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Maternal Nutrient Restriction in Pregnant Guinea Pigs and the Impact on Fetal Growth and Brain Development

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ABSTRACT

Maternal nutrient restriction (MNR) in guinea pigs results in placental structural abnormalities that reduce nutrient transport contributing to fetal growth restriction (FGR). However, whether brain weights are similarly reduced, or preserved by "brain sparing" mechanisms, and whether energy levels are depleted leading to membrane failure and overt injury remains unknown. Guinea pig sows were fed ad libitum (Controls) or 70% of the control diet pre-pregnant switching to 90% at mid-pregnancy (MNR). Animals were necropsied near term for fetal growth measures and fetal brains were assessed for markers of necrotic cell injury, apoptotic cell injury, endoplasmic reticulum stress, and altered development proteins. MNR resulted in FGR with brains that are large relative to body weight and livers that are small relative to body weight, which suggests a degree of blood flow redistribution. These fetuses have reduced brain weights, but with substantial brain sparing, and with no increased necrotic cell injury and no changes in synaptic development, indicating that the threshold for membrane failure or aberrant development with energy depletion has likely not been reached. However, apoptotic indices were increased in FGR-MNR cohort compared to appropriate for gestational age (AGA)-control cohort and more so in males than females. Changes in apoptosis were primarily in hippocampal regions and were not accompanied by significant changes of protein levels of investigated pro-apoptotic factors.

Keywords: Maternal Nutrient Restriction (MNR), Fetal Growth Restriction (FGR), Fetal Programming, Undernutrition, Brain Injury, Brain Development, Hypoxia.

CO-AUTHORSHIP STATEMENT

All work presented in this thesis was performed by Andrew Ghaly under the supervision of Dr. Bryan Richardson. Animal care and drug administration during necropsy were in part carried out by Brad Matushewski. Establishment of the animal model, as described in chapter 3, was carried out by Alexander Elias. Immunohistochemistry staining, imaging, and analysis were in part carried out by Evan Formosa and with the help of Karen Nygard at the Western University Biotron. The pathology core at the Robarts Institute, London, Ontario, performed organ tissue blocking. Western Blots were carried out with the help of Dr. Daniel Hardy, Dr. Nicole Barra, and Michael Wong. Catherine Nevin, Dr. Nica Borradaile, Dr. Robert Hammond, and Dr. John Ciriello contributed to editing the content of this thesis.

To everyone I've called friend or family along the way:
Nothing would be possible without you.

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LIST OF ABBREVIATIONS

aFGR	Asymmetrical Fetal Growth Restriction
AGA	Appropriate for Gestational Age
AIF	Apoptosis-Inducing Factor
BAX	Bcl-2 Associated X Protein
BCL2	B-Cell Lymphoma 2
CV	Coefficient of Variation
DAB	Diaminobenzidine
ER	Endoplasmic Reticulum
FGR	Fetal Growth Restriction
Grp78	G-protein Coupled Receptor 78
H&E	Haematoxylin and Eosin
HPF	High-Power Field
IGF	Insulin-like Growth Factor
IR	Immunoreactivity
IQ	Intelligence Quotient
IUGR	Intrauterine Growth Restriction
MNR	Maternal Nutrient Restriction
MRI	Magnetic Resonance Imaging
PARP1	Poly ADP Ribose Polymerase 1
sFGR	Symmetrical Fetal Growth Restriction
SGA	Small for Gestational Age
SYN	Synaptophysin
TBST	Tris-buffered saline-Tween 20
TDT	Terminal Deoxynucleotidyl Transferase
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

CHAPTER 1
INTRODUCTION

1.1 CLINICAL RELEVANCE

1.1.1 Incidence and Classification of FGR

Fetal growth restriction (FGR), also known as intrauterine growth restriction (IUGR), and formerly as fetal growth retardation, is defined clinically as birth weight below the 3rd, 5th, or 10th percentile for gestational age and is indicative of a reduction in growth rate that prevents a newborn from achieving their complete growth potential (Lackman, Capewell, Richardson, DaSilva, & Gagnon, 2001). FGR pregnancies have up to a 6-fold increase in fetal mortality and a 5-30 fold increase in neonatal mortality which correlates with the severity of FGR (Piper et al., 1996; Seeds et al., 1998), as well as increased neonatal morbidity (Bernstein, Horbar, Badger, Ohlsson, & Golan, 2000; Kramer, Olivier, McLean, Willis, & Usher, 1990). Approximately 30% of pregnancies with weights below the 10th percentile have a pathological origin, whereas the remaining 70% are constitutionally small. This incongruity highlights that these measures are not always able to accurately delineate genuine FGR from infants that are simply small for gestational age (SGA).

Early differentiation between FGR and constitutionally small fetuses arising from non-pathological conditions *in utero* is important, as misdiagnosing small, healthy fetuses as FGR may waste valuable resources. As such, other measures of fetal growth are used to provide supplementary criteria for a clearer definition of FGR, rather than constitutional SGA. Customized growth curves, which account for maternal, environmental, and socioeconomic status may be beneficial for the identification of pathological FGR (Arbuckle, Wilkins, & Sherman, 1993; Figueras et al., 2007; Gardosi & Francis, 2009; Hutcheon, Walker, & Platt, 2011; Kierans et al., 2008; Kramer et al., 2001). The child of a mother that is 10 cm taller and 10 kg heavier than an average

mother would have an expected mean body weight at term that is 140 g heavier than a child born to an average sized mother (Gardosi & Francis, 2009). Therefore, identification of FGR would require a higher threshold based on maternal size to avoid misdiagnosis of FGR. These customized growth curves result in reclassifying 2.7-4.3% of babies (Figueras et al., 2007).

Other measures of fetal growth are used in conjunction with fetal growth curves to produce a clearer definition of FGR vs. SGA. Ratios of fetal weight/length, are used as a measure of leanness and fetal nutrition at birth. Additional studies have found placental weight and placental function to positively correlate with birth weight and thus relate to FGR (Lackman, Capewell, Richardson, et al., 2001; Pollack & Divon, 1992). Markers of fetal size and shape at a particular gestational age are useful predictors of fetal health and postnatal outcome.

1.1.2 Symmetrical vs. Asymmetrical Growth

FGR presents phenotypically as symmetrical (sFGR) or asymmetrical (aFGR) fetal growth restriction; this distinction is based primarily on head and body proportions. An sFGR fetus is typically impacted very early in pregnancy, with all growth parameters being affected equally. This presents with brain growth inhibition remaining proportional to reductions in weight, length, and head circumference and with a normal ponderal index at birth (al Riyami et al., 2011; Halliday, 2009; Pollack & Divon, 1992) In aFGR, growth restriction onsets later in pregnancy, which tends to occur in a more severe fashion, however the later onset is associated with better health outcomes compared to the very early-onsetting growth restriction seen in sFGR cases. aFGR presents with average head dimensions but a small abdomen (due to decreased visceral organ sizes), decreased

muscle mass, and low weight/length ratios (due to increased leanness reflective of disproportionate body dimensions) (al Riyami et al., 2011; Campbell & Thoms, 1977; Halliday, 2009; Jones & Parer, 1983). This "brain sparing" effect occurs as a mechanism of adaptation when chronically exposed to a hypoxic *in utero* environment to ensure fetal survival. There is a redistribution of blood towards essential organs such as the brain, the heart, and the adrenal glands at the expense of other organs such as the liver, kidneys, and skeletal muscle (Dubiel, Breborowicz, & Gudmundsson, 2003; Dubiel, Breborowicz, Marsal, & Gudmundsson, 2000; Poudel, McMillen, Dunn, Zhang, & Morrison, 2015; Salihagic-Kadic et al., 2006). This redistribution is intended to ensure that the brain continues to receive sufficient nutrient supply and maintain relative growth. The effects of this are visible at birth, with the size of the fetal head being relatively large compared than that of the abdomen, giving rise to the observable asymmetry observed in aFGR fetuses (Jensen, Storgaard, Madsbad, Richter, & Vaag, 2007).

Unlike aFGR, sFGR can be difficult to distinguish from inherently small fetuses because they are proportionally similar, it is the presence of pathological processes that causes an infant to be considered sFGR rather than constitutionally SGA; therefore, additional tests, such as umbilical artery Doppler assessment or extreme discrepancy from a growth curve may be necessary to infer pathological sFGR. The most reliable means of differentiating aFGR and sFGR is by measuring brain-to-liver weight ratios. An aFGR fetus will have a disproportional decrease in liver weight, but a relatively normal brain weight and therefore an increased brain-to-liver weight ratio. Whereas sFGR fetuses will have small livers but with the brain being similarly reduced and thus similar brain-to-liver weight ratios compared to

appropriate for gestational age (AGA) fetuses (P. Cox & Marton, 2009). Consequently, the brain-to-liver weight ratio is used in many animal studies to identify aFGR at the time of necropsy. In clinical settings, head circumference-to-abdominal circumference ratio measurements can serve as a proxy in a newborn or human fetus.

There is a higher incidence of aFGR, reportedly occurring in 70% of growth restricted fetuses (Campbell & Thoms, 1977). Population studies report that 60-80% of growth restricted babies born in developed countries are aFGR whereas 70-80% of growth restricted babies in developing countries are classified as sFGR (Villar, Altobelli, Kestler, & Belizan, 1986). This indicates that both aFGR and sFGR do not arise as the result of a single anatomical dysfunction or abnormality during development; as such, there is a large amount of research dedicated to learning more about the exact combination of conditions that contribute to FGR.

