Bilateral Uterine Artery Ligation in Rats cultivates Long Term Neurological Deficits reminiscent of Human Cerebral Palsy: A model for Therapeutic Intervention.

by

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Abstract

Introduction

Cerebral Palsy (CP) is a ubiquitous term used to describe a group of permanent, non-progressive disorders of movement, posture, and behaviour. Despite countless advances in neonatal medicine, the incidence of CP has remained constant in term infants over the last three decades. More so, the overall incidence has increased due to the survival of premature births. With the exception of hypothermia, which is restricted to a subset of patients, no effective prevention of CP exists. Recent literature has implicated placental insufficiency in the development of CP.

Objectives

The purpose of this experiment is to validate the use of bilateral uterine artery ligation (BUAL) in Long-Evans rats as a model of CP using functional and histological assessment. Specific objectives include determining whether BUAL results in offspring that exhibit (1) Intrauterine growth restriction (IUGR) at birth; (2) motor, cognitive and behaviour impairment after P35 and (3) white and grey matter injury at P80.

Methods

Timed pregnancy was achieved in virgin rats. On gestational day 20 (E20), pregnant dams were randomly allocated to either BUAL or SHAM surgery. Offspring were divided based on treatment and sex to form the following four study groups: 1) SHAM Males, 2) SHAM Females, 3) IUGR Males, and 4) IUGR Females. Each group underwent one month of functional assessment commencing on post-natal day 35 (P 35). Functional assessment included: 1) motor

testing using beam walking, skilled reaching, and gait analysis; 2) cognitive testing using object recognition and Morris water maze and 3) behaviour testing using elevated plus maze and open field. Animals were euthanized on P80 and brains were collected and fresh frozen for histological evaluation. Fresh frozen brains were stained with haematoxylin and eosin (H+E), myelin basic protein (MBP) and Olig-2. Hippocampal cell counting was used to detect grey matter injury. Lateral ventricle volumes, immunodensity of MBP and Olig-2+ cell counts were used to assess for white matter injury.

Results

BUAL resulted in a statistically significant increase in mortality and growth restriction (IUGR) in surviving rat pups. Both IUGR males and females made significantly more faults than SHAM controls in beam walking and skilled reaching. IUGR males were found to have a significantly wider hind limb stance than SHAM males. IUGR males utilized a significantly less efficient swim strategy in the Morris water maze than controls. No differences in open field or object recognition were detected. Both IUGR males and females made significantly more entries into the open arms of the elevated plus maze than their respective controls. The number of pyramidal neurons in the CA3 region of the hippocampus in IUGR males was significantly reduced. No differences in white matter were detected.

Conclusion

BUAL in the pregnant Long Evans rat leads to IUGR and permanent neurological deficits in offspring that are reminiscent of CP. Both males and females demonstrated significant motor impairment. Cognitive impairment and neuropathology were only significant in males. This is congruent with a higher prevalence of CP in human males. Moreover, the etiological and

functional similarities of this model to CP, in combination with its long term sustainability, make it an excellent template for the pre-clinical testing of therapeutic interventions.

Preface

This thesis is an original work by Jennifer Corrigan. Some elements of this work have been reproduced in a manuscript that has not yet been submitted for publication.

This research project received ethics approval from the Animal Care and Use Committee at University of Alberta.

Dedication

To my family, for enabling me to embark on this project.

To my mentor, for enabling me to complete it.

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List of Abbreviations

ANOVA - Analysis of Variance

ATP - Adenosine triphosphate

BUAL - Bilateral Uterine Artery Ligation

CP - Cerebral Palsy

IUGR - Intrauterine Growth Restriction

PI - Placental Insufficiency

H_2O_2 - Hydrogen Peroxide

H+E - Haematoxylin and Eosin

HI - Hypoxia Ischemia

HIE - Hypoxic Ischemic Encephaplopathy

MBP - Myelin Basic Protein

NFkB - Nuclear Factor k B

O_2^- - Superoxide Radical

$OH^{\cdot}$ - Hydroxyl Radical

ROS - Reactive Oxygen Species

SEM - Standard Error of the Mean

Chapter I: Introduction

Cerebral Palsy

Clinical Significance and Rationale

Countless advances in neonatal medicine have been made over the past fifty years. Most notably, the mortality rate of extremely low birth weight infants (<1000g) has dropped significantly from 95% in the 1960s to less than 5% today (Philip, 2005). In spite of these advances, the prevalence of neurodevelopmental disabilities such as Cerebral Palsy (CP) has not declined. Over the last three decades the incidence of CP has remained constant at 2-4 per 1000 live births; rendering it the most common physical disability of childhood (Paneth, Hong, & Korzeniewski, 2006). Moreover, in the premature infant, even though it has declined somewhat, remains at about 22/1000 live births, and is inversely proportional to gestational age.(Robertson 2000, Robertson, Watt et al. 2009)

CP is associated with tremendous physical, emotional and financial costs. Use of well validated quality of life scales has confirmed that children with CP have significantly reduced quality of life when compared to developmentally typical peers (Colver et al., 2015; Vles et al., 2015). Financially, CP presents a challenge from both an individual and a societal perspective, as each individual with CP will cost the healthcare system in excess of $1,000,000 throughout the course of their lifetime (Eunson, 2015). The growing persistent prevalence of CP combined with its high financial and emotional burdens calls for continued research into preventative and therapeutic strategies.

Classification and Definition

Cerebral palsy is a heterogeneous disease consisting of non-progressive motor disorders that result from injury to the developing fetal or infant brain. It was first described in the early 19th century by William John Little, an English surgeon, who referred to the lower limb hypertonia and spasticity he observed as "Little's disease." This entity is now known as spastic diplegia and represents the most common form of CP. Little attributed the condition to prematurity as well as difficult delivery resulting in intra-partum asphyxia (Panteliadis, Panteliadis, & Vassilyadi, 2013). This notion that CP occurs primarily as a result of asphyxia has persisted into the 21st century and to some extent is still present today. However, new research suggests that less than 10% of children with CP suffered an asphyxial event at the time of birth (McIntyre, Blair, Badawi, Keogh, & Nelson, 2013).

In 1888, Sir William Osler coined the term Cerebral Palsy. He divided CP into different subtypes based on clinically apparent motor disorder phenotypes, and then began to correlate these phenotypes with neuro-anatomical pathology (McIntyre et al., 2013). The four major subtypes of CP are spastic, dyskinetic, ataxic and mixed. Spastic CP is characterized by hypertonia and affects greater than 80% of patients with CP. It can be further described as diplegic, hemiplegic or quadriplegic depending on the topographical distribution (Mandaleson, Lee, Kerr, & Graham, 2015). Although these terms are commonly used in practice today, a new classification system was recently introduced that demonstrates superior validity and reliability in predicting severity of motor impairment in CP. The Gross Motor Function Classification System (GMFCS) is a five level classification tool that categorizes patients based on the severity of their gross motor limitations. GMFCS Level I patients are able to walk without limitations whereas Level V patients are wheel chair bound. The vast majority of patients with CP (>60%)

have mild motor impairment, with approximately 35% falling into GMFCS Level I and 25% into GMFCS Level II (Reid, Carlin, & Reddihough, 2011). In addition to constituting the majority of patients with CP, GMFCS Level I and II patients are the most likely to benefit from therapeutic intervention (Chen et al., 2013). Notwithstanding this, there are very few pre-clinical models designed to mimic the degree of severity that reflects the most common CP phenotypes. Developing an animal model reminiscent of mild to moderate CP is crucial, therefore, in the translation of novel therapeutic agents.

In addition to motor dysfunction, cognitive and behavioural co-morbidities are prominent among patients with CP and contribute considerably to the disease's outcome and quality of life (Hou et al., 2010). According to the 2008 Centers for Disease Control (CDC) report, greater than 60% of children with CP have at least one associated neurodevelopmental disorder including, but not limited to, intellectual disability (40%), epilepsy (35%) and vision impairment (15%). While the prevalence of co-morbidities increases with GMFCS level, the relationship is by no means linear. Recent studies have demonstrated executive dysfunction is present in approximately half of patients with CP (Bodimeade, Whittingham, Lloyd, & Boyd, 2013; Bottcher, Flachs, & Uldall, 2010; Hakkarainen, Pirila, Kaartinen, & Meere, 2012; Pirila, van der Meere, Rantanen, Jokiluoma, & Eriksson, 2011). The prevalence increases with prematurity and bilateral brain lesions (Pirila et al., 2011). The Executive Committee for the Definition and Classification of CP released an updated definition in 2006 to more accurately reflect these associated neurodevelopmental disabilities. CP is now defined as "a group of permanent disorders of the development of movement and posture, causing activity limitations, attributed to non-progressive disturbances …often accompanied by disturbances of sensation, perception, cognition,

communication, and behaviour—epilepsy and musculoskeletal problems" (Rosenbaum et al., 2007).

Intrauterine Growth Restriction

Etiology of CP

Cerebral palsy is a heterogeneous and complex disease with multiple aetiologies. Until recently, the neurological disturbances observed in CP had been primarily attributed to acute intrapartum asphyxia that resulted in hypoxic ischemic encephalopathy (HIE). Acute oxygen and glucose deprivation undoubtedly results in neuronal cell death and varying degrees of neurological sequela. Low APGAR scores, which are indicative of intra-partum hypoxia, are a risk factor for the development of CP. The majority of children with CP, however, do not have low APGAR scores at the time of birth (Lie, Groholt, & Eskild, 2010). Furthermore, increased fetal surveillance and prompt intervention at the time of delivery has not decreased the incidence of CP. One explanation for this is that the majority of HI insults actually occur prior to birth.

Placental Insufficiency

Several independent risk factors have been identified for CP. The most significant of these is low birth weight (Zhonghua 2013), which is frequently associated with prematurity and intrauterine growth restriction (IUGR). IUGR refers to a fetus that is small for gestational age (weight $<10^{th}$ percentile) as a result of a pathological process that prevents it from meeting its genetic growth potential (Sehested & Pedersen, 2014). IUGR can often be attributed to one or more fetal, placental, or maternal factors. With the exception of intrinsic fetal factors such as a genetic anomaly that impedes normal development, the most common cause of IUGR is placental insufficiency (PI). PI, also known as utero-placental vascular insufficiency, precludes

adequate delivery of blood to the fetus resulting in chronic hypoxemia and hypoglycemia or fetal ischemia. The relationship between placental blood flow, IUGR and neurological outcomes has been demonstrated clinically and in animal models. Doppler studies consistently reveal high placental vascular resistance in IUGR human fetuses. If umbilical artery resistance increases to the point of impairment, loss or ultimately reversal of end diastolic flow; the rates of poor neurological outcome and neonatal death increase exponentially (Volpe, 2008).

Pathophysiology of Cell Death

Neuronal cell death in the face of ischemia is triggered initially by a lack oxygen and glucose supply to the tissue. This results in a loss of energy reserves, and subsequently to the loss cell membrane potential. Energy failure occurs when adenosine triphosphate (ATP) production is insufficient to sustain cell metabolism. Unlike adults, the fetus has very little glucose reserve. Therefore, the developing brain is highly reliant on a constant supply of oxygen and glucose from the placenta. When both oxygen and glucose are present, energy is efficiently produced via glycolysis. Aerobic glycolysis generates 32 molecules of ATP per molecule of glucose. During hypoxia, there is a shift to anaerobic glycolysis which produces only two ATP for every one glucose that is broken down. The rate of energy depletion is hastened in PI due to a reduced glucose supply, in addition to lack of oxygen. Lack of glucose limits the rate at which glycolysis can occur. Energy depletion leads to failure of ATP dependent sodium and potassium pumps resulting in cell swelling and imminent cell death, by necrosis. With continued energy depletion, calcium enters the cell, along with the accumulation of toxic metabolites. These metabolites include extracellular glutamate and intracellular calcium which trigger secondary apoptosis (Volpe, 2008).

Oxygen-free radicals play an important role in the pathogenesis of post-ischemic injury(Chan 1996, Chan 2001). A free radical is a molecule that contains an uneven number of electrons, and is unstable. The major site of free-radical production is the mitochondria, where reactive intermediates including the superoxide radical (O_2^-), and hydrogen peroxide (H_2O_2) arise from the oxygen used in the electron transport chain. These intermediates interact with transition metals such as iron (Fe^{2+}) which mediate the dismutation of H_2O_2 to result in the formation of the highly reactive hydroxyl radical (OH^-). Under physiologic conditions, the brain contains endogenous mechanisms by which scavenging of free-radicals takes place. These include enzymes such as superoxide dismutase, and glutathione peroxidase. Endogenous free radical scavengers also include glutathione, and Vitamin E.

During ischemia and following resuscitation, reactive oxygen species (ROS) directly perpetuate brain injury by causing the disruption of cell membranes through lipid peroxidation, DNA damage, reduced glutamate re-uptake, and inhibition of sodium-potassium ATPase. Free radicals also contribute to the continued elevation of intracellular calcium (Ca^{++}). The propagation of these events following HI result in the phenomenon of cell necrosis(Charriaut-Marlangue, Pollard et al. 1996). In this regard, HI insults not only render the mitochondria dysfunctional, it also causes the release of cytochrome c into the cytoplasm. Here, it activates a series of caspases, leading to DNA fragmentation and apoptotic cell death. Nuclear factor -k B (NFkB), and other transcription factors are regulated by the redox state of the cell. Under conditions of ischemia or pro-inflammatory stimuli, NFkB is activated and targets downstream inducible genes such as iNOS, COX-2 and cytokines, which are also involved in neuronal injury and the inflammatory response following cerebral ischemia(Kadhim, Tabarki et al. 2001).

Cytokines play a pivotal role in physiologic and pathologic conditions. In pathologic conditions, cytokines are neurotoxic through stimulation of microglia and astroglia to produce nitric oxide, reactive-oxygen species, and EAAs. Additionally, TNF may be directly toxic to neurons and glia. Cytokines, especially TNF, can also induce endothelial cells to promote the expression of adhesion molecules and enhance coagulant activity, potentiating free radical production, and eventual cell death(Kadhim, Khalifa et al. 2006).

The inflammatory response to brain injury has been characterized by a cycle of genomic responses. Hence, in the first minutes following MCA occlusion, c-fos, c-jun and others are transiently increased. This is followed by the heat-shock proteins (HSP-70, HSP-72) at 1 to 2 hours of recovery, which lasts up to 2 days. The third wave of gene expression, peaking at about 12 hours after ischemia, and lasting up to 5 days, is largely comprised of the pro-inflammatory cytokines TNF-a, IL-1B, and IL-6 and the anti-inflammatory cytokine IL-1ra. Chemokine expression is also elevated along with a host of "adhesion molecules" (ICAM-1, ELAM-1, P-selectin, CD11/CD18, and MAC-1). Not surprisingly, the noted elevation of inflammatory genes occurs in concert with the infiltration of inflammatory cells in the form of neutrophils, polymorphonuclear leukocytes and monocytes/macrophages. In an apparent attempt to begin the process of repair, neurotrophic factors (NGF, BDNF, and p53) also appear at the site of injury. The inflammatory reaction not only contributes to lipid-membrane peroxidation, but also exacerbates the degree of tissue injury by further occluding blood flow with vascular plugging. Moreover, leukocytes enhance the generation of ROS, which perpetuate the events leading to necrosis and apoptosis(Bona, Andersson et al. 1999, Kadhim and Sebire 2002, Kadhim, Tabarki et al. 2003).

Neural cell death is a continuum that ranges from necrosis to apoptosis (Portera-Cailliau, Price & Martin1997; Portera et al. 1997). Necrosis is characterized by the loss of membrane integrity, signs of organelle damage, and release of lysosomal contents. Apoptosis is characterized by the preservation of membrane integrity, organelle structure and lysosomal contents, but with diminution of cell volume, chromatin condensation, and nuclear fragmentation. This latter type of cell death appears to be a form of "programmed cell death". Generally, two major pathways of programmed cell death have been identified: intrinsic and extrinsic pathways. The intrinsic pathway involves initiation of apoptosis as a result of disturbances in intracellular homeostasis. This pathway has also been termed the "mitochondrial cell death pathway". The extrinsic pathway involves the initiation of apoptosis through the activation of plasma membrane death receptors. Although the initiation stage of intrinsic and extrinsic pathways are different, most recent evidence shows that they converge on a group of pro-enzymes known as caspases, the final "executioners" of apoptosis (MacManus, Buchan, Hill, Rasquinha, Preston 1993; MacManus & Linnik 1997; Eldadah & Faden 2001).

Neuropathology

Hypoxic Ischemic Encephalopathy

The association between anatomical neuropathology and clinically relevant neurodevelopmental outcomes in CP is complex. Timing and chronicity of HIE have been postulated as an explanation for the variable outcomes in both neuropathology and neurodevelopment in CP.

Hypoxic-Ischemic Encephalopathy (HIE) is recognized as a major source of neurological disabilities. Classically, HIE is said to arise from intrapartum asphyxia which ultimately culminates in CP. If HI occurs early in development (before 32 weeks) it commonly results in periventricular leukomalacia (PVL). The term PVL was first used by Banker and Larroche in 1962 to describe the softening ("malacia") of white ("leuko") matter seen in the periventricular region of the brain of premature infants on autopsy (de Vries et al., 1998). Specifically, PVL denotes focal cystic lesions in the white matter surrounding the lateral ventricles. It is considered to be the anatomical substrate for CP in preterm infants.

Pathologically, PVL occurs in a distribution around the anterior horns and trigone of the lateral ventricles(Volpe 2001); sites recognized as border zones between penetrating branches of the anterior, middle, and posterior cerebral arteries. Within 1 to 3 weeks of the HI insult, multiple cavities can be visualized (Figure). Subsequently, subcortical white matter loss and areas of focal ventricular dilation can be seen. Microscopically, oligodendroglial deficiency along with loss of myelin and astrogliosis are dominant.

Several factors contribute to the production of PVL in the premature infant. As indicated, vascular development plays a major role in the selective distribution of PVL. Thus, vessels derived largely from the middle cerebral artery, penetrate the cerebral wall from the pial surface. These long penetrators are *end arteries*, and do not anastomose with surrounding vessels, leaving the region vulnerable to a reduction in perfusion pressure. As a result, these "watershed regions" are most prominent in the least mature infants, accounting for the prevalence of this disease in the 26 - 36 week gestation neonate (Miyawaki, Matsui et al. 1998, Inage, Itoh et al. 2000).(Inder et al., 1999).

Interestingly, the latter is not true of IUGR infants that are born at term (Cabaj, Bekiesinska-Figatowska, & Madzik, 2012). Instead, IUGR infants maintain the predilection for white matter injury. This can in part be explained by the stage of development of oligodendrocytes at the time of HI insult. Using normal control neonatal human brains obtained from autopsy, Back et al. highlighted the stages of oligodendrocyte development as it pertains to gestational age and vulnerability to white matter injury. The highest propensity for white matter injury, particularly PVL, occurs between 23-32 weeks of gestational age. Three distinct types of oligodendrocytes are present in the fetal brain during this time: 1) late oligodendrocyte progenitor cells 2) pre-myelinating oligodendroctyes and 3) mature myelinating oligodendrocytes. Of these, the non-myelin producing late oligodendrocyte progenitor cell predominates. In comparison to their mature counterparts, progenitor cells are more vulnerable to HI. Consequently, HI occurring in the early to mid-third trimester results in extensive myelination abnormalities. (Back et al., 2002; Back, 2006; Back, Riddle, & McClure, 2007; Back et al., 2007). Injury to progenitor cells leads to a subsequent reduction in the number of normally functioning mature myelinating oligodendrocytes. This in turn leads to hypomyelination and loss of white matter or, more subtly, preserved white matter volume with conduction impendence secondary to disorganized and dysfunctional myelin sheaths (Back & Miller, 2014).

Pathophysiology of Periventricular White Matter Injury

While periventricular leukomalacia is the classic anatomical substrate for cerebral palsy, it does not accurately depict the more common underlying neuropathology. Recent literature suggests that the majority of white matter injury is actually non-cystic and non-focal in nature (Khwaja & Volpe, 2008). It is more precisely described as a diffuse reduction or alteration in the

quality of white matter surrounding the lateral ventricles. As such, the term “white matter brain injury” to describe the changes observed following intrauterine HI has come into favour.

White matter brain injury is typically initiated by cerebral ischemia as outlined above. HI leads both to immediate cell death via necrosis and delayed cell death via apoptosis. The latter is triggered by downstream mechanisms that are activated by ischemia. In particular, the generation of the reactive oxygen species and free radicals are now recognized as a major proponent of white matter injury (Haynes et al., 2005; Volpe, 2008). The immature brain is deficient in free radical scavengers and thus more vulnerable to oxidative stress than the mature brain (Folkerth et al., 2004). Hypothermia is believed to achieve neuroprotection through halting the deleterious cycle of inflammation and reactive oxygen species generation that occurs in the reperfusion phase of HI brain injury (Basu, 2014; Fernandez, 2011; Garfinkle et al., 2015; Nguyen, Armstrong, & Yager, 2013). Because the use of hypothermia is limited to those infants that suffer an acute HI injury around the time of birth, the search for a method of preventing the inflammatory cascade prenatally has become an area of research focus.

Grey Matter Injury

The motor deficits of CP are, more often than not, accompanied by cognitive and behavioural deficits. Although reduced or improper myelination may account for some cognitive slowing, the presence of significant cognitive abnormalities suggests a concurrent cortical grey matter injury. A 2003 article from the journal of neuroscience put forth subplate neurons as the grey matter equivalent to oligodrendrocyte progenitor cells (McQuillen, Sheldon, Shatz, & Ferriero, 2003) . Subplate neurons are located below the cortical plate and are essential in the establishment of normal cortical circuitry. Subplate neurons are exclusive to the fetal brain and

peak in prevalence around 24 weeks gestational age, coinciding with the peak in oligodendrocyte progenitor cells. Multiple in vitro and in vivo animal studies have shown that subplate neurons appear to be, similarly, selectively vulnerable to HI (Miller & Ferriero, 2009; Volpe, 2005). T1 and T2 diffusion weighted images reveal that under conditions of mild hypoxia loss of white matter prevails as the most dominant type of injury. Conversely, under more severe hypoxia, the loss of grey matter in the striatum and parietal cortex is more pronounced (Qiao, Meng, Scobie, Foniok, & Tuor, 2004).

Paradoxically, epidemiological studies suggest that children exposed to intrauterine HI who do not develop CP, remain at increased risk for learning disabilities and other neurological abnormalities; irrespective of their motor function. In keeping with this line of thought, other authors have hypothesized that two different patterns of HI brain injury exist (Steinman et al., 2009). The first is the watershed pattern of white matter injury, described above, where cortical injury is only observed in the more severe cases. The second is a basal ganglia predominant pattern of injury. In the latter pattern, the hippocampus and deep grey nucleus are principally affected. This incongruence highlights the need for ongoing research in the area.

Translational Research

Animal Models of CP

In light of our improved understanding, our ability to prevent the occurrence of, or provide curative treatment for, CP remains static. With the exception of hypothermia, treatment for CP to date is generally symptomatic and supportive. Hypothermia is accepted as the standard of care for HI brain injury; however this is only effective in the case of acute intrapartum asphyxia or neonatal stroke (Azzopardi et al., 2014; Basu, 2014; Chalak et al., 2014; Fernandez,

2011). As will be described below, the timing of this intervention limits its applicability to only a small subset of those affected by CP. The need for an intervention that accurately targets the more common intrauterine etiology is supreme. One of the major obstacles to doing so is the absence of an appropriate animal model for testing potential therapies in (Kinney & Volpe, 2012). Models to date can largely be ground into one of three categories: 1) inaccurate inciting etiology, 2) too severe, 3) too subtle. Historical animal models of CP largely fall into the first category, as they were designed to mimic birth asphyxia, the proposed mechanism of injury at the time. Vannucci's model of neonatal stroke in rats is the classic example (Vannucci & Vannucci, 1997). The Vannucci model was based off the Levine model of adult stroke. Briefly, HI brain injury is accomplished by performing a unilateral carotid artery ligation followed by systemic hypoxia, in post-natal day seven (P7) rat pups. Developmentally, a seven day old rat approximates that of a human infant at term (37 weeks gestational age). Although this serves as an excellent model for stroke occurring at the time of birth, it cannot be used to test therapeutic interventions geared at prenatal insults. A new model that more accurately reflects the prenatal origin of CP is needed.

In 2009, the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations were updated to address translational barriers between animal models and clinical medicine. The ideal model needs not only to accurately mimic developmental stage and etiology of insult, it needs to be 1) translatable 2) sustainable long term and 3) reproducible, so that it can be utilized for the testing of therapeutic interventions in the future (Fisher et al., 2009). Bearing this in mind, several investigators have focused their attention on developing pre-natal models of CP. Many have exploited the well-established relationship between placental insufficiency and IUGR in order to study the neurodevelopmental outcome in growth restricted fetuses. Different methods

of placental insufficiency have been tried in multiple species, each with unique strengths, challenges and limitations. Endeavours in the rabbit, sheep, guinea pig and rat will be highlighted in brief.

Derrick et. al developed a rabbit model that is perhaps the animal model that is most reminiscent of spastic cerebral palsy to date (Derrick, Drobyshevsky, Ji, & Tan, 2007; Derrick et al., 2009). Acute placental insufficiency was accomplished by temporarily occluding the descending aorta of the pregnant rabbit. Kits displayed significant neurodevelopmental delay and white matter injury on magnetic resonance imaging. The severity of the models imposes 24 hour intensive care requirements and significantly shortened lifespan for the kits. This poses limitations both in reproducibility of the model by others and testing of therapeutic interventions long term.

IUGR and associated placental pathologies have been extensively studied in sheep. Placental insufficiency has been derived from numerous techniques including umbilical artery ligation, placenta embolization and maternal hyperthermia. The Ewe's large size facilitates ease of monitoring and has generated a wealth of knowledge regarding oxygen saturation and metabolic abnormalities during placental insufficiency. From a neurological perspective, sheep have provided significant information in regards to brain pathology, but little in terms of clinical function (cognition, behaviour, rehabilitation). Mallard et. al demonstrated increasing neuronal loss in the hippocampus, following umbilical artery occlusion, with increasing gestational age (Mallard, Williams, Johnston, & Gluckman, 1994). The precocial nature of the sheep makes timing and consequences of intrauterine insults difficult to extrapolate to a human condition that is characterized by prematurity. Back et. al have characterized the maturation of oligodendrocytes in sheep and correlated it to their work in humans (Back, Riddle, & Hohimer,

2006; Back, Riddle, Dean, & Hohimer, 2012). This information would prove useful for ensuring that intrauterine insults are accurately timed; however this work has not translated into examination of clinically significant endpoints such as physical or cognitive disability. Other more apparent disadvantages of sheep models are their high cost and housing requirements, as well as their long gestation.

Unilateral uterine artery ligation has been used in both guinea pigs and rats to study the effects of IUGR on the brain. This procedure was first described by Wigglesworth in 1974 (Wigglesworth, 1974). Using this method, Rehn et al. published a study that found decreased brain weight and increased ventricle size in IUGR guinea pigs at 8 weeks of age (Rehn et al., 2004). Delcour et al. used unilateral artery ligation to measure long-term cognitive function in IUGR rats (Delcour et al., 2012). This study did not look at any measures of gross or fine motor impairment. This may be due to the fact that the injury produced by unilateral artery ligation is not severe enough to cause overt physical disability. This may be due to 1) cross contamination of litters with pups from the control horn that are merely SGA or 2) neuroprotection from collateral blood flow from the contralateral uterine artery as well as the ipsilateral ovarian artery.

While each of these animal models has some value, few provide the necessary ingredients that will fulfill the criteria required for testing the long term viability of a therapeutic intervention. In this regard, models in the sheep provide excellent physiologic and pathophysiologic determination of the interaction between the mother, placenta and fetus. However, long term monitoring and behavioural assessment is not practical. Similarly, utilization of the rabbit as a model provides an excellent phenotype for severe CP, but has no long term viability. The rodent, by contrast, is a very hardy animal. In-utero assessment, mechanistic investigation, and long term behavioural assessment are not only viable, but reliable.

Preliminary Results

With the exception of Lane, we are the only group to perform bilateral uterine artery ligation to induce IUGR in rats, and the first to use Long-Evans rats. Long-Evans rats are recognized by the Canadian Centre for Behavioural Neuroscience (CCBN) as being the most robust rats for behavioural testing (Whishaw, Gorny, Foroud, & Kleim, 2003). Previous work in our laboratory has demonstrated consistent asymmetrical growth restriction, developmental delay, and white matter and hippocampal neuronal cell reductions in post-natal day 21 rat pups (Black, 2010).

Objectives

The overarching goal of this study is to create a clinically relevant long term animal model of CP that can be used for pre-clinical testing of therapies. The purpose of this experiment is to validate the use of bilateral uterine artery ligation (BUAL) in Long-Evans rats as a model of CP using functional and histological assessment. Specific objectives include determining whether BUAL results in offspring that exhibit (1) Intrauterine growth restriction (IUGR) at birth; (2) motor, cognitive and behaviour impairment after P35 and (3) white and grey matter injury at P80.

Hypotheses

The following hypotheses have been generated: 1) Bilateral uterine artery ligation (BUAL) will result in offspring that exhibit (a) Intrauterine growth restriction (IUGR), (b) motor impairment. (c) cognitive impairment, (d) behaviour impairment, (e) white matter injury and (f) grey matter injury. 2) The pattern of functional impairment and histological brain injury observed will be consistent with our previous short term study. 3) The pattern of functional impairment and histological brain injury observed will be reminiscent of the clinical phenotype and neuroanatomical lesions seen in CP.

Chapter 2: Methods

Experimental Paradigm

All procedures were approved by the Animal Care and Use Committee for Health Sciences at the University of Alberta. Long-Evans rats were purchased from Charles River Laboratory, then subsequently housed and bred at the University of Alberta. Animals were on a 12-hour light and dark cycle and allowed to feed ab libitum, in keeping with the Nature Protocol for housing, husbandry and handling of rodents for behavioural experiments (Deacon, 2006) . Timed-pregnancy was accomplished by housing one male and two female rats together for 16 hours and then subsequently examining vaginal smears. The first sperm positive day was denoted as embryonic day 1 (E1) of a 23 day gestation. All dams were 12 to 16 weeks old and nulliparous prior to this conception.

Sample size calculation was performed using STATA 10 software prior to commencing the study. Sample size (n) was defined as the number of individual rats needed to undergo functional and histological assessment in order to detect an effect size (r) of 0.45. Effect size refers to the magnitude of difference seen between two groups. Effect size can be reported in a multitude of ways including as an equivalence correlation coefficient (r). The equivalence correlation coefficient (r) is equal to the difference in percentage of "successes" in each of the two groups (Sullivan & Feinn, 2012). In our previous study using this model, the difference in success (r) between IUGR and SHAM rats on functional assessment was approximately 0.45. We therefore powered our current study to detect a similar effect size. Use of the individual rat, as opposed to dam or litter, for the calculation of required sample size is in accordance with current animal model experimental design literature (Johnson & Besselen, 2002). In experiments where the treatment is expected to have a variable effect on the offspring such as a surgical

procedure or drug therapy, the individual offspring is considered to be the entity under study or experimental unit. In contrast, if the treatment is expected to have a more uniform effect on the offspring, such as a teratogen administer to the mother (ex. Caffeine), then the dam should be considered the experimental unit. Our previous study demonstrated that bilateral uterine artery ligation produces varying degrees of mortality, growth restriction and concomitant neurological injury based on proximity of rat fetus to the uterine artery. Therefore, the experimental unit in this study was deemed to be the individual rat undergoing assessment. Group size was calculated on that basis. Despite this, we recognize that an interaction between dam and pup is present in our model and may have an effect on treatment. The surgery itself is performed on the dam and therefore inter-litter variability is a potential factor. Furthermore, maternal affection and nutrition are known to impact animal development and therefore inter-dam variability must also be accounted for. In order to reduce variability secondary to these factors, each group of animals was comprised of 16-18 animals arising from 7-8 different dams. In other words, no more than two males and two females from the same dam were included in the study. The number of dams used were based on numbers used in previous literature as well as ethical considerations. Sample size using dams as the experimental unit was not formally calculated. Wherever possible, offspring excluded from this study were allocated to a short term histological study in attempt to minimize waste of animal lives. Ultimately, A sample size (n) of 16 rats/ group was determined in order to be able to detect an effect size (r) of 0.45, with a power of 0.80 and an alpha of 0.05.

Surgery was performed on a total 22 rat dams, to obtain 15 litters (8 BUAL surgery litters and 7 SHAM surgery litters) that met the inclusion criteria highlighted below. The animals were divided into groups based on treatment and sex, yielding the following four groups: 1) SHAM Males 2) SHAM Females 3) BUAL Males and 4) BUAL Females. As described above, and

outlined in Figure 1, each group was compromised of 16-18 rats. Ultimately, 68 animals underwent functional and histological assessment (Figure 1).

Pups were weighed and sexed on postnatal day 1 (P1), immediately following delivery. IUGR criteria was determined as a P1 weight 2 SD below the mean SHAM weight (<5.52g) (Pschirrer & Yeomans, 2000). The inclusion criteria were 1) Dam must not have experienced any intra or post-operative complications; 2) Pups must be born on E23; 3) SHAM animals must weigh >5.52g on P1; 4) BUAL animals must weigh <5.52g on P1; 5) Litter size must be > 4 pups. All litters were culled to 6 on P1, with an even distribution of males and females when possible.

Surgeries were performed on ED20, in a sterile surgical suite. Dams were selected, in alternating order, to undergo either BUAL or SHAM surgery. The BUAL model utilized was adapted from Wigglesworth's original model of chronic placental insufficiency and fetal growth restriction (Buser et al., 2012). In brief, dams were induced and maintained under anesthesia for the duration of the procedure using Isoflurane. A longitudinal skin incision approximately 5cm long was made along the midline of the abdomen. Both horns of the uterus were externalized and left and right uterine arteries were ligated at the point most proximal to the bifurcation with 4-0 vicryl (Figure 2). Following ligation, the uterine horns were gently placed back inside the body and the muscle and skin layers were closed with 4-0 vicryl and 5-0 silk, respectively. Prior to closing the skin layer, 0.1mL of 0.5% bupivacaine was administered topically to the muscle layer for analgesia. The same anesthesia and surgical procedure was followed in SHAM animals but both uterine arteries were left intact. Dams were allowed to deliver vaginally on ED23.

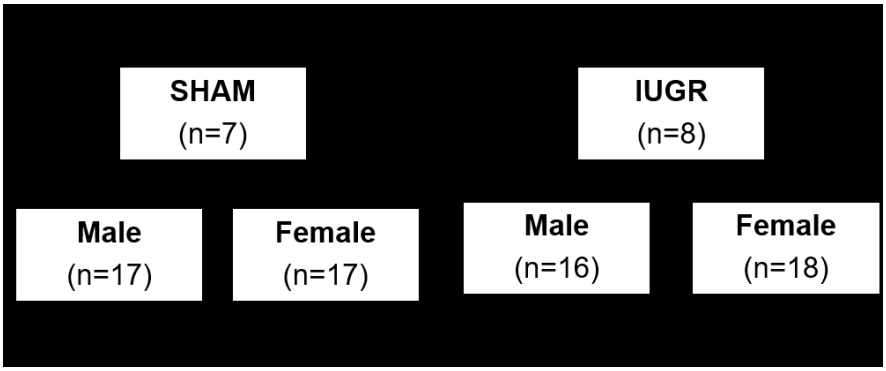

Figure 1. Study Groups. Pregnant dams underwent SHAM (n=7) or IUGR (n=8) surgery. Offspring were divided, based on treatment and sex, to form the following four study groups: 1) SHAM Males 2) SHAM Females 3) IUGR Males and 4) IUGR Females. Sample size of each is reported. In total, 68 animals underwent functional and histological assessment.

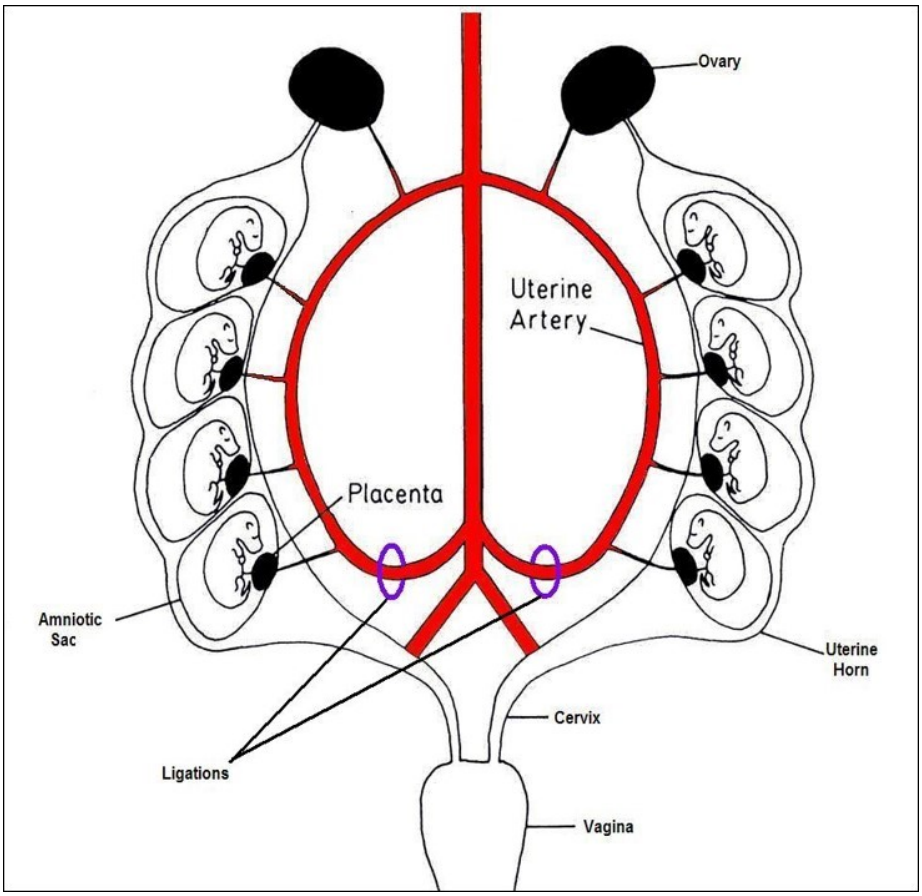

Figure 2. Bilateral uterine artery ligation schematic.

Animal Preparation

On P21 pups were weaned from their dam and housed two females or two males per cage. One week prior to behavioural testing, animals were handled and food rewarded daily. Behavioural assessment was instituted on the first Monday following P35, and was completed over 28 consecutive days (Figure 3). For the duration of testing, animals were food restricted to 95% of their projected body weight. Food restriction was used to increase the rats' motivation for food reward during skilled pellet reaching training. Animals were given approximately 1 hour to reacclimatize to the behaviour room before commencing testing each morning. All behavioural tests were recorded with a Sony HDR-CX700 Handycam video camera for later analysis.

In order to mitigate inter-researcher variability, the same researcher was responsible for animal handling and administration of functional assessment for the duration of the experiment. Due to the primary researcher's familiarity with the rats, double blinding was not always possible during functional assessment. Objective scoring was achieved in Morris water maze, elevated plus maze and open field by using Panlab Smart video tracking and analysis software. Beam walking and skilled pellet reaching were video recorded and then scored independently by two researchers. The average of the two scores was used for statistical analysis. Animals were coded to conceal sex and treatment during scoring. Although animals were coded to conceal sex and treatment during scoring, size discrepancy between males and females as well as the presence of visible genitals precluded reliable blinding to sex during the scoring of beam walking and skilled reaching. There was no visually detectable difference in size between IUGR and SHAM rats at time of functional assessment.

All histological evaluations were performed double-blind, and cell counts were verified by two independent experimenters. Brain specimens were re-coded by third party so that histological evaluation could not be biased by preceding functional assessment.

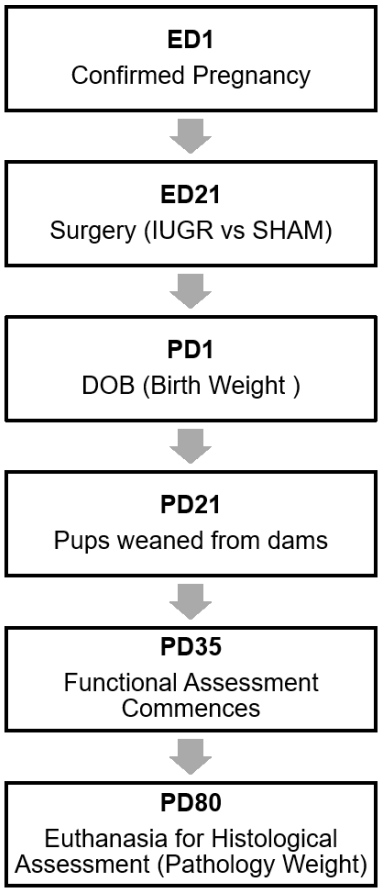

Figure 3. Experimental Paradigm.

Motor Assessment

Beam Walking

Hindlimb function was assessed by having animals traverse a raised, tapering beam. The beam is composed of a two tier ledge structure, which allowed for unintentional foot slips or "faults", from the top ledge to be assessed safely. Each animal underwent two consecutive trials,

and both trials were video-taped. The second trial was scored, using a method previously described by Schallert and Woodlee (Schallert & Woodlee, 2005). In brief, half faults were given a score of 1 and defined as anytime a portion of the hindfoot came off the top beam and bore weight on the lower ledge. Full faults were given a score of 2 and defined as anytime the entire hindpaw slipped off the top beam and landed on the bottom ledge.

Skilled Reaching

Box construction, training, and scoring was done in accordance with skilled reaching protocol outlined by Whishsaw (Whishaw et al., 2003; Whishaw & Kolb, 2005). Forty five mg dustless precision banana flavoured sucrose pellets (BioServ) were used for reaching.

Animals were trained 5 days per week for 4 consecutive weeks to reach for the pellet, eat it, and then return to the back of the cage in order for the next pellet to be placed.

Animals were scored on 10 distinct movements, based on the Eshkol-Wachmann movement scale that was adapted by Wishsaw (Metz & Whishaw, 2000) (Table 1). A 3-point rating system was used to grade each movement. If the movement was present and normal, then the animal received a score of 1 for that movement. If the movement was present but abnormal, then the animal received a score of point 0.5. If the movement was absent, then the animal was given a score of 0. On the day of testing, the animals were given ten attempts to reach and the first three "successful" reaches were scored. The average of these three reaches was used for statistical calculations. If the animal failed to complete three reaches it was excluded.

Table 1. Skilled Reaching steps. Adapted from Eshkol-Wachmann movement analysis.

Step	Movement	Description
2	Digits flexed	Paw is relaxed in supinated and semi-flexed position.
4	Advance	Forelimb is extended through the reaching slot. Head is raised in order to accommodate the limb.
6	Pronation	The paw is pronated and opened. Digits 2 through 5 all touch the shelf, so that paw is covering the pellet.
8	Supination I	Supination I occurs when the animal begins to withdraw the paw. Paw faces medially as it is withdrawn from through the reaching slot.
10	Release	The digits are opened, allowing the pellet to be eaten.

Gait

Footprint analysis, otherwise referred to as walking track analysis, was executed and analyzed following the methodology first described by de Mendinaceli et al., and well outlined by Sarikcioglu et al. (de Medinaceli, Freed, & Wyatt, 1982; Sarikcioglu, Demirel, & Utuk, 2009). In short, animals were trained to walk straight down a 10cm wide x 100cm long hallway with white paper lining the floor. Next, the animal's forepaws were dipped in blue ink and allowed to walk the length of the hallway. The forepaws were cleaned, and the same procedure was repeated after dipping the hindpaws in black ink. Stance (distance to opposite foot) and toe spread (distance from second to the fourth digit) were analyzed for both the front and hindpaw footprints.

