct Volumes
Comparing Different Delays in Drug Delivery
P = 0.016
6-12 hour Delay in Drug Delivery
20-26 hour Delay in Drug Delivery
Infarct Volume (mm3)
40
30
20
10
0
N = 8
N = 8
N = 11
#
N = 6
Control
5 mg/kg Fluoxetine +
1 mg/kg Simvastatin

Figure 17.

Sample infarct showing a simple infarct, easily identifiable at the injection site. Both a cartoon diagram and an actual image of an example of this type of infarct are shown. Cartoon outlines of the brain slice are provided for reference. The infarct is outlined in red and labeled. The corpus callosum and edges of the brain slice are outlined in blue for reference.

Figure 17.



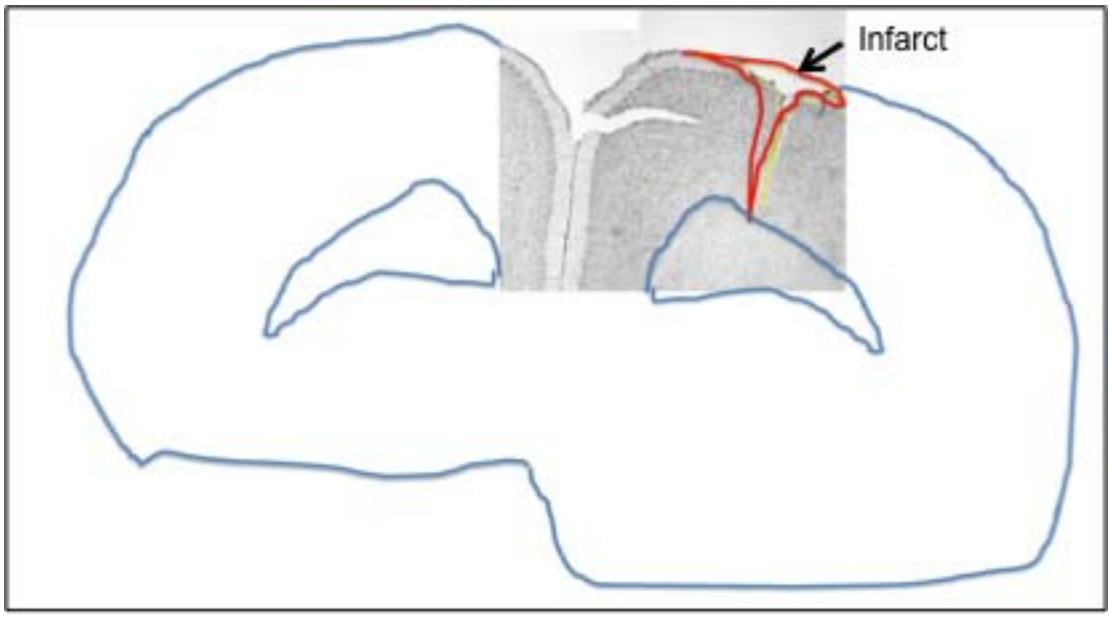
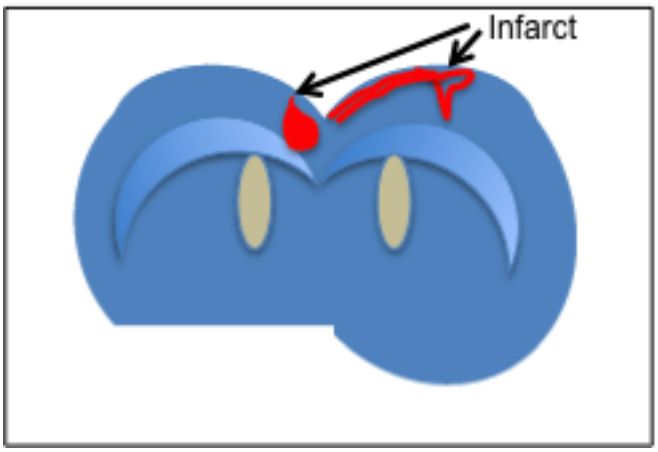


Figure 18.