1.1.3 Etiology of FGR

Fetal growth is dependent on the interplay between maternal, placental, and genetic factors. Optimal fetal growth is achieved when there is a balance of genetic growth potential of the fetus, efficiency of the placenta in nutrient and oxygen transport, and the state of the maternal environment. These maternal-placental-fetal factors work synergistically to provide a healthy intrauterine environment for the development of the fetus throughout gestation while supporting physiological changes in the mother. When one or more of these factors is impacted, a sub-optimal intrauterine environment is created that is incapable of supporting normal growth and development (Cetin et al.,

2004; Han, 1993; Pollack & Divon, 1992). Maternal causes for FGR can arise pre-pregnancy or during pregnancy; maternal etiologic factors include weight, age, and parity as well as variables like undernutrition, hypertension, hypoxic conditions, vascular disease and environmental choices such as cigarette smoking and substance abuse (Fang, 2005; Pollack & Divon, 1992). Placental factors that can contribute to FGR and sub-optimal development include abnormal cord insertion, nuchal cord, placental infarcts, placenta previa, and multiple gestations (Pollack & Divon, 1992). Fetal factors, though relatively uncommon, are capable of contributing to FGR; fetal etiologic factors include genetic factors such as fetal aneuploidy, congenital irregularities such as major heart abnormalities, and infections such as cytomegalovirus (Demirci et al., 2015). Any one of these factors is sufficient to cause FGR, however it is estimated that 60% of FGR is idiopathic in nature and likely derived from a combination of factors, the majority of which involve some form of abnormal placental development, commonly referred to as placental insufficiency (Ghidini, 1996; Pallotto & Kilbride, 2006; Pollack & Divon, 1992).

1.1.4 Placental Insufficiency

The placenta is responsible for the transfer of nutrients between maternal and fetal tissue throughout gestation. The human placenta has a complex system of blood vessels that constitute the main area of fetal-maternal exchange. At approximately 21 days of gestation, there is an increase in fetal-placental blood vessels that allows for vascularization and transforms the initially hypoxic *in utero* environment to one that is capable of supporting fetal growth and organ system development during later gestation (J. Kingdom, Huppertz, Seaward, & Kaufmann, 2000). This increase in vasculature incudes a 10-fold increase in volume, an increase in surface area of the

placental labyrinth, and a decrease in trophoblast thickness, all of which promote elevated exchange of nutrients, essential substrates, and oxygen between maternal and fetal tissue (Myatt, 2006).

The majority of conditions compromising fetal growth, especially in developed countries, involve idiopathic aberrant development of the placenta referred to as placental insufficiency (Hashimoto et al., 2012; Lumey, 1998). Placental insufficiency is a term used to describe conditions in which the placenta is unable to transfer appropriate amounts of nutrients or oxygen for full fetal growth. Clinical studies of placental insufficiency with FGR have shown aberrant placental vascularization, involving insufficient or incomplete trophoblastic invasion of spiral arteries in the placental bed, and decreases in umbilical blood flow (J. Roberts, 1998; Salafia, Charles, & Maas, 2006; Wang, Walsh, & Kay, 1992). This decrease in umbilical blood flow may result in inadequate nutrition and oxygen reaching the fetus, resulting in a hypoxic, nutrient deprived *in utero* environment (Economides & Nicolaides, 1989; Giussani, Salinas, Villena, & Blanco, 2007; Romo, Carceller, & Tobajas, 2009; Salafia et al., 2006); thus resulting in poor fetal development and therefore FGR. Under these conditions, the fetus adapts by altering metabolic and developmental processes at the expense of complete growth (T Jansson & Persson, 1990; Jones & Parer, 1983; Myatt, 2006). It should be noted that independent of reductions in nutrient supply, hypoxia alone has been shown to have a significant impact on fetal growth, therefore hypoxia may be a major contributor to impaired fetal growth and FGR (Giussani et al., 2007).

1.1.5 Maternal Undernourishment

As previously stated, FGR is a prevalent problem worldwide, however there are differences in FGR etiology between developed and developing countries. While the majority of conditions negatively affecting fetal growth in developed countries involve placental insufficiency, in developing countries maternal nutrition plays a more prominent role (Hashimoto et al., 2012; Lumey, 1998). It is estimated that 11% of offspring in developing countries fail to reach their full growth potential due to nutritional, social, and health factors related to poverty (de Onis, Blossner, & Villar, 1998; S. P. Walker et al., 2007a). Early evidence of the impact of maternal nutrition on fetal health outcomes was observed in population studies during the Dutch famine of the 1940s; studies of infants whose mothers' nutrition was compromised at the time of conception or in early pregnancy showed increases in placental weight but not in birth weight (Lumey, 1998). These increases in placental weight are possibly an attempt to compensate for the reduced availability of substrates in the maternal circulation, which is what is seen in examination of human FGR cases (Lumey, 1998). Clinical study in populations subjected to food restriction (Lumey, 1998) and in mothers deemed underweight with low body mass index (Ehrenberg, Dierker, Milluzzi, & Mercer, 2003; Kalk et al., 2009; Li et al., 2013; Sebastián Manzanares et al., 2012; Sebire, Jolly, Harris, Regan, & Robinson, 2001) also support the notion that maternal undernourishment is undoubtedly causative for FGR.

It has been found that nutritional supplementation throughout pregnancy led to better outcomes in motor development of the child at 8 months, but not at the 5-year mark. Similar supplementation given during the final trimester of pregnancy and throughout infancy provided no benefit from 6 months to 3 years (Adair & Pollitt, 1985; Waber et al., 1981). Additional studies have shown that maternal weight and nutrition

prior to pregnancy are actually better determinants of fetal growth and development compared to changes in weight during pregnancy (Stevens-Simon, Metlay, & McAnarney, 1995), reflecting the fact that the majority of women do not significantly improve lifestyle habits and nutrition over the course of pregnancy (Crozier, Robinson, Godfrey, Cooper, & Inskip, 2009). These studies show evidence that the placenta adapts to maternal nutrient supply early on in pregnancy and is not easily altered or rescued in response to supplementation later on in pregnancy. This shows the importance of maternal nutrition in fetal programming and development as it has the potential to be a chronic stressor on the mother, placenta, and developing fetus.

In cases of maternal nutrient restriction (MNR), the mother's nutrient reserves are depleted which results in a reduction of nutrient transport to the fetus and the placenta, increasing the risk of FGR. In addition to this, MNR has been shown to generate placentas with altered vascular development and increased barrier thickness, in both animal models (Belkacemi, Nelson, Desai, & Ross, 2010; Redmer, Wallace, & Reynolds, 2004; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001) and human cases (Aherne & Dunnill, 1966) of maternal undernourishment; which will further diminish the transport of glucose, amino acids and lipids to the fetus (Belkacemi et al., 2010; Gaccioli, Lager, Powell, & Jansson, 2012) in a manner similar to that seen in placental insufficiency induced FGR.

1.1.6 FGR and long-term health

FGR remains the second most common adverse condition, behind preterm birth, that arises from complications in pregnancy; and studies have shown that FGR is actually associated with an increased risk of spontaneous preterm delivery (Lackman,

Capewell, Richardson, et al., 2001). FGR pregnancies have up to a 6-fold increase in fetal mortality and a 5-30 fold increase in neonatal mortality which correlates with the severity of FGR (Piper et al., 1996; Seeds et al., 1998), as well as increased neonatal morbidity. In addition to risk of adverse fetal and neonatal outcomes, FGR is also a recognized risk factor for long term adverse health outcomes including heart disease, diabetes and neurodevelopmental disability, with the greatest risk observed in those with severe and early-onsetting FGR (Barker, 2004; Thomas Jansson & Powell, 2007; Low et al., 1992; Pryor, Silva, & Brooke, 1995; Zaw, Gagnon, & da Silva, 2003). Studies on sex differences in FGR offspring found that there is a significantly higher incidence of FGR in females, which suggests that there is a higher tendency for adaptation to a sub-optimal intrauterine environment in females relative to males (J. Liu, 2014); however females tend to have more favourable outcomes and a better likelihood of surviving FGR related outcomes compared to males (Synnes et al., 2010). Studies of MNR in mice showed a higher susceptibility to FGR in males, with a higher incidence of impaired cognitive function (Akitake et al., 2015) and this sex difference persists in adults, as less tissue damage for an equivalent insult has been reported in global and focal cerebral ischemia in adult female rodents compared to males (Alkayed et al., 1998; Hall, Pazara, & Linseman, 1991).

At the time of birth, FGR newborns are at high risk for hypoxia, hypoglycemia, and hypothermia due to limited nutrient supply and storage, low tissue adiposity, diminished gluconeogenesis and impaired lipid metabolism (Fang, 2005; Rodríguez et al., 2011). Interventions in early pregnancy are aimed at promotion of fetal growth and survival. Mothers often receive glucocorticoid supplementation if they are at risk of preterm delivery in order to attempt to replicate the endogenous glucocorticoid burst

that is necessary for lung development. Another major goal of intervention in postnatal care is the achievement of catch-up growth, whereby there is rapid growth after birth to match the growth curves of AGA infants. Previous studies have demonstrated that aFGR infants are capable of full catch-up growth within 3 years, however sFGR infants have a reduced rate of weight gain and growth up to 40 weeks, followed by an increased linear weight gain until approximately 8 months (Strauss & Dietz, 1997). The same study demonstrated that 70% of sFGR infants had weights greater than that of the 10th percentile but remained at a lower weight compared to AGA infants up to 3 years old (Strauss & Dietz, 1997). A related study, which examined growth of sFGR infants, found that weight, height, and head circumference were still reduced even up to 14 years of age (Indredavik et al., 2010). While post-natal treatment may aid in catch-up growth, it does not lessen the long-term morbidity that arises due to sub-optimal *in utero* conditions and aberrant development.

1.1.7 Developmental Programming

Fetal programming is the theory that there are critical periods of organ development, during which the system is highly sensitive to environmental cues, which are capable of setting the platform for health outcomes in later life (Barker, 2004). This is known as the "Barker hypothesis" or the "developmental origins of health and disease" in later life. The adaptations that occur in response to adverse intrauterine conditions during these critical periods of development are capable of causing permanent structural and functional changes to several of the body's systems. The three primary means by which fetal programming can occur are: 1) direct damage, such as early loss of a limb; 2) induction, deletion, or impaired development of a somatic

structure resulting from insult during a critical period of development; or 3) physiological re-setting by an early stimulus or insult at a critical period with long term consequences for endocrine/autocrine/paracrine axes (Lucas, 1994). Barker described these periods as most likely occurring in the fetus during phases of rapid cell division, and being more impacted by intrauterine environment than genetics (Cosmi, Fanelli, Visentin, Trevisanuto, & Zanardo, 2011). Early evidence which supported claims that undernutrition *in utero,* resulting in FGR, increased the risk of cardiovascular disease in adult life lead to the popularization of the idea of fetal programming (Barker, 2004). More recently, studies have found a positive correlation between low birth weight, caused by either FGR or preterm birth, and increased rates of cardiovascular disease and type-2 diabetes in adult life (Langley-Evans, 2009). There is growing evidence that supports Barker's hypothesis of developmental adaptations *in utero* having long-term changes on the "programming" of later life health outcomes (Barker, 2004), and there is an increasing amount of research that suggest that the etiology of many neurological disorders can also be attributed to the effects of the suboptimal intrauterine environment seen in FGR.

Maternal undernutrition gives rise to fetal programming by means of the above mentioned physiological re-setting. The early stimulus of placental compromises in nutrient/oxygen delivery causes growth restriction to occur as an adaptive process by which energy needs are decreased in order to ensure survival (Hochachka, Buck, Doll, & Land, 1996). However, this has consequences for aberrant growth processes which lead to an increased predisposition to later life disease.

1.2 FGR AND THE BRAIN

1.2.1 FGR and neurological outcome

The link between FGR and neurological disorders and cognitive deficits is apparent in all age groups and has been reported in many studies. FGR is associated with a higher risk of development of a number of neurological disorders that manifest in early childhood including cerebral palsy, attention deficit hyperactivity disorder, and autism spectrum disorder (Halliday, 2009; Indredavik et al., 2010; Rodrigues, Mello, & Fonseca, 2006). The effects of FGR in early childhood can be observed with reductions in cognitive skills, impaired memory, learning difficulties, difficulties reading, writing, and with adaptive skills, inattention, reduced psychosocial function, behavioural problems, sensorineural deterioration, reduced mathematics abilities, and reduced intelligent quotient (IQ) scores (Geva, Eshel, Leitner, Fattal-Valevski, & Harel, 2008; Pallotto & Kilbride, 2006; Rodrigues et al., 2006; Synnes et al., 2010; D.-M. Walker & Marlow, 2008). More severe effects can be seen with altered brain development later in life as FGR-born adults are at a higher risk of schizophrenia, epilepsy, and psychiatric hospitalization (M. Cannon, Jones, & Murray, 2002); studies of these neurological disabilities show that their severity correlates positively with the severity of FGR.

Neurological difficulties in FGR-born children continue through adolescence and into early adult life. At 10 years of age, late-onset aFGR-born children were found to have continued deficiencies in verbal short term memory of auditory or visiospatially presented information and reduced IQ scores (Geva et al., 2008). Studies comparing 13 year-old FGR-born children to age matched controls showed poor everyday memory, mathematical reasoning and numerical operation skills even after learning-skills interventions (Isaacs et al., 2000). At 14 years of age a cohort of sFGR-born children

were reported to still have reduced head circumference as well as increased hyperactivity, inattention, psychiatric diagnosis, autism spectrum disorder scores and reduced psychosocial function (Indredavik et al., 2010). These studies indicate that behavioural and cognitive difficulties seem to become more complex and quite possibly more severe with age for FGR-born offspring.

Regional differences exist in the brain's development and thus, certain areas can be affected differently by *in utero* insults. For example, the hippocampus is the least genetically regulated region of the brain and is therefore more vulnerable to environmental and developmental influences compared to areas of the brain under higher levels of genetic influence (Lodygensky et al., 2008). A study by Lodygensky *et al.* showed reductions in hippocampal volume of FGR-born infants at 2 years of age, with a significant correlation between total hippocampal volume and size at birth, this cohort was also found to have poor performance in motor, attention-interaction, and self-regulation behavioural function maturation assessments (Lodygensky et al., 2008). Hippocampal differences have been observed in other studies as well, with reduced hippocampal volume and enlarged ventricles appearing on magnetic resonance imaging (MRI) scans in FGR-born infants compared to age matched controls (Isaacs et al., 2000). These irregularities in the development of the hippocampus may, in part, explain the basis for several of the neurological deficits observed in FGR as the hippocampus and associated areas are critical for learning, memory, and proper cognitive function (Reed & Squire, 1997).

1.2.2 Fetal Brain Growth and Development

Brain growth and development are intricate processes that occur throughout the entirety of gestation and continue to be refined after birth. At term, the developing human fetal brain is responsible for approximately 20% of body mass and 80% of energy expenditure (Gilles, 2011). The rate of brain growth peaks around term; this involves the formation and migration of neurons, and once neurons reach their final migration point myelination begins and there is a notable decrease in the rate of brain growth which plateaus at about two years after birth (Gilles, 2011); after which brain alteration is largely dependent upon experience and environmental factors.

Complete growth of the brain requires neuronal generation and differentiation, navigation and organization of axonal projections between neurons, and the formation and maturation of synaptic contacts (Bourgeois, 1997). It is theorized that there is an initial formation of large amounts of synaptic contacts by means of intrinsic mechanisms of growth; however, subsequent maturation of specific neuronal connections occurs by means of selective usage (Bourgeois, 1997). As a result, it is believed that environmental influences play a major role in the development of these processes and can have significant impacts on later life neurological health.

1.2.3 Synaptogenesis

Synaptogenesis is a process that occurs during gestation and through to about puberty in humans (Bourgeois, 1997). The early phases involve neurogenesis, neuronal migration, individualization of cortical layers and synapse formation that are controlled by intrinsic mechanisms which are considered experience-independent (Bourgeois, 1997). In later phases synapses are formed at a much faster rate, driven by an experience expectant mechanism, which generates an abundance of synapses in the

brain and pre-adapts the brain for an experience dependent phase with individual customization on the basis of experience and environmental cues (Bourgeois, 1997). A plateau phase exists, in which there is elimination of select synapses and selective maturation of others, in order to fine tune and establish certain neuronal connections. (Bourgeois, 1997). In order to mature, a synapse requires an abundant amount of energy. Areas with newly forming synapses have high levels of mitochondria in order to supply the large amount of energy necessary for protein synthesis and synapse formation (Mjaatvedt & Wong-Riley, 1988); as such compromises in nutrient/oxygen delivery as a result of a poor intrauterine environment are capable of affecting the earlier phases of synaptogenesis and may predispose the fetal brain to deficiencies in later life.

Synaptophysin (SYN) is a 38 kDa presynaptic protein marker present in all presynaptic boutons on presynaptic vesicle membranes in the central nervous system (Calhoun et al., 1996; Jahn, Schiebler, Ouimet, & Greengard, 1985). Histological staining using SYN antibodies shows punctate staining localized to presynaptic boutons (Calhoun et al., 1996; Fletcher, Cameron, De Camilli, & Banker, 1991). This presynaptic protein marker has been used in animal studies of FGR as a measure of synaptic numbers in the brain (Camm, Gibbs, Harding, Mulder, & Rees, 2005; Tolcos et al., 2003). It is also found in immature synapses and small synaptic vesicles, and its protein levels directly increase with synapse formation and development (Daly & Ziff, 1997; Fletcher et al., 1991). Using immunohistochemistry, the immunoreactivity (IR) of SYN has been shown to be an accurate presynaptic marker for the detection of synapse formation.

1.2.4 Myelination

Myelin is necessary for the insulation of electrochemical signals that are sent along neurons; proper myelination throughout development ensures neuronal communication and connectivity between areas of the brain. The presence of neurons and the electrical activity of axons perpetuates myelination in a specific axon (Barres & Raff, 1993). The degree to which an axon is myelinated dictates the conduction velocity of an axonal signal and is critical to the synchronous firing of action potentials, which is necessary to strengthen neuronal connections (Fields, 2008). As such, the increasing electrical activity in a maturing neuron aids in myelination which in turn establishes saltatory conduction and aids in the coordination of synaptogenesis between neuronal connections.

In humans, the rate of myelination of the brain increases towards the end of gestation and into the 2nd year of life. Myelin sheath proteins are seen in the brain as early as the 5th week of gestation and become more abundant with advancing gestational age (Jakovcevski, Mo, & Zecevic, 2007; Kinney, Brody, Kloman, & Gilles, 1988). The high rate of signaling and protein turnover required for myelination makes it a process with high energy demand; therefore, extended periods of compromised nutrient/oxygen delivery may impact on the process leading to poor myelination and therefore poor synaptogenesis and aberrant development of the brain.

1.2.5 Apoptosis

Apoptosis, or programmed cell death, has a physiological basis during the fetal/neonatal period of brain development that coincides with neuronal differentiation and synaptogenesis, and possibly relates to the competition for trophic factors produced by target cells and the establishment of axonal-target connectivity (Blaschke,

Staley, & Chun, 1996). The process of apoptosis involves stimulation of intrinsic and/or extrinsic signaling pathways leading to the downstream cleavage of caspase-3, which together with effector caspases can target multiple proteins for proteolysis causing a resultant fragmentation of the cell's DNA and dismantling of cellular structures (Banasiak, Xia, & Haddad, 2000; D'Amelio, Cavallucci, & Cecconi, 2010). Apoptosis can be pathologically activated in the brain in cases of chronic hypoxia and result in selective neuronal loss (Burke et al., 2006; Yue et al., 1997) with immature neurons and/or milder insults more likely to result in apoptotic death, while terminally differentiated neurons and/or severe insults are more likely to result in death by necrosis (a D. Edwards et al., 1997; Scott & Hegyi, 1997; Yue et al., 1997).

1.2.6 Mechanisms of Damage and Aberrant Development

The histo-pathological alterations seen in human pregnancies with undernourishment resulting in FGR (Aherne & Dunnill, 1966), have been shown to result in reductions in nutrient transport to the fetus for glucose, amino acids, and lipids (Crozier et al., 2009). Recent studies have also shown evidence for chronic hypoxia with MNR-FGR in guinea pigs (Elias, Matushewski, Zhao, Regnault, & Richardson, 2013), low grade oxidative stress may play a mechanistic role here given its known role in the pathogenesis of chronic disorders (Hracsko, Orvos, Novak, Pal, & Varga, 2008; Mohn et al., 2007). If these insults occur during critical time points in brain growth and development, there is a high risk for potential derangements.

As previously stated, in FGR with compromised nutrient/oxygen delivery there is adaptive programming in which growth restriction occurs and select developmental processes are shut down in order to conserve limited energy stores for the maintenance

of essential processes necessary for survival. (Hochachka et al., 1996). Variable alterations to brain development and injury may be observed on the basis of the insult. Selective neuronal loss has been observed in developing brain cells upon exposure to hypoxia or ischemia; with milder insults likely to impact immature neurons and result in apoptotic death and more severe insults likely to affect terminally differentiated neurons and result in death by necrosis (Scott & Hegyi, 1997; Yue et al., 1997). Sensitivity to insults and brain injury also varies on the basis of energy demand, with areas that have a higher energy demand being more sensitive; as such hippocampal cells are expected to see the largest change (Zhao & Flavin, 2000). Additionally, there is regional variation in susceptibility, with the cortex and striatum being more sensitive than the thalamus (Dirnagl, Iadecola, & Moskowitz, 1999). In prenatal brain developers such as guinea pigs, and for humans as perinatal brain developers, the most rapid phase of synapse formation and neuronal myelination onsets during the latter half of pregnancy (McIntosh, Baghurst, Potter, & Hetze, 1979; Penn & Shatz, 1999). As such, FGR with nutrient transport impairment may disrupt synapse formation and neuronal myelination, as they are energy demanding growth processes (Jiang & Schuman, 2002). This may lead to alterations in brain development and underlie risk for later cognitive impairment and neuropathology.

Additionally, hypoxia, a decrease in amino acid levels, and/or oxidative stress can lead to the accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER), which if prolonged can initiate a pro-apoptotic cascade (Koumenis et al., 2002; Marciniak & Ron, 2006; Szegezdi, Logue, Gorman, & Samali, 2006). Hypoxia and low amino acid supply, as seen in the MNR guinea pig model (Elias et al., 2013; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; C. T. Roberts, Sohlstrom, Kind, Grant, et al.,

2001), have been demonstrated to hinder disulfide bond formation – one of the key processes underlying protein maturation and folding within the ER lumen, thus augmenting ER stress (Benham, van Lith, Sitia, & Braakman, 2013; Frand & Kaiser, 1999; Yu et al., 2012; Zhang et al., 2014). In cases where ER stress is not sufficiently alleviated, downstream apoptotic pathways will be activated (D. Liu, Zhang, & Yin, 2013; Matsumoto, Minami, Takeda, Sakao, & Akira, 1996). G-protein coupled receptor 78 (Grp78) is an essential component of the ER translocation machinery and plays a significant role in retrograde transport of aberrant proteins destined for degradation by the proteasome; as such, synthesis of Gpr78 is induced under conditions that lead to the accumulation of unfolded polypeptides in the ER (Hendershot, Valentine, Lee, Morris, & Shapiro, 1994; Ting & Lee, 1988); Consequently, Grp78 is used as an early marker of ER stress. Since the ER is the primary site of protein synthesis and maturation within the cell, prolonged ER stress could negatively impact essential signaling and transport function necessary for proper development of the fetal brain (Braakman, Hoover-Litty, Wagner, & Helenius, 1991; Frand & Kaiser, 1999; Kawakami et al., 2014; Red-Horse et al., 2004).

1.2.7 Mechanisms of Adaptation

Previous studies have shown that there are compensatory mechanisms in action that deal with prolonged episodes of lowered energy and oxygen that involve suppression of energy-demanding pathways to regulate the cell at new steady state levels, even during drastic energy depletion. These mechanisms include channel arrest of membrane ion pumps, cessation of synaptic transmission, and decreased protein synthesis (Hochachka et al., 1996) In some cases, if the nutrient impairment is severe

enough, it is possible that these compensatory mechanisms, which are usually protective for the brain, may become limited and brain energy levels may be sufficiently impacted, leading to membrane failure with an increase in necrotic cell injury and/or changes in apoptotic regulators (Rocha, Hammond, & Richardson, 2004). While these mechanisms have yet to be elucidated, regional differences in the balance of pro-apoptotic and anti-apoptotic gene expression and activity-dependent changes in this balance with the strengthening of incoming afferent activity are likely to be involved (Anand & Scalzo, 2000).

Some of these pro- and anti-apoptotic factors include Bcl-2 associated X protein (Bax), B-cell Lymphoma 2 (Bcl-2), poly ADP ribose polymerase 1 (PARP1), and cleaved caspase-3. Pro-apoptotic Bax and anti-apoptotic Bcl-2 are known to synergistically regulate apoptosis (D. Liu et al., 2013; McCullough, Martindale, Klotz, Aw, & Holbrook, 2001). PARP1 is required for translocation of apoptosis-inducing factor (AIF) from the mitochondria into the nucleus, in which it induces large scale DNA fragmentation (Daugas et al., 2000). Extrinsic and intrinsic apoptotic pathways both converge on caspase-3, with the downstream event consisting of substrate cleavage. There is abundant evidence that pathways leading to caspase-3 cleavage/activation are engaged following neonatal hypoxia-ischemia (Blomgren et al., 2001; Felderhoff-Mueser et al., 2002; Hu, Liu, Ouyang, Blomgren, & Siesjö, 2000; Northington, Ferriero, Flock, & Martin, 2001). It is of note that caspase-3 activation does not always lead to DNA fragmentation, in the developing brain caspase-3 appears to also play a non-apoptotic role in cellular differentiation and remodeling of neuroplasticity through selective removal of existing synaptic connections (D'Amelio et al., 2010; Xu et al., 2015).

1.3 ANIMAL MODELS OF FGR AND ADVERSE DEVELOPMENT

1.3.1 Guinea pig brain development

Guinea pigs and sheep have been used to model human FGR due to their delivery of precocious young after a relatively long gestational period (Dobbing & Sand, 1970). These species demonstrate a similar timeline of developmental events in the brain to those occurring *in utero* during human pregnancy; therefore, intrauterine insults will coincide with critical periods of development and the associated long-term outcomes more accurately parallel that of the human situation. Additionally, both humans and guinea pigs are thought to be born with the full complement of neuronal and synaptic numbers (Lennon, Francon, Fellous, & Nunez, 1980). This is in contrast to rodents, which have a shorter gestation and altricial young; therefore many of their developmental processes occur in the postnatal period and are less affected by the *in utero* environment.

In comparison to humans, the guinea pig brain is larger and at a more developed stage of functionality at birth. In the human situation brain growth plateaus at approximately 2 years of age, this plateau occurs just prior to birth in the guinea pig (Dobbing & Sand, 1970). At birth, guinea pig brain functions include motor, cognitive and regulatory processes that are comparable to that of a human toddler, this is due to peak neurogenesis occurring in several brain areas much earlier in guinea pigs than in humans (Clancy et al., 2007). Therefore, *in utero* insults may actually be magnified in the guinea pig brain in comparison to humans; while the impact of post-natal environmental influences that play a role in ongoing brain development will be lessened in the mature guinea pig brain. The precocious function and comparable anatomy of the guinea pig brain in comparison to the human brain are major assets in the

determination of changes in neurodevelopment and their extrapolation to the human situation.

1.3.2 Animal Studies of FGR with Placental Insufficiency

Clinical studies have shown that a major contributor to placental insufficiency, and therefore FGR, is aberrant placental vascularization and the accompanying changes in umbilical blood flow. As a result of this, research on animal models of placental insufficiency has been a growing area of research. The guinea pig is a good model of human placental insufficiency because it has a very similar placenta; both human and guinea pigs have a discoid haemochorial placenta in which fetal vessels and maternal blood are in direct contact. Placental insufficiency can be induced by a variety of interventions at pre-pregnancy, mid-pregnancy, and late pregnancy. Some of these include: uterine caruncletomy, the removal of endometrial tissue, exposure to hypothermic environment in pregnant sheep, placental embolization midway through gestation, or uterine artery ligation/ablation midway through gestation; these techniques have successfully resulted in decreased fetal weight, increased brain-to-fetal weight ratios in sheep, and increased markers of hypoxemia and hypoglycemia (Gagnon, Murotsuki, Challis, Fraher, & Richardson, 1997; Harding, Jones, & Robinson, 1985; Murotsuki, Challis, Han, Fraher, & Gagnon, 1997; Regnault, Orbus, Battaglia, Wilkening, & Anthony, 1999). Of particular interest to this study, are reports of uterine artery ligation in rodents and sheep that have successfully induced FGR with accompanying reductions in fetal brain development and long-term brain function (Olivier et al., 2007; Rees, Breen, Loeliger, McCrabb, & Harding, 1999). Guinea pig models of uterine artery ligation have resulted in decreased placental size, and

decreased transfer of oxygen and nutrients (T Jansson & Persson, 1990; Jones & Parer, 1983; Lafeber HN, Rolph TP, 1984), which successfully models human placental insufficiency. Recent studies have additionally shown that uterine artery ligation is capable of leading to disruptions in synapse formation, regional synapse maturation, and myelination (Piorkowska et al., 2014). These animal models that mimic placental insufficiency are highly effective in producing FGR with variable levels of hypoxemia and nutrient deficiency, however they are not without their flaws. There have been high reported rates of mortality in the pups in uterine artery ligation and ablation models (a. J. Turner & Trudinger, 2009), and they involve a one-time, invasive procedure, which doesn't ideally parallel the human condition of a chronic, non-invasive insult.

1.3.3 Animal Studies of FGR with Maternal Undernourishment

As previously stated, clinical studies have also shown that maternal nutrition is a major contributor to the health outcomes of a fetus. As a result, there are many animal models that aim to investigate the impacts of maternal undernourishment on fetal and placental growth and development. These models use a variety of levels of nutrient restriction and different time points for restriction relative to gestation resulting in a large range of different levels of growth restriction and altered development. These models can range from "moderate" restrictions, whereby animals are fed 70-85% of a control *ad libitum* diet, to "severe" restriction studies, whereby animals are provided with 50% of a control *ad libitum* diet to mimic cases of extreme undernourishment. Animal models of MNR have been used to study developmental outcomes with induced FGR (Belkacemi et al., 2010; Thomas Jansson & Powell, 2007; Redmer et al., 2004; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Vonnahme et al., 2003). MNR has been

shown to generate placentas with altered vascular development and increased barrier thickness, in both animal models (Belkacemi et al., 2010; Redmer et al., 2004; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001) and human cases (Aherne & Dunnill, 1966) of maternal undernourishment; recent studies have also shown evidence for chronic hypoxia with MNR-FGR in guinea pigs (Elias et al., 2013).

Studies conducted in sheep and guinea pig animal models, ranging from moderate to severe nutrient restriction, result in a range of different levels of growth restriction and altered development. MNR starting pre-conception and lasting over the entire course of pregnancy in guinea pigs has been shown to be sufficient in causing altered fetal and placental weights consistent with FGR (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001). Studies that utilized pre-conception moderate MNR showed a significant impact on fetal-placental growth. MacLaughlin *et al.* demonstrated that MNR, applied pre-conception through to gestational day 7 in sheep, was sufficient to impact fetal and placental weight at birth. Previous studies have used moderate MNR in guinea pigs, applied pre-conception and through pregnancy, and have observed reduced fetal weight and altered fetal development, as well as structural and functional changes in the placenta, including increased barrier thickness, decreased size of the placental labyrinth, and therefore decreased availability for nutrient exchange between maternal and fetal tissue (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001).

1.3.4 Animal Studies of FGR and Altered Brain Development

Animal studies of aFGR have demonstrated that, despite brain weights being relatively similar to age matched controls, there are maladaptive changes in brain areas

such as the hippocampus, retina, ventricles, cerebellum, brain stem, and cortex (Mallard, Loeliger, Copolov, & Rees, 2000; Rees & Bainbridge, 1992; Rees et al., 1999; Rehn et al., 2004; Tolcos et al., 2003; Tolcos & Rees, 1997). There have been several studies in FGR guinea pigs showing that synapse formation and myelination are reduced (Dieni & Rees, 2003; Mallard et al., 2000; Piorkowska et al., 2014). Guinea pig offspring that utilized a uterine artery ligation model of placental insufficiency were found to have reduced neuronal numbers in select brain regions at 7 days of age; these changes significantly correlated with reductions in brain weight (Mallard et al., 2000). Another study using a uterine artery ligation model in guinea pigs found FGR-born animals to have increased lateral ventricle size and reduced basal ganglia volume at 8 weeks of age (Rehn et al., 2004). Additional studies in guinea pig models of placental insufficiency have found reduced myelination and a reduced number of myelinated fibers in FGR offspring compared to control animals, as well as disproportionally thin myelin sheath in growth restricted fetuses (Nitsos & Rees, 1990) A number of structural changes occur in the brain with a great deal of regional variability, and these changes persist after birth.

1.4 SUMMARY

Fetal growth restriction is a failure to attain full genetic growth potential during fetal life. It can manifest as asymmetrical or symmetrical growth restriction, based on the incidence of brain sparing. FGR occurs due to a combination of maternal, placental, and genetic factors; the most common being the occurrence of placental insufficiency in developed countries or undernutrition in developing countries. In both cases there is a reduction in the transport of nutrients and oxygen that reach the fetus from the mother;

either by reduced capacity of the placenta to transport nutrients, and/or by reduced concentration of nutrients in maternal circulation. FGR leads to an elevated risk of cognitive deficits and neurological disorders in later life. This includes increased risk of autism spectrum disorder, attention deficit hyperactivity disorder and schizophrenia. Cognitive deficits may present as poor academic performance, learning disabilities and memory deficiencies in childhood and throughout life. Clinical study and research on potential mechanisms behind FGR have led to the creation and use of many animal models to mimic the human situation. Previous studies have shown that there is a connection between FGR and altered growth and development of the fetal brain; however, there has been little study in models of MNR. As such, determining the effects on brain growth and development resulting from MNR leading to FGR may lead to a better understanding of the human situation and may be utilized in extrapolation of potential treatments and interventions in human FGR.

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CHAPTER 2
RATIONALE, HYPOTHESIS, AND RESEARCH OBJECTIVES

2.1 RATIONALE

Next to premature birth, fetal growth restriction (FGR) is the most common antenatal complication in developed countries contributing to increased risk for fetal/neonatal morbidity and mortality (Ghidini, 1996; Lackman, Capewell, Richardson, et al., 2001). Furthermore, FGR-born offspring have an increased risk for the development of adverse health outcomes in later life including heart disease, diabetes, and neurodevelopmental disability, with the greatest risk observed in those with severe and early-onsetting FGR (Barker, 2004). A majority of FGR cases arise as the result of an unknown combination of maternal, fetal, and placental factors giving rise to incomplete growth; though many cases are associated with idiopathic placental insufficiency (Ghidini, 1996; Pallotto & Kilbride, 2006; Pollack & Divon, 1992).

Clinical studies of placental insufficiency with FGR have shown aberrant placental vascularization, involving insufficient or incomplete trophoblastic invasion of spiral arteries in the placental bed, and decreases in umbilical blood flow (J. Roberts, 1998; Salafia et al., 2006; Wang et al., 1992). These studies have led to animal models of placental insufficiency induced by uterine artery ligation, placental embolization, or carunclectomy, primarily in sheep and guinea pigs, producing FGR offspring with reduced fetal weight, and often associated with asymmetrical fetal growth restriction (aFGR) with increased brain-to-body weight ratios, and increased polycythemia and hypoglycemia as commonly seen with human FGR (Gagnon et al., 1997; Harding et al., 1985; Murotsuki et al., 1997; Regnault et al., 1999). These animal models are relevant for representation of human FGR and developmental programming, however they mainly affect the latter portion of pregnancy and also limit the ability of researchers to observe placental responses with artifactual blood flow manipulations. The study of

maternal nutrition and FGR is complex, and while maternal undernourishment is causative for FGR, its full effect will depend upon severity and timing both pre-conception and throughout pregnancy. This has led to animal models of maternal nutrient restriction (MNR) induced pre-conception, peri-conception, and throughout pregnancy. These models have been found to be sufficient to cause FGR (Lumey, 1998; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001), are relevant for representing human FGR and have the additional benefit of targeting the insult in a chronic and non-invasive way, which more closely parallels the human situation with severe FGR (Low et al., 1992). However, the specific impact that these changes have on the brain in MNR-FGR fetuses is yet to be determined.

Growth restricted infants have a higher risk for the development of short and long term neurological deficits. FGR-born children have a higher chance of developing cerebral palsy, attention deficit hyperactivity disorder, and autism spectrum disorder (Halliday, 2009; Indredavik et al., 2010; Rodrigues et al., 2006; D.-M. Walker & Marlow, 2008). In later life, higher risks of schizophrenia, epilepsy, and psychiatric hospitalization are observed in FGR-born offspring than offspring of normal size (T. D. Cannon et al., 2003). Deficits of cognitive function associated with FGR can be observed from a very young age; FGR-born children are observed to have reductions in cognitive skills, impaired memory, learning difficulties, difficulties reading, writing, and with adaptive skills, inattention, reduced psychosocial function, behavioural problems, sensorineural deterioration, reduced mathematics abilities, and reduced intelligence quotient scores (Geva et al., 2008; Pallotto & Kilbride, 2006; Rodrigues et al., 2006; Synnes et al., 2010; D.-M. Walker & Marlow, 2008). These neurological skills are associated with particular areas of the brain, such as the hippocampus, that have an

abundance of neuronal connections, which develop prenatally (Bourgeois, 1997). Neuronal connection is partially dependent upon the creation of an immature synapse and selective maturation of the synapse at the synaptic cleft (Bourgeois, 1997). Therefore, a sub-optimal uterine environment may lead to altered development of synapses and therefore altered development of these neuronal connections and consequently, contribute to these observed pathologies.

Development of the fetal brain is an intricate process that involves successful completion of many processes. An interruption in these processes could lead to aberrant neuronal communication between brain regions. A sub-optimal intrauterine environment may lead to reduced energy levels, accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER), and the exhaustion of adaptive mechanisms leading to altered synapse formation or membrane failure with an increase in necrotic cell injury and/or changes in apoptotic regulators. These *in utero* changes may underlie the cognitive deficiencies observed in F GR-born children. Examination of the expression of markers of necrosis, apoptosis, ER stress, and synaptogenesis may provide some insight to their contribution to these neurological deficits associated with FGR.

2.2 HYPOTHESES

1. Maternal nutrient restriction in guinea pigs beginning pre-conception and continuing throughout pregnancy will result in aberrant placental growth and fetal growth restriction in near term pups; with the growth restriction being asymmetrical with increased brain-to-body weight and brain-to-liver weight ratios compared to control animals.

2. Maternal nutrient restriction in guinea pigs leading to fetal growth restriction with evidence for chronic hypoxia/nutritional impairment will have threshold effects on brain growth and development, depending on severity and timing, ranging from occult injury with altered growth processes including synapse formation to overt injury with cellular necrosis/apoptosis.

3. Maternal nutrient restriction in guinea pigs leading to fetal growth restriction with evidence for chronic hypoxia/nutritional impairment will show increased pro-apoptotic factors and increased endoplasmic reticulum stress as mechanistic pathways for occult and/or overt brain injury.

2.3 OBJECTIVES

1. To further characterize pregnancy outcomes and fetal and brain growth in a well-established model of FGR by application of moderate MNR in guinea pig sows (70% of *ad libitum* diet at least 4 weeks prior to pregnancy and continuing throughout and increased to 90% of *ad libitum* diet at mid gestation until near term put-down).

2. To determine differences in structural cell damage in the grey matter, periventricular white matter, thalamus, CA1, CA4 and the dentate gyrus between control appropriate for gestational age (AGA) and MNR-FGR guinea pig fetuses, by examining cellular necrosis and apoptosis using Hematoxylin and Eosin (H&E) staining and ApopTag® Peroxidase In Situ Apoptosis Detection Kit, respectively.

3. To determine differences in the expression of endoplasmic reticulum stress proteins and pro-apoptotic proteins in subcortical regions of the brain between AGA-control and MNR-FGR guinea pig fetuses, by examining levels of Bcl-2-associated X protein (Bax),

cleaved caspase 3, G protein-coupled receptor 78 (Grp78) and Poly ADP Ribose Polymerase 1 (PARP1) via Western Blot.

4. To determine differences in synaptic protein expression in the grey matter, thalamus, and CA1, CA4 and the dentate gyrus between AGA-control and FGR-MNR guinea pig fetuses, by examining Synaptophysin immunoreactivity using immunohistochemistry techniques.

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CHAPTER 3
MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS AS AN ANIMAL MODEL FOR INDUCING FETAL GROWTH RESTRICTION

3.1 INTRODUCTION

Fetal growth restriction (FGR) with infants small for their gestational age when born is a major contributor to perinatal morbidity and mortality as well as for later adverse health outcomes, including heart disease, diabetes, and neurodevelopmental disability (Barker, 2004; Kramer et al., 1990; Lackman, Capewell, Richardson, et al., 2001; Piper et al., 1996; Pryor et al., 1995). This has led to the notion that the intrauterine environment can "program" the development of risk factors for these later adverse outcomes during fetal life and an increasing number of human and animal based studies examining mechanisms underlying this relationship support this concept of fetal programming (Armitage, Khan, Taylor, Nathanielsz, & Poston, 2004; Barker, 2004; Fowden, Giussani, & Forhead, 2006; K. M. Godfrey & Barker, 2000).

Fetal growth restriction remains widespread in developed countries where aberrant placental development or placental insufficiency is a major cause, and in developing countries where maternal undernourishment plays a more prominent role (Thomas Jansson & Powell, 2007; S. P. Walker et al., 2007b). Clinical study of placental insufficiency with FGR demonstrates aberrant placental vascularization and associated decreases in umbilical blood flow (Ferrazzi et al., 2000; J. C. P. Kingdom & Kaufmann, 1997). This has led to animal models of placental insufficiency induced by pre-pregnancy uterine carunclectomy, mid-pregnancy exposure to hypothermic environments, uterine artery ligation/ablation, or placental embolization later in pregnancy (Detmer & Carter, 1992; T Jansson & Persson, 1990; Lafeber HN, Rolph TP, 1984; McIntosh et al., 1979; Murotsuki et al., 1997; Regnault et al., 1999; a. J. Turner & Trudinger, 2009). These models are primarily in sheep, rats, and guinea pigs, and lead to FGR with variable hypoxemia and nutrient restriction. While relevant for

representing human FGR and developmental programming, these vascular models are variable in their timing and severity throughout pregnancy and can limit the study of placental responses with artifactual blood flow manipulations.

Clinical study of maternal nutrition and FGR is complex, and while maternal undernourishment is indeed causative for FGR, the impact will depend upon severity and timing pre-conception as well as throughout pregnancy (K. Godfrey & Robinson, 1998; Thomas Jansson & Powell, 2007; Lumey, 1998; S. P. Walker et al., 2007b). This has led to animal models of maternal nutrient restriction (MNR) induced by global nutrient restriction or protein restriction, pre-conception, peri-conception, and through pregnancy, in a number of animal species and leading to variable degrees of FGR (Belkacemi et al., 2010; Clarke, Heasman, Juniper, & Symonds, 1998; L. J. Edwards & McMillen, 2001; Thomas Jansson & Powell, 2007; MacLaughlin, Walker, Roberts, Kleemann, & McMillen, 2005; Redmer et al., 2004; Sohlstrom et al., 1998; Soo et al., 2012; Vonnahme et al., 2003). These models of MNR are also relevant for representing human FGR and developmental programming, and have the advantage of targeting the insult throughout pregnancy, which is analogous to the human situation where intrauterine deprivation is likely onset early in pregnancy (J. C. P. Kingdom & Kaufmann, 1997; MacLaughlin et al., 2005; Wienerroither, Steiner, Tomaselli, Lobendanz, & Thun-Hohenstein, 2001).

Guinea pigs deliver precocious young after a relatively long gestational period with many developmental events occurring during fetal life, similar to what is seen in humans (Carter, 2007). They have therefore proved useful for modeling human FGR with uterine artery ligation and ablation, but with high fetal loss rates and variable occurrence of growth restriction (Detmer & Carter, 1992; T Jansson & Persson, 1990;

Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009). Moderate MNR in guinea pigs at 70% of an *ad libitum* diet from four weeks pre-conception until mid-pregnancy increasing to 90% thereafter, has also been well studied for modeling human FGR (Kind et al., 2003, 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998). Here the capacity of the mother to deliver nutrients to the fetus is further impaired since her own fuel reserves are depleted prior to conception (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001). This better reflects the human situation with maternal undernourishment where pre-pregnancy weight is a better determinant of fetal growth and development than weight gain during pregnancy (Stevens-Simon et al., 1995) and the majority of women do not make improvements to their dietary and lifestyle patterns during pregnancy (Crozier et al., 2009). Studies with moderate MNR in guinea pigs have shown fetal weights to be decreased by as much as 40% in animals near term in association with maternal insulin-like growth factor (IGF) and insulin-like growth factor-binding protein (IGFBP) alterations (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998), and leading to insulin resistance in male offspring as also seen in FGR-born humans (Barker, 2004; Kind et al., 2003). Of note, placental weights are decreased less than fetal weights suggesting compensatory growth, but with reduced surface area for nutrient exchange, increased barrier thickness, and altered vascular development (C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001; Sung, Vohr, & Oh, 1993). These structural alterations in the placenta indicate functional impairment and can be seen in human FGR with maternal undernourishment (Aherne & Dunnill, 1966; Belkacemi et al., 2010) and with pre-eclampsia associated placental insufficiency (Aherne & Dunnill, 1966; Teasdale & Jean-Jacques, 1988). Accordingly, moderate MNR in guinea pigs may result in adverse *in utero* conditions similar to that

seen with maternal undernourishment or placental insufficiency during human pregnancy and prove useful for modeling FGR in both conditions.

Moderate MNR in guinea pigs has proved useful for inducing FGR and studying maternal, placental and fetal growth characteristics, and offspring outcomes (Kind et al., 2003, 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998). However, there has been little study of breeding and pregnancy success, the distribution of fetal weights and means by which FGR might be denoted, and the impact on fetal organ weights and blood metabolites. We have therefore studied moderate MNR in guinea pigs and report on our breeding and pregnancy success, and distribution of fetal weights near term and means for denoting FGR to further characterize the utility of this model. We have also determined the impact on fetal crown rump length, organ weights, and blood hemoglobin and glucose, hypothesizing that MNR-FGR animals will be lean with asymmetrical growth restriction, and polycythemia and hypoglycemia, as often seen in human pregnancy with moderate to severe FGR (K. Godfrey & Robinson, 1998; Kramer et al., 1990; Piorkowska et al., 2014).

3.2 MATERIALS AND METHODS

3.2.1 Animal Feeding, Breeding and Pregnancy

A previously established model of moderate MNR in guinea pigs (Sohlstrom et al., 1998) was used with all experimental procedures approved by The University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. Nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were housed in individual cages in a dedicated small animal care facility with a 12 hour light/dark cycle and temperature at

25ºC. Animals were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked (Lilley, Epping, & Hafner, 1997).

Thirty guinea pig sows were randomly assigned to either a control group fed *ad libitum* or an MNR group fed 70% of the average food intake per kilogram of body weight of the *ad libitum* fed animals as described by Sohlstrom *et al.* (1998). After four weeks of adaptation to respective feeding regimens, animals were mated. A female found to be in estrous was placed in a cage with a male for 48-72 hours and removed when the vaginal membrane was again closed. Animal pregnancies were confirmed by ultrasound 14-21 days later with conception calculated as the day prior to membrane closure and thereby day zero of gestation. Animals that were not pregnant were rebred at their next estrous cycle. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the control animals per kilogram body weight. Throughout the experiment, food intake was monitored daily and body weights were monitored 3-4 times per week and the dietary intake of the MNR animals was adjusted as needed to maintain their food intake at 70% or 90% of the average food intake per kilogram of body weight of the *ad libitum* fed animals.

3.2.2 Necropsy, Tissue Collection and Blood Analytes

On day 60-61 of pregnancy (term = ~68 days), guinea pig sows were weighed and then sedated with an intramuscular injection of Versed (midazolam, 5 mg/kg; Sandoz Canada Inc., Boucherville, Que., Canada) and after 10 minutes an intramuscular

injection of Vetalar (ketamine, 50 mg/kg; Bioniche Animal Health, Belleville, Ont., Canada) and Rompun (xylazine, 3 mg/kg; Bayer Inc., Toronto, Ont., Canada). A sub-umbilical midline incision was made after local infiltration of the abdominal skin with lidocaine (2%; Pfizer Animal Health, Kirkland, Que., Canada) followed by uterine incision and delivery of each of the fetuses. Approximately 1cc of amniotic fluid was obtained from each gestational sac prior to delivery, which was cold-centrifuged and stored at -80ºC for later analysis. All liveborn fetuses were treated with Vetalar as above, with body and placental weights then obtained along with crown rump length measurements. The number of live and demised fetuses in each uterine horn was also noted. Fetuses were considered to be appropriate for gestational age (AGA) if ≥ 80 g and FGR if < 80 g, which is in accord with the criteria we (Piorkowska et al., 2014) and others (T Jansson & Persson, 1990) have used for categorizing AGA and FGR fetal weights in guinea pigs near-term. Subsequently, only AGA fetuses from control group litters and FGR fetuses from MNR group litters were subjected to full necropsy, with priority given to the medial fetuses in each uterine horn meeting these criteria and with no more than three full necropsies per litter to ensure rapid tissue collection. Full necropsy consisted of a cardiac puncture to obtain ~1cc of blood in a heparinized syringe which was then placed on ice until analysis for glucose and hemoglobin using an ABL 725 Blood Gas Analyzer (Radiometer, Copenhagen, Denmark). This was followed by dissection and weighing of the brain, heart, liver and kidneys, extraction of the gonads for determining fetal sex, and extraction of skeletal muscle and peri-renal adipose tissue. These organs/tissues along with the placenta were partitioned and both fixed in 4% paraformaldehyde and frozen in liquid nitrogen for later analysis.

3.2.3 Data Acquisition and Statistical Analysis

Litter size was based on the number of liveborn and demised fetuses noted at necropsy. Fetal body weight (g)/crown rump length (cm) was calculated as a measure of leanness. Overall control and MNR Population characteristics included data from all control sows and their liveborn fetuses, as well as all MNR sows and their liveborn fetuses, excluding data from animals who failed to conceive, and those delivering prior to necropsy. Select AGA-control and FGR-MNR population characteristics included data from all AGA-control and FGR-MNR fetuses that were liveborn and underwent full necropsy. Maternal and fetal characteristic findings are presented as group means ± SEM. Overall control and MNR population characteristics and select AGA-control and FGR-MNR population characteristics were compared using analysis of variance and non-paired student`s t-test which were also nested for litter size (Graphpad Software, San Diego, CA). For all analysis, statistical significance was assumed for $p<0.05$.

3.3 RESULTS

3.3.1 Breeding and Pregnancy Outcomes

Of the 30 guinea pig sows, 12 were bred under *ad libitum* feeding conditions and 18 under MNR feeding conditions assuming breeding and pregnancy outcomes would be more adverse for the MNR animals and with the smaller litter size reported for MNR pregnancies. Of the 12 animals bred under *ad libitum* feeding conditions, three or 25% failed to become pregnant despite up to four breeding attempts while the remaining animals took 2.4 breeding attempts on average to conceive. All nine of these control animals had continuing pregnancies to necropsy at day 60/61 of gestation with 31 liveborn fetuses and one fetal demise, forming the overall control population. Of the 18

animals bred under MNR feeding conditions, three or 17% failed to become pregnant despite up to four breeding attempts while the remaining animals took 2.6 attempts on average to conceive. However, three of these pregnant MNR sows delivered preterm and prior to necropsy at day 60/61 of gestation; one at 54 days with three fetuses weighing 21, 27, and 35 g, one at 56 days with three fetuses weighing 50, 60, and 75 g, and one at 57 days with 4 fetuses weighing 22, 33, 51, and 66 g. The remaining 12 MNR animals had continuing pregnancies to the time of necropsy with 42 liveborn fetuses and one fetal demise which formed the overall MNR population.

3.3.2 Maternal and Fetal Population Characteristics

The overall maternal and fetal population characteristics from all *ad libitum* fed control pregnancies and all MNR pregnancies excepting the three MNR sows delivering preterm, are shown in Table 3.1. These data are presented for all fetuses to indicate the population variance and allow for comparison with past studies. While maternal weights were not different at conception averaging 800 g, by 60/61 days gestation MNR sows were ~17% lighter at 1046±g than control sows at 1253 ± 60 g ($p<0.01$). Food consumption for both animal groups increased through pregnancy as maternal weight increased, with the actual food consumption of MNR sows at conception and at 60/61 days gestation being ~65 % and 70 %, respectively, of that consumed by the control sows. While litter size did not differ between the two study groups averaging 3-4, the combined fetal weight per litter was less for the MNR animals at 242 ± 21 g than that of the control animals at 331 ± 30 g ($p<0.05$). This was due to fetal weights at necropsy being ~28% less in the MNR pregnancies at 69 ± g than in the Control pregnancies at 96 ± 2 g ($p<0.01$). The 31 liveborn control fetuses ranged in weight from 119 g to 70 g with

the 50th and 10th percentiles being ~96 g and 78 g, respectively, while the 42 liveborn MNR fetuses ranged in weight from 102 to 41 g with the 50th and 10th percentiles being ~69 g and 50 g, respectively (Figure 3.1). Placental weights were also decreased in the MNR pregnancies by ~23% at 5.1 ± 0.2 g compared to that of the control pregnancies at 6.6 ± 0.3 g ($p<0.01$). As such, placental weights were decreased less than the fetal weights with the placental-to-fetal weight ratio thereby increased in the MNR pregnancies at 7.6 ± 0.3% vs. that of the control pregnancies at 6.8 ± 0.3% ($p<0.05$). Fetal crown rump lengths were also decreased in the MNR pregnancies by ~15% at 10.6 ± 0.1 cm vs. that of the control pregnancies at 12.3 ± 0.2 cm ($p<0.01$), but again less than the decrease in fetal weights. As such, fetal weight-to-length ratio as a measure of leanness was also decreased in the MNR pregnancies by ~15% at 6.4 ± 0.1 g/cm vs. that of the control pregnancies at 7.8 ± 0.1 g/cm ($p<0.01$).

While MNR fetuses were on average smaller than control fetuses, there was considerable overlap in the population weight distributions as seen in Figure 3.1. This is not surprising since litter size, number of fetuses per uterine horn, and fetal position within the horn are all known to impact fetal growth (Piorkowska et al., 2014; A. J. Turner & Trudinger, 2000). We therefore chose to establish a cohort of AGA fetuses from the control group pregnancies and a cohort of FGR fetuses from the MNR group pregnancies to allow for more in-depth comparative study of growth related parameters. As noted, we used 80 g as our threshold for categorizing AGA and FGR fetal weights at 60/61 days gestation and determining which control and MNR fetuses were to be subjected to full necropsy with priority given to the medial fetuses in each uterine horn and with no more than three full necropsies per litter. This resulted in 20 AGA-control fetuses and 25 FGR-MNR fetuses with the select population characteristics from

these animals shown in Table 3.2 and Figure 3.1. After examining the gonads for sexing animals, it was determined there were 10 AGA-control males and 10 AGA-control females, and 11 FGR-MNR males and 14 FGR-MNR females with no sex differences evident for any of the select fetal population characteristics as assessed using analysis of variance. Accordingly, these data are presented for all males and females combined as a measure of the population variance and to allow for comparison with past studies. While all fetal weights in MNR pregnancies were decreased ~28% on average compared to control pregnancies, FGR-MNR fetal weights were decreased by ~37% at 64 ± 2 g compared to that of the AGA-control fetuses at 101 ± 2 g ($p<0.01$). FGR-MNR brain weights were also decreased, but less so, by ~12% at 2.39 ± 0.04 g vs. that of the AGA-controls at 2.73 ± 0.05 g ($p<0.01$) while FGR-MNR liver weights were markedly decreased by ~40% at 2.8 ± 0.1 g vs. that of the AGA-controls at 4.7 ± 0.2 g ($p<0.01$). Accordingly, the brain-to-fetal weight ratio and brain-to-liver weight ratio, as measures of asymmetrical growth, were increased 40-50% in the FGR-MNR fetuses at 3.8 ± 0.1% and 0.90 ± 0.03% compared to that of the AGA-control fetuses at 2.7 ± 0.1% and 0.61 ± 0.03%, respectively (both $p<0.01$). FGR-MNR heart weights also showed a smaller decrease than the corresponding decrease in fetal weights, by ~23% at 0.46 ± 0.03 g vs. that of AGA-controls at 0.60 ± 0.03 ($p<0.01$). As such, the heart-to-fetal weight ratios were increased ~22% in the FGR-MNR fetuses at 0.72 ± 0.05% compared to that of the AGA-controls at 0.59 ± 0.03% ($p<0.05$). It is also of note that the threshold of 80 g here used at 60/61 days gestation for denoting AGA and FGR fetal weights, is close to the 10th percentile for the population weight distribution of the 31 liveborn Control fetuses at ~78 g and thereby in accord with the FGR definition often used for human pregnancies (Lackman, Capewell, Gagnon, & Richardson, 2001). Using this 80 g

threshold, 26 of the 31 control fetuses or ~85% met weight criteria for AGA study, while 32 of the 42 MNR fetuses or ~75% met weight criteria for FGR study (Figure 3.1).

3.3.3 Blood Analytes

Blood glucose and hemoglobin values obtained by cardiac puncture at the time of full necropsy in the select AGA-Control and FGR-MNR animals are shown in Table 3.2. FGR-MNR glucose values were decreased by ~27% at 4.3 ± 0.2 mmol/L vs. that of the AGA-control values at 5.9 ± 0.4 mmol/L ($p<0.01$). Conversely, FGR-MNR hemoglobin values were increased by ~9% at 15.9 ± 0.2 g/dL vs. that of the AGA-control values at 14.6 ± 0.4 g/dL ($p<0.05$).

Table 3.1 Overall Maternal and Fetal Population Characteristics

	Control (9/31)*	MNR (12/42)*	Control (9/9)*	MNR (12/12)*
Maternal wt (gms)				
Conception	816±33	778±9		
GA 60/61	1253±60	1046±25‡		
Food (gm/day)				
Conception	38±2	28±1‡		
GA 60/61	54±4	37±1‡		
Litter size	3.4±0.3	3.5±0.3		
Total litter wt (gms)	331±30	242±21†		
Fetal wt (gms)	96±2	69±2‡	96±3	71±4‡
Placental wt (gms)	6.6±0.3	5.1±0.2‡	6.5±0.4	5.3±0.2‡
Placental/fetal wt (%)	6.8±0.3	7.6±0.3†	6.8±0.4	7.7±0.4
Crown rump length (cm)	12.3±0.2	10.6±0.1‡	12.3±0.2	10.8±0.2‡
Fetal wt /length (gm/cm)	7.8±0.1	6.4±0.1‡	7.8±0.2	6.5±0.2‡

Data presented as means ± SEM; † $p < 0.05$, ‡ $p < 0.01$ vs. corresponding control group value analyzed using non-paired Student's t-test, both for the overall populations and nested for litter size; * n values were 9 and 12 for maternal and 31 and 42 (overall populations) or 9 and 12 (nested) for fetal control and Maternal Nutrient Restricted characteristics, respectively, except for litter size where demised fetuses were also counted; MNR = maternal nutrient restriction; GA = gestational age.

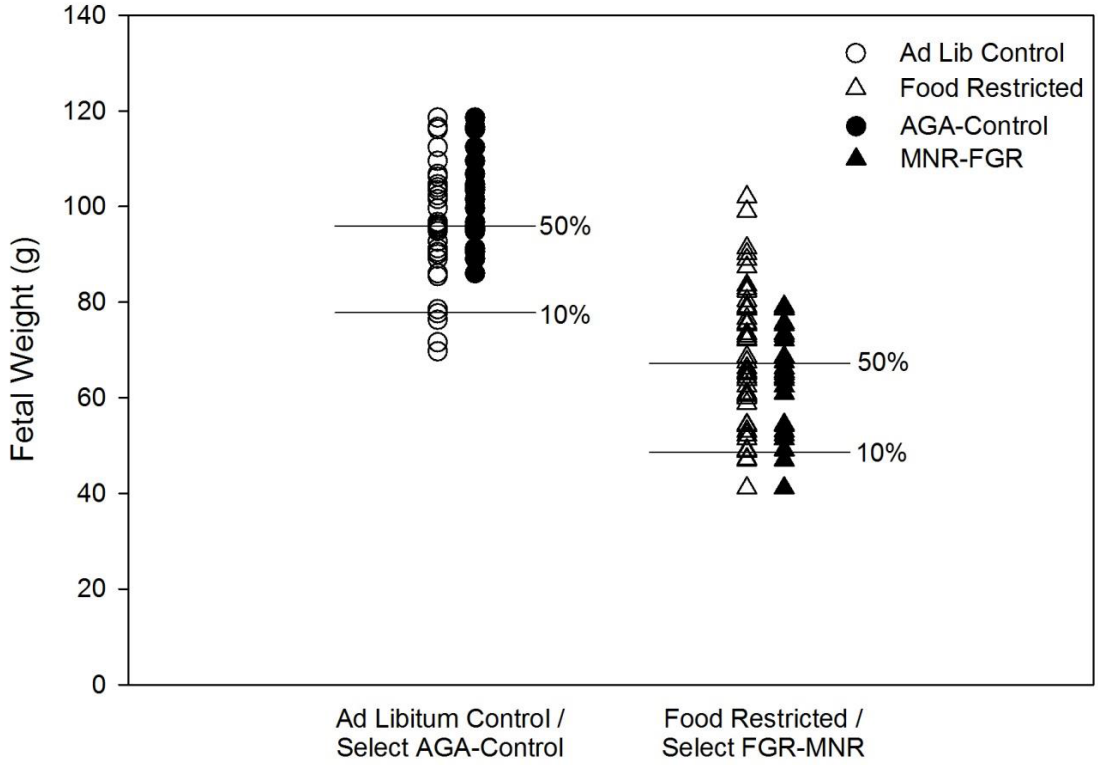

Figure 3.1 Scatter plot showing the fetal weights for all 31 liveborn control fetuses (open circles) and all 42 liveborn maternal nutrient restricted (MNR) fetuses (open triangles) along with the 50th and 10th percentiles for each of these cohort populations. Additionally shown are the distribution of fetal weights for the select 20 AGA-control fetuses (closed circles) and 25 FGR-MNR fetuses (closed triangles).

Table 3.2 Select Fetal Population Characteristics

	AGA-Control (20)*	FGR-MNR (25)*	AGA-Control (9)*	FGR-MNR (11)*
Fetal wt (gms)	101±2	64±2‡	99±2	65±3‡
Brain wt (gms)	2.73±0.05	2.39±0.04‡	2.72±0.06	2.42±0.05‡
Heart wt (gms)	0.60±0.03	0.46±0.03‡	0.59±0.04	0.47±0.03†
Liver wt (gms)	4.7±0.2	2.8±0.1‡	4.6±0.2	2.9±0.2‡
Brain/fetal wt (%)	2.7±0.1	3.8±0.1‡	2.8±0.1	3.8±0.1‡
Brain/liver wt ratio	0.61±0.03	0.90±0.03‡	0.61±0.03	0.89±0.05‡
Heart/fetal wt (%)	0.59±0.03	0.72±0.05†	0.60±0.04	0.73±0.07
Blood glucose (mmol/l)	5.9±0.4	4.3±0.2‡	5.9±0.5	4.4±0.3†
Blood Hb (gm/dl)	14.6±0.4	15.9±0.2†	14.6±0.4	15.8±0.3†

Data presented as means ± SEM; † $p < 0.05$, ‡ $p < 0.01$ vs. corresponding AGA-control group value analyzed using non-paired Student's t-test, both for the select populations and nested for litter size; *n values were 20 and 25 (select populations) or 9 and 11 (nested) for fetal AGA-control and FGR-MNR characteristics, respectively, except for blood glucose and hemoglobin where these were instead 15 and 19 (select populations) or 8 and 11 (nested), respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted, Hb = hemoglobin.

3.4 DISCUSSION

In the present study, we have characterized pregnancy outcomes in guinea pigs subjected to moderate nutrient restriction both before and through pregnancy as a useful model for inducing FGR with similarities to that seen in humans with maternal undernourishment and idiopathic placental insufficiency. Both control and MNR sows had comparable fertility with successful pregnancies in ~80% of the animals, and requiring ~2.5 breeding attempts on average. Maternal age did not have an impact on pregnancy success, although no animals were bred prior to four months which is presumed adulthood for guinea pigs. However, only two animals, one control and one MNR, became pregnant with a conception weight less than 750 grams, suggesting an effect of maternal weight on pregnancy success which has also been noted in guinea pig studies with uterine artery ligation (Detmer & Carter, 1992). Since MNR animals took anywhere from 1-4 breeding attempts before becoming pregnant, the duration of preconception undernourishment was also impacted, and could be from four weeks up to 12 weeks. However, there was no evidence that an increased duration of MNR impacted maternal weight at conception, litter size, fetal weights, or the risk of preterm delivery. As such, four weeks of moderate MNR prior to breeding in guinea pigs is sufficient for inducing FGR, while longer periods of moderate MNR as studied did not appear to worsen pregnancy outcomes. The fetal demise rate was low at 1/32 control fetuses and 1/43 MNR fetuses and much lower than that reported with uterine artery ligation or ablation models, where demise rates upwards of 70–80% have been noted (Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009). This demonstrates the early and gradual growth restriction that occurs in response to MNR (Belkacemi et al., 2010; K. Godfrey & Robinson, 1998; Redmer et al., 2004; C. T. Roberts, Sohlstrom, Kind,

Earl, et al., 2001; Sohlstrom et al., 1998) compared to the relatively abrupt nature of uterine artery ligation/ablation-FGR with normal fetal growth then a sudden reduction in placental blood flow and a variable mismatch between metabolic needs and nutrient delivery. However, 3/15 MNR sows delivered preterm and prior to the planned necropsy at 60/61 days gestation, with two of these mothers having the lowest maternal weights adjusted for gestational age and with low fetal weights at delivery. This finding has also been reported in guinea pigs by Kind *et al.* (2003) with moderate MNR-FGR and by Palliser *et al.* with uterine artery ablation-FGR and likely involves FGR associated increases in inflammatory processes with a shift in prostaglandin production over metabolism (Palliser, Kelleher, Welsh, Zakar, & Hirst, 2014). In the present study the overall fetal loss rate whether from demise or preterm birth was therefore 1/32 or 3% for the control group and 11/ 53 or 20% for the MNR group which is still considerably less than that reported for uterine artery ligation/ablation models as noted. Additionally, some of the MNR fetuses delivering preterm were liveborn and did survive offering the opportunity for study of longer-term outcomes with FGR and preterm birth which is also well known to occur in humans (Lackman, Capewell, Richardson, et al., 2001).

Moderate MNR at 70% of the *ad libitum* diet beginning at least four weeks pre-pregnancy and increasing to 90% of the *ad libitum* diet at mid-pregnancy, resulted in a decrease in maternal weights by ~5% and 17% at conception and 60/61 days gestation, respectively. This decrease in maternal weight is somewhat less than that previously reported by Sohlstrom *et al.* and Roberts *et al.* at ~10% and 28% using the same moderate MNR dietary regime; however, their animals were smaller to begin with, averaging 550 g at mating and likely indicate strain differences to the guinea pigs

presently used. Likewise, the actual decrease in food consumption at ~26% and 32% in MNR sows at conception and 60/61 days gestation, respectively, was somewhat less than that reported by Roberts *et al.* at ~37% and 36% in their MNR sows which may be attributable to their smaller animals. Interestingly, litter size was unchanged in the present study which differs from the findings of Sohlstrom *et al.* where moderate MNR decreased litter number from ~3 to 2 when necropsied at 60 days. This may again be attributable to their smaller animals and indicates that moderate MNR as outlined may adversely affect early developmental events leading to failure pending initial maternal weight and thereby fuel reserves for mobilization (Abrams & Newman, 1991; Belkacemi et al., 2010; Clarke et al., 1998; K. Godfrey & Robinson, 1998; Kramer, 1987). Fetal weights were decreased by 28% on average for all MNR pregnancies necropsied near term at 60/61 days gestation, which not surprisingly is less than that reported by Sohlstrom *et al.* at ~40% given their smaller animals. This again emphasizes the importance of maternal pre-pregnancy weight as a measure of nutritional availability for fetal/placental growth and development during pregnancy (Abrams & Newman, 1991; Belkacemi et al., 2010; Clarke et al., 1998; K. Godfrey & Robinson, 1998; Kramer, 1987; Redmer et al., 2004). Of interest, the fetal weight variation in these animal cohort populations can be assessed using the coefficient of variation (CV) and calculated as the standard deviation divided by the population mean. In the present study and that of Sohlstrom *et al.*, this was comparable at ~12% for all fetal weights from control animals fed *ad libitum*, and somewhat less than that at ~18% for control fetuses in the untreated uterine horn of animals subjected to uterine artery ligation/ablation, suggesting an impact of the surgical procedure here. In both the present and Sohlstrom studies, the CV for all fetal weights from MNR animals was substantially increased at ~22%, and now

similar to that in the treated horn of uterine artery ligation/ablation animals (a. J. Turner & Trudinger, 2009) and indicating an increase in growth variance with these animal models for inducing FGR. Placental weights were decreased by 23% on average for all MNR pregnancies and less than the corresponding decrease in fetal weights thereby resulting in an ~12% increase in the placental/fetal weight ratio which is similar to that noted by Sohlstrom *et al.* with moderate MNR (Sohlstrom et al., 1998). Notably this increase in placental-to-body weight ratio is also seen in human pregnancies leading to FGR both with maternal undernourishment and presumed placental insufficiency and is believed to indicate a degree of compensatory growth by the placenta to minimize FGR (Belkacemi et al., 2010; K. Godfrey & Robinson, 1998; Thomas Jansson & Powell, