Dedication:

I want to dedicate this work to all the young children and their families suffering from focal cerebral ischemia. I know that whatever knowledge my research offers to the field, no matter how small, is a step closer towards effective treatments for these babies to live healthy, productive lives.

Drew University

College of Liberal Arts

Neurofunctional assessment in a neonatal rat model of focal cerebral ischemia following caffeine exposure

A Thesis in Neuroscience

by

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Abstract:

While much is known about perinatal brain injury as a result of focal cerebral ischemia (FCI), there is a need for diagnostic criteria to detect the variable symptomology as well as effective treatments following injury. There are currently no effective treatments for focal cerebral ischemia in the developing neonate. Recent evidence suggests that caffeine citrate may offer neuroprotection for ischemic injury. I utilized a novel neonatal rat model of focal cerebral ischemia (FCI) to assess the behavioral outcomes as well as determine the effects of caffeine citrate on these deficits. FCI animals were significantly impaired in weight gain ($p<0.0005$), startle response ($p<0.0005$), and righting reflex at P10 ($p=0.007$). Caffeine-treated FCI animals exhibited a marked weight decrease ($p<0.0005$) compared to FCI animals treated with saline. No other statistically significant differences with caffeine were found and behavioral data were replicated. The growth impairment shown in caffeine-treated FCI animals may be an increase in endogenous corticosterone, but further investigation is necessary to assess caffeine's efficacy as a potential treatment. Such a dramatic loss in weight for FCI animals treated with caffeine is indicative of a negative potentiation of FCI effects and must be investigated further.

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NMDA: N-methyl-D-aspartate receptor

AMDA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

FCI: Focal Cerebral Ischemia

SO: Sham-Operated

HI: Hypoxic-Ischemia

MCA: Middle Cerebral Artery

ICA: Internal Carotid Artery

ECA: External Carotid Artery

CCA: Common Carotid Artery

Caff: Caffeine Citrate

CORT: Corticosterone

Introduction

Although there is substantial research on ischemia stroke in the elderly, the neonatal population is in need of well-defined criteria for diagnosis and effective treatment strategies. In order to investigate ischemia in neonates, there must be a clinically relevant animal model system to offer experimental manipulation. Our group developed a novel model for focal cerebral ischemia (FCI) in the neonatal rat to exhibit similar pathology to human neonates in the NICU. It was hypothesized that animals with FCI would be significantly impaired in a number of sensorimotor tasks and that these deficits would be attenuated by caffeine citrate administration. Before describing my experiments, it is important to have a comprehensive understanding of stroke pathology and etiology, potential risk factors, long-term impairments, current animal models, and potential drug therapies for focal cerebral ischemia.

Stroke History and Etiology

Stroke is defined as a set of deficits resulting from focal (or global) damage to the central nervous system via the vascular system (Sacco et al. 2013). Damage or death to neuronal systems occurs when there is a disruption in oxygenated blood flow. The impairments as a result from such an injury are widely variable and depend largely on the region in which the insult occurs. However, the most common signs of stroke are contralateral paralysis and speech impairments (Goldstein 2013).

Hippocrates was the first to diagnose these types of impairment in the 3rd century B.C.E. (Nilsen 2010). He noted that patients would exhibit contralateral paralysis and

impaired speech after a traumatic injury to the brain. Due to the sudden and violent onset of these deficits, he referred to the impairment as apoplexy, which in Greek means, "struck with sudden violence" (Thompson 1999). Around the same time, Galen had the important observation that the severity of the deficits depended on the severity of the injury (Karenberg 1994). Several treatments emerged during the Middle Ages including bloodletting, with hopes of alleviating the excess blood associated with apoplexy. However, without a proper knowledge of brain structure and function, such treatments were ineffective.

It took several hundred years, but in the 17th century Dr. Jacob Wepfur noted that patients who died of apoplexy exhibited bleeding in the brains and a smaller subpopulation had an occlusion in the middle cerebral artery (Karenburg 2004). This observation hinted at different mechanisms for a similar impairment, which were further fleshed out by the work of Rudolph Virchow (Demarin et al. 2011). In the 18th century, he was the first to categorize the patients with apoplexy resulting from a clot as suffering from thromboembolism. Patients had thromboses that could break off into a free-floating embolus that could fatally injury the individual by cutting off the brains blood supply. It was not until the early 1900's that these subtypes were classified as hemorrhagic and ischemic injuries, associated with a bleed or a blockage, respectively. This was also the time period when the nomenclature shifted from apoplexy to stroke (Nilsan 2010).

Today stroke accounts for 9% of the deaths worldwide and the reported incidence is expected to grow as diagnosis criteria improve (Murray and Lopez 1997). An estimated 6.8 million Americans older than 20 years, or about 3% of the general population, have

had a stroke (Go et al 2013). Older adults, African Americans, and individuals with lower socioeconomic status have a higher prevalence of stroke. In 2011, the United States government spent about $17 billion on stroke and other cerebrovascular disorders, which is a marked increase from the $13 billion spent in 2006 (Medical Expenditure Survey Table 4, 2011). Nearly $8 billion of these funds are spent on hospital inpatient care and about $5 billion was spent on in-home hospice care. It is estimated that in the United States, an individual has a stroke every 40 seconds and a stroke related death occurs every 4 minutes. Unfortunately, it is projected that by 2030, an additional 4 million people will have had a stroke in the US, which is a 21.9% increase in the national prevalence rate of today.

While the media has popularized the typical stroke as a "bleed in the brain", about 87% of all strokes are ischemic and 10% are intracerebral hemorrhage with the remaining 3% is attributed to subarachnoid hemorrhage strokes (American Heart Association, 2009). It is important to understand the pathology of these subtypes in order to effectively develop therapeutic treatments and lower the burden of this disease.

Stroke Pathology

The different subtypes of stroke may be generated by different mechanisms in the brain but they ultimately follow a similar pathway towards impairment. The hemorrhagic injury, or intracerebral hemorrhage (ICH), is caused by a rupture of weakened blood vessels deep within the brain (Fewel et al. 2003). This extravascular blood, or hematoma, accumulates in the region of the rupture and deprives that region of substrates necessary for normal cell functioning. This accumulation results in an increase in intracranial

pressure as the hematoma expands, directly damaging the local brain tissue (Sutherland and Auer, 2006).

The primary compression and damage to tissue is traumatic, but the secondary injuries may be just as significant (Wang, 2010). The inflammatory response following ICH further damages vulnerable tissue and is accounts for in 15-20% of all cases. The ICH allows for immune factors, such as leukocytes and macrophages to invade the damaged region and release pro-inflammatory cytokines in response to the damage (Wang and Tsirka 2005). These cytokines activate microglia in the brain, which provide a defense against foreign agents and damaged tissue. This event happens over a matter of minutes; however, the prolonged activation of microglia observed in ICH (often for 2-3 hours) can result in severe neuronal damage by triggering apoptotic cascades (van Rossum and Hanisch 2004, Zhang et al. 2009). While the contributions of these pathways are significant, the ICH eventually develops into ischemia (as there is prolonged loss of blood to the brain) and the main contributions to neuronal death originate from the ischemic pathology, which is examined in detail below.

Ischemic stroke pathology is fairly complex and can be divided into two broad classes based on the location of the damage: focal and global. The focal ischemic injuries can be further subdivided based on pathology. About 45% of ischemic strokes are caused by thrombosis, and another 20% occur through embolism, while the rest are attributable to global causes or else the causes remain unknown (Hickey 2003). Thrombosis is most commonly caused by atherosclerosis of an artery from calcium or fat deposits (Ross 1993). The roughing of the of the arterial wall allows platelets to aggregate to the plaque

which results in slowed or disrupted blood flow to many regions of the body, including the brain via the middle cerebral artery (MCA). Some other general risk factors for ischemia are diabetes and a history of hypertension (American Heart Association, 2009).

If a thrombosis occurs in the periphery, and depending on the consistency and size of the plaque, a small portion may break off, or embolize, into the blood stream and form a blockage in another artery, most commonly in the MCA as it carries more than 80% of the blood to the brain (Hinkle and Guanci 2007). While it is easier to disrupt a small embolism than a mature thrombus, the emboli are more difficult to locate due to their small size.

Ischemic Stroke Pathology: The Ischemic Cascade

Global ischemic stroke, often referred to as hypoxic-ischemia or systematic hypoperfusion (SHP), results from a reduction of blood flow throughout all regions of the body (Jefferson et al. 2007). Cardiac arrest or heart failure results in a collective reduction in blood flow to the brain, which may cause damage over prolonged periods of time (Malhotra et al. 2001). While hemorrhagic, focal ischemic, and global ischemic strokes have quite different etiologies, there is evidence to suggest that all strokes have similar pathology of damage via the ischemic cascade (Choi 1990, Sutherland and Auer 2006, Donnan et al 2008, Banasiak et al 2000). I will highlight the key features of the ischemic cascade, including blood-oxygen deprivation, lack of glucose to cells, glutamate excitoxicitity, the generation of reactive oxygen species, and the secondary inflammatory response.

As blood flow is reduced to the brain the neurons accommodate for the loss of glucose by shifting to anaerobic metabolism. In the first several minutes, neurons break down lactose into lactic acid, which can be toxic to the cell in high concentrations (Luft 2001). If left untreated for three or more hours the region of the brain affected by the ischemia will suffer irreversible damage or death (Donnan et al 2008). It is this region, the ischemic penumbra, in which the ischemic cascade occurs.

Glucose is quickly used up and not reproduced as a result of a depletion of oxygen rich blood and the neurons fail to complete basic cell processes. The generation of high-energy phosphate molecules like adenosine triphosphate (ATP) is absolutely essential to cell function and cannot occur without an abundance of glucose (Berg et al. 2012). ATP hydrolysis provides necessary energy for a multitude of enzymes to function within the cell, but membrane-bound Na/K-ATPase enzyme is of particular interest for the ischemic cascade.

Glutamate is an excitatory neurotransmitter and is the primary cell signaling molecule in about 80% of neurons in the CNS. In high concentrations, glutamate has toxic properties referred to as excitotoxicity (Mark et al. 2001). Glutamate is normally kept in low concentrations outside the cell by glutamate transporters, proteins that are regulated by sodium ions in the extracellular space. This ion gradient is generated by the Na/K ATP-ase enzyme and results in no excitotoxicity under normal conditions (Rose et al 2009). However, with the low concentration of ATP inside the cell as a result of the ischemic insult, the ATP-ase pump cannot function properly and cannot generate an ionic gradient across the membrane. This imbalance of sodium ions (more sodium inside the

cell) leads to a dysfunction in the glutamate reuptake proteins EAAT1 and EAAT2 (Rose et al. 2009). Furthermore, the depolarized presynaptic neuron allows for an influx of calcium and further release of glutamate into the extracellular space. If glutamate excitotoxicity were indeed involved in ischemic damage, then the excessive stimulation would result in seizures, which is the primary symptom present after insult (American Heart Association, 2009). While selective NMDA receptor antagonists like MK-801 offer neuroprotective effects against ischemia in the cat, such antagonists are not viable therapeutic options (Ozyurt et al. 2008). Clinical trials using MK-801 have largely failed because while they inhibit the effects of glutamate excitotxicity, they also inhibit vital neuronal communications using glutamate, resulting in hallucinations.

As glutamate begins to accumulate in the extracellular space, it binds to NMDA and AMDA receptors on the postsynaptic cells in the penumbra and again induces an influx of calcium. Calcium is known to participate in several cell processes like neurotransmitter release and cytoskeletal formation, but prolonged calcium stimulation can lead to apoptosis (Pinton et al 2008, Nicotera and Orrenius 1998). Excess intracellular calcium activates various proteases and phospholipases, which lead to cleavage and inactivation of enzymes. Furthermore, excess calcium influx results in the release of cytochrome C from the mitochondria and the initiation of the caspase cascade for apoptosis. Therefore, the loss of oxygen-rich blood to the brain initiates a chain of events that results in neuronal death after a period of time via a glutamate excitotoxic mechanism.

In addition to the glutamate excitotoxicity following ischemia there is a secondary risk of damage following reperfusion. When the occlusion is removed, either spontaneously or by intervention, the sudden return of oxygen-rich blood induces additional damage to the ischemic penumbra and blood brain barrier (Zweier et al 2006). It was initially thought that the inflammatory response from cytokines released by the white blood cells carried in the blood resulted in the damage, but additional work suggests a strong role of oxidative free radicals (Forsyth and Guilford 1995). The white blood cells also release reactive oxygen species (ROS), molecules that are highly reactive and unstable due to an unpaired electron in the valence shell. The free ROS take part in a peroxidation reaction with the lipid membrane and break it down, exposing additional free radicals in the cytosol (Park and Lucchesi 1999). ROS continue to damage cells by denaturing enzymes and ion channels as well as tearing DNA strands. Ultimately, the ROS contribute to cell death after the primary glutamate excitotoxic pathway. Given the high incidence rate and the shared pathology among stroke subtypes, I chose to investigate ischemic stroke exclusively.

Neonatal Stroke: Burden of Diseases and Prognosis

I am interested in the incidence and diagnosis of acute ischemic stroke during the perinatal/neonatal period of development. This population includes all individuals between 20 weeks of fetal life to 28 days of postnatal life (Raju et al. 2007). Neonatal arterial ischemic stroke outnumbers the cases of ischemia in older children by nearly 10-fold. This injury involves a focal arterial occlusion most commonly in the middle cerebral artery (MCA). The estimated incidence of ischemic stroke in neonates is second only to

the estimated incidence of ischemic stroke in the elderly (Govaert et al. 2009). This is a sobering realization when assessing the diagnosis and recognition of ischemia in the two populations. Older adults have a wide variety of risk factors and symptoms that are readily apparent to both the individual and the clinician. In a recent study, elderly patients were able to personally identify a stroke symptom 60.5% of the time (Zerwic et al. 2007). Furthermore, elderly individuals have characteristic signs of stroke that can be easily recognized by friends and family, such as facial paralysis, speech impairment, and cognitive decline. Patient recognition of symptoms is not realistic in the neonatal population, and if symptoms are present at all, they may be very difficult to identify by clinicians. The phenotype of stroke is often hemiplegia, which is almost undetectable in an infant in an incubator. This symptom is only noted later on in development and by that time it is often to late to repair any permanent damage done by the insult.

The long-term effects of ischemia are pronounced in the neonatal population. Ischemic stroke in neonates is the strongest predictor of cognitive impairment by age 12 in the United States (Allen 2008). A comprehensive study of survivors of neonatal stroke found that 219 of 308 individuals had lower test scores in cognitive ability, reading, and mathematics compared to controls (Johnson et al. 2009). This impairment was confirmed in another study that found that about 47% of survivors of an ischemic insult during the neonatal period require state aid for school services such as reading, writing, and math compared to controls (6%). In addition, neonatal ischemic stroke accounts for nearly 30% of all known cases of cerebral palsy in the United States (Wu et al. 2006). Stroke deficits depend on size of the lesion and location in the brain, but due to the proximity of motor

regions, such as the basal ganglia, internal capsule, and primary motor cortex, to the MCA, occlusion in this area results in nearly 45% of survivors suffering from a range (mild-severe) of cerebral palsy symptoms (Boardman et al. 2005).

However, not all infants who suffer this type of stroke have poor prognosis. A study done by Westmacott et al. investigated the performance of neonatal stroke survivors both in preschool (3-5 years) and grade school (6-12 years) on several cognitive and verbal tests (Westmacott et al. 2009). At the preschool level, they found that there was no significant difference in cognitive impairment between stroke and control individuals. This result suggests that infants who suffer a stroke during the neonatal period have the potential to live normal lives, possibly due to the increased neuronal plasticity during this critical time of development (Bukowski et al. 2002). However, the Westmacott study also found that these same individuals, particularly the males, had significantly lower working memory and verbal performance compared to controls. This result suggests that all infants might be impaired by the initial neonatal stroke but the delay in these impairments is a function of the complexity of cognitive processing. This study is one of the only longitudinal studies of its kind, so more research investigating these outcomes is necessary.

Due to larger variability between individual behavioral responses to injury, clinicians struggle to diagnose ischemic events. Neonatal ischemic risk factors, symptomology, and diagnosis are all in need of further investigation. These topics will be discussed in more detail below.

Risk Factors for Ischemia

There are numerous risk factors that increase the likelihood for a neonate to have an ischemic stroke. Gender plays a role, as boys have a 1.28-fold greater risk of an ischemic stroke (Fullerton et al. 2002). Race also contributes as black children have a 2-fold greater risk for stroke than white children, which can be partially explained by the increased prevalence of sickle cell anemia in that population as the misshapen red blood cells have a higher propensity to form thrombi (Fullerton et al. 2003). Congenital heart disease (CHD) is known to increase the risk of ischemic stroke in neonates. In fact, neonates with this disease, in particular single-ventricle malfunctions, are at a 16-fold increased risk of having an arterial ischemic stroke than age-matched controls (Hoffman et al. 2011).

Genetic risk factors play a large role in arterial ischemic stroke, especially Factor V Leiden mutation, resulting in activated protein C resistance and coagulation disorders (Bertina, 1999). The mutation results in a replacement of a glutamine for an arginine residue at the 506 position on the Factor V protein. In a healthy individual, the anticoagulation pathway begins with activation of thrombomodulin cofactor as shown in Figure 1. This activated enzyme converts the procoagulant enzyme thrombin into an anticoagulant enzyme. Thrombin activates protein C, which then combines with protein S attached to platelets to form an anticoagulation complex. This complex normally degrades the procoagulant enzymes factor V and factor VIII to slow down the clotting process.

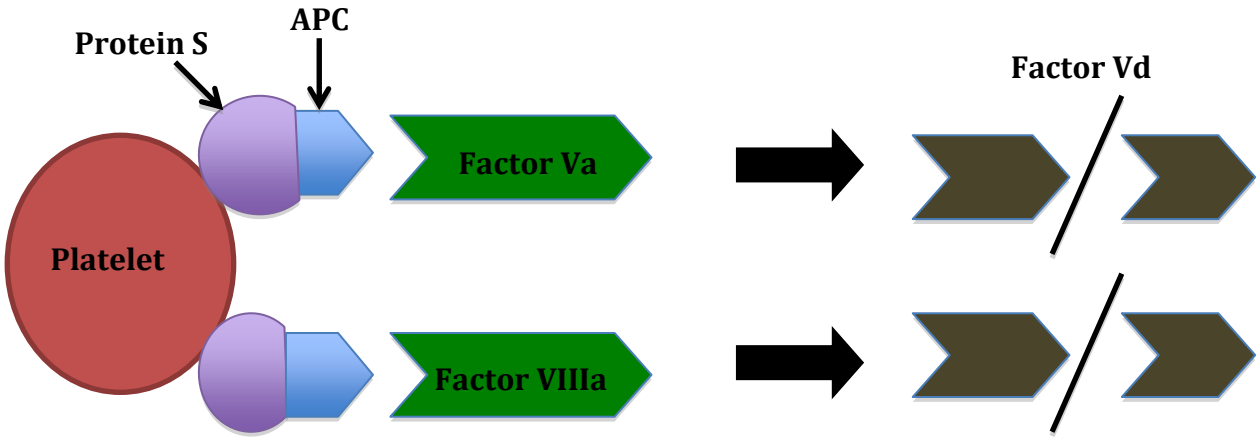

Figure 1: Role of Activated Protein C (APC) in healthy individuals. Factor VIIId Factor Vd found in human blood have a phospholipid, which allows proteins to bind to, such as protein S. Protein S has a high affinity for anticoagulant proteins like Protein C, which activate upon binding. The Protein S/APC complex can bind to the active site of procoagulants Factor V and Factor VIII in their active forms (a). This results in degradation of the enzymes (d) and a lower likelihood of thrombosis.

However, Figure 2 depicts the malfunctions in the pathway with Factor V mutation. Factor V Leiden is resistant to the activated protein C/protein S complex and cannot be degraded. Thus, factor V will be inactivated by protein C at a much slower rate, which increases the likelihood of a clot to form. Interestingly, Factor V Leiden is a risk factor for neonatal stroke but it is not found in older adults, suggesting a valuable target for diagnosis and drug therapy (Thorarensen et al. 2004).

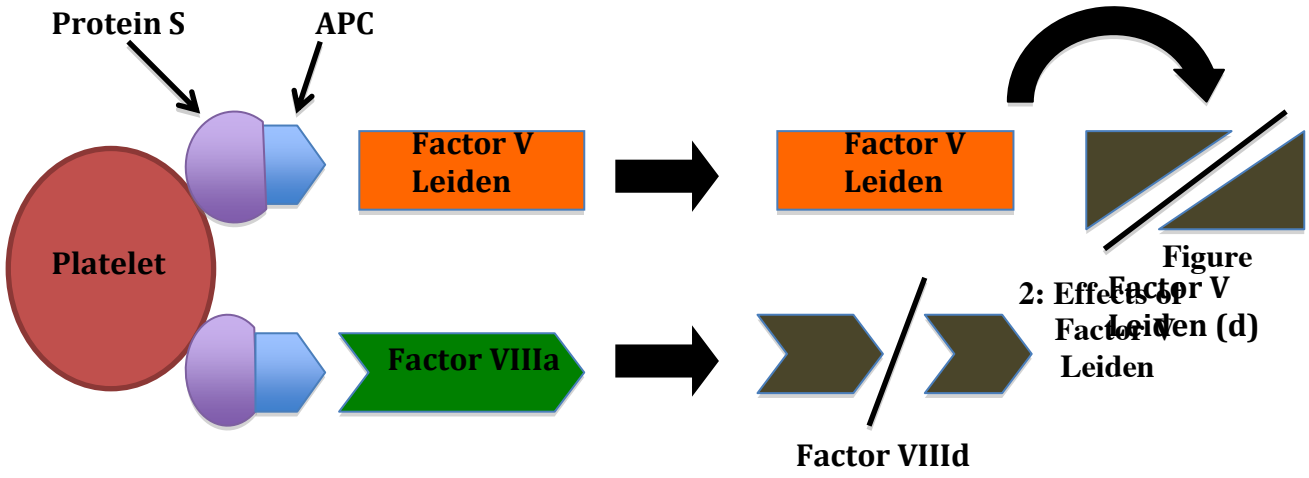

Figure 2: Effects of Factor V Leiden (d)

mutation. The replacement of an arginine for a glutamate residue at the 506 position on Factor V results in the Factor V Leiden mutation. This does not affect the affinity of Protein C to Protein S in anyway. Rather, the mutation alters the structure of the Factor V protein in its active state, which lows the binding affinity for APC. While Factor VIII is still broken down by APC at a steady rate, it takes significantly higher APC concentrations to degrade Factor V Leiden. This is known as activated protein C resistance (APCR) and greatly increases an individuals risk for thrombosis and thus an ischemic event.

This mutation may be important during fetal development as well. In a recent study, it was shown that placental thromboembolism might play a contributing role in the risk for neonatal stroke (Elbers et al. 2011). Ten out of the twelve neonates studied had signs of arterial ischemic stroke as well as placental thrombosis. Mothers with Factor V Leiden had placentas that were more likely to clot while feeding the baby, resulting in a embolus breaking off and traveling to the babies MCA. This would easily result in an arterial ischemic stroke even if the child did not have the mutation for Factor V.

However, placental dysfunction is not the only maternal risk factor identified for ischemic stroke. Neonates who suffer infections of the central nervous system in the womb, such as meningitis, are also susceptible to ischemic stroke (Ment, Ehrenkranz, and Duncan 1986). Infection leads to hypercoagulation states through the rapid degradation of protein C, which results in increased thrombosis, as explained above. Other important maternal risk factors include a history of infertility, preeclampsia, prolonged rupture of membranes, cocaine exposure, and chorioamnionitis (Lee J et al. 2005). However, one study reported that nearly 50% of pregnancies are normal with no complications upon delivery (Kirton et al. 2011). These infants have evidence of an ischemic event with no discernable risk factors. No cause can be found in about one fourth of all ischemic infarctions detected in neonates (deVeber et al. 2000). Since risk factors may not be the

best predictors of an ischemia episode, advances in neuroimaging techniques like cranial ultrasounds may help to detect a cerebral infarction to offer earlier detection and a better prognosis for recovery.

Ischemia Symptomology and Diagnosis

Cranial ultrasounds (CUS) are an inexpensive alternative to standard MRI techniques to determine the presence of a cerebral infarct after the onset of seizures. Perhaps the most common symptom present in neonates following a cerebral infarction, focal seizures usually occur contralateral to the affected hemisphere and are the most pronounced symptom in 48%-85% of neonates with unilateral ischemic stroke (Volpe 2008). Brain imaging is necessary to confirm the seizure is related to an ischemic event and not another insult, but MRI scans can be expensive and traumatic to the baby. Therefore, cranial ultrasounds are an attractive alternative. However some reports claim they are only successful in identifying cerebral infarctions 30% of the time (Golumb et al. 2003). In order to assess this claim, Cowan et al. investigated the sensitivity of cranial ultrasounds for identifying cerebral infarctions in the neonate (Cowan et al. 2004). They ran CUS scans on full term infants (n=47) at both an early (1-3 days post birth) and a late (4-14 days post birth) time point and compared them to neonatal MRI scans conducted on the same babies. The goal was to assess the accuracy of the CUS scans in detecting the infarct that the MRI scans found. They found that the CUS scans were abnormal in 68% of neonates at the early time point and 87% of neonates at the late time point. This result suggests nearly a 3-fold increase in sensitivity from previous reports. However, the CUS scans increased in sensitivity as the infarct increased in size over time, suggesting they

are not the most effective method for early diagnosis. Regardless, imaging techniques are invaluable for neonatal stroke diagnosis. It was recently reported that due to advances in imaging techniques the reported incidence of ischemic stroke has nearly doubled due to improvements in initial diagnosis of the insult (Agrawal et al. 2009).

There are other symptoms that are indicative of ischemia in neonates that can be detected later in life. Since most ischemic infarctions occur following the occlusion of the middle cerebral artery, one may expect to see deficits in the primary motor cortex. The MCA wraps around this region, and since proximal and distal limb control are mapped topographically on that region of the cortex, the contralateral side of the body should experience limb paralysis. Indeed, hemiplegia is observed in young neonates but it often goes unrecognized due to the sedentary nature of early infant development. One study investigated the emergence of these deficits and their correlation to hemiplegia later in life (Bouza et al. 1994). They followed five full-term neonates whom were diagnosed with a cerebral infarction via CUS and MRI for a period of 24 months. Their aim was to observe the incidence and evolution of any hemiplegic symptoms. Interestingly, abnormities were not visible during the neonatal period, but rather they emerged six months later in children who developed hemiplegia. This suggests that any outward signs of trauma as a result of an ischemic insult are not helpful for early diagnosis and intervention. Instead, they are more likely to aid in the treatment of any complications as a result of the primary injury but not the injury itself.

It seems that a well-defined criteria of symptomology for ischemic stroke in neonates is necessary for clinicians. While seizures are the most common symptom

following injury, there is still 15% of the population that is asymptomatic entirely. Furthermore, advances in imaging techniques have boosted the recognition of ischemia in neonates, but they have also identified the shortcomings of the current criteria presented in the International Classification of Disease Handbook, 9th Revision. The current criteria severely underestimate the incidence of ischemic stroke in neonates as nearly 25% of infants with ischemia go undiagnosed until later in life (Agrawal et al. 2009). For example, a subpopulation of children are asymptomatic, with no seizures or hemiplegia, during the neonatal period and only show impairment in fine motor control at about 4-5 months. By using the current clinical criteria of symptomology, an ischemic event would easily be missed in these children and a full recovery would be difficult without early intervention.

With inadequate diagnosis criteria and variability in the presentation of symptoms, it follows that the incidence statistics would be variable and under representative of the population. The few population based studies done to investigate neonatal stroke support this idea. A study done by the Kaiser Permanente Medical Care Program in 2005 used a cohort of 199,176 babies born from 1997-2003 to determine the maternal and infant characteristics associated with perinatal arterial stroke (Lee et al. 2005). The babies were selected based on a review of brain imaging data and medical records from their stay in the hospital. They found the population prevalence of ischemic stroke to be 20 per every 100,000, or 1/5,000 with 85% falling into the neonatal category. Another population study was performed by Javier Estan and Peter Hope to investigate a similar question of unilateral neonatal stroke (Estan and Hope, 1997). They determined

that the prevalence of ischemia in neonates was about 1/4,000 live births using only medical records from 1987-1993. Most recently, the retrospective study combining MRI, CUS, and diagnostic code searches done by Agrawal et al. in 2009 offers a different picture (Agrawal et al. 2009). They used a population of 2.3 million children from 1997-2003 in Northern California with this new combination of diagnosis techniques and estimated the incidence of ischemic stroke to be 29 per 100,000 live births, or 1/3,500. However, the incidence of 1/4,000 live births is taken to be the standard in most literature.

Together, these studies suggest that the incidence of ischemic stroke is highly variable and most likely underestimated in the neonatal population. This is due in part to a lack of consistency in diagnosis criteria as well as the asymptomatic profile of many babies in the neonatal population. Without diagnosis and early treatment, neonates with ischemic stroke have a poor long-term prognosis with an increased risk in cerebral palsy and neurological deficits. Risk factors can aid in diagnosis, but even they fall short, as 25% of ischemic events have no official cause. This stresses the importance for a strong understanding of the deficits associated with an ischemic event in a neonate to increase diagnosis and administer the proper treatment, which will be discussed in detail later. In order to investigate the neurofunctional outcomes of neonates after ischemia, a reliable model of the disease in an animal is paramount. After a comprehensive study of the deficits associated with the injury, any possible novel treatment methods may be assessed in the model.