Sample infarct showing a simple infarct, easily identifiable at the injection site, with damage that crosses the midline. This additional damage could represent original damage caused by the initial infarct or evidence of a secondary hemorrhagic stroke. Both a cartoon diagram and an actual image of an example of this type of infarct are shown. Cartoon outlines of the brain slice are provided for reference. The infarct is outlined in red and labeled. The corpus callosum and edges of the brain slice are outlined in blue for reference.

Figure 18.



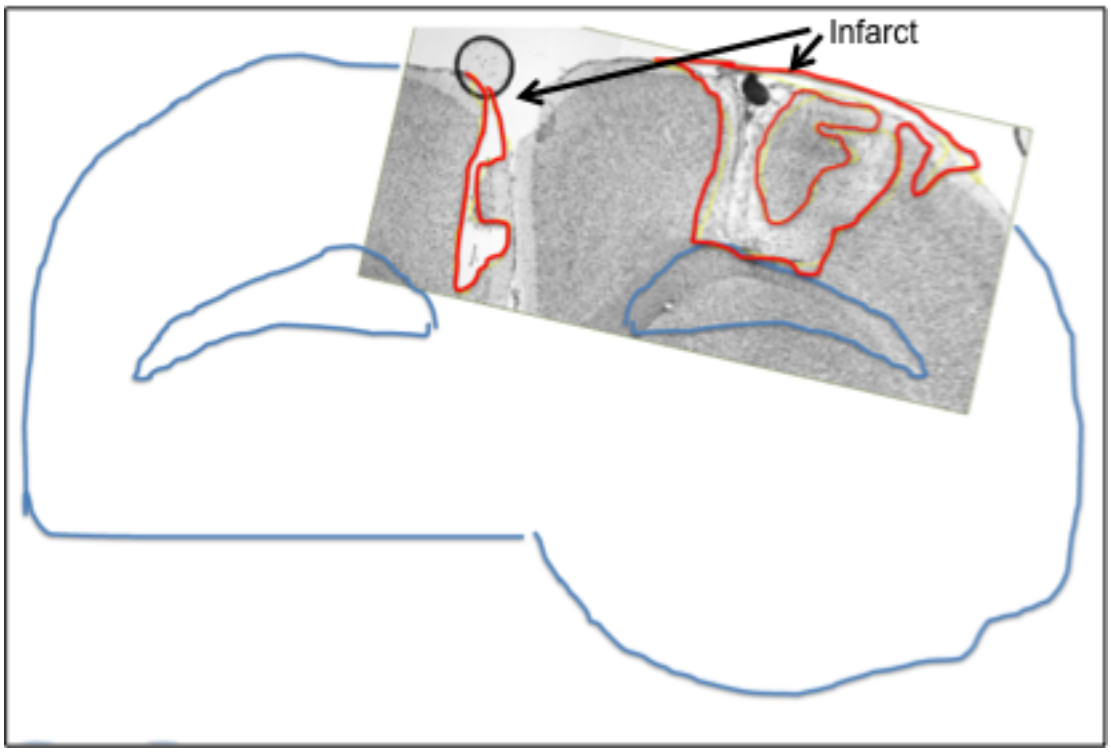
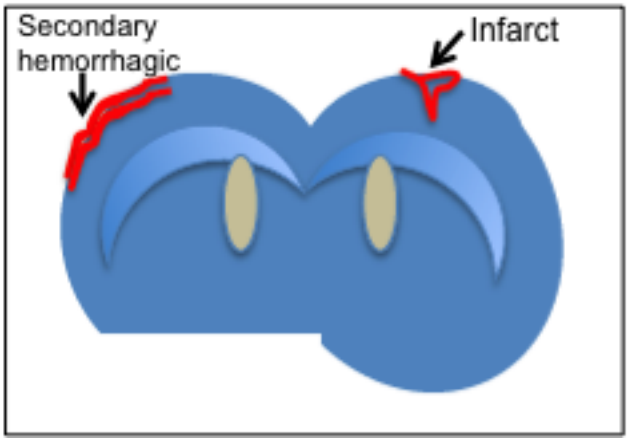


Figure 19.

Sample infarct showing a simple infarct, easily identifiable at the injection site, with obvious secondary hemorrhagic stroke damage contralateral to the initial infarct. Both a cartoon diagram and an actual image of an example of this type of infarct are shown. Cartoon outlines of the brain slice are provided for reference. The infarct and secondary hemorrhagic stroke damage are outlined in red and labeled. The corpus callosum and edges of the brain slice are outlined in blue for reference.

Figure 19.


Secondary
hemorrhagic
Infarct

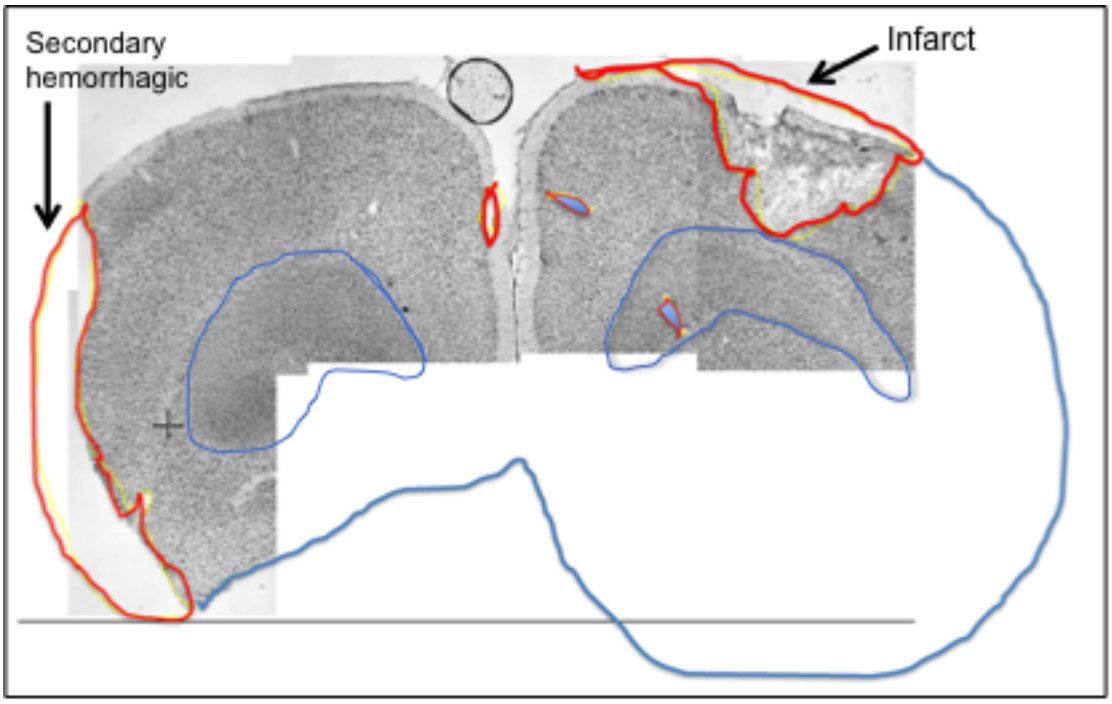
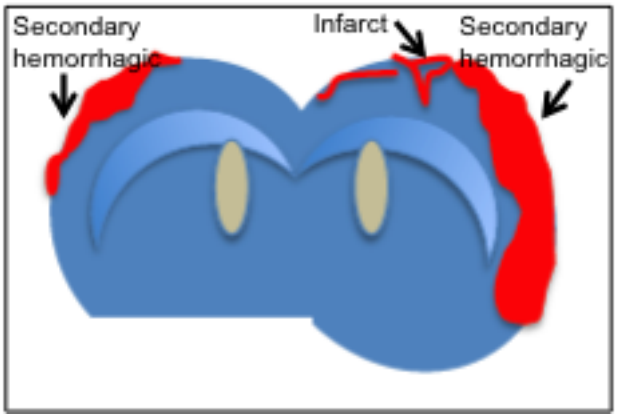
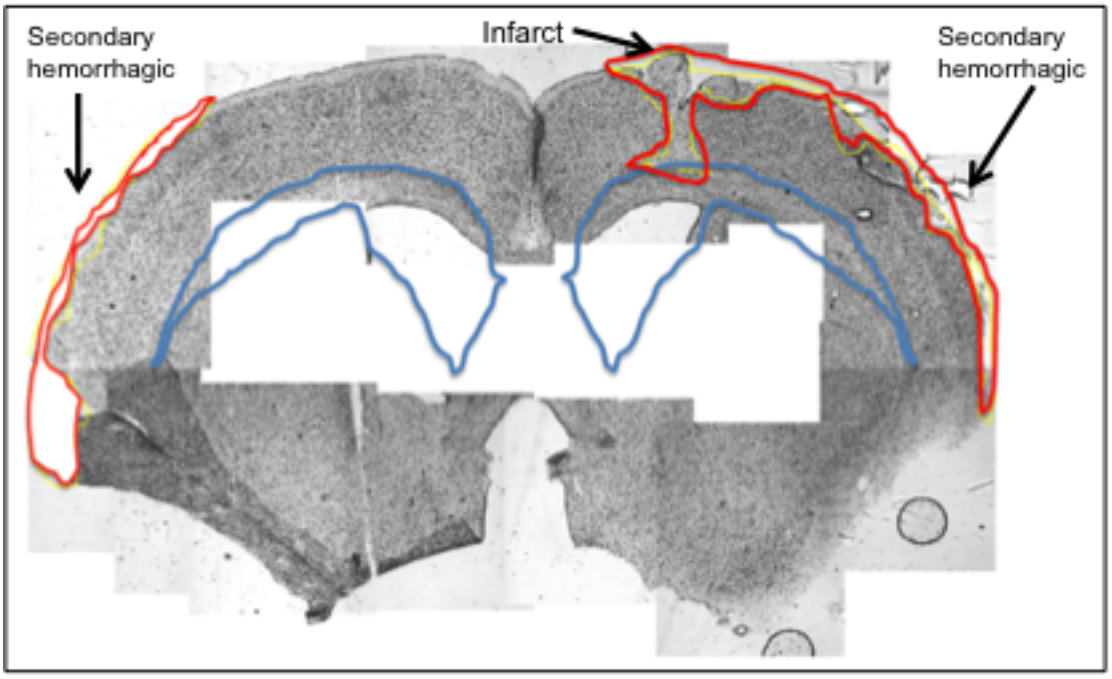

Secondary
hemorrhagic
Infarct

Figure 20.

Sample infarct showing a simple infarct, easily identifiable at the injection site, with obvious secondary hemorrhagic stroke damage both ipsilateral and contralateral to the initial infarct. Both a cartoon diagram and an actual image of an example of this type of infarct are shown. Cartoon outlines of the brain slice are provided for reference. The infarct and secondary hemorrhagic stroke damage are outlined in red and labeled. The corpus callosum is outlined in blue for reference.

Figure 20.


Secondary hemorrhagic
Infarct
Secondary hemorrhagic


Secondary hemorrhagic
Infarct
Secondary hemorrhagic

An initial thought regarding the cause of the observed secondary hemorrhagic strokes was the possibility of endogenous tPA production. This thought surfaced because statins provide protection against ischemic injury by upregulating endogenous tPA (Asahi *et al*, 2005). However, this does not appear to be the best theory because 25 million people currently take a statin (Associated Press, 2014), and there is no evidence that anyone taking a statin stops taking it when they have a stroke. In fact, one study showed people taking statin did worse if the statins were stopped once the stroke occurred (Blanco *et al*, 2007). Additionally, the dose of simvastatin administered to rats in our study is extremely small when compared to the scope of human doses (Rx List, 2014b). Because human stroke patients receiving statins do not experience these complications, it is thought that the amount of tPA produced in conjunction with our rat dose would equate to much less than that produced in conjunction with the normal human dose. If this is the case, then it is unlikely that the amount of tPA produced would be enough to initiate hemorrhagic transformation.