Behaviour Assessment

Elevated plus maze

Elevated plus maze was conducted in accordance with Walf and Frye (Walf & Frye, 2007). The elevated plus maze apparatus consists of two opposing open and two opposing closed arms, constructed in a plus shape, that are raised above the ground. Animals were placed in the center of the maze, with the head pointing to an open arm. The animals were video recorded from above for 5 minutes. Panlab Smart (San Diego Instruments, San Diego CA) video tracking software was employed to count the number of entries made into each of the arms, as well as the amount of time spent in each arm.

Open field

The open field apparatus was a 60cm x 60cm x60cm Plexiglas box, with opaque sides. Animals were released in the center of the box, always facing the same direction. The trial was video recorded from above for 5 minutes. Panlab smart video tracking software was used to calculate percent of time spent in the center and percent of time spent in the perimeter (Schmitt & Hiemke, 1998a).

Cognitive Testing

Object Recognition

Object recognition was always performed the day after open field testing, in the same Plexiglas box. Animals were placed in the apparatus with two identical objects for five minutes. One hour later they were replaced in the apparatus with one object from the first trial and a new

object. Object recognition was manually scored from video footage. The amount of time (s) spent with the familiar object and the amount of time spent with the novel object in the final phase was analyzed. Score = time spent with new object/time spent with both objects (Bevins & Besheer, 2006).

Morris Water Maze

Apparatuses were constructed using the original Morris protocol, with incorporation of modifications from by Anisman and McIntyre (Anisman & McIntyre, 2002; Morris, 1984) . Testing was carried out in a large white, circular pool measuring 180 cm in diameter, located in a designated water maze room with minimal extraneous visual cues. The extra maze objects that were present, including the tracking apparatuses and the experimenter, were considered visual cues for spatial navigation and remained in the same position in the room throughout all the trials. The platform, 10 cm by 10 cm, was constructed of clear Plexiglas, such that it was invisible when it was submerged under 2cm of 20 degree Celsius water. A solid black rectangular box was used as the platform cue, and hung from the ceiling approximately 60cm above the platform. Distracter cues were similar in size to the platform cue but black and white striped. The distracter cues included a black and white circle, square, triangle and ring. The water maze, as originally described by Morris in 1984, assesses memory by calculating the amount of time the animal spends swimming before it finds the hidden platform and escapes from the water. Our lab followed the original Morris protocol, including use of the hidden platform, with two major adaptations to differentiate between allocentric and egocentric swim strategies. The first major adaptation was use of varying animal start positions, as described by Dupret et al. (Dupret et al., 2008) The second notable change was use of varying platform

quadrant as described by Schallert et al. (Choi, Woodlee, Hong, & Schallert, 2006). All trials were video recorded from above and tracked using Panlab Smart Video Tracking Software. The following parameters were recorded, extract rated and analyzed: latency to platform, distance travelled, swim speed and Whishaw's error index.

Neuropathology

Euthanasia

Following completion of neurobehavioural assessment, animals were euthanized at P80. Animals were first anesthetized in a plastic induction box using 5% isoflurane in 30% oxygen and balanced nitrogen. Immediately following the excitatory phase of induction, animals were decapitated using a guillotine. Skulls were split down the midline and the brain removed, carefully, as not to disrupt the tissue. Brains were fresh frozen in isopentane, in an ethanol/dry ice bath, and stored at -80°C until analyzed.

Brain sectioning

Fresh frozen brains were cut into 14 μm sections at -15°C using a *Recichert Jung Cryocut 1800* cryostat. Serial sections were collected every 0.5 mm starting from where the corpus collosum joins in the anterior region of the brain and continuing throughout the cerebrum. All sections were stained with hemotoxylin and eosin but only three sections/brain were used for Olig-2 and Myelin Basic Protein (MBP) immunohistochemistry. The three points of interest were determined based on the level of the brain at which the following structures appeared 1) the anterior commissure pars posterior, 0.5mm anterior to bregma, 2) the complete hippocampus,

3.0mm posterior to bregma, and 3) the lateral ventricles, 3.5mm posterior to bregma (Paxinos & Watson, 2005).

Hematoxylin + Eosin (H&E) Staining

Sections of brain, described above, were stained with Harris Hematoxylin and Eosin (H&E) following a standard staining protocol.

Hippocampal pyramidal neurons were counted, directly under the microscopic, in CA1 and CA3 of the hippocampus (Figure 4). Cell counting methodology was described previously by our laboratory which was adapted from Colbourne and Corbett (Black, 2010; Colbourne & Corbett, 1995).

H&E stained sections were then photographed using a Leica MC170 HD camera attached to a Leica GZ6E stereoscope. The areas of both right and left lateral ventricles was then measured in 2 sections anterior to bregma, bregma, and 6 sections posterior to bregma with ImageJ ver. 1.41(Schneider, Rasband, & Eliceiri, 2012). The ventricle volumes were then calculated by integrating the distance between sections and the ventricle areas.

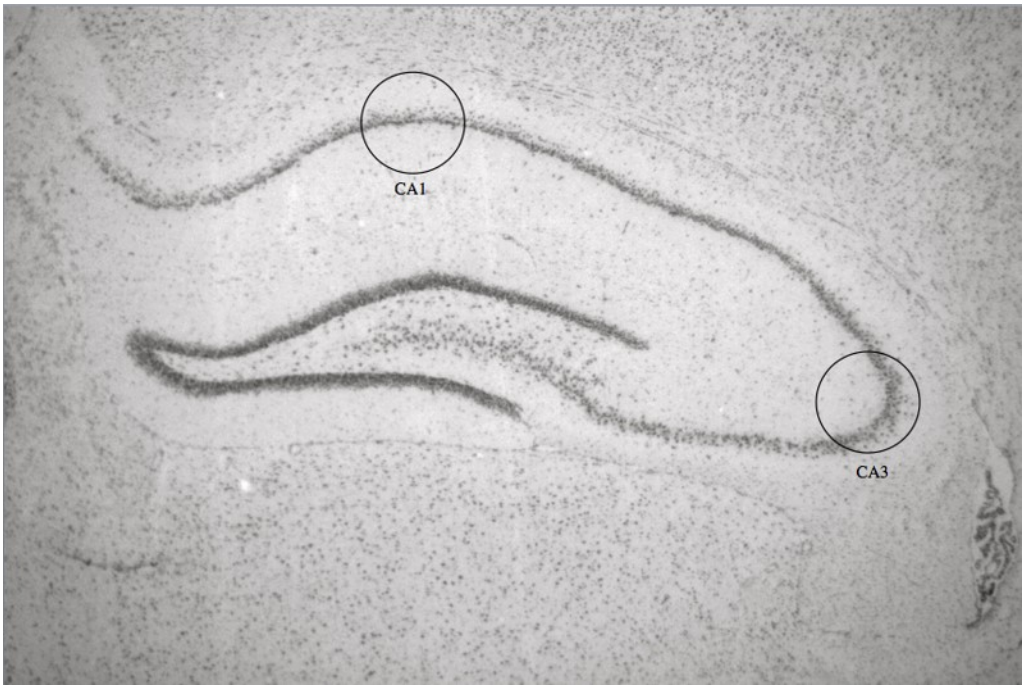

Figure 4. CA1 and CA3 regions of the hippocampus. Number of neuronal cells in each outlined area was counted.

Myelin Basic Protein (MBP)

MBP is a chief constituent of the myelin sheath of oligodendrocytes, and therefore can be used to detect and quantify white matter if present (Baud et al., 2004). Immunohistochemistry was performed on 14 μm sections of fresh frozen brain, described above, using a standard ABC detection kit and protocol. Anti-MBP antibody (Covance, Princeton NJ) was used as the primary antibody at a dilution of 1:2000. Densitometry was performed on 6 areas of the brain including an anterior and posterior section of the medial corpus callosum, cingulum, and lateral corpus callosum. All densitometry was obtained using ImageJ calibrated with a Kodak No. 3 Calibrated Step Tablet scanned with an Epson Expression 1680 Professional scanner. The average density of all regions was calculated, after correcting for the background, and used for statistical analysis.

Olig-2

Oligodendrocyte and their progenitor cells were stained for using Olig-2 (Vontell et al., 2013). Anti-Olig-2, (Millipore, Etobicoke ON) at a dilution of 1:2000 was used as the primary antibody, followed by detection with Vectastain ABC kit. Images of the periventricular white matter and corpus callosum, in the anterior and posterior brain, were taken at 400x magnification with a Leica MC170 HD camera attached to a Leica DM2000LED microscope.

Statistical Analyses

Two-way analysis of variance (ANOVA) was used first to determine if an overall effect, and interaction, of treatment and sex on functional performance and neurological pathology were present. Results generated by two-way ANOVA were reported as: ($F(1, df)= F$, p-value). Next, an unpaired t-test was performed on interval data to quantify the size of treatment effect. Unpaired t-tests were performed in males and females separately. For all unpaired t-tests, an F-test was used to compare variances. If variance was not significant ($p \geq 0.05$), then results were reported as: ($t(df)= t$, p-value). If variance was significant ($p < 0.05$), then means were re-evaluated using an unpaired t-test with Welch's correction. Magnitude of difference between SHAM and IUGR was reported as (Mean ± SEM, N of SHAM, Mean ± SEM, N of IUGR). For ordinal data, which included beam walking and skilled pellet reaching, results were analyzed using Mann Whitney U test. Results were reported as (SHAM median, IUGR median, U statistic, p-value). Graphs were provided for significant functional results. Graphs for significant findings and pertinent negative findings were included for histological results.

Chapter 3: Results

Intrauterine Growth Restriction (IUGR) and Mortality

BUAL surgery had a significant overall effect on birth weight ($F(1, 57)=54.53$, $p<0.0001$). There was no effect of sex ($F(1, 57)=1.78$, $p= 0.1877$), or interaction between treatment and sex ($F(1, 57)=0.05$, $p=0.8191$), on birth weight (Figure 5A). Male IUGR pups weighed significantly less than male Sham pups on P1 ($t(27)=5.522$, $p<0.0001$). Female IUGR pups also weighed significantly less than female Sham pups on P1 ($t(22)= 5.495$, $p<0.0001$). IUGR animals demonstrated catch up growth by the end of the study (Figure 5B). At P80, treatment had no effect overall on weight ($F(1, 41)=1.99$, $p=0.1660$). There was a significant effect of sex on P80 weight ($F(1, 14)= 199.47$, $p=0.0001$). There was no significant interaction between treatment and sex on P80 weights ($F(1,41)= 0.05$, $p=0.8201$). *In utero* mortality was estimated based on number of viable pups born on P1. As seen in Figure 6 and Table 2, litter size was significantly smaller following BUAL surgery ($t(11)=6.722$, $p<0.0001$).

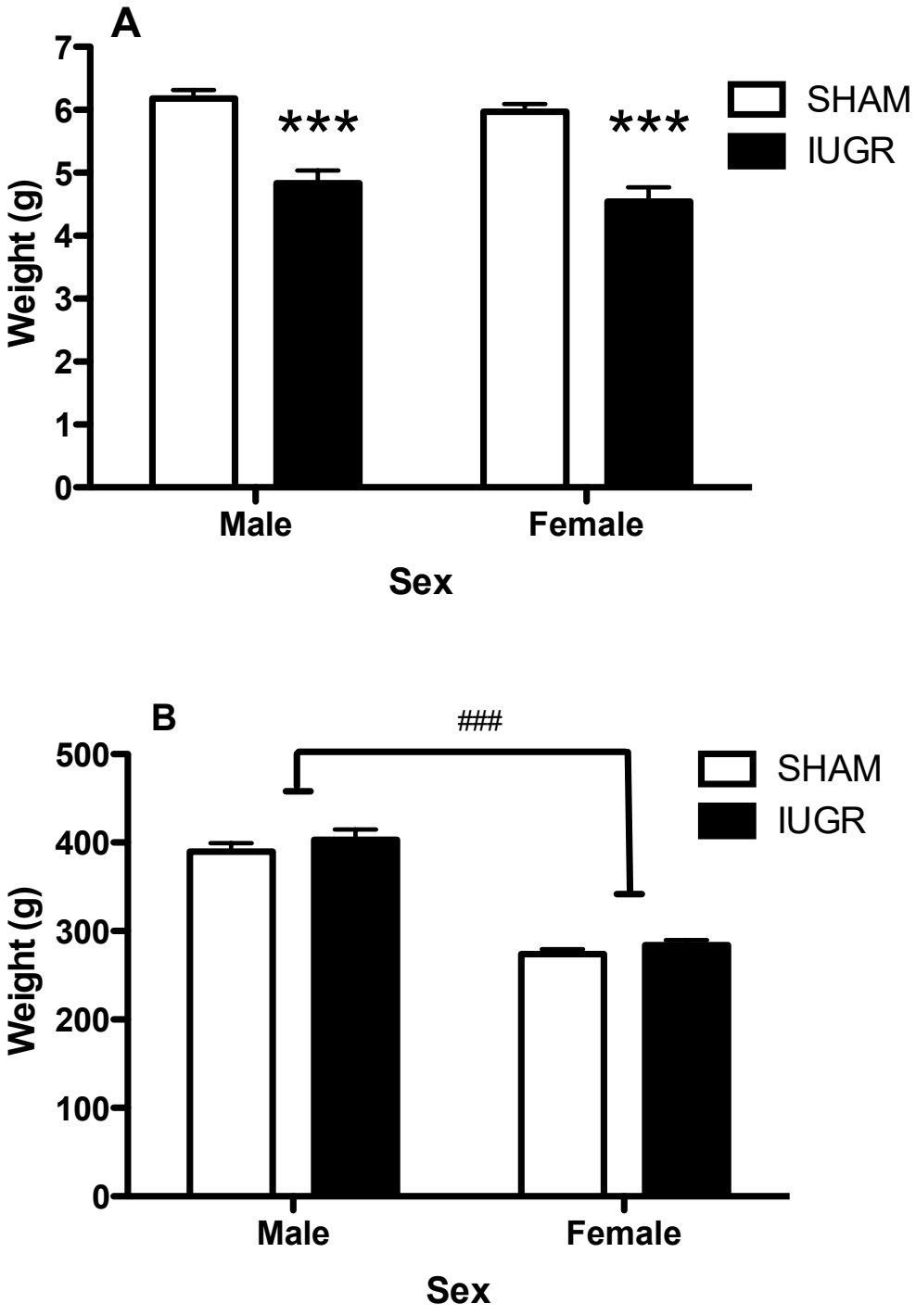

Figure 5. Weights of rats at birth (A) and at 80 days old (B). IUGR rats show catch up growth as they get older. Mean ± SEM, *** - Significantly different from sham animals ($p<0.001$) by Student's t-test, ### - Significant difference between male and female rats ($p<0.001$) by 2-way ANOVA.

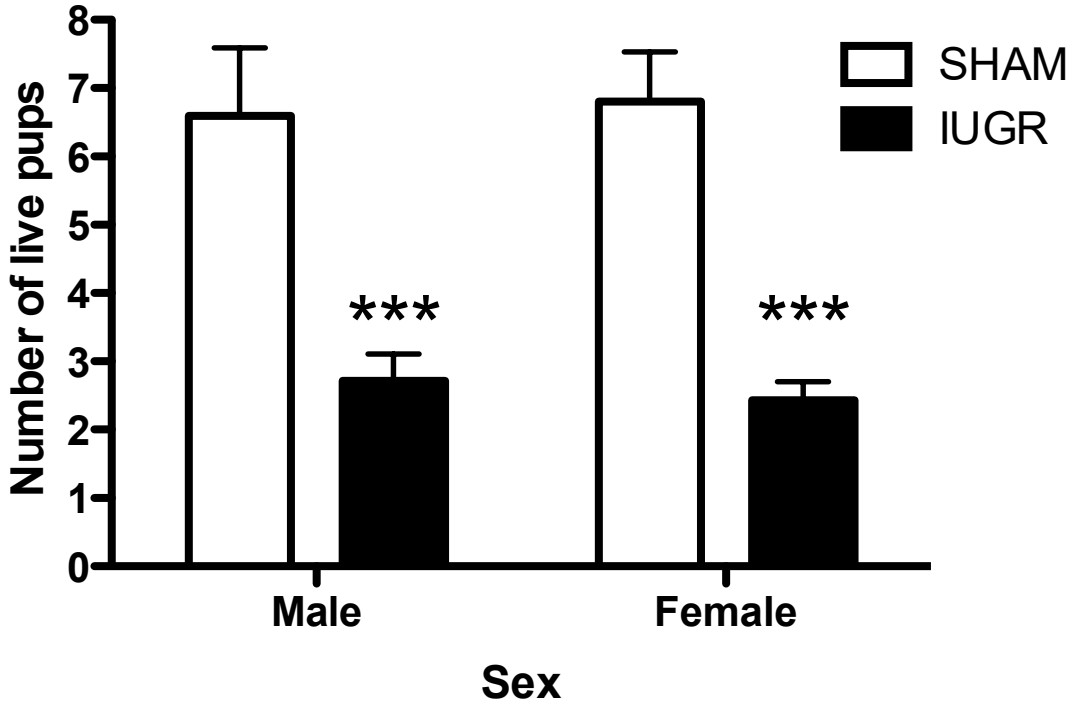

Figure 6. Effect of BUAL surgery on litter size. The number of pups born to Sham and BUAL treated pregnant rats were counted. Mean ± SEM, *** - Significantly different from Sham animals by Student's t-Test ($p<0.001$).

Table 2. Birth and Pathology Weight by Litter. Average weight in grams for sex is reported. Dams are labelled in the chronological order in which they underwent surgery. Pups that did not meet the weight criteria for their respective groups were excluded from the study. Litters were further culled to ensure uniformity in maternal attention and nutrition prior to weaning. Where ever possible P1 brains were collected and allocated to alterative studies. Approximately two males and two females from each dam underwent long term functional and histological assessment. *N/A-Pathology weights were not documented for litter 4.

Dam	Treatment	Litter Size	Birth Weight (M)	Birth Weight (F)	Pathology Weight (M)	Pathology Weight (F)
2	SHAM	17	6.44 (n=10)	6.12 (n=7)	377 (n=3)	291 (n=2)
4	SHAM	10	6.80 (n=5)	6.41 (n=5)	N/A (n=2)	N/A (n=2)
6	IUGR	13	5.47 (n=6)	5.12 (n=7)	442 (n=4)	276 (n=4)
8	IUGR	5	4.44 (n=3)	3.59 (n=2)	417 (n=2)	246 (n=2)
10	IUGR	4	4.07 (n=2)	5.48 (n=2)	398 (n=2)	288 (n=2)
12	IUGR	5	4.50 (n=2)	3.41 (n=3)	350 (n=1)	276 (n=1)
14	SHAM	10	6.54 (n=5)	6.11 (n=5)	401 (n=2)	268 (n=3)

Motor Results

Beam Walking

Treatment had a significant effect overall on beam walking performance ($F(1,58)= 25.91$, $p<0.0001$). No interaction or effect of sex was observed (Figure 7). IUGR males made significantly more hindlimb foot faults traversing the tapered beam than Sham males (Sham Median=1.5, IUGR Median=8.5, Mann-Whitney U=46.00, p=0.064). Likewise, IUGR females made significantly more hindlimb foot faults than Sham females (Sham Median=3, IUGR Median=6.0, Mann Whitney U=59.50, p=0.0107).

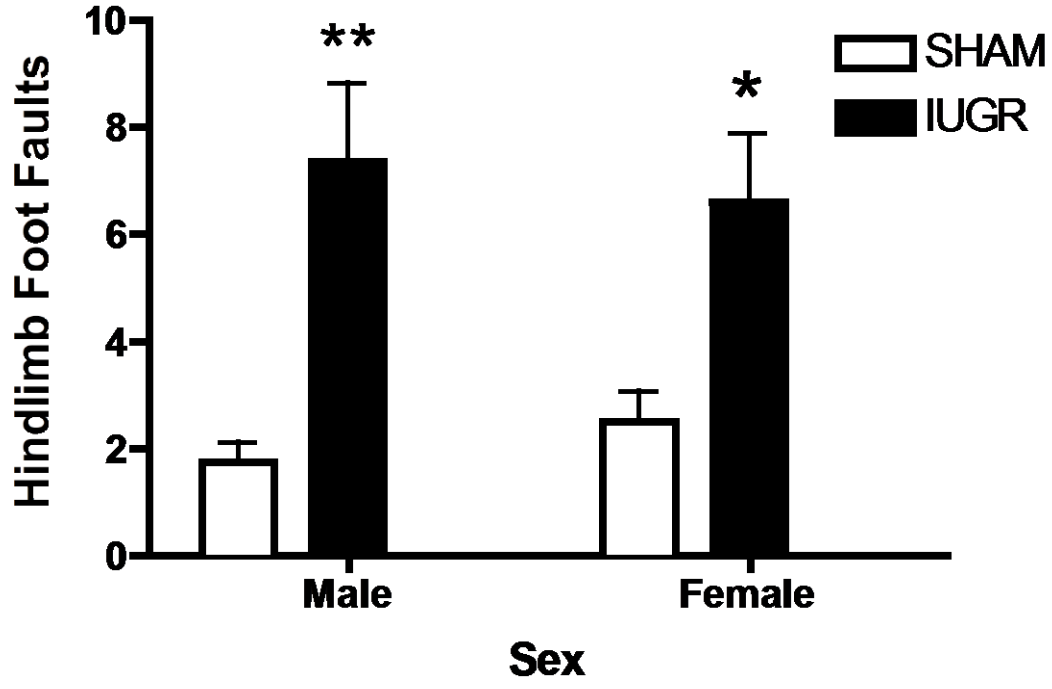

Figure 7. Beam Walking. The number of foot faults made during one trial of tapered beam walking was scored. Each step with the hind foot was given a score of 0, 0.5 or 1. The total number of foot faults were added together. Mean ±SEM, *Significantly different from Sham by Mann-Whitney U Test (** - $p<0.01$, *$p<0.05$).

Skilled Reaching

Treatment had a significant overall effect on skilled reaching performance in five of the ten steps of reaching, defined as digits to midline, elbow to midline, digits extended, supination II and release (Table 3). There was a significant ($p=0.0194$) interaction between treatment and sex on step 5 (digits extend). Treatment effect was quantified in males and females separately using a Mann-Whitney U test. Results for each step are presented in Table 4 and Figure 8. Both male and female IUGR rats performed significantly worse than Sham rats on step 5 (digits extend).

Step 1 (Digits to midline): IUGR animals made significantly more errors bringing their digits to midline than Sham animals ($F(1, 51)=4.32$, $p=0.0427$). There was no interaction, or effect of sex.

Step 3 (Elbow to midline): A significant overall effect of treatment ($F(1, 49)= 9.78$, $p=0.0030$) and sex ($F(1,49)=11.71$, $p=0.0013$), with females performing worse, on ability to bring the elbow to the midline of the body. The interaction was not significant. IUGR females made significantly more errors than Sham females (Sham Median=0.17, IUGR Median=0.50, Mann-Whitney $U=45.00$, $p=0.0471$). There was no difference in the ability of Sham and IUGR male rats to complete this step.

Step 5 (Digits extended): There was a significant effect of treatment ($F(1,51)= 5.57$, $p=0.0221$) on extension of digits. The effect of sex was not quite significant ($F(1,51)=3.23$, $p=0.0782$); however a significant interaction ($F(1,51)= 5.83$, $p=0.0194$) between treatment and sex was noted. IUGR males made significantly more errors in digit extension than Sham males (Sham Median= 0.00, IUGR median=0.17, Mann-Whitney $U=51.00$, $p=0.0243$). Similarly,

IUGR females also made more errors than Sham females (Sham Median=0.00, IUGR Median=0.17, Mann- Whitney U=40.50, p=0.0249).

Step 9 (Supination II): There was a significant effect of both treatment ($F(1,51)=11.89$, $p=0.0011$) and sex ($F(1,51)=4.55$, $p=0.0011$) on the completion of supination. There was no significant interaction between sex and treatment. IUGR males made significantly more errors than Sham males (Sham Median=0.00, IUGR median=0.33, Mann-Whitney U=29.50, p=0.0014). The difference between Sham females and IUGR females was not significant.

Step 10 (Release): There was a significant effect of both treatment ($F(1,50)=6.06$, $p=0.0173$) and sex ($F(1,50)=6.43$, $p=0.0144$) on release of pellet to mouth (Figure 9). The interaction was not significant. IUGR males made significantly more errors than Sham males (Sham Median=0.00, IUGR median=0.50, Mann-Whitney U=39.50, p=0.0117). There was no difference between SHAM females and IUGR females.

Table 3. Skilled pellet reaching results from two-way ANOVA. P-values pertaining to overall effect of treatment, sex and interaction for each step of skilled reaching.

Step	Movement	Interaction	Treatment	Sex
2		p=0.7059		p=0.0310
4		p=0.5973		p=0.8183
6		p=0.1230		p=0.2137
8		p=0.1448		p=0.1041
10		p=0.0537		p=0.0144

Table 4. Skilled Pellet Reaching results from Mann- Whitney U test. P-values indicate difference between IUGR and SHAM median performance on each step of skilled reaching. Significant p-values are italicised.

Step	Movement	Male	Female
1	Digits to Midline	p=0.1396	p=0.2638
2	Digits Flexed	p=0.8196	p=0.1954
3	Elbow to Midline	p=0.0844	***p=0.0471***
4	Advance	p=0.5555	p=0.8131
5	Digits Extend	***p=0.0243***	***p=0.0249***
6	Pronation	p=0.1052	p=0.4745
7	Grasp	p=0.3698	p=0.5871
8	Supination I	p=0.3719	p=0.9783
9	Supination II	***p=0.0014***	p=0.3447
10	Release	***p=0.0117***	p=0.3911

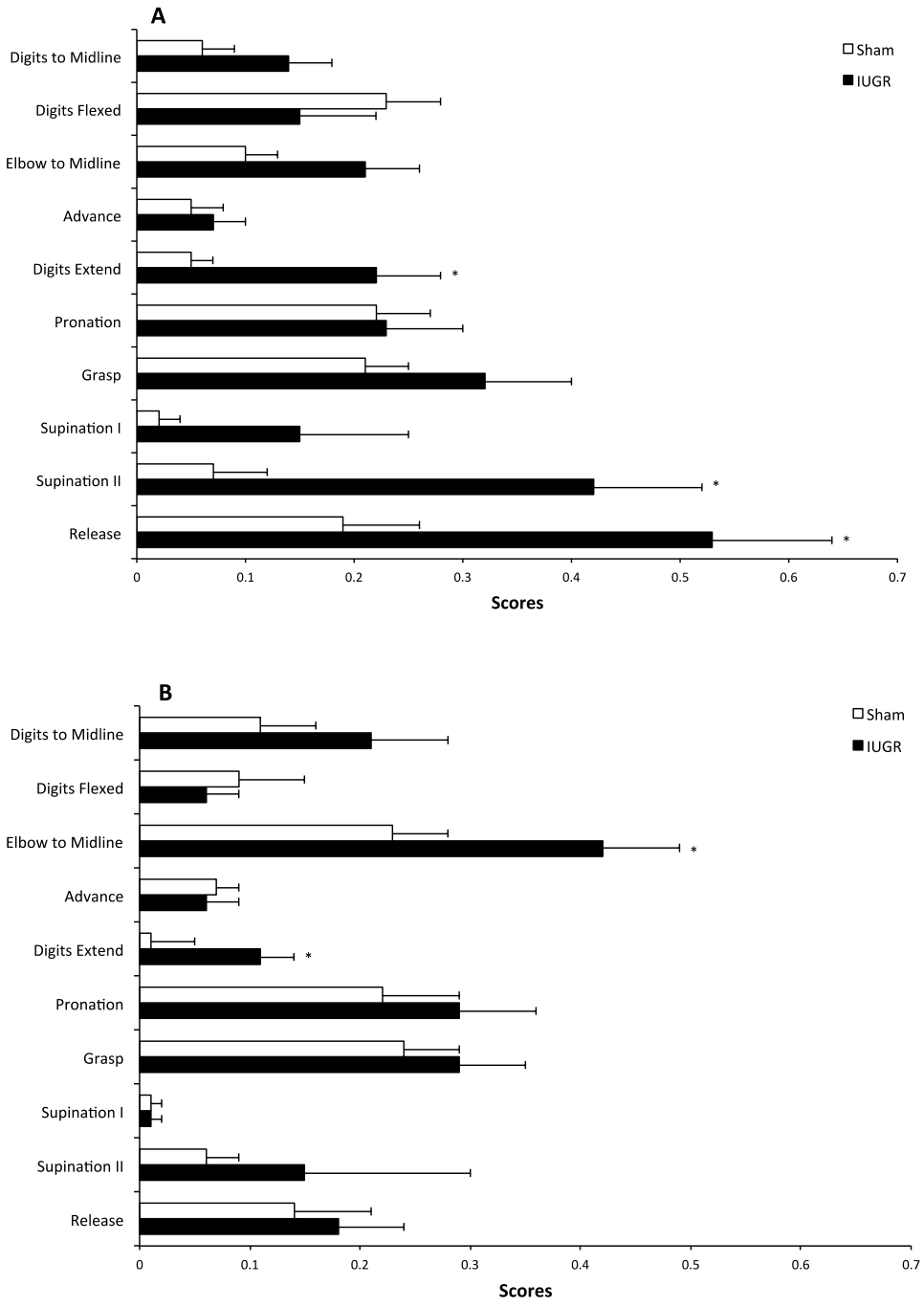

Figure 8. Skilled Reaching. The ability of male (A) and female (B) rats to reach for and ingest was scored for 10 different movement parameters in 3 successful reaches. Mean ± SEM, * - Significantly different from Sham by Mann- Whitney U Test ($p<0.05$).

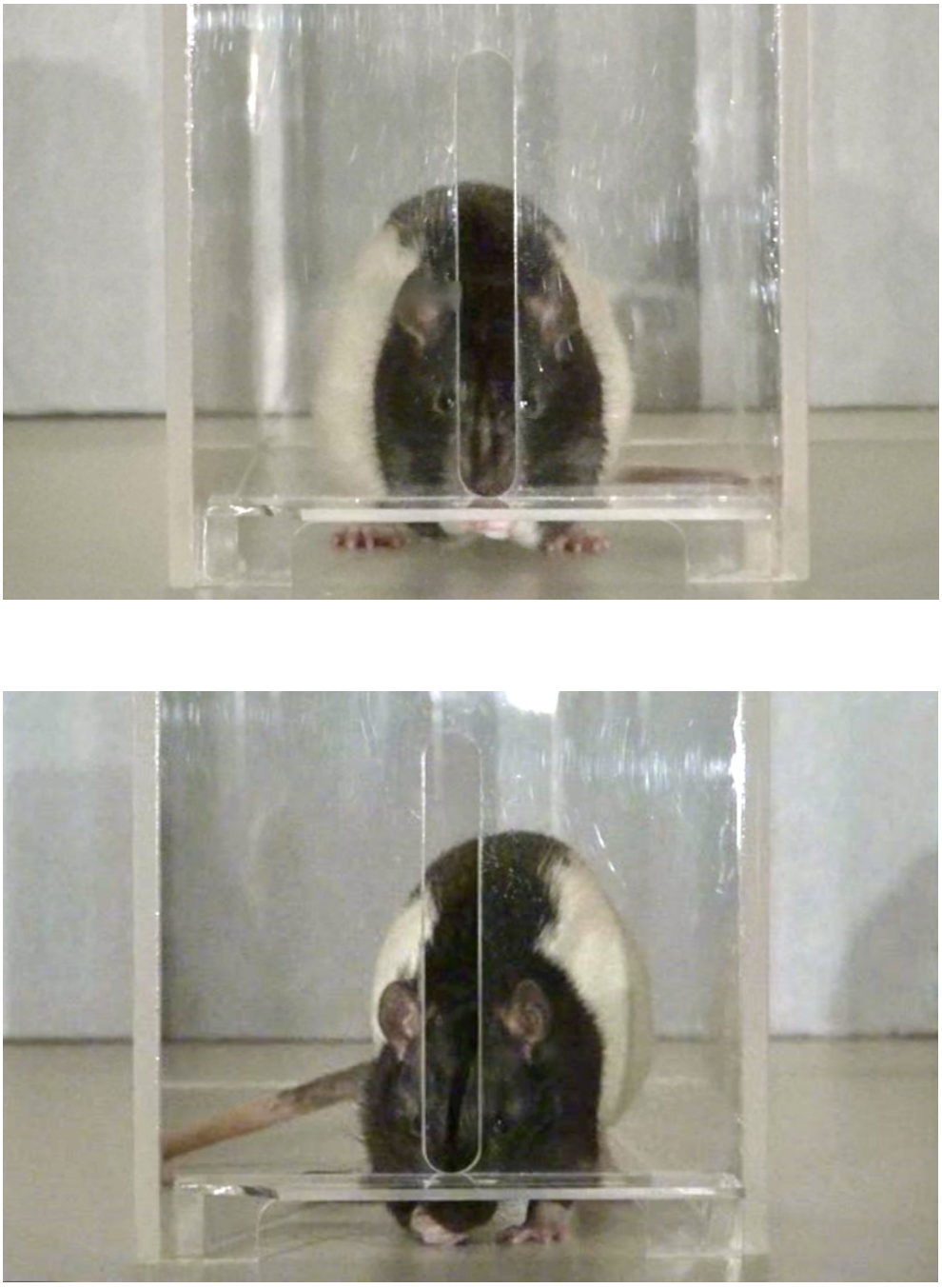

Figure 9. Skilled reaching step 10: Normal release performed by SHAM male (A) and absent release by IUGR male (B). In (A) the animal is well balanced, with both forepaws off the ground. He brings the food pellet towards the mouth with supinated paws, and releases it normally. In (B) the animal does not independently move his forelimb/forepaw in order to accomplish the step. Instead the step is completed by lowering of the head to retrieve the food pellet from a forepaw that is stabilized on the ground. Note also that the non-reaching limb remains on the ground, throughout the release, for stability.

Gait Analysis

No overall effect of treatment was observed for forelimb gait analysis. However, an overall effect of sex was observed in hindlimb stance ($F(1,42)= 14.67$, $p=0.0004$), with male rats having a wider stance than female rats. The spread of toes 3 -5 on the rats' hind paw also showed an effect of treatment ($F(1,42)=14.21$, $p=0.0005$, sex ($F(1,42)=16.14$, $p=0.0002$) and an interaction between sex and treatment, as shown in Figure 5. When the difference in means between treatment groups was quantified using an unpaired t-test, IUGR males were observed to have a significantly larger toe spread ($t(21)=3.465$, $p=0.0023$) than Sham males.

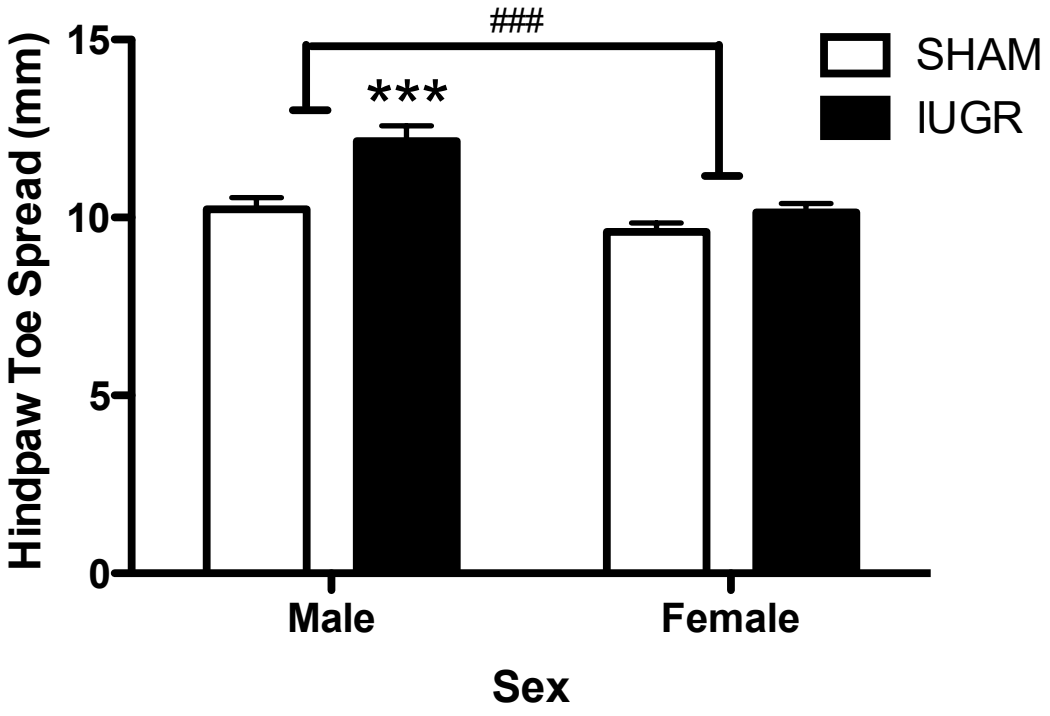

Figure 10. Gait analysis. The distance between toes 3 and 5 on the hindpaw were measured in 4 consecutive strides. Mean ± SEM, *** - Significantly different from Sham animals ($p<0.001$) by Student's t-test, ### - Significant difference between male and female rats ($p<0.001$) by 2-way ANOVA.

Behaviour Results

Elevated Plus Maze

Treatment had a significant effect overall on the percent of time animals spent in the open arms ($F(1,48)=12.01$, $p=0.0011$) of the elevated plus maze, with no effect of sex, or interaction. IUGR males spent significantly less time in the open arms than Sham males ($t(25)=2.47$, $p=0.0207$). Likewise, IUGR females spent significantly less time in the open arms than Sham females ($t(18)=2.387$, $p=0.0281$). Furthermore, the treatment had a significant effect overall on the number of discrete entries made in to the open arms of the maze ($F(1,48)=7.51$, $p=0.0086$). Although they spent significantly more time in the open arms than IUGR males, SHAM males did not make significantly more entries. SHAM females did make significantly more entries than IUGR females into the open arms ($t(1, 16)=2.414$, $p=0.0281$).

Open Field

There was no overall effect of treatment, sex or interaction on time spent in the center of the apparatus, after Welch's correction was applied to the unpaired t-tests to account for significant variation.

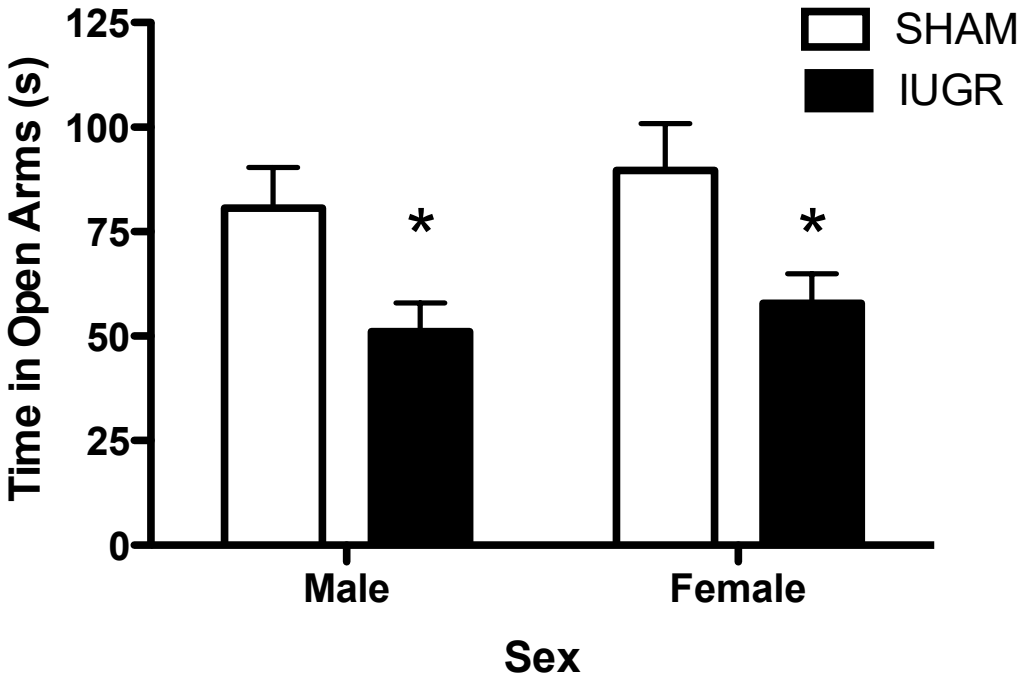

Figure 11. Elevated Plus Maze. The amount of time spent in the open arms of the elevated plus maze during a 5 minute trial was measured. Mean ± SEM, * - Significantly different than Sham by Student's t-Test ($p < 0.05$).

Cognition Results

Object Recognition

There was no overall effect of treatment, sex or interaction on percent time spent with the novel object.

Morris Water Maze

Males and females were evaluated independently in regards to latency to target, distance travelled and Whishaw's index. There was no effect of treatment on latency to target in males or females. There was a significant effect of day on latency to target in both males ($F(1, 118)=0.30$, $p=0.5866$) and females ($F(1, 131)= 78.38$, $p<0.0001$). There was no interaction between treatment and day in males or females. IUGR males traveled a greater distance to reach the platform, but the result was not significant. Conversely, female Shams travelled a greater distance to reach the platform ($F(1,119)=8.47$, $p=0.0043$). Spatial learning, as depicted by swim strategy (efficiency of the swim path to reach the goal location), was quantified using Whishaw's index. Treatment had a significant effect overall on Whishaw's index in males ($F(1,124)= 14.52$, $p=0.0002$) but not females. The effect of day and the interaction was not significant. SHAM males had a significantly better Whishaw's index, indicating more efficient swim path, on days 1 ($t(27)=2.686$, $p=0.0122$) and 4 ($t(30)=3.041$, $p=0.0049$) of the water maze than IUGR males. The Whishaw's index on days two and three were not statistically significant; however a consistent trend was observed where SHAM males continued to improve their swim strategy over time while IUGR males did not.

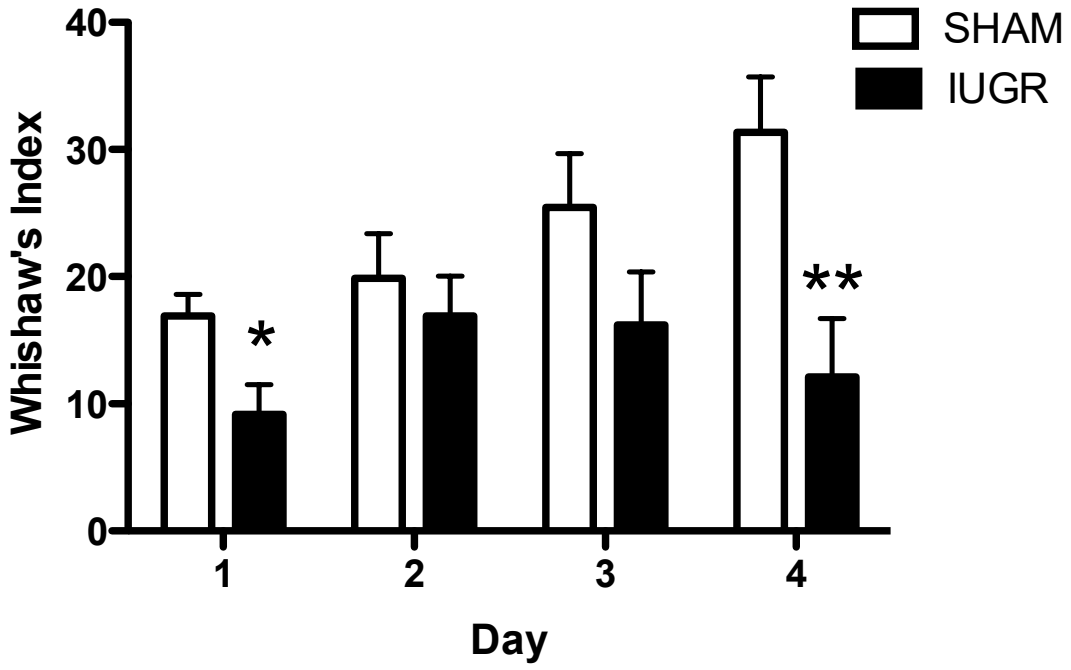

Figure 12. Morris water maze: Whishaw index in male rats. The Whishaw index during four days of water maze was calculated. Mean ± SEM, ** - Significantly different than Sham by Student's - Test ($p < 0.01$), * - Significantly different from Sham by Student's t-test ($p<0.05$).

Neuropathology

Hippocampal Cell Counting

An overall effect of treatment on the number of pyramidal neurons was observed in both the CA1 ($F(1, 59)=6.11$, $p=0.0163$) and CA3 ($F(1, 59)=8.90$, $p=0.0041$) region of the hippocampus. No overall effect of sex or interaction was observed in either the CA1 or CA3 region of the hippocampus. IUGR males had significant loss of pyramidal neurons in the CA3 region ($t(27)=2.680$, $p=0.0124$). 52), but not the CA1 region, compared to Sham males. SHAM females had more pyramidal neurons in both the CA1 and CA3 region than IUGR females; however neither difference was significant.

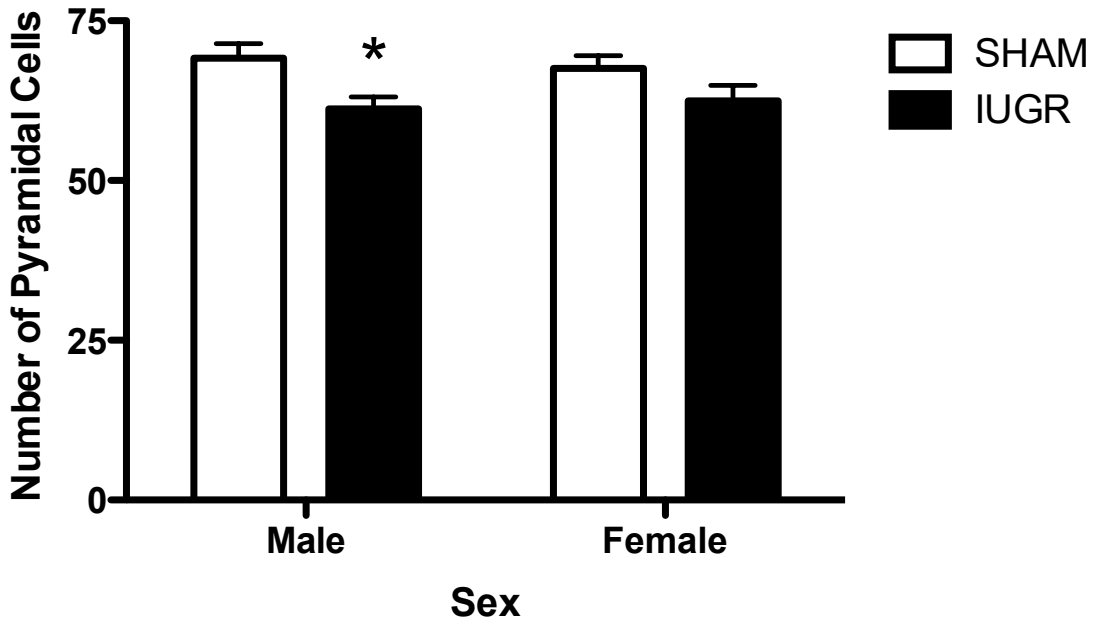

Figure 13. Number of Pyramidal Cells in 0.238 mm2 area of CA3. Mean ± SEM, * - Significantly different from Sham by Student's t Test ($p < 0.05$).

Lateral Ventricle Volume

No effect overall of treatment, sex or interaction was observed (Figure 14).

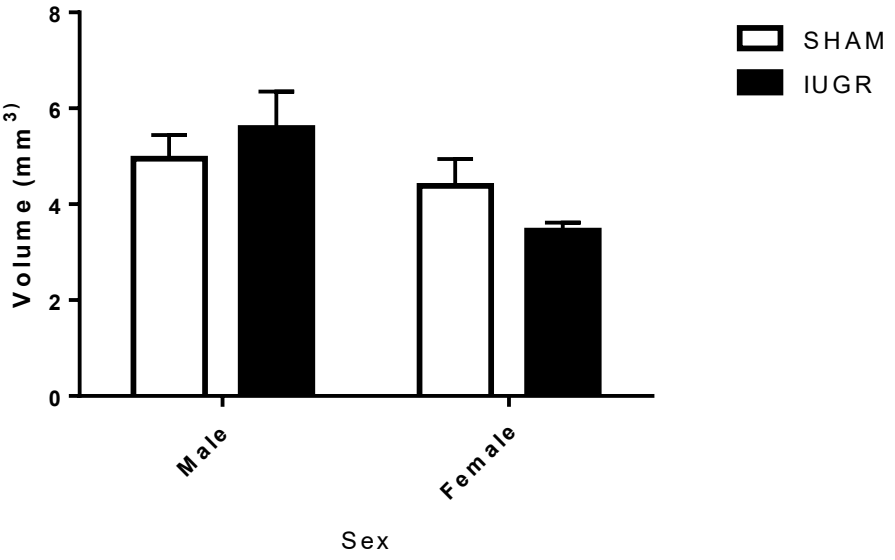

Figure 14. Lateral Ventricle Volume. No significant differences were detected between SHAM and IUGR rats. Mean ± SEM, * - Significantly different from Sham by Student's t-Test ($p<0.05$).

Immunodensity of MBP

No overall effect of treatment, sex or interaction on immunodensity of MBP was observed in the anterior (Figure 15) or posterior (Figure 16) aspects of the medial corpus callosum (CC), cingulum, nor lateral CC.

Olig 2+ Cell Counting

There was no effect overall of treatment, sex or interaction on number of Olig2+ cells counted in the periventricular white matter or corpus callosum.

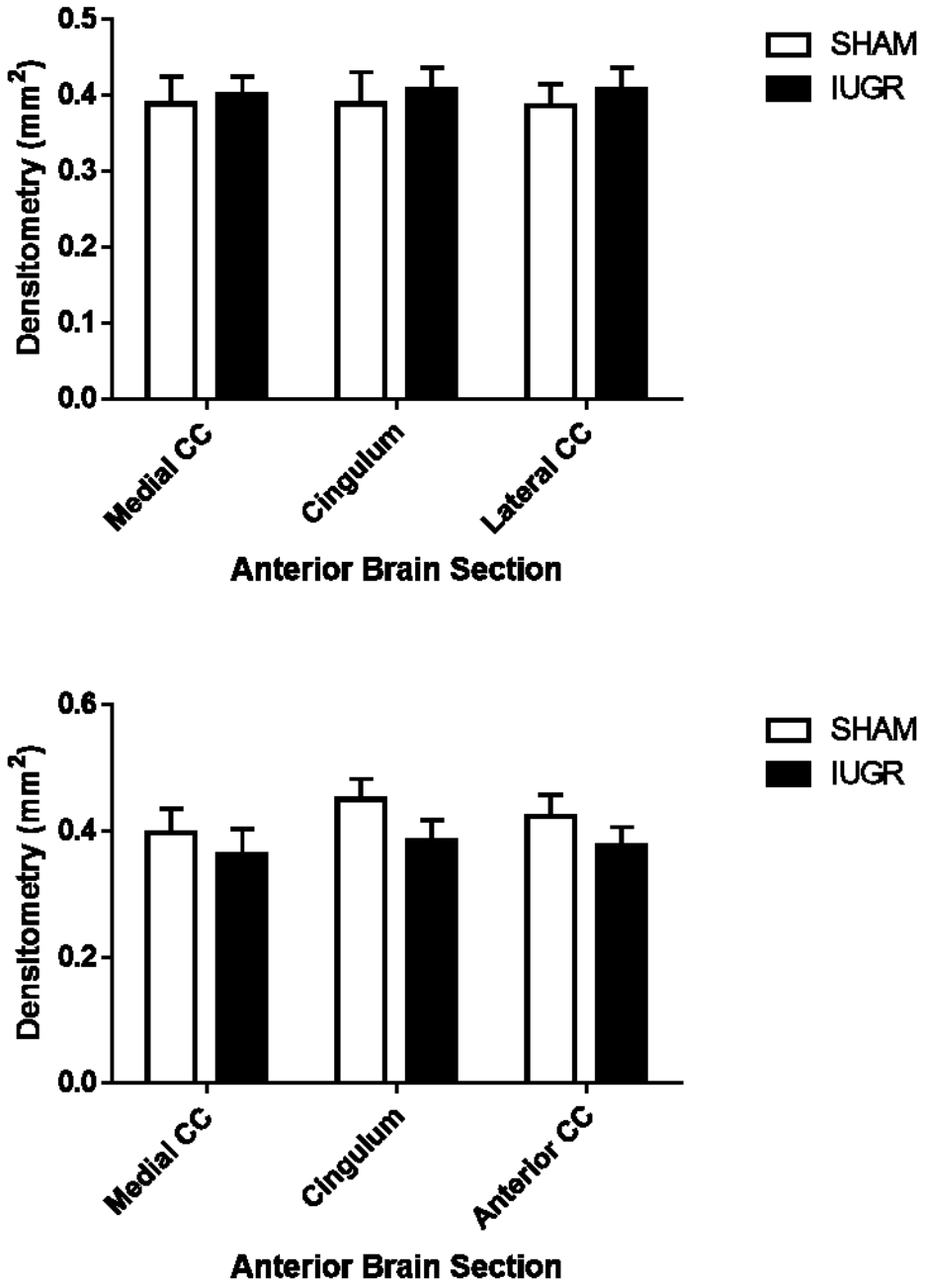

Figure 15. Myelin basic protein (MBP). Density of MBP in the anterior brain in male (A) and female (B) rats. No significant differences were detected between SHAM and IUGR rats. Mean ± SEM, * - Significantly different from Sham by Student's t-Test ($p<0.05$).

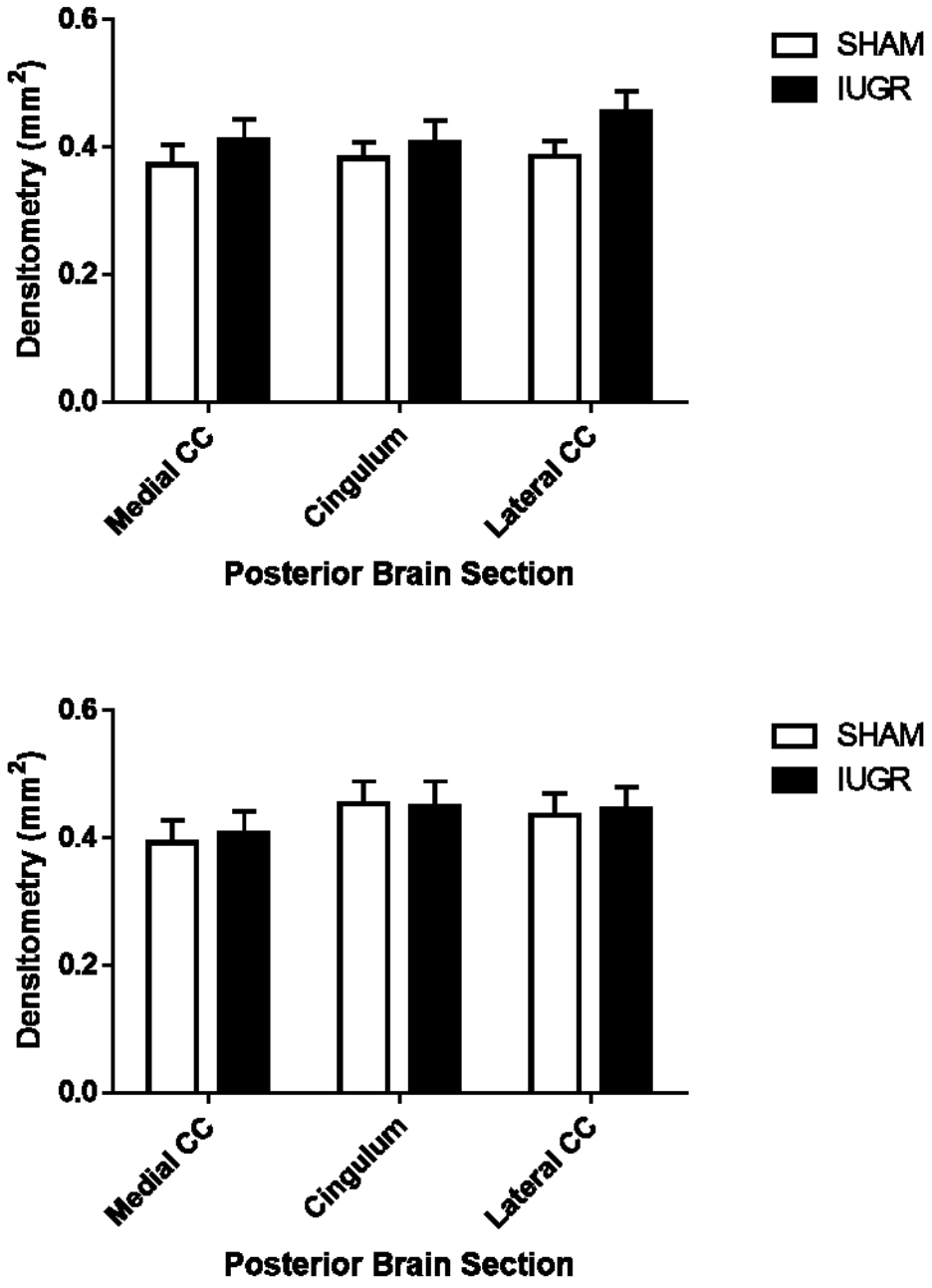

Figure 16. Myelin basic protein (MBP). Density of MBP in the posterior brain in male (A) and female (B) rats. No significant differences were detected between SHAM and IUGR rats. Mean ± SEM, * - Significantly different from Sham by Student's t-Test ($p<0.05$).

Chapter 4: Discussion

The purpose of this study was to determine whether bilateral uterine artery ligation (BUAL) in the Long-Evans rat results in permanent neuro-deficits that are reminiscent of CP. Furthermore, it aimed to ascertain whether or not these deficits can be discriminated using functional and histological assessment tools. This experiment verified that BUAL on E20 consistently results in IUGR. The most likely mechanism of this is intrauterine ischemia secondary to placental insufficiency following ligation of the uterine arteries. It further established that adult IUGR offspring exhibit significant motor, behavioural and cognitive impairments. The degree of functional impairment observed was not reflected by pathology. Underlying brain pathology was not readily detected by the histological techniques used. Interestingly, the only statistically significant pathology observed was a reduction in the number neurons of the CA3 region of the hippocampus. Quantitative reduction in white matter was not identified.

Previously, our laboratory used BUAL to study neurodevelopmental outcomes in rat pups up to P21. Results from that study demonstrated that BUAL resulted in IUGR offspring whom exhibited delayed acquisition of newborn reflexes as well as behavioural abnormalities up to P21 (Black, 2010). Our laboratory hypothesized that these findings were due to underlying permanent hypoxic ischemic brain injury. Due to the short term nature of the study, it was impossible to exclude transiently delayed myelination or strength discrepancy secondary to smaller size as a possible explanation for the neurodevelopmental delay. The current study was conducted to prove that the neurological deficits observed were secondary to hypoxic ischemic cell death, not simply delayed myelination, and will therefore persist long term. As anticipated, this study confirmed that without early intervention neurological deficits persist in the mature rat.

Clinically, IUGR rats remain significantly impaired in all three areas of functional testing. Surprisingly, the majority of brain pathology observed at P21 is no longer detectable at post-natal day 80.

The overall goal of this project was to establish an animal model of CP. The reason for doing so is to fill a clinically relevant gap in the literature. Currently there is no ideal model that exists for the testing of therapeutic interventions in CP (Fisher et al., 2009). In fact, proposed neuroprotective agents have one of the lowest translational success rates in current medical literature. Two possible explanations have been postulated for the repeated clinical failure of agents with preclinical success. These include: 1) overestimated efficacy in animal models due to clinically insignificant histological endpoints and 2) use of animal models for preclinical testing that are too dissimilar to the human condition in terms of pathophysiology or functional outcome. In attempts to increase the success rate of therapy translation from bench to bedside, numerous criteria for animal models have been proposed. In addition to routine quality control issues such as randomization and reproducibility, criteria include assessment of both histological and functional outcomes in both males and females for a period of at least one month (Sena, van der Worp, Howells, & Macleod, 2007). Our current project has met all of the above criteria.

The current study yielded IUGR pups, with the same asymmetrical phenotype previously observed, indicating the model`s reproducibility. Birth weights did not differ significantly from before. Similarly, mortality was once again significantly increased following BUAL, as evidence by litter sizes that were significantly reduced compared to SHAM. Of note, IUGR animals did display catch up growth. In our previous study, accelerated growth weight was also observed in the IUGR pups, however a significant size discrepancy was still present for the majority of testing (Black, 2010). This created ambiguity around whether or not ailments

observed could be attributed, at least in part, to smaller physical stature. In distinction to our previous study, IUGR and SHAM weights were equal for their respective sex throughout testing and at the time of pathology.

From a functional standpoint, IUGR animals performed worse than SHAM animals overall. This confirmed our hypothesis that the deficits observed up to P21 were not simply a by-product of transient developmental delay, but rather the manifestation of permanent brain injury. The persistence of neurological deficits into adulthood is evidenced by significant discrepancies in motor, behavioural and cognitive testing in this study. Gross and fine motor skills were assessed via beam walking and skilled reaching. Beam walking is a sensitive and reliable tool for detecting hindlimb placing errors in rodents (Schaar, Brenneman, & Savitz, 2010). Both male and female IUGR animals made significantly more foot faults (hindlimb placing errors) than their SHAM counterparts indicating impaired hindlimb function. From a translation perspective, confirming the presence of gross motor function abnormalities is essential for a representative model of CP. Clinically this test parallels the Gross Motor Function Measure (GMFM), which has become the international gold standard for quantifying progress in gross abilities in children with CP (Ko & Kim, 2013). What's more, the large effect size seen in beam walking is ideal for testing therapeutic interventions, as it provides large enough margins for detecting improvement. In addition to gross motor deficits, IUGR animals performed significantly worse on 5 out of 10 skilled reaching movements, indicating fine motor impairment of the forelimbs. The skilled reaching task is referenced as one of the most sensitive tests for detecting mild lesions and is useful for grading improvement over time (MacLellan, Gyawali, & Colbourne, 2006). The maximum utility of this test will be in detecting therapeutic effect. In the present study, both sexes demonstrated reduced function however there was a discrepancy in

both the degree and type of movement that was affected. The degree of disability was more pronounced in IUGR males, suggesting that male rats develop a more severe motor phenotype than females when exposed to the same insult. A similar phenomenon was observed in beam walking, with a more significant difference occurring in males. This is consistent both with previous animal models of hypoxic ischemia as well as with the higher clinical prevalence of CP in male children (Chounti, Hagglund, Wagner, & Westbom, 2013; Johnston & Hagberg, 2007; Skiold et al., 2014; Smith, Alexander, Rosenkrantz, Sadek, & Fitch, 2014).

The phenotype of IUGR animals was further developed through gait analysis which generated information on movement and stance patterns. Children with CP classically demonstrated a wide base gait or stance due to impaired balance. We expected IUGR rats to exhibit a similar phenotype. Surprisingly, there was no difference in forelimb or hindlimb stance between groups. However, our hypothesis was not entirely disproven as IUGR males did have a significantly wider toe spread than SHAM males. The fact that toe stance and not overall stance was widened may be ascribed largely to the four legged nature of the rat, which in and of itself increases stability and minimizes the need for further compensation. Alternatively, it may indicate that our model is representative of a milder CP phenotype. The explanation for these findings may be better elucidated by further investigations with more sensitive technology such as CatWalk (Bozkurt et al., 2008). CatWalk would also allow for us to see more sophisticated aspects of gait, including differences in acceleration and asymmetry which are observed in children with CP (Saether et al., 2014).

In addition to delayed acquisition of motor reflexes, our previous study illustrated anxious and hyperactive behaviour in IUGR pups at P21 (Black, 2010). Specifically, P21 IUGR rats demonstrated increased activity in the open field apparatus. We sought to better characterize

these behaviours in the adult rat using an elevated plus maze and a larger open field apparatus. Elevated plus maze and open field are well validated for the measurement of anxiety and hyperactivity, respectively (Schmitt & Hiemke, 1998a; Schmitt & Hiemke, 1998b; Walf & Frye, 2007). The elevated plus maze capitalizes on a rodent's natural aversion to open spaces, where the degree of anxiety is proportional to the amount of time spent in the closed arms. IUGR animals spent significantly more time in the closed arms than SHAM, indicating underdeveloped coping skills despite identical rearing conditions. Interestingly, IUGR animals did not demonstrate evidence of hyperactive behaviour in either the elevated plus maze or open field test. IUGR animals generally appear to be more hyperactive to the experimenters during handling and testing; however this may have been a manifestation of their anxiety. Conversely, it may be that their anxiety was masking hyperactivity, and repeated testing is required in order to properly desensitize and reveal it. This phenomenon is clearly demonstrated by Dubovicky et al. in a rat model of neonatal hypoxic ischemia whereby rats underwent repeated open field testing. On the first day of open field testing affected rats were significantly less active than controls, however with repeated trials they demonstrated significantly higher levels of hyperactive behaviour (Dubovicky, 2010). Moreover, when well habituated, animals with hypoxic ischemic brain injury are more hyperactive than controls. However, these animals are slow to habituate and experience disabling anxiety in unfamiliar environments. Despite our previous success with younger animals, it is probable that a single open field trial was inadequate to assess hyperactivity in adult rats. Similarly, object recognition may have been confounded by anxiety given that it was performed after only one habituation session in the open field box. This would provide an explanation for the lack of results attained in the object recognition task, which was

unanticipated given the significant learning impairments observed in Morris water and supporting hippocampal damage.

Morris water maze is a well-studied and accepted method of evaluating spatial learning and memory in rodents. Classically, latency to target was used to measure spatial learning performance. With successive trials, the unimpaired animal creates a spatial map using visual cues located outside the maze, allowing it to navigate to the platform more efficiently and thereby reducing the amount of time it takes to reach the platform. While latency to target may be used as a gross screening tool for learning deficits, it is unreliable as it does not take into account swim strategy. In general, spatial memories are formed using two discrete processing systems: allocentric and egocentric. Allocentric learning involves using external cues, such as visual cues in the room, as a frame of reference in order to generate a spatial map. Allocentric learning is dependent on a functioning hippocampus. Conversely, egocentric learning uses oneself as the frame of reference and is dependent on the caudate nucleus of the striatum. In the water maze, animals typically use allocentric and/or egocentric swim strategies to navigate to the platform. While allocentric strategies are more efficient initially, over time rodents with hippocampal lesions learn to swim directly to the platform by developing an egocentric strategy (Garthe & Kempermann, 2013; Kealy et al., 2008). Wishaw's index, a measure of percentage of the path travelled within a straight corridor from the starting point to the platform, can be used as a measure to decipher swim strategy (Valero, Mastrella, Neiva, Sanchez, & Malva, 2014). Our data did not show any significant differences in latency to target, however it did show significant differences in Wishaw's index. IUGR males exhibited significantly less efficient swim strategies than SHAM males, indicating impaired allocentric, or hippocampal dependent, spatial learning and memory.

Compromised allocentric spatial learning witnessed in the water maze is supported histologically by loss of pyramidal neurons in both the CA1 and CA3 region of the hippocampus. Both male and female IUGR animals demonstrated a loss of neurons compared to their SHAM counterparts, with only males reaching significance. This is consistent with the water maze data in which a significant difference in swim strategy was only observed in males. Furthermore, these findings confirm that reduced hippocampal cell counts detected at P21 were indicative of a permanent neurological injury and not merely the reflection of developmental delay.

Despite seeing a reduction in MBP immunodensity at P21, we were unable to detect a loss of white matter in the current study (Black, 2010). Based on the extent of motor disability observed, it is unlikely that the lack of significant results indicates an absence of white matter pathology. Instead it is likely the result of two processes: 1) the severity of the model and 2) the type and sensitivity of immunochemical and histological tests. First, the phenotype of the surviving animals here is probably most reminiscent of a child with GFMCS 1-2 CP. While significant motor and cognitive disabilities are appreciable in this study, survivability into adulthood is not precluded by severe brain pathology. Animals that demonstrate evidence of true periventricular leukomalacia, such as the Derrick laboratory rabbit model, are more substantially affected and do not survive into adulthood (Derrick et al., 2007). In the past PVL was considered the anatomical substrate of CP. However, with advances in neonatal medicine and more sensitive diagnostic technology, it can now be appreciated that children with true PVL and the accompanying severe phenotype comprise a minority of the CP population. Recent studies using diffusion tension magnetic resonance imaging (DT-MRI) have illustrated changes in the microarchitecture of white matter resulting in significant functional outcomes without the presence of quantitative white matter loss (Brehmer et al., 2012; Lodygensky et al., 2011).

Furthermore, a study published in 2014 has demonstrated the neurological significance of arrested development of oligodendrocyte progenitor cells and altered dendrite maturation, without actual death or reduction in cell numbers (Back & Miller, 2014). Bearing this in mind, it is unsurprising that lateral ventricle volume was not increased, nor was MBP density or number of Olig 2 positive cells decreased in the corpus callosum. All of these are quantitative measures of white matter and therefore do not detect qualitative changes in white matter. Unsuitable histological evaluation was undoubtedly the largest limitation of this study. The challenge this poses is a lack of concrete histological targets when evaluating therapeutic effect in the future. The decision to use histological techniques that merely screen for gross loss in white matter was based on preliminary results that were conducted at an early age. The decrease in MBP seen at P21, a snap shot in time, may have been a consequence of a transient delay in myelination, rather than absolute reduction. Nevertheless, this delayed myelination is still an abnormal phenomenon and may in fact be an indicator of a pathological myelination process. This issue may be easily rectified by use of more qualitative and sensitive imaging and histopathology techniques, such as DT-MRI and black-gold or golgi staining, respectively (Mayoral, Omar, & Penn, 2009; Schmued et al., 2008; Tomassy et al., 2014).

An interesting finding from our study was the gender difference found. Our study showed that, in general males do more poorly than females. In this regard, males were significantly poorer in the Morris Water maze, an indication of working memory. The latter was perhaps provided a pathologic substrate based in the hippocampus. Similarly, tapered beam was more poorly done by the males than the females, worsened motor control in this sex group. Several studies have recently explored the differences in sex. At the level of ischemia, research has indicated a differentiation between males and females in the pathway to cell death. In this

regard, males appear to prefer a caspase dependent cell death, compared to females, which prefer a non-caspase dependant pathway. (Hurn, Vannucci et al. 2005, Johnston and Hagberg 2007)

Moreover, this study fills a gap in the literature by providing a model of CP derived from an appropriate etiology and yielding motor, behaviour and cognitive results that are consistent with CP. It affirms our hypothesis that developmental delay observed at P21 persists as permanent neurological deficits, and that functional recovery does not occur in adulthood. The degree of functional impairment in motor, behavioural and cognitive was clearly established, providing an excellent target for clinically significant improvement. Findings of cognitive impairment on functional testing were reinforced by histological evidence of hippocampal neuron death.

Future directions include both further characterization of the model and testing of therapeutic interventions. The underling neuropathology for observed motor deficits may be better explained using qualitative and functional techniques for white matter assessment. As referenced above, diffusion weighted tensor imaging as well as myelin conduction studies are two potential methods for evaluating structure and functionality of white matter respectively. Similarly, subtle cognitive and behavioural findings may not be the result of significant cell death. Our lab has already begun using Golgi staining to evaluate neuronal structure and interconnectivity. The primary goal of this experiment was to develop a model that could be used for pre-clinical testing of potential therapies for CP. Potential therapies include anti-oxidants and stem cells. Broccoli sprouts have been identified by our laboratory as a potential preventative therapy for CP. Of all cruciferous vegetables, broccoli sprouts contain the highest concentration of glucoraphanin, the precursor for the the isothiocyanate sulforaphane. Sulforaphane works by up regulating anti-enzymes such as glutathione transferases via increased expression of Nrf2.

This results in increased endogenous free radical scavenging (Black, 2015). The ability to provide anti-oxidant capabilities to the developing brain that is typically devoid may play a pivotal role in halting cell death in hypoxic ischemic brain injury (Zeisel, 2004). Our laboratory recently published a study demonstrating the effectiveness of broccoli sprouts, based on functional and histological assessment up to 21 days, at preventing brain injury following BUAL. The next steps will be to test broccoli sprout therapy in our current long term model with the incorporation of the more sensitive white matter and neural connectivity assessment methodology described above. The use of this model should not be limited to the testing of therapies intended exclusively for CP. Long term results of this study are congruent with numerous aspects of developmental delay and mental illness. In the future, the battery of functional tests administered to the IUGR rats can be adjusted to best assess for the impairment of interest. For example, BUAL reliably produces anxiety in rats that can be quantified using elevated plus maze. This model could therefore easily be used for testing of anxiolytic agents. Moreover, BUAL in Long-Evans rats provides an excellent template for the pre-clinical testing of therapeutic interventions for CP and associated developmental delays.

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