Animal Models of Neonatal Stroke

Animal modeling of focal cerebral ischemia is absolutely essential in further understanding the pathology and potential treatment strategies. The most widely used model of neonatal stroke is the Rice-Vinnucci model of hypoxic-ischemia (Lynch et al. 2002). The model is an adaptation of the Levine preparation (Levine 1960) for hypoxic-ischemia in adult animals, which involved the unilateral occlusion of cerebral arteries as well as prolonged exposure to anoxia (nitrous oxide) conditions. It was noted that the brains of immature rats cannot be damaged by nitrous oxide alone (Hicks et al. 1962), so the Levine preparation of unilateral arterial occlusion was applied to immature rats to produce an ischemic infarction (Rice et al 1981). Seven-day-old rat pups (P7) are anesthetized and the left common ceratoid artery is exposed and ligated. After a 4-8 hour recovery period, the pups are placed in airtight chambers and exposed to 8% oxygen for 3.5 hours to create sufficient hypoxic conditions. This resulted in highly consistent infarcts in animals (92%) and a low mortality rate with no convulsions or abnormal turning behavior for the first 48 hours after injury. This model, therefore, is attractive when investigating the immediate effects of the hypoxic-ischemic insult on brain morphology. Rice et al. found that the areas most damaged in this model included the posterior cerebral cortex, the hippocampus, and basal ganglia, which is similar to injury in a human neonate.

Since its introduction, the Rice-Vannucci model has been used in a wide-variety of experiments to assess the pathophysiology of neonatal hypoxic-ischemia. Various treatments for reduction of injury size, including hypothermia (Saeed et al. 1993), nitrous

oxide inhibitors (Trifiletti 1992), and free radical inhibitors (Thordstein et al. 1993), were discovered using the Rice-Vannucci model. One study found that posthypoxic glucose supplements reduce the size of infarct in immature rats (Hattori and Westerlain 1990). P7 rats were exposed to a bilateral ligation of the ICA and 8% oxygen for 1 hour, a slight modification of the original model. Glucose supplements were immediately administered via IP injections and brain damage was assessed histologically 72 hours later. Glucose administration significantly decreased infarct size in the cortex by 37% and attenuated further injury to the striatum and the hippocampus. This result suggests that glucose administration may be influential in management of ischemia as close to the onset of symptoms as possible.

The Rice-Vannucci model is associated with a large degree of variability in juvenile and adult rats (and little work has been done to investigate the impairments during the neonatal stage that lead to these deficits later in development). A recent study was performed to examine the development of these neurological reflexes in neonatal rats following hypoxic ischemia (Lubics et al. 2004). Twelve P7 rats were exposed to unilateral ligation of the common carotid artery and 8% oxygen for 2 hours. Temperature was controlled at 37°C throughout surgery. Physical characteristics such as eye opening and weight were observed, as well as a variety of neurological reflexes, such as righting reflex, auditory startle, and negative geotaxis. These tests were all measures of neurological development used by our lab and will be described in greater detail in the methodology. The experimenters found neonatal pups exposed to hypoxic-ischemic conditions were severely delayed in all measures of development and somatic growth

during the two-week observation period post surgery. Interestingly, while animals were exhibited retardation of motor coordination they reached normal performance during a follow-up at 5 weeks. This result supports previous findings that neurological deficits are not present in neonatal stroke survivors when observed years after injury (Westmacott et al. 2009).

However, there are problems with the Rice-Vannucci model. Perhaps the most important is the lack of clinical relevance. In a recent review, it was determined that the most common cause of neonatal ischemic stroke is occlusion of the middle cerebral artery via thromboembolism from placental circulation with global hypoxia (Nelson and Lynch, 2004). The Rice-Vannucci model essentially creates thrombosis through ligation of extracranial arteries and starvation of the entire brain of oxygen via hypoxia. Despite the high level of reproducibility, one may question if the Rice-Vannucci model is an accurate model of actual events that transpire during neonatal stroke. An alternative model involving MCA occlusion would be more relevant to actual neonatal ischemic events.

The middle cerebral artery occlusion model (MCAO) of focal cerebral ischemia has been well documented in adult rats (Koizumi et al. 1986). The model involves the introduction and advancement of a 0.25 mm nylon filament into the external carotid artery (ECA) to occlude the MCA. Reperfusion is incorporated in this model by removal of the filament 2-4 hours after introduction and brains are harvested and histologically examined for infarct size. It is highly reproducible and results in neocortical and

caudoputamen damage (Memezawa et al. 1992). Given the nature of the injury induced, a neonatal adaptation seems appropriate.

A pioneer study performed by Ashwal et al. used the MCAO model in neonatal rats to model the effects of reperfusion after injury, which is a common practice in neonatal intensive care (Ashwal et al. 1995). They anesthetized P14-P18 spontaneously hypertensive rats using 2% isoflurane and maintained body temperature at 37°C for the duration of surgery. An incision was made on the ECA and a 0.07mm nylon filament was passed through it into the MCA to occlude it. The filament was sutured into place only to be removed 1.5, 2, 3, or 4 hours later for a 24-hour reperfusion period. After reperfusion, the animals were sacrificed and the brains were stained using 2,3,5-triphenltetrazolium chloride (TTC). In the healthy brain, TTC is converted to a red-formazan product by mitochondrial oxidative enzymes (Saeed et al. 1993). However, after prolonged exposure to ischemic conditions the mitochondrial enzymes are rendered inactive, resulting in failure of TTC conversion and a pale stain in the area of infarction (Isayama et al. 1991). Results indicate consistent, well-defined infarct volumes (38%-54%) in 80% of animals exposed to MCAO for up to 4 hours. Only 25% and 50% of animals exposed to MCAO for 2 and 3 hours respectively showed signs of cerebral infarction. The 4 hours exposure to MCAO occlusion followed by a 24-48 hour reperfusion period has become the standard when used to model neonatal focal cerebral ischemia.

This model more accurately depicts injury following a focal occlusion of the MCA that is commonly seen in neonatal ischemic events (Nelson and Lynch 2004). In addition, the use of TTC staining allows for well-defined analysis of infarct size,

suggesting high reproducibility with infarcts present in 90% of cases (Memezawa et al. 1992). However, perhaps the most glaring problem with the model is the age of MCAO in the neonatal rat. When this study was performed, the introduction of the nylon filament was limited by the size of the arteries that encapsulated it. Therefore, the filament was too large to be used on pups younger than 14-18 postnatal days without fatally damaging the artery. It has been noted that a P7 rat is developmentally similar to that of a newborn human (Donaldson 1918; McIlwain and Bachelard 1971) and that many biochemical and pathological changes occur within the rat between P7 and P14, such as maturation of receptor systems (McDonald et al. 1990), cerebral blood flow (Nehlig et al. 1989), and overall energy consumption (Yager et al. 1997). Therefore, it seems that the MCAO model of focal cerebral ischemia is more a model of juvenile stroke than neonatal stroke.

Shortly after this model was introduced, Derugin et al. developed a new model with the goal of inducing focal cerebral ischemic strokes via transient occlusion in the middle cerebral artery in P7 Sprague-Dawley rat pups (Derugin et al. 1998). It involves the induction of a 7-0 suture with a silicon tip into the MCA via the ECA to induce ischemia and removal after 3 hours for a 24-hour reperfusion period. Following reperfusion, animals were sacrificed and their infarcts were measured using the TTC method as previously described. They found that 50% of the pups exhibited infarctions following surgery with about 35% of the ipsilateral hemisphere damaged. While these numbers are smaller than those observed in the Ashwal et al. model, a comparison might be unwarranted due to the differences in age. However, the main drawback to this model is the technical complexity involved. Sutures must be inserted, secured, and then

removed without damaging the delicate vessels, which is an extremely difficult task. In addition, the variation in rat size at P7 (9-13g) means that the vessels are at various sizes as well, making the model inherently variable in outcome. Due to low success rate and technical difficulties, this model is not frequently used to model neonatal FCI.

The demand for a reproducible, clinically relevant, technically accessible model has lead the Eckman lab to develop our own model of focal cerebral ischemia in the P7 Sprague-Dawley rat (Wen et al. 2004). Similar to the model developed by Derugin et al, our model involves occlusion of the MCA by induction of a suture embolus with cerebral infarction measured by TTC staining, but with some notable differences. First, we use a smaller 8-0 suture tailor-made to each pup according to size. We coat the sutures in silicone according to the procedure used by Derugin et al, however, the diameter of the coated end was selected based on the body weight of the pup. This allowed for a smoother presentation of the suture into the cranial arteries with less chance of damaging them. Second, we make the incision on the central carotid artery (CCA) near the CCA-ECA-ICA junction compared to the ECA incision in the previous model, which allows for easy feed of the embolus into the MCA. Lastly, there is no reperfusion in our model. We do this for two reasons. First, reperfusion injury is well documented in both adults and children (Fellman and Raivio 1997). By limiting our model to damage caused by the ischemic event, we can get a more accurate observe the pathology of deficits caused by the embolism. Secondly, there is a large population of neonates who go undiagnosed for FCI until later in life so immediate reperfusion is not an option for them (Agrawal et al 2009). Furthermore, only children diagnosed with FCI caused by genetic factors or heart

malfunctions are given anticoagulant drugs and not children with a single event, so reperfusion is not a common practice for the majority of neonates suffering from FCI (Monagle et al. 2012). A more comprehensive review of treatment options for FCI will be discussed later. For these reasons, we feel that a permanent embolism is more clinically relevant than reperfusion. Our model results in damage to the ipsilateral neocortex and striatum with an 89% success rate, however there are no long-term behavioral data associated with this model. With a well-defined set of behavioral criteria, our model may then be used to assess novel therapeutic agents for focal cerebral ischemia.

Treatment Strategies

The treatment of focal cerebral ischemia for neonates is primarily focused on observation and monitoring to detect an ischemic event. While clinicians can treat the seizures associated with ischemia, many of the neonates are asymptomatic, suggesting that this therapy may not target the primary issue of ischemia. The neonatal intensive care unit utilizes anticoagulation therapies, such as unfractionated heparin, in some neonates with arterial ischemic stroke. The 2012 American Association of Chest Physician guidelines suggests a regimen of heparin treatment for neonates with arterial ischemic stroke and a documented cardioembolic source for the impairment (Monagle et al. 2012). There is a risk when taking anticoagulants as they may promote bleeding and further brain damage. If a source for the embolism is found, then it more likely that the neonate will have a reoccurrence of ischemic and the risks of unfractionated heparin are outweighed by the benefits. However, for a neonate with a single ischemic event, which is a majority of the population of interest, there is currently no therapy administered by

the NICU other than treatment of symptoms. However, recent work suggests that caffeine citrate may be an effective neuroprotective agent for ischemic stroke damage in the neonate.

Caffeine citrate is among the top ten medications used in the neonatal intensive care unit (Clark et al. 2006) and is primarily used for the treatment of apnea of prematurity. Apnea of prematurity is a condition in which premature infants stop breathing for short intervals during sleep. Murat and colleagues determined the efficacy of caffeine on preterm infants (gestational age 29-35 weeks in well-controlled study (Murat et al. 1981). They divided 18 infants into a control and treatment group and studied the effects of caffeine of apneic episodes at 24-hours and 5 days. They determined that there were significantly fewer instances of severe and mild apnea in the treatment group. Furthermore, no treatment other than caffeine was required in in the first group, while six of the nine infants from the control group were treated with caffeine for their severe apnea since lack of such an effective treatment would be unethical. Since then, caffeine has been used in the NICU as the primary treatment of apnea of prematurity. The citric acid group was added to increase solubility and transport across the blood brain barrier and the improvement in efficacy has been well documented (Buck et al. 2008). The proposed mechanism for caffeine citrate is activation of respiratory systems, resulting in increased oxygen consumption in apneic babies, but the actual mechanism has yet to be worked out.

Interestingly, apnea in the neonate is intimately linked with ischemic stroke. Neonates with apnea have a 2-fold higher risk for ischemia and death, with risk

increasing with severity of apnea (Yaggi et al. 2005). A study by Martinez-Garcia and colleagues found that in a population of neonates with apnea and ischemia, an increase in positive airway pressure resulted in a 81.4% reduced risk for a recurrent ischemic event to occur (Martinez-Garcia et al. 2005). Furthermore, treatment with caffeine for apnea in the neonate is associated with a decrease in incidence of cerebral palsy and cognitive delay, 4.4% and 34% respectively (Benitz, 2008). Caffeine citrate treats the long-term deficits associated with FCI as well as aids in prevention of recurring ischemic events. For this reason, along with its prevalence in the NICU, caffeine citrate is an attractive candidate for ischemic stroke therapy.

A recent study confirmed caffeine's neuroprotective effects on long-term behavioral deficits in a model of hypoxic-ischemia (Alexander et al. 2013). Alexander and colleagues examined the effects of caffeine administration on behavior in a P7 adaptation of the Rice-Vannucci model of hypoxic ischemia. Following a 2-hour hypoxic period, pups were immediately received an ip injection of caffeine (10mg/kg) or sterile saline for controls. Behavior was monitored on P85 with a water escape task and on P90-P95 with a Morris Water Maze (MWM) task. This task required the animals to find a partially submerged platform in order to stop swimming. This test was used as a measure of spatial memory and motor control. Statistical analysis showed a significant effect of caffeine treatment following hypoxic-ischemia with treated animals having a lower latency to find the platform in the MWM than the vehicle-treated hypoxic animals. Hypoxic animals treated with caffeine showed increased spatial memory and motor control than those hypoxic animals treated with vehicle. The volumetric analysis as well

as the water escape task did not exhibit significant differences, but this is most likely due to the small sample size of the treatment (n=10) and control groups (n=9).

This study supports the neuroprotective hypothesis for neonatal ischemic stroke, however the clinical relevance is again in question. Hypoxic-ischemia is not the most prevalent mechanism for ischemic stroke in the neonate, so a model of MCAO in the P7 rat may be more relevant. Furthermore, the experimenters administered one dose of caffeine immediately after injury, but that would most likely not be the case in the NICU. Infants in incubators are most often placed on a caffeine drip to prevent apnea (Clark et al. 2006). Any further beneficial or deleterious effects of caffeine accumulation in the neonate cannot be accounted for by this study. Finally, caffeine citrate was not used, which can only improve the ability of caffeine to cross the blood brain barrier. No study has been performed to address these further questions and requires further research.

Research Goals

The new model of focal cerebral ischemia in the neonatal P7 rat developed by Wen and colleagues may be the most clinically relevant and reproducible model to date (Wen et al. 2004). However, there is no evidence regarding the short-term or long-term behavioral outcomes of these rats following FCI. While an *in vitro* study allows experimenters to see the effects of ischemia on the cellular level such as the ischemic cascade, an *in vivo* study allows experimenters to assess the efficacy of both the MCAO method as a model system as well as caffeine citrate for treatment. By monitoring behavioral changes over development, we can assess the comprehensive effects of the initial FCI event as well as any therapeutic potential of caffeine thereafter. The goal of

my study was to investigate the neurofunctional outcomes of neonatal rats in a model of focal cerebral ischemia following caffeine treatment. Various sensorimotor and physiological outcomes were assessed with a battery of behavioral tests in our model. There is no research to date investigating the long-term neurofunctional outcomes of neonatal rats following this method of MCAO. Furthermore, no data regarding the efficacy of caffeine citrate is present for this model.

In my study the following questions of interest were posed: 1) whether the new model of MCAO induces significant impairment in behavior compared to controls, 2) whether caffeine citrate may be used to rescue deficits in FCI pups compared to FCI pups treated with saline, and 3) whether there are any adverse effects of caffeine administration in healthy sham animals. To test these questions, a battery of non-invasive behavior tests was compiled from literature and FCI and sham-operated animals were assessed for two weeks post surgery. Once deficits were characterized, either caffeine citrate or saline control was administered to the pups via ip injections. After a 14-day period post-FCI, the rat brains were harvested and infarct volume was assessed to ensure successful surgery. Based on previous studies, it was hypothesized that our model of MCAO will result in physiological, motor, and sensory developmental impairments compared to sham animals. It was also hypothesized that caffeine will improve the behavioral outcomes in FCI animals as compared to sham animals. Finally, it was predicted that there would be no adverse behavioral effects on sham animals treated with caffeine.

Methodology

Behavior Experiment

Subjects

A total of 62 Sprague-Dawley rats (29 female, 33 male) were used for the behavioral portion of this study. Rats were obtained either from breeding animals on site at Drew University or from Harlan Laboratories at P1. Litters ranged from 8-14 rats with an average size of 10 pups. All animals were treated ethically in accordance with Drew University's Internal Review Board standards.

FCI Surgery

Focal cerebral ischemia was induced in P7 rats via and introduction of a suture embolus into the middle cerebral artery (MCA). For the most successful indication of FCI, each suture was tailored to each individual pup's weight. A thorough description can be found in Wen et al. 2004 and a visual schematic can be found in Figure 3. Pups were anesthetized using 2% isoflurane in oxygen in a anesthesia chamber with vapomatic. After 5 minutes in the chamber, the animals were fixed on their backs and flow rate was reduced to 1% isoflurane. A midline incision was made at the neck to expose both the central carotid artery (CCA) and external carotid artery (ECA), which were then ligated using a 6-0 silk suture to limit collateral branching from the MCA. The internal carotid artery was isolated and a microaneurysm clip was placed toward the rostral end. A small incision was made near the caudal end of the CCA and the corresponding suture embolus was advanced up to the microaneurysm clip. The clip was then removed and the suture was advanced another 7 mm to fully occlude the MCA. The clip acted as a guide to

ensure the suture reached target and did not branch off into any adjacent arteries. A final 6-0 silk suture was used to ligate the ICA around the suture embolus to hold it in place. This method ensured a permanent injury with no chance of spontaneous reperfusion. The midline incision was closed with adhesion glue and pups were then placed in a heated cage. Since hypothermia is a very real concern when conducting surgical procedures on animals, the surgery was performed under a heat lamp and animals were kept warm until they awoke. Sham-operated animals received the same surgical procedure without the induction of the suture embolus to the MCA. To determine the success of surgery, brains were harvested and tissue health was assessed using a triphenyl tetrazolium chloride (TTC) stain. This compound interacts with metabolically active tissue, staining it red, while staining metabolically inactive tissue white. An example can be found in Figure 4.

Behavioral Tests

A battery of behavioral tests were conducted on both FCI and sham animals starting 24 hours post-surgery and ending two weeks post-surgery (P22). A detailed behavioral schedule can be found in Table 1. This schedule was generated using the expected emergence of the desired behavior as outlined in Lubics et al. 2005. A detailed description of each test is included below.

Eye Opening: Each day whether or not the pup's eyes were open was recorded with a yes or no.

Body Weight: Each day the pups were weighed and their body weight was recorded in grams.

Righting reflex (surface): Pups are placed on their backs and allowed to roll over. Time (sec) and direction of turn are recorded. This test measures motor development with special attention paid to the hind limbs.

Righting reflex (air): Pups will be dropped on their backs from a height of 20cm and allowed to right themselves in mid-air. Time (sec) and direction of the turn are recorded. Similar to the previous test, the air adaptation assess the pups' reaction time to a fall.

Negative Geotaxis: A ramp was constructed at a 30-degree angle from table top. The pups were placed on it facing downward and allowed to orient themselves the opposite direction. Time (sec) and direction of turn was recorded with a maximum of 90 seconds. This test measures the development of proprioception in each pup.

Cliff Avoidance: The front paws of each pup were placed over the edge of a tabletop and they were observed as they tried to avoid the cliff using their hind limbs. If accomplished, it was recorded as yes (Y), if not a no was recorded (N). Direction of avoidance was also recorded. Another test of proprioception, this test also assesses the motor development in the hind limbs as they are necessary for successful completion.

Startle Response: The experimenter administers a loud noise or yell and a startle reaction (flinching, freezing, or avoidance) is observed in the rat pup. Recorded as a yes (Y) or no (N). This test assesses the sensory development of the pup.

Tactile Startle: A puff of air is administered to the face of each pup, and a startle reaction is observed. Recorded by a yes or no. This test assesses the sensory development of the pup.

Walking Initiation: Each pup is placed in the center of two concentric circles (10cm and 30cm respectively) drawn by chalk. The pups were allowed to roam freely throughout the circles and time (sec) to leave each circle as well as any unilateral directional preference was recorded. This test assesses both propensity of motor initiation of the pups and a crude measure of cognitive ability if they can recognize the chalked line.

Square Bridge: Rat pups are placed in the center of a bridge (3.5cm width) connected to two square platforms raised about 20 cm of the ground. The time (sec) to traverse the bridge as well as the platform chosen was recorded with a maximum of 90 seconds. If the pup did not complete the task after 90 seconds or fell off it was considered a failure and was marked by an “F”. This test assesses the balance and coordination of the pups.

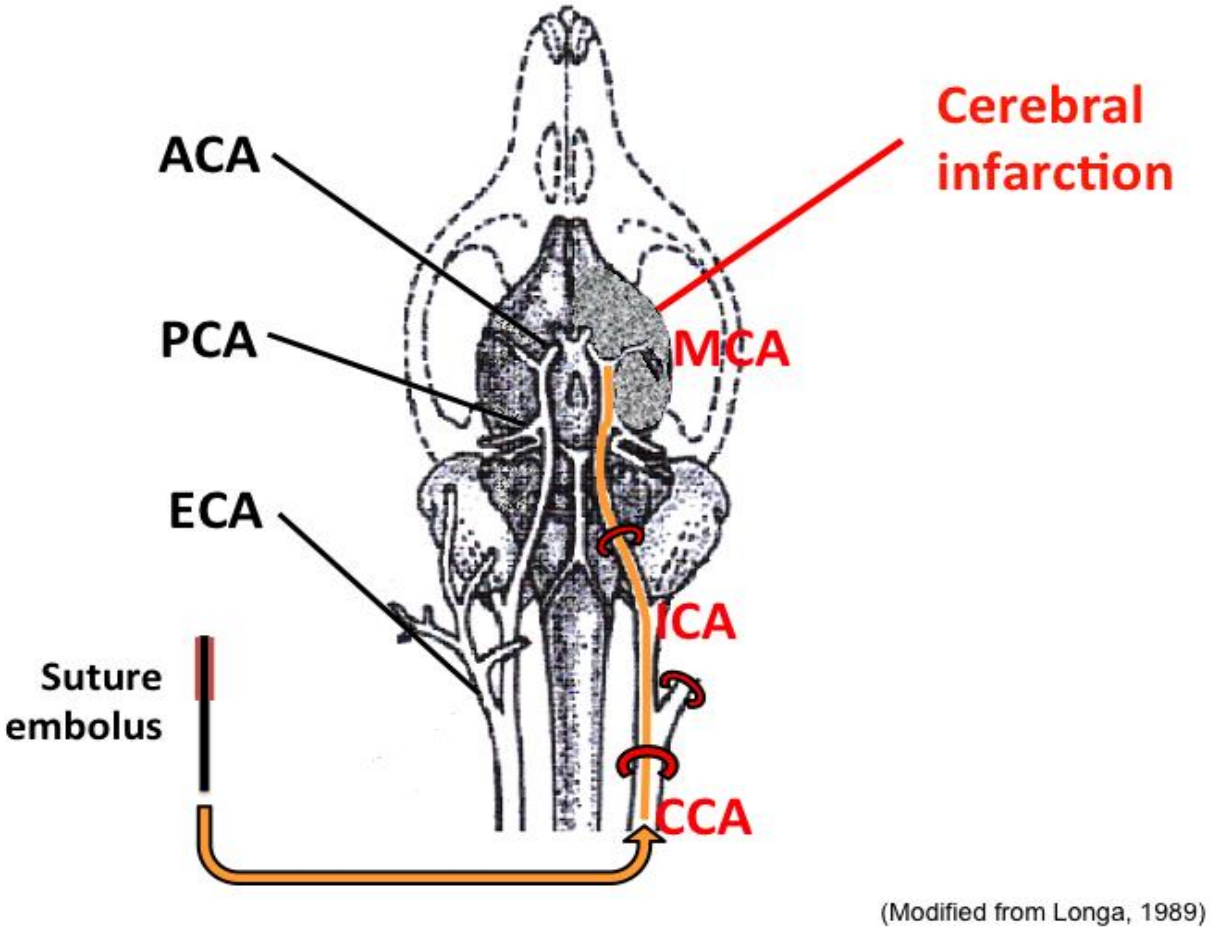

Figure 3: Schematic of FCI surgery. The custom suture embolus is introduced to the CCA and advanced to occlude the MCA. Sham-operated animals are done the same way minus the suture embolus.

Table 1: Behavior schedule for FCI and SO rats. The days indicate day of life for the animal. Checks indicate the test was performed on that day.

Test	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
Eye Opening (Y or N)	✔	✔	✔	✔	✔	✔	✔	✔
Body Weight (g)	✔	✔	✔	✔	✔	✔	✔	✔
Righting Reflex Surface (sec)	✔	✔	✔	✔	✔	✔	✔	✔
Righting Reflex Air (sec)					✔	✔	✔	✔
Negative Geotaxis (sec)		✔		✔		✔		✔
Cliff Avoidance (Y or N)	✔		✔		✔		✔	
Startle Response (Y or N)		✔	✔	✔	✔	✔	✔	✔
Tactile Startle (Y or N)	✔		✔		✔		✔	
Walking Initiation (sec)				✔		✔		✔
Square Bridge (sec)			✔		✔		✔	

Caffeine Experiment

Animal surgery and behavioral assessments were preformed as described above. Only those tests which detected significant differences between FCI and SO animals were used which can be found in the results section. After reviewing the data on the pilot behavioral study it was determined that the dynamic changes in behavior could not be captured with every other day assessment, so a consecutive schedule was developed and can be found in Table 2. A total of 30 FCI (17 A-treated, 13 B-treated) and 31 Sham (13 A-treated, 18 B-treated) were used in this experiment.

Drug Administration

For the duration of drug administration, experimenters were blinded to the identity of the drug. The Principal Investigator prepared two solutions, A and B, and the blind was broken only after data collection had ceased. Solution A was sterile saline

while solution B was caffeine citrate. In order to aid in solubility, a stock solution of caffeine citrate was prepared at 20 mg/ml according to a protocol provided by Bedford Laboratories. Caffeine citrate was injected daily via intraperitoneal injection beginning with a loading dose of 2 mg/kg at P5 and maintained with a 1 mg/kg injection until P14. Since the mechanism of action of caffeine is unknown, this dosing paradigm was used to capture any effects caffeine may have on the pups, both protective and restorative. Solutions were injected using 100 unit insulin syringes with half unit markings for higher dosing accuracy with smaller animals.

Sample Collection

At P28, all animals were sacrificed and brain tissue was extracted. Animals were given a lethal flow of isoflurane, decapitated, and the brain was removed and weighed. The suture embolus was also removed and sterilized for use in further surgical procedures. The brains were stored on dry ice for later analysis using western blots. In addition, several animals were sacrificed via a standard perfusion procedure. The rib cage was removed, the heart was punctured with a 16-gauge needle and the animal was perfused with phosphate buffered saline (0.01M PO_4, 0.0027M KCl, 0.138M NaCl, pH = 7.4) followed by 4% paraformaldehyde in PBS. The brain was removed and stored in sucrose for 48 hours then was kept in 4% paraformaldehyde until further analysis in a cryostat.

Statistical Analysis

All data were analyzed using SPSS version 18 courtesy of Drew University. The majority of the data, such as the weight gain and righting reflex tests, were assessed using

mixed model ANOVA to assess the interactions between surgery, drug, behavior, and time.

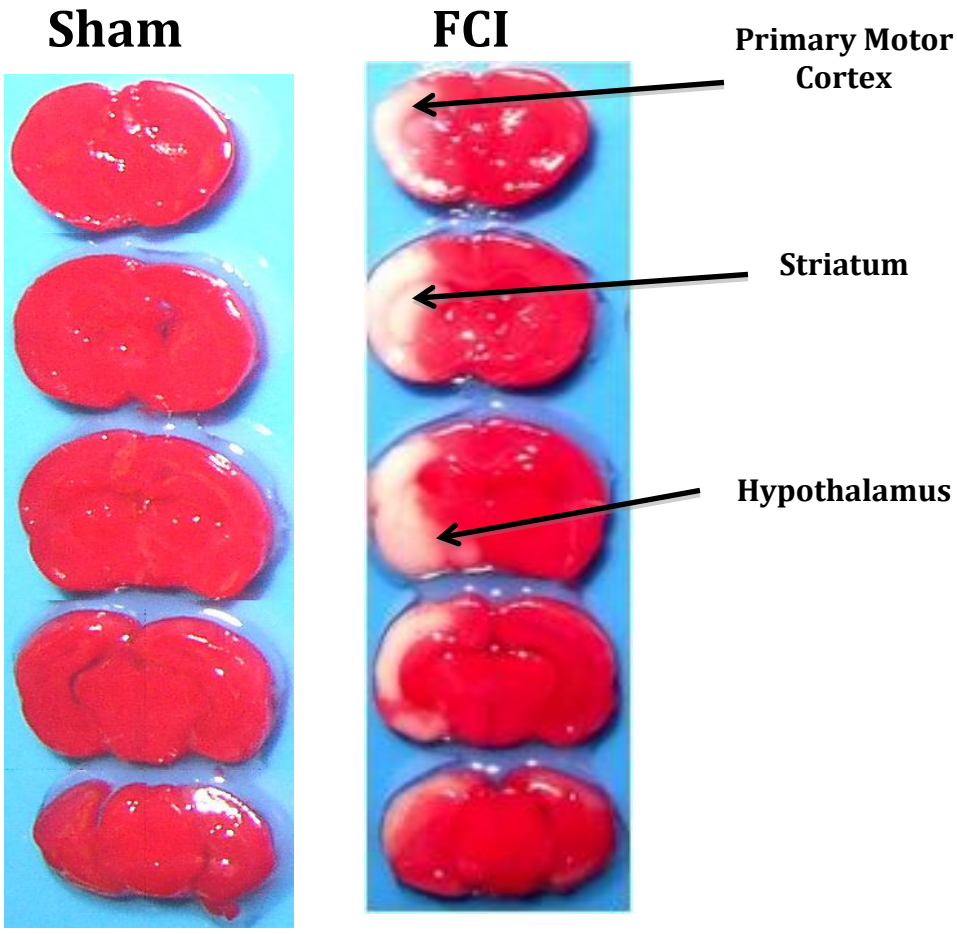

Figure 4: Coronal TTC stain following MCAO surgery to induce FCI. The surgery described above produces pronounced damage, most notably to the left primary motor cortex, striatum, and hypothalamus (shown in white). As expected, this damage was not seen in sham animals, which suggests it is a product of suture embolus induction.

Table 2: Revised behavioral schedule for FCI and SO animals. Days indicate days of life of the animal. Checks indicate the test was done on that day.

Test	Day 8 - Day 16		Day 17 - Day 22	
Eye Opening (Y or N)	✔	✔	✔	✔
Body Weight (g)	✔	✔	✔	✔
Righting Reflex Surface (sec)	✔	✔	✔	✔
Startle Response (Y or N)	✔	✔	✔	✔
Walking Initiation (sec)			✔	✔

Results

Experiment 1: Behavioral Results

FCI animals are significantly impaired in weight gain, righting reflex-surface, and auditory startle

A total of 62 animals (30 FCI, 32 Sham) were used in this experiment. According to the results of the mixed model ANOVAs, there is a statistically significant interaction between surgery groups and time for weight gain ($F_{(1.35, 785.78)} = 10.03$, $p = 0.001$). A post-hoc analysis was conducted to determine the nature of the interaction. As shown in Figure 5, Sham animals gained and maintained weight at a consistent level throughout the observation period. FCI animals followed a similar pattern of weight gain, however they maintained a significantly lower weight than sham animals throughout observation. It is

important to note that there was no difference in the average P7 weight prior to surgery for animals in the FCI and sham groups.

The mixed model ANOVA indicates a trend in the interaction between surgery group and time for righting reflex surface ($F_{(2.79, 2.18)} = 2.68$, $p = 0.054$). Post-hoc analysis showed that FCI animals are significantly impaired with larger latencies to right compared to sham animals at P10 ($p = 0.007$) and a trend at P8 ($p = 0.059$). However, as Figure 6 illustrates, this difference is quickly corrected by P12 ($p = 0.089$) and FCI animals were performing at the same level as sham by P18 ($p = 0.144$). FCI pups had a significantly more difficult time righting themselves than sham pups at P10, but soon corrected this deficit.

Figure 7 shows the auditory startle response for FCI vs. sham-operated animals. There was a significant interaction between surgery group and time for auditory startle response ($F_{(1.74, 289.35)} = 8.98$, $p = 0.001$). Post-hoc analysis indicates that FCI animals were significantly delayed in the development of a startle response at both P10 ($p < 0.005$) and P12 ($p < 0.038$). At P10, about 46% of FCI animals exhibited a response compared to 99% of sham animals. At P12, about 67% of FCI animals exhibited a response compared to 100% of sham animals. All FCI animals exhibited a response by P14.

The ANOVA results for the walking initiation test indicate a significant main effect of time. As shown in Figure 8, animals' latency to leave both the inner and the outer circle significantly lowered on subsequent days (P18 and P22) compared to the first

exposure to the test (P14). However, there was no significant effect of surgery on walking initiation.

As shown in Figure 9, the results of the ANOVA indicate a significant main effect of time ($p<0.005$) for the righting reflex-air test as all animals became faster over time. However, there was no significant effect of surgery. The cliff avoidance test, as shown in Figure 10, also exhibited a main effect of time ($p<0.005$), as well as a significant main effect of surgery ($p=0.012$) at one time point (P16). More sham animals, on average, were able to complete the task at P16 than FCI animals. Otherwise, there was no difference in rate of test completion. Finally, Figure 11 depicts the results of the tactile startle test and it is evident that there are no significant differences between the two groups. Only four time points are shown but there was no change over subsequent days.

The remaining tests, negative geotaxis and square bridge, did not yield meaningful data. Negative geotaxis is a measure of proprioception and was performed according to the above schedule (see Table 1). Animals successfully performed the test on the first day, but as their eyes began to open (P12), they preferred to explore rather than perform the test. They would run down the ramp rather than right themselves towards the top. This resulted in only one day of data and eliminated the analysis by time. Similarly, the square bridge test involved animals placed in the center of a bridge placed 20cm above the ground where they were required to traverse the beam to a platform on either side. Animals were too afraid to move and almost all attempts by both sham and FCI animals resulted in freezing behavior for over 90 seconds, which was denoted as a failure.

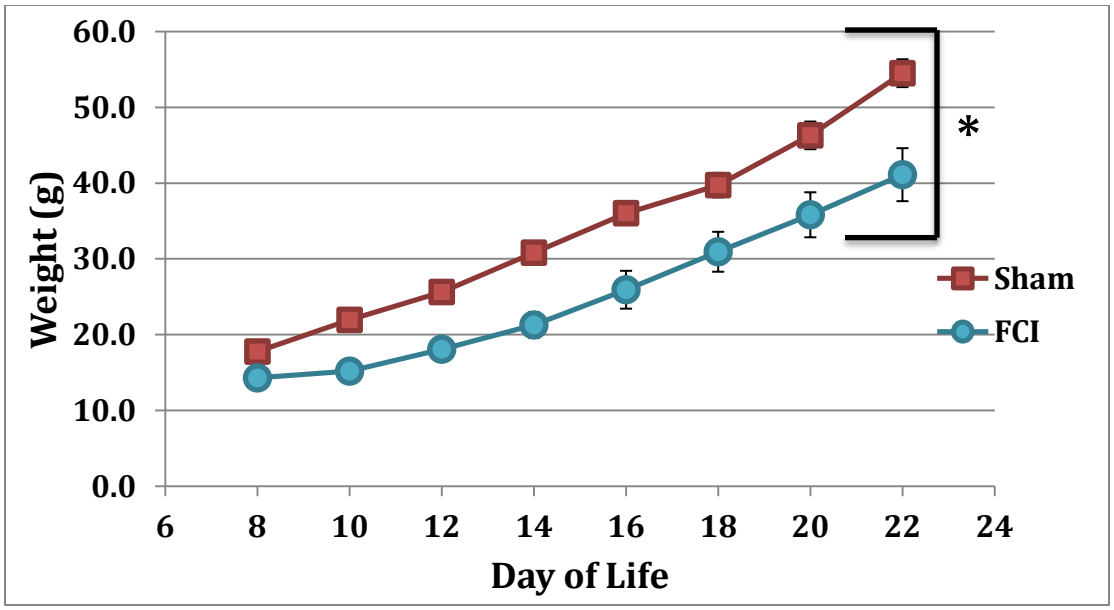

Figure 5: Weight gain for FCI and Sham-operated animals in Expt 1. The asterisk (*) denotes a significant interaction between surgery and time (p = 0.001). Sham animals gain more weight on average compared to FCI animals over time. All animals increased in weight over time, albeit at different rates. Data are mean ± SEM. Sham: n = 32. FCI: n = 30

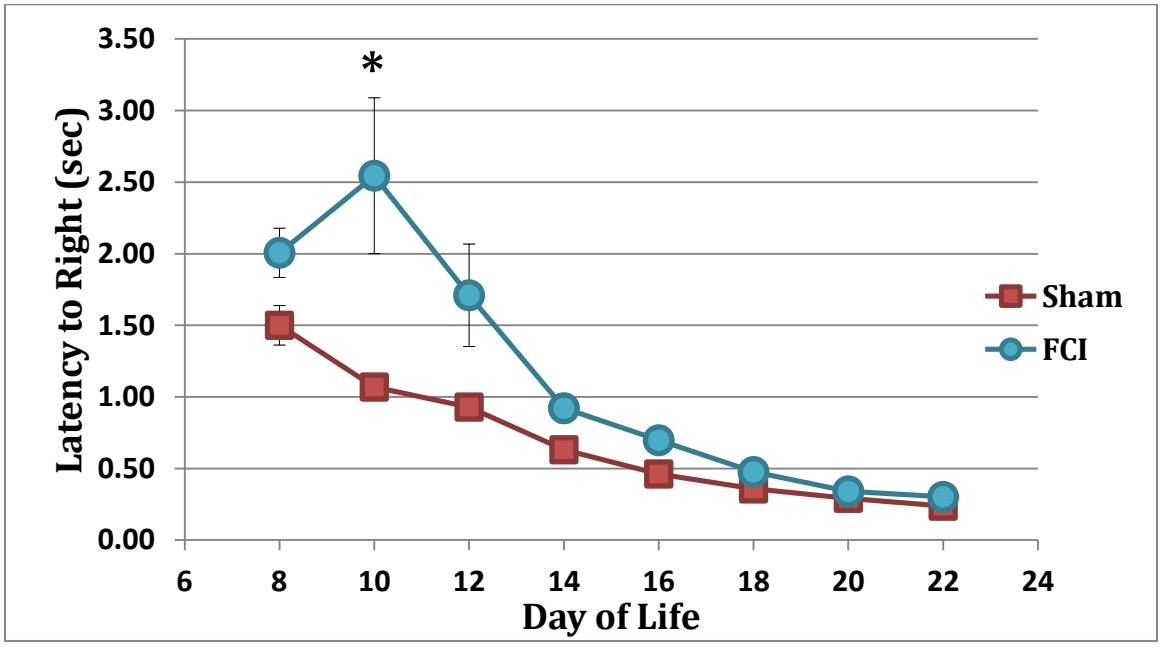

Figure 6: Righting reflex surface for FCI and Sham-operated animals in Expt 1. While there was a trend in the interaction between surgery group and time for this test (p = 0.054), it was really driven by a significant impairment in FCI pups (*) compared to controls at P10 (p = 0.007). Data are mean ± SEM. Sham: n = 32. FCI: n = 30

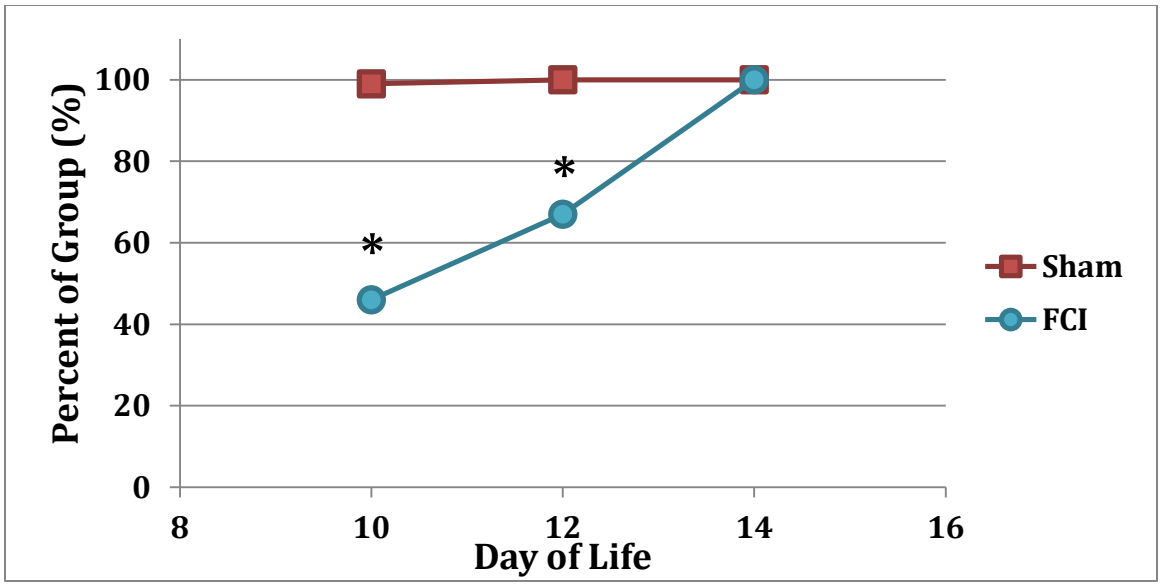

Figure 7: Startle response for FCI and Sham animals in Expt 1. The asterisk (*) denotes a significant interaction between surgery and time for startle response (p = 0.001). About 46% and 67% of FCI pups exhibit a startle response at P10 and P12 respectively. All animals exhibit a response by P14. Data are percent of total group members exhibiting a response for that day. Sham: n = 32. FCI: n = 30

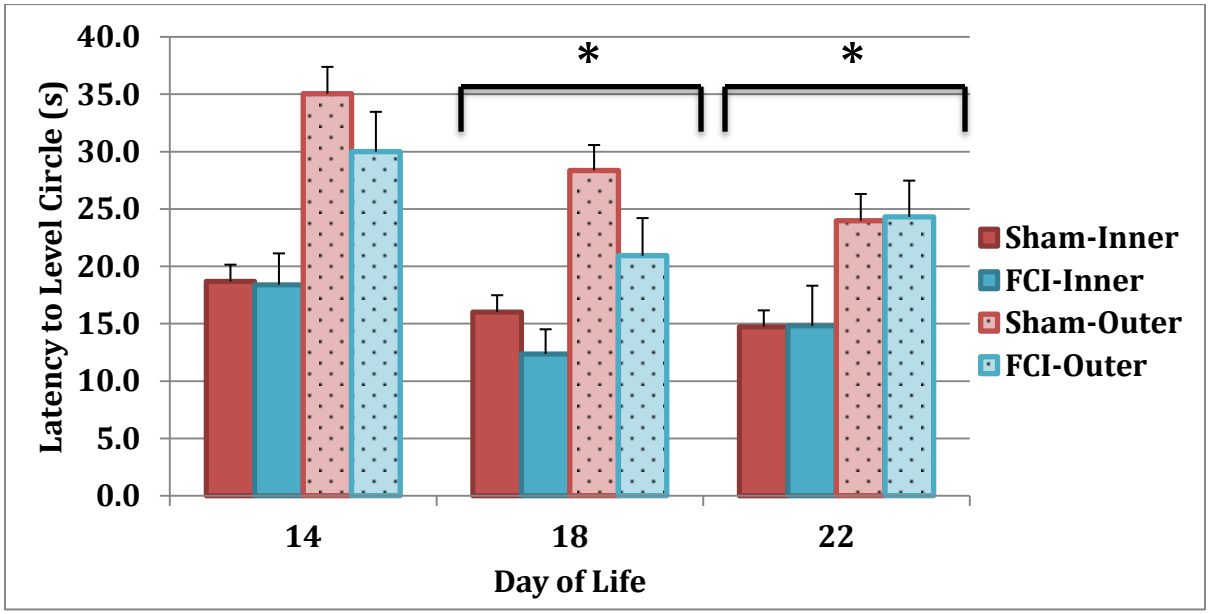

Figure 8: Walking initiation (inner and outer) for sham and FCI animals in Expt 1. There was a significant main effect of time animals' latency to leave both the inner and outer circles ($p<0.005$). The asterisk (*) indicates significant difference from corresponding group compared to P14. All animals left both the inner and the outer circles quicker on P18 and P22 compared to P14. However, there was no effect of surgery on latency to leave. Data are mean ± SEM. Sham: n = 32. FCI: n = 30

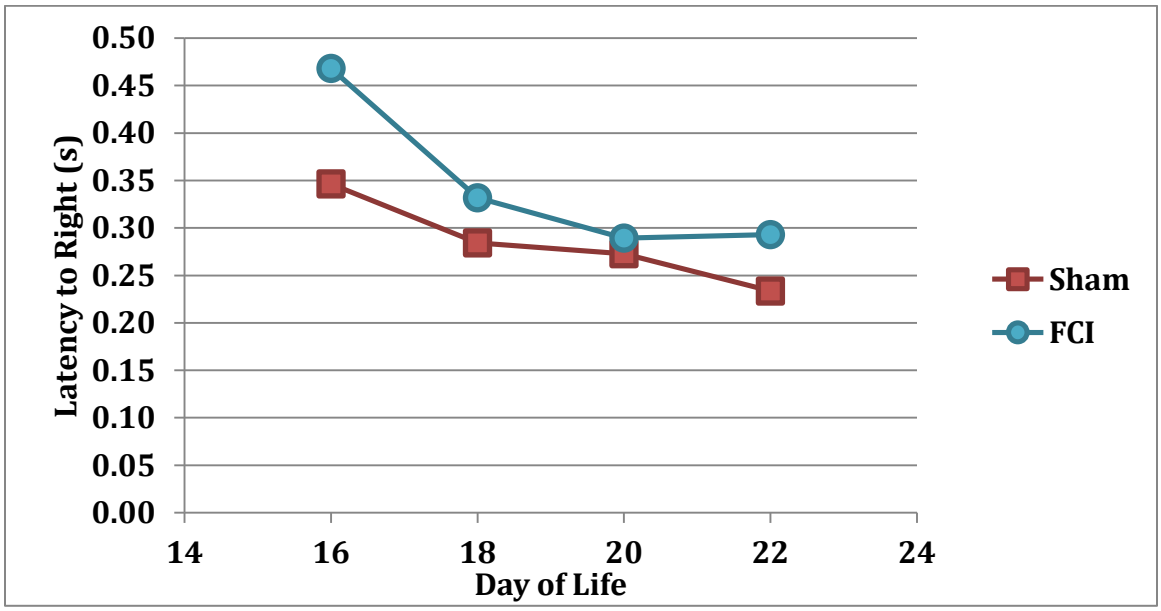

Figure 9: Righting reflex air for sham and FCI animals in Expt 1. There is a significant main effect of time on righting in the air ($p<0.005$). All animals righted faster over time compared to P16. There was no main effect of surgery. Data are mean ± SEM. Sham: n = 32. FCI: n = 30

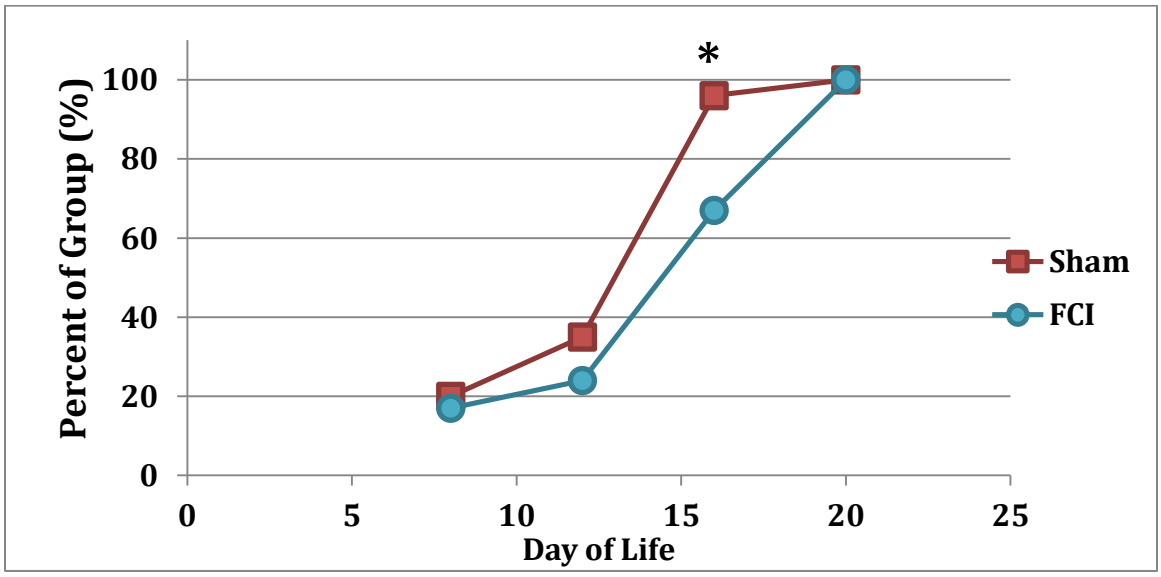

Figure 10: Cliff avoidance task for sham and FCI animals in Expt 1. There was a significant main effect of time on the amount of animals completing the cliff avoidance task ($p<0.005$). Significantly more animals completed the task over time compared to P8. In addition, there was significantly more sham animals ($p=0.012$) completing the task by P16 compared to FCI animals, denoted by an asterisk (*). Data are mean ± SEM. Sham: n = 32. FCI: n = 30

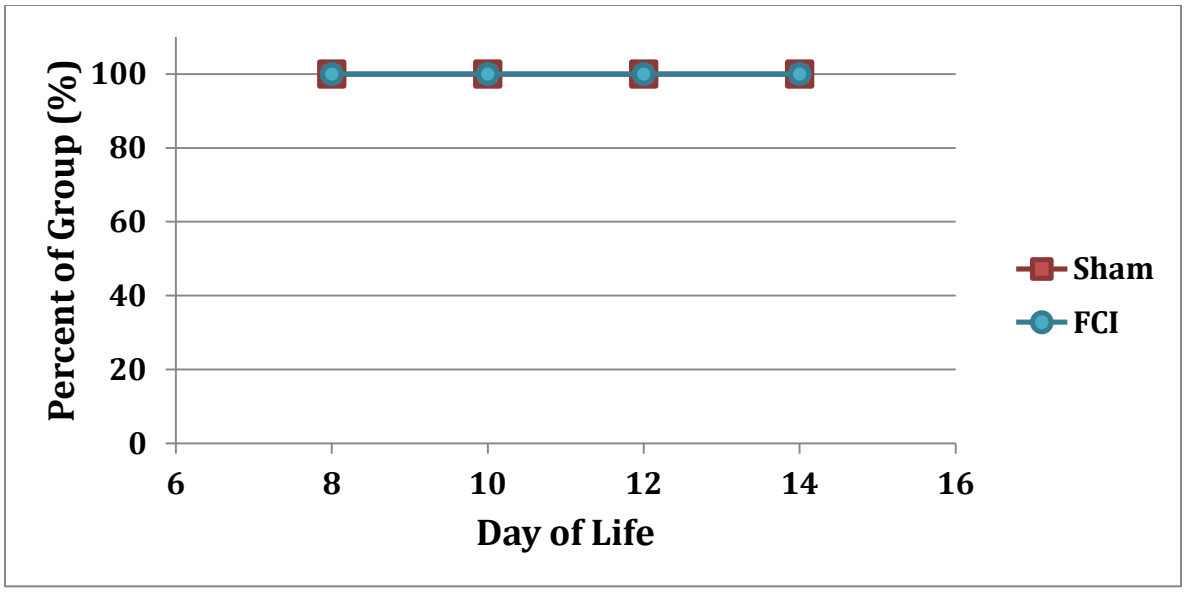

Figure 11: Tactile startle response for sham and FCI animals in Expt 1. There were no significant differences between FCI and sham animals in the emergence of tactile startle response. Data are percent of total group exhibiting response on that day. Sham: n = 32. FCI: n = 30

Experiment 2: Caffeine Results

A total of 61 animals (30 FCI, 31 Sham) were used in this experiment. All 61 animals received injections: FCI (17 caffeine-treated, 13 saline treated) and Sham (13 caffeine-treated, 18 saline-treated).

<u>FCI animals treated with caffeine are significantly smaller than surgery-matched controls</u>

According to the results of ANOVA, there is a significant 3-way interaction between surgery and drug treatment for weight gain over time ($F_{(1.78, 477.35)} = 6.36$, $p = 0.004$). The data shown in Figure 12 are labeled with different letters to denote significant differences between those groups. There is a significant main effect of time ($p<0.005$), as all groups increased in weight over time, albeit at different rates. Sham animals treated with caffeine (A) do not gain weight at a significantly different rate, on average,

compared to sham animals treated with saline (A) for all time points. Therefore, these two groups will be collectively referred to as sham to aid in comparisons.

FCI animals treated with saline (B) were significantly smaller than sham animals (A) for all time points. This finding is consistent with previous results (see Figure 5). FCI animals treated with caffeine (C) were significantly smaller than FCI animals treated with saline (B) for all time points with the exception of P8. It follows then that FCI animals treated with caffeine (C) gained weight at a significantly slower rate, on average, compared to sham control groups (A) throughout the observational period.

Caffeine treatment had no effect on FCI righting reflex impairments

The ANOVA results indicate a significant main effect of time ($p<0.005$) and a trend of surgery ($p=0.062$) for the righting reflex test. However, multivariate ANOVA showed no interaction between surgery and drug treatment over time. Figure 13 shows the pattern of change for the four groups of the observation period and it is evident that all groups righted faster over time.

However, analysis of the data from only the first week of testing (P8-15) shows a significant interaction between time and surgery ($F_{3.16,1.08} = 3.78$, $p = 0.011$). Sham animals were able to right themselves faster than FCI animals at all time points. It is likely that the lack of effect from P16 onwards masked this interaction when the entire data were analyzed together. Drug treatment had no effect on righting ability across groups.

FCI animals are significantly delayed in startle response regardless of drug treatment

There is a significant interaction between surgery group and time for startle response ($F_{1.88,2.11} = 20.64$, $p < 0.005$). As shown in Figure 14, FCI animals were significantly delayed in the emergence of an auditory startle response compared to sham animals until P11 when the animals caught up and the difference disappeared. However, FCI animals improved significantly from each day to the next, ultimately catching up to the sham animals completely by P12. Drug treatment had no effect on this developmental delay in FCI animals. It is important to note that caffeine had no negative effects on startle response emergence in the sham controls.

No difference of any kind in walking initiation task

After analysis by multivariate ANOVA, there was no effect main effect of surgery, drug or time on the walking initiation test. This was most likely due to the large degree of variability in all groups. The latency to leave the inner circle and outer circle were recorded in the same time trial so comparison of the two is irrelevant; the results are displayed separately in Figures 15 and 16 respectively. Neither the FCI insult or caffeine treatment effected animals' latency to explore a novel environment on any of the three test days.

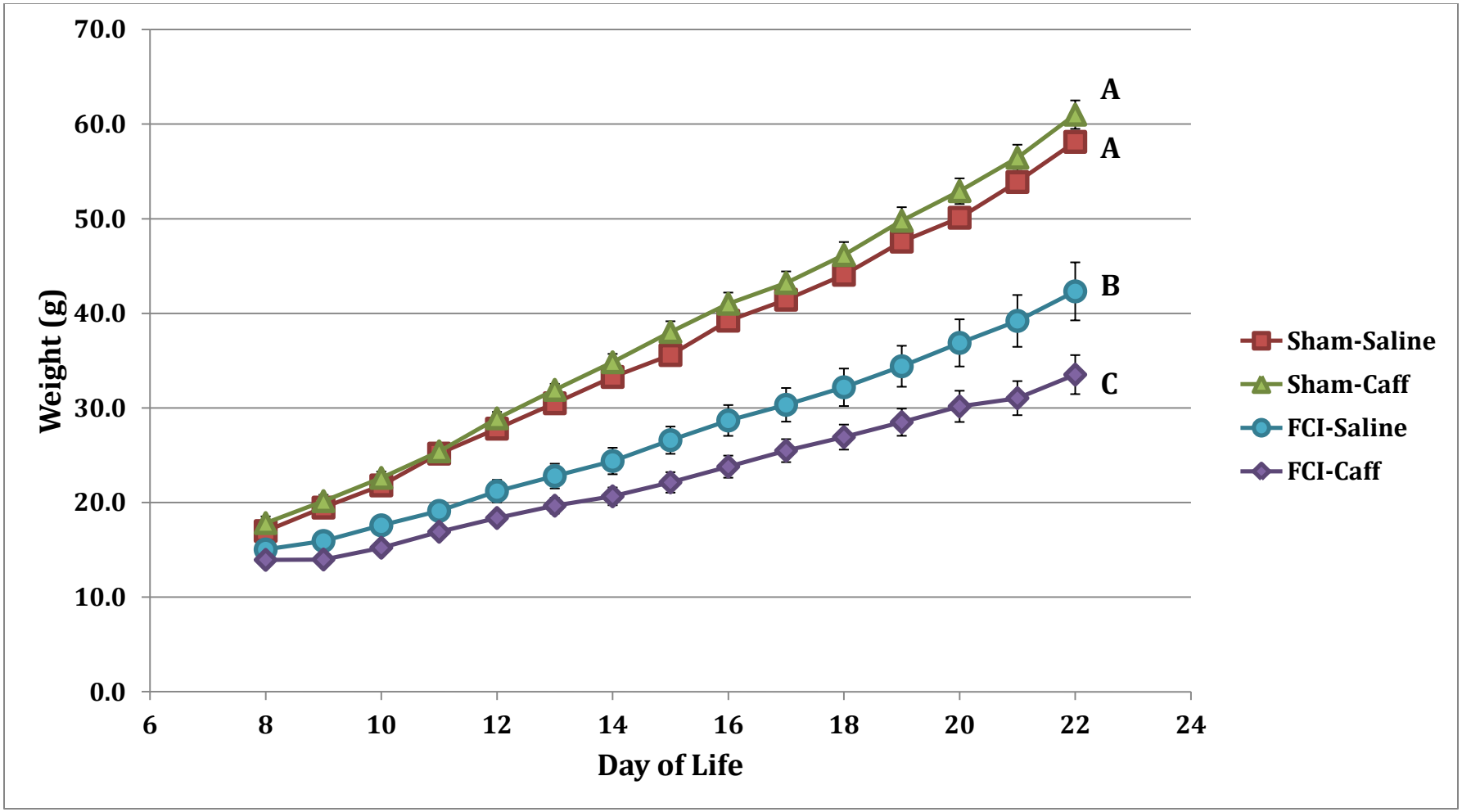

Figure 12: Weight gain rates for sham and FCI animals exposed to caffeine or vehicle in Expt 2. There was a significant 3-way interaction between surgery, drug, and time. Different letters are used to denote significant difference in rate of weight gain for all other groups ($p<0.005$). All groups significantly increased in weight over time. Data are mean±SEM. Sham: n = 31. FCI: n = 30.

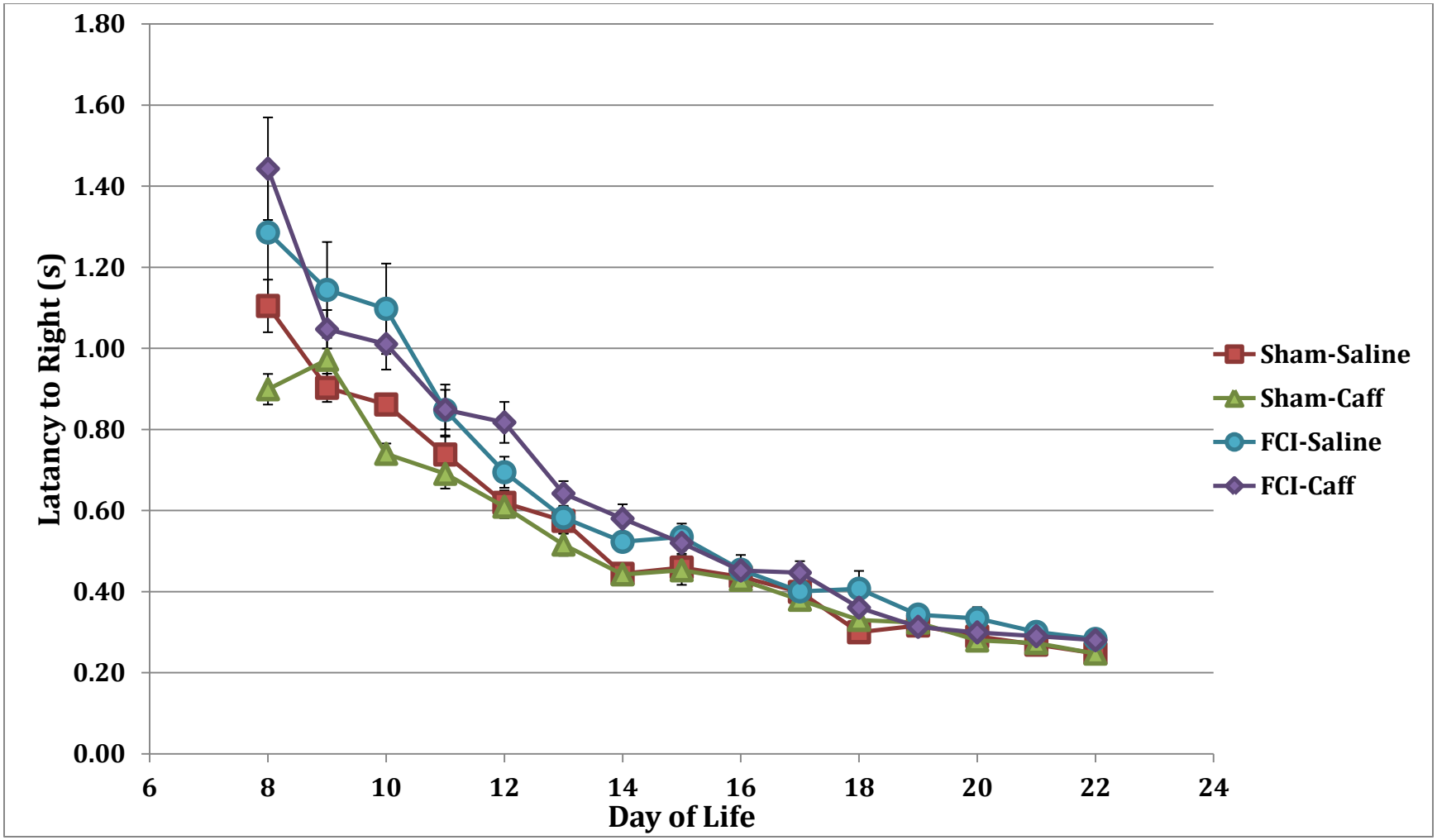

Figure 13: Righting reflex surface for sham and FCI animals treated with caffeine and saline in Expt 2. There was a significant effect of time ($p<0.005$) as all animals improved in righting from the first time point. There was a trend of surgery ($p=0.062$) and no significant effect of drug treatment. Data are mean±SEM. Sham: n = 31. FCI: n = 30.

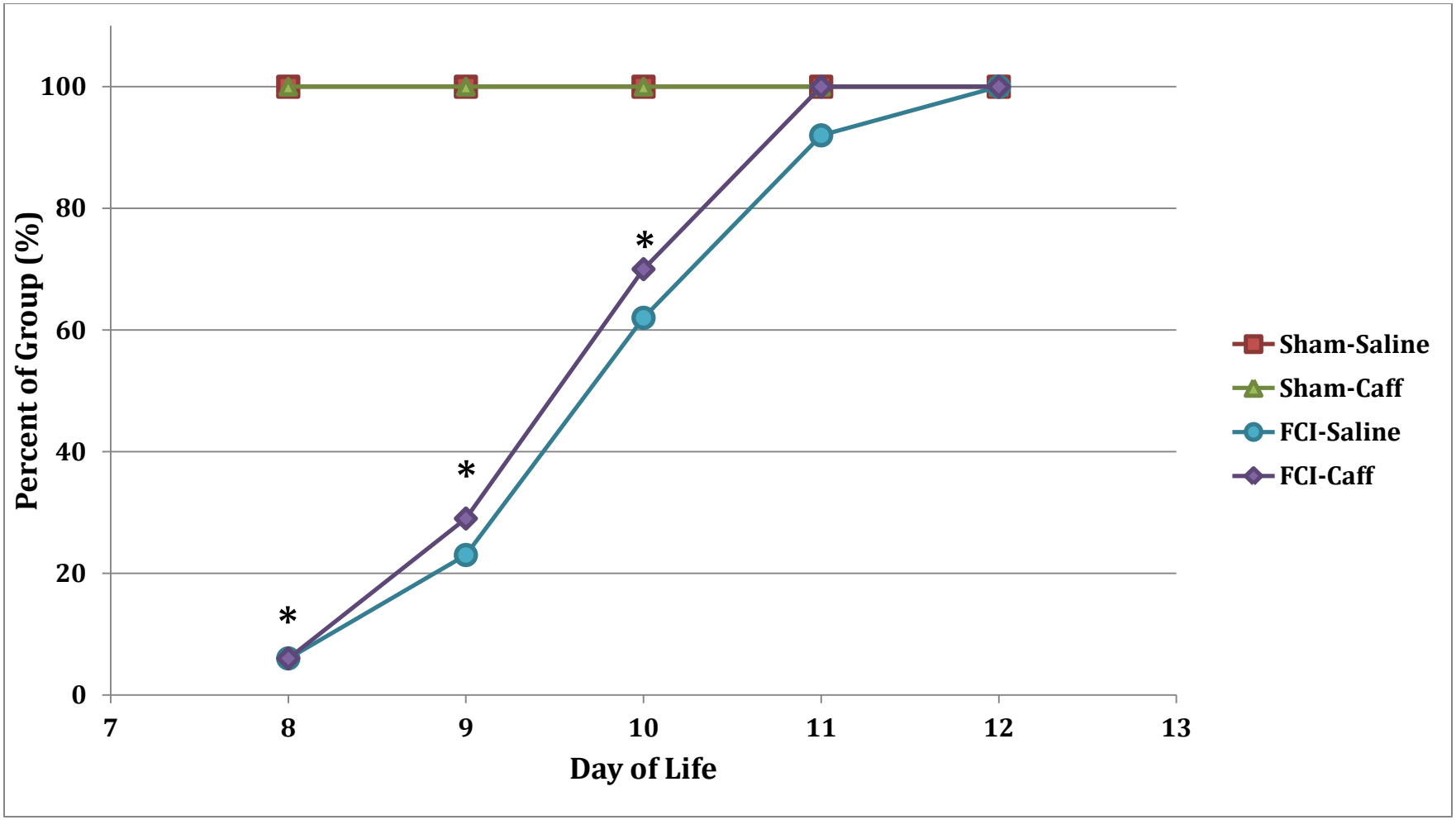

Figure 14: Auditory startle response for sham and FCI animals treated with caffeine and saline in Expt 2. The asterisk (*) denotes a significant interaction between surgery and time ($p<0.005$). FCI animals were significantly delayed in the emergence of the auditory startle response. There was also a significant main effect of time ($p<0.005$) as the number of FCI animals significantly increased over time from P8. There was no effect of drug treatment. Data are mean±SEM. Sham: n = 31. FCI: n = 30.

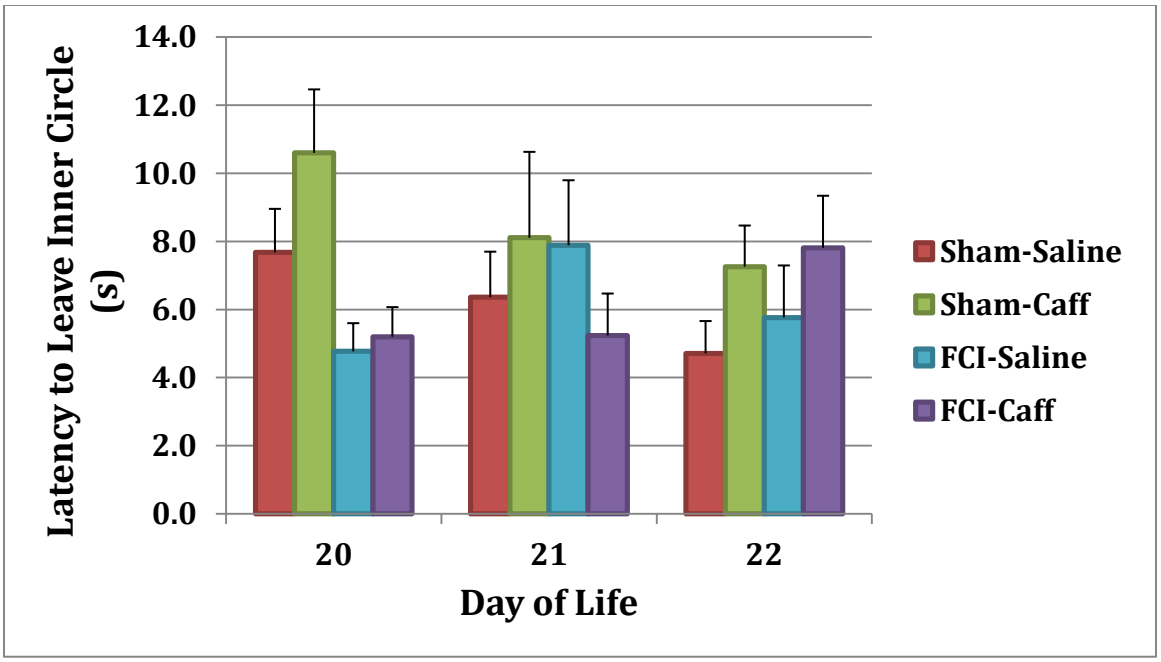

Figure 15: Latency to leave inner circle of walking initiation task for sham and FCI treated with caffeine and saline in Expt 2. There were no differences between surgery, drug treatment, or time for any group. Data are mean±SEM. Sham: n = 31. FCI: n = 30.

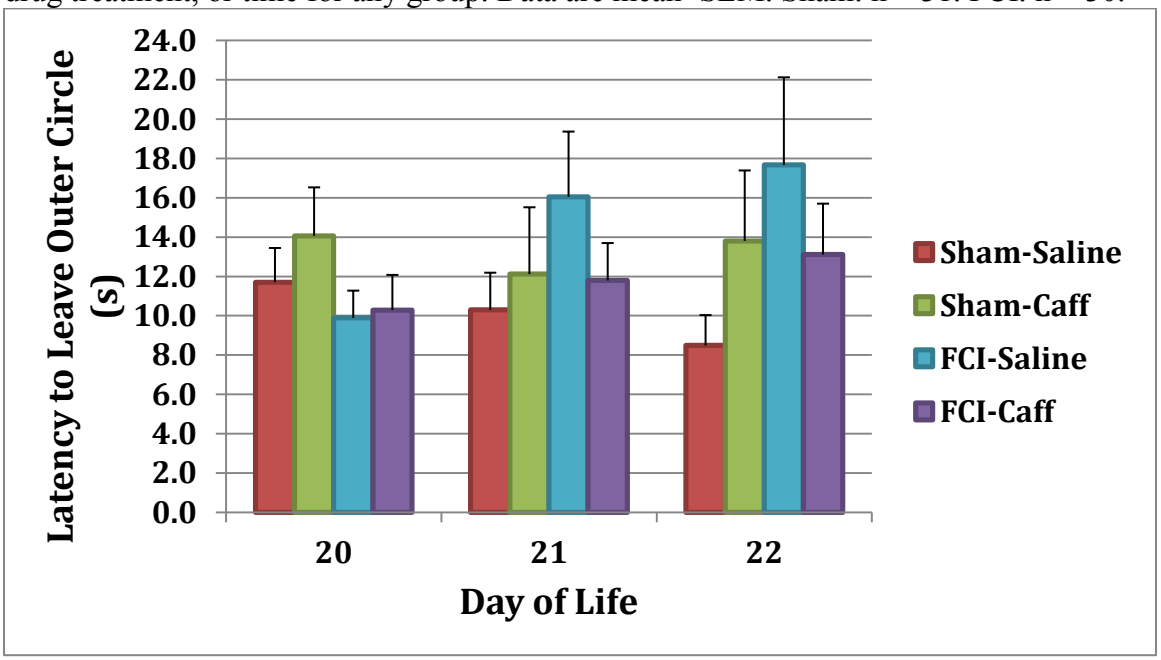

Figure 16: Latency to leave outer circle of walking initiation task for sham and FCI treated with caffeine and saline in Expt 2. There were no differences between surgery, drug treatment, or time for any group. Data are mean±SEM. Sham: n = 31. FCI: n = 30.

Discussion

In this study, I aimed to investigate the neurofunctional outcomes of neonatal rats in a model of focal cerebral ischemia and examine the therapeutic potential of caffeine treatment. I hypothesized that our model of MCAO will result in physiological, motor, and sensory developmental impairments in FCI animals compared to sham animals. I also hypothesized that caffeine will improve the behavioral deficits exhibited by FCI animals as compared to vehicle-treated FCI. Finally, I hypothesized that there would be no adverse behavioral effects on sham animals treated with caffeine compared to sham animals treated with saline. In the following discussion, I will examine each hypothesis in the order presented above.

It was hypothesized that FCI animals would be significantly impaired in tests of physiological and sensorimotor development as compared to sham animals following MCAO. This hypothesis was supported by the results of the first behavioral experiment. Although both groups significantly increased in weight throughout the experiment, the FCI animals grew at a slower rate and were smaller than sham animals at all time points. Lubics et al. used a similar behavior paradigm to the present study to assess a model of hypoxic-ischemia (Lubics et al. 2005). This group also noted this weight differential in their assessment of animals following hypoxic-ischemia (HI). In their experiment, a total of 12 P7 Wistar rats were placed in a hypoxic chamber for 2 hours following induction of a suture embolus via the MCA. The sham animals were identical to the ones used in the present study as they received the same ligations without induction of suture embolus or hypoxia. These results were further validated in other studies using hypoxic-ischemia (Andine et al 1990, Wagnet et al. 2002). This suggests that an ischemic infarction in the

absence of hypoxia is sufficient to cause persistent growth impairments in developing animals. This is also in concordance with what is observed in the clinic. A recent study identified that neonates with ischemic episodes are significantly smaller than healthy individuals (Bukowski et al. 2003). This effect remained even after controlling for gestational age and birth weight. These findings suggest our model of FCI produces a robust effect that is clinically relevant.

While the weight differential was long lasting, other deficits were more transient. The righting reflex data suggest that FCI animals are significantly impaired in righting at P10 compared to sham controls. FCI animals' hind limb development was delayed due to the insult, yet this deficit disappeared by P14. The Lubics group showed a similar pattern of FCI impaired; however, the differences emerged at P8 and persisted throughout their experiment. This result may be explained by the difference in models. The hypoxic-ischemia and MCAO ischemia models produce different deficits and therefore different behavioral responses. It is possible that the focal injury used in the current model does not impact the development of hind motor systems as dramatically as a global hypoxic insult would.

The righting reflex is not the only test that detected significant difference between surgical groups in this experiment. The startle response test shows a significant delay in sensory development in FCI animals compared to sham. About 67% of FCI animals exhibited a response by P12 while nearly 100% of sham animals exhibited a response. Interestingly, Lubics et al. found that the there was no difference between the development of startle response in HI and sham animals and that the average day of emergence of this hallmark in both groups was P13. In addition, Lubics found that HI

animals opened their eyes significantly later (P15) than control animals (P14); however, the present study found no difference. The Lubicis group also found significant impairments in HI animal proprioception as measured by negative geotaxis while the present study could not replicate those differences. This was due primarily to difficulty with the test subjects. Once the animals opened their eyes around P14, they would rather explore the new environment rather than right themselves up the ramp. The animals would walk down the ramp rather than right themselves upward even after multiple attempts per day. Some animals would attempt to right themselves up the ramp, but would then run down the sides. The Lubics study obtained significant data well beyond this point until P20 so more information on their test methodology is necessary to conclusively account for this difference.

All animals exhibited a tactile startle from P8, suggesting this is a feature that develops early in life and is resistant to FCI damage. Animals that performed the walking initiation, righting reflex air, and cliff avoidance tasks all showed a significant increase over time, which is expected as the animals develop. However, there was no main effect of surgery for these tests. The lack of difference between FCI and sham groups may be due to lack of test sensitivity. The deficits may in fact be there during the critical developmental period, but our tests lacked the power to detect them. Some tests that showed no difference at P8, like the tactile startle response, may find a significant difference if performed prior to P8. Furthermore, additional tests like the forelimb grasp tests and hang test may offer further insight into motor development in FCI animals.

It is likely that the majority of these differences in behavioral deficits can be explained by the pathology of the injuries induced in the different model systems. A

comprehensive review of hypoxic-ischemia pathology in the developing rat points out the wide range of damage associated with the injury (Johnston et al. 2001). Glutamate excitotoxicity is believed to be the primary method of damage to neuronal systems involved in various sensorimotor behaviors. It has been shown that focal cerebral ischemia also involves glutamate excitotoxicity, as the glutamate antagonist MK-801 reduces infarct size in the cat (Ozyurt et al. 1988). It has been reported that the developing rat will not exhibit significant behavioral deficits from hypoxia without the induction of ischemia as well (Johnston et al. 1995). Ischemia, therefore, is the primary insult to these animals that produces behavior effects; however, the excess glutamate toxicity is exacerbated by the global hypoxia affecting more areas than the local ischemic infarction. With more areas damaged by excessive glutamate stimulation, it is reasonable to expect more dramatic behavioral deficits in hypoxic-ischemia compared to focal cerebral ischemia alone. To investigate this further, I would run behavior tests in both a model of FCI and a model of hypoxic-ischemia with various exposure times to hypoxic (45 minutes, 70 minutes, 90 minutes). I suspect that behavior deficits will increase as a function of hypoxic exposure. Furthermore, I would administer MK-801 *in vivo* and monitor its effects on behavior following FCI.

It is also possible that these deficits are model specific and would not be seen in a clinical setting. The FCI animals generated by the current model may be significantly smaller than sham animals because it is easier for the shams for to compete for feeding from the mother. This would result in smaller FCI animals not from any neurologic injury but from sheer weakness and inability to compete with healthy pups for nutrients. Furthermore, babies in the NICU are supplied nutrients artificially, so the somatic growth

impairment seen in FCI individuals would not be seen if it is model specific. However, this weight differential is present in all ischemic stroke models to date and seems to be a real neurologic effect of the injury. The current model of focal cerebral ischemia produced poor growth, impairment in righting, and developmental delay in startle response in the developing rat and these deficits are primary targets for therapeutic intervention.

My second hypothesis was that caffeine would attenuate the deficits produced by FCI as identified by experiment 1. Before discussing the weight gain data for this hypothesis, however, it is important to examine the weight gain data for saline-treated animals first. I found that there was a significant difference between saline-treated FCI and sham animals with FCI animals markedly smaller than sham animals. The rates of increase were very similar between sham saline (2.87 g/day) and sham caffeine (3.03 g/day) groups. This finding is a replication of the previous experiment that the novel model of MCAO produces a persistent difference in weight gain between surgery groups, which is most likely permanent according to the slopes of the weight gain rates. Surprisingly, the FCI animals treated with caffeine gained weight at a significantly smaller rate (1.43 g/day) than FCI animals treated with saline (1.94 g/day) throughout the observation period. This result is directly opposed to my initial hypothesis that caffeine would reduce the weight loss exhibited by vehicle FCI animals. In addition, the caffeine-treated FCI animals were significantly smaller than sham animals.

The weight differential seen between saline groups may be explained by corticosterone exacerbating the ischemic injury. The FCI surgery and the recovery thereafter may act as a stressor leading to a prolonged elevation of stress hormones such

as corticosterone (CORT). A study by Mulholland et al. (2005) suggests an interesting relationship between CORT and oxygen-deprivation on the developing rat. In their experiment, neonatal (P7) rats were exposed to 90-minutes of oxygen-glucose deprivation and their brains were harvested. The brains were cultured and treated with corticosterone and treated with propidium iodide to measure oxidative stress. In the control animals, CORT alone did not increase propidium iodide uptake of the cells or the relative synaptic number in hippocampus. However, exposure to oxygen-glucose depravation and corticosterone together potentiated the effective of the oxidative stress on the cells as well as lower synaptic density in the hippocampus. This finding suggests that corticosterone exacerbated the effect of oxygen-deprivation on neonatal neurons. Chronic levels of CORT then would produce a larger cerebral infarction in FCI animals, possibly explaining the pronounced difference in somatic growth seen between sham and FCI in experiment 1 and 2.

It is widely accepted that caffeine, as well as other stimulants, suppress appetite and may account for the lower weight gain seen in the FCI animals treated with caffeine (Safer et al. 1972). However, since this depression in growth was not noted in the sham animals treated with caffeine, the lower somatic growth in the caffeine-treated FCI group is more likely due to an interaction between caffeine treatment and the FCI insult. This interaction may be represented by one of two mechanisms. There is evidence that i.p. injected caffeine markedly increased serum corticosterone levels in rats (Spindel et al. 1983). As noted above, corticosterone alone will not produce dramatic synaptic loss in the hippocampus. An oxygen deprivation event, such as FCI, interacts with serum corticosterone levels to produce more pronounced infarcts. Caffeine in turn raises serum

corticosterone levels and potentiates the neuronal damage to have a greater effect. This effect can be assessed in the current model by a TTC stain to compare infarct size in caffeine and saline treated sham and FCI animals. However, since somatic growth was the only observed feature negatively effected by caffeine administration and the more complex tasks like righting reflex and startle response were spared, I would suspect that a larger infarct is less likely.

Caffeine may also be altering the amount of growth hormone in the body via a corticosterone-mediated mechanism. There is strong evidence to suggest corticosterone inhibits the production of growth hormone (Hadley and Levine, 2007), so it is reasonable to see how this interaction between caffeine and FCI insult may negatively impact somatic growth. Saline treated FCI animals would be smaller than sham controls due to a decrease in growth hormone production from the stress of surgery. The caffeine would exacerbate this effect as it raises endogenous levels of corticosterone. In order to test this further, I would need to induce FCI in animals treated with either saline or caffeine and take serum corticosterone and growth hormone levels at various time points (24 hours, 48 hours, 96 hours, etc.) I would suspect that there would be a marked increase in corticosterone in the FCI population compared to sham animals, and even larger increase within the caffeine-treated FCI group. There would also be markedly less growth hormone in FCI animals, especially caffeine treated, compared to sham controls. Furthermore, this spike would then be maintained at a constant level to induce the constant weight differential shown in the data. This interaction may also be assessed using a selective glucocorticoid receptor antagonist to block corticosterone binding. I

suspect that the difference between saline and caffeine-treated somatic growth would decrease, as well as the difference between FCI saline-treated animals and sham groups.

As shown in experiment 1, FCI animals were significantly impaired in hind limb development as measured by the righting reflex task, but these deficits were soon corrected. While these differences were not significant in experiment 2, the FCI animals (saline and caffeine-treated) exhibited the same pattern of recovery to sham level. This observation is a testament to the plasticity of the neonatal brain. Neuroplasticity is well documented in learning and memory processes but it is also critical in traumatic brain damage recovery (Pascual-Leone et al. 2005). The TTC data provided above shown how pronounced this injury is and the fact that these animals can recover almost entirely is a remarkable testament to neuronal plasticity. Selectively increasing plasticity after ischemia may be an interesting avenue of research that needs further investigation.

FCI animals exhibited significant delay in auditory startle response development compared to sham animals regardless of caffeine or saline treatment. The vehicle results are similar to what was observed in the previous experiment; however, the FCI animals in experiment 2 exhibit a response faster then those in experiment 1. In the first experiment, about 67% of FCI animals exhibited a startle response at P12, however in this experiment about 92% of vehicle-treated FCI animals exhibited a response at P11 with a similar effect seen in caffeine-treated FCI animals. There are two possible explanations for this phenomenon. Firstly, the behavior and drug experiments were conducted nearly one year apart from one another. It is quite possible that the conditions in the animal facility (i.e. mothering, number of rats, number of litter mates) added additional variables that supported faster recovery. Unfortunately, variables such as these were not taken into

consideration and were not recorded. Secondly, there may be potential experimental error in the administration of these tests. With one year of practice prior to the drug experiment, I had an ample amount of experience conducting the auditory startle task and developed a better knowledge of a positive result. This hyper-vigilance may have led to observing more subtle changes than previously noted. Additional rounds of drug testing are necessary to assess the validity of not just these observations, but all measures acquired in the experiment.

My final hypothesis stated that there would be no significant difference in any developmental measure between sham animals treated with caffeine and saline. The results support this hypothesis as no developmental measure showed a significant difference between the two sham groups. This finding is consistent to what is seen in the human neonatal population over long-term observation. Schmidt et al. (2007) investigated the incidence of apnea in low birth weight infants following caffeine treatment. In order assess incidence of apnea, as well as long-term somatic and behavioral development, 2006 infants were randomly allocated into caffeine and placebo groups. The results suggest that caffeine significantly lowers apnea at a young age, but more importantly there were no significant differences in somatic development between the non-apneic babies in the caffeine and placebo groups. The lack of difference in rate gain weight between sham groups suggests a similar outcome.

Caffeine citrate did not have the effect on FCI animals that was previous hypothesized. A future study may investigate a wider range of dosing paradigms due to caffeine's wide therapeutic window. However, the dose used in our experiment is considered the standard dosage in the clinic, so a study using higher levels may lose

clinical relevance (Gary et al. 2011). I would be more interested in studying another member of the methylxanthine family, pentoxifylline (PTX). Pentoxifylline is a widely studied compound and is currently marketed in Europe as Trental for treatment multi-infarct dementia (Ward 1987). Its neuroprotective effects on transient ischemic stroke are well documented and a recent study suggests a greater efficacy than caffeine citrate (Sliwa et al. 2004). Assessment of PTX in the present model of FCI is an attractive alternative to caffeine and deserves further investigation.

Nevertheless, caffeine's negative effects on the somatic growth of FCI animals have important implications for neonates being treated by clinicians in the NICU today. At Morristown Medical Center, the current standard of care for premature or full term infants entering the NICU is immediate incubation and caffeine drip. This is done as a precautionary measure to protect against potential apneic episodes developing in these neonates. However, my data suggest that this may be causing more detriment to the developing neonates suffering from ischemia. No study to date has investigated the effects of caffeine on the incidence of ischemia in neonates. Since ischemia was induced in the current model, this is not a relevant question, albeit an important one. Further studies must be conducted to investigate caffeine's preventative potential against ischemic stroke. Furthermore, If an ischemic event were to occur and go undiagnosed until later in development, which accounts for nearly 25% of neonatal ischemic stroke incidence, then clinicians may be producing smaller neonates and therefore exacerbating the effects of the ischemia. One of the only longitudinal studies investigating neonatal caffeine administration found no significant difference in weight between apneic and non-apneic groups, however ischemia was no analyzed (Schmidt et al. 2007). It is evident

from my research that further longitudinal studies are paramount to assess the effects of chronic caffeine exposure on somatic growth of neonates suffering from FCI during the critical period.

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