A more promising idea is that the hemorrhagic transformation involves the drug combination's mechanism of action that increases VEGF in the brain. VEGF is a mediator of neurogenesis, neuroprotection, and angiogenesis after ischemia (Gaillard and Mir, 2001). VEGF is one mediator released by oxygen-deprived cells. It binds to receptors on endothelial cells and, in response, the associated vessels dilate and leak. The endothelial cells respond to the VEGF signal by producing other mediators that aid in degrading the vascular basement membrane and reorganizing the extracellular matrix, among other events. This permits and encourages endothelial cell migration and, ultimately, proliferation and assembly into new vasculature (Mayes, 2006).

In addition to promoting neurogenesis by increasing BDNF levels, fluoxetine may induce expression of VEGF (Gaillard and Mir, 2001). Simvastatin also upregulates BDNF and VEGF (Wu *et al*, 2008). VEGF increases angiogenesis, and it is possible that vessel leakage is occurring during the period of increased angiogenesis. One study showed that VEGF given to rats shortly after stroke resulted in a hemorrhagic transformation; however, VEGF given later after a stroke proved to be beneficial (Zhang *et al*, 2000). If our drug combination, given early at 6-12 hours post-stroke, is causing abnormally-early expression of VEGF and vessel leakage, it is possible that this is the mechanism behind the hemorrhagic transformation observed in this study.

Our study used a 5 mg/kg dose of fluoxetine. This rat dose equates to a human dose of approximately 40 mg/kg, which is moderate when reviewing the dose range of 20-80 mg for this drug (Rx List, 2014a). Our rat dose of 1 mg/kg dose of simvastatin equates to a human dose of 10 mg, which is low in the dose range of 5-40 mg for this drug (Rx List, 2014b). Because of this, it is possible that fluoxetine would contribute more to the VEGF timing production issue. More research is needed to determine the exact culprit behind the secondary hemorrhagic strokes observed in this study. Changing the administration time of the drug combination to one already proven to be safe would also be encouraged.

FUTURE EXPERIMENTS

Many questions were raised during analysis of the data presented in this thesis. Several possible future experiments are discussed.

Repeat This Study

The findings of this study suggest possible benefits with drug treatment alone, rehabilitation alone, and a combination of the two variables. However, a secondary hemorrhagic stroke may be occurring as a result of early drug delivery at 6-12 hours post-stroke. An essential future study would be to repeat this study, analyzing drug-treated animals and control animals in both rehabilitation and non-rehabilitation groups with drug delivery at 6-12 hours post-stroke, with a larger sample size. By redoing this study with increased sample sizes, it could be determined if our findings in this study are consistent and if earlier drug delivery may in fact be causing the hemorrhagic transformation. Steps could also be taken to measure endogenous tPA and VEGF production to identify the exact culprit behind the secondary hemorrhagic strokes.

Drug Administration at 20-26 Hours Post-Stroke Plus Rehabilitation Component

It has been shown in previous studies from our laboratory that drug administration at 20-26 hours post-stroke is beneficial and provides better results than earlier delivery at 6-12 hours post-stroke (Corbett *et al*, 2013). An important future study would be to duplicate Experiment 2 from this study by comparing the four animal groups – drug-treated and control animals in both rehabilitation and non-rehabilitation groups – while providing drug administration at 20-26 hours instead of 6-12 hours post-stroke.

This would revisit a successful administration timeline with the added rehabilitation component and ideally eliminate the possible issue of secondary hemorrhagic stroke due to too-early drug delivery.

Change Drug Duration

In our study, drug-treated animals continued receiving treatment for the full length of the study. No investigation has been performed on determining the proper duration of drug-treatment. Initiating future experiments to test different durations of treatment would be valuable because it is unknown whether an animal's recovery would continue, plateau, or regress after the drug-treatment is stopped. It is also valuable because, in human stroke patients, it would be necessary to know whether this treatment would be temporary or permanent.

Lengthen Study to Better Determine Rehabilitative Effects

Animals in the rehabilitation group received rehabilitation beginning at post-stroke day 8 for five-and-one-half weeks. While rehabilitation was occurring during the Montoya testing for functional recovery at post-stroke days 29-31, rehabilitation had been stopped already when tests were performed at post-stroke days 59-61 and 89-91. Animals - even the control animals – appeared to retain functional recovery across the duration of the study if they received rehabilitation, despite the fact that the rehabilitation had been stopped much earlier (Figure 9). Additional studies conducted at longer length would be beneficial to map whether functional recovery is retained or decreases after a substantial amount of time.

Measure Infarct Size Throughout Study

This study measured infarct volume at the end of the study using histological analysis of the brain tissue. It would be interesting to measure infarct size at different points throughout the study. One approach could involve inducing strokes in a large sample size of animals followed by infarct analysis at different increments. For example, brain tissue of one group of animals could be analyzed at 24 hours post-stroke to understand the initial infarct size. Another possible approach could involve infarct measurement in the same animals at different times throughout the study by using magnetic resonance imaging (MRI), as seen in other studies (Christoforidis *et al*, 2011).

Use of Other Stains

To examine if infarct size could be used as a good variable in measuring the effects of our drug combination, a future experiment could employ the use of different stains. While the Nissl stain allows normal neurons to be observed, other stains may benefit future studies by helping to determine other neuronal states, such as neurons that are functional as compared to those that have grown and made new connections but are not yet functional.

Employing Somatosensory Tests

The experiments examined in this thesis were completed using functional behavioral tests to measure the effects of the stroke and, later, our drug combination on functional – that is, motor – recovery. No analysis was done to also analyze effects of the stroke and our drug treatment on somatosensory behavior. It would be interesting to

employ the use of an added behavioral somatosensory test to determine if a somatosensory deficit is present in addition to a motor deficit. This could give insight precisely as to how the larger infarcts observed in this thesis study affected the animals' brains. If a large infarct greatly affected somatosensory areas, this could provide implications as to why little functional motor deficit was detected. Additionally, an animal with impaired somatosensory function may have intact motor function, but it would have difficulty grasping the sucrose pellets if it cannot feel or smell them. Adding a somatosensory component to the study may help answer the question presented earlier in this section regarding heterogeneity in damage.

CONCLUSIONS

Differences in functional recovery across groups suggest several possible treatment options. Without rehabilitation, drug treatment provides better functional recovery (Figure 13). This proposes that drug treatment alone to be an option, which is beneficial because patients incapable of performing physical therapy could still have hope for recovery. With rehabilitation, there was no difference in functional recovery between drug-treated and control animals, suggesting rehabilitation – regardless of drug administration – may provide an opportunity for recovery (Figure 14). If rehabilitation alone is an option, this could help patients with pharmaceutical restrictions. Rehabilitation also provides the opportunity for greater recovery even in the occurrence of larger infarcts (Figure 15), which may be beneficial to hemorrhagic stroke patients. Finally, drug treatment combined with rehabilitation may initiate faster recovery early on (Figure 9), but further investigation is needed.

The issue of heterogeneity in damage raises questions of whether the forelimb motor cortex alone was affected. With possible somatosensory areas involved, especially with the larger infarct volumes, additional parameters such as somatosensory testing could be employed in future studies.

Larger infarcts presented in this study with earlier drug delivery at 6-12 hours post-stroke may be the result of hemorrhagic transformation due to a too-early induction of VEGF expression by our drug combination. Further exploration into the mechanisms involved is necessary, as is duplicating this study and performing new experiments at the later drug delivery of 20-26 hours post-stroke.

Ultimately, further investigation is needed to increase sample size to better quantify the results. Additional research will also aid in properly measuring the effects of our drug combination on functional recovery and infarct size, the timing of drug delivery, and the effects of limb rehabilitation on functional recovery.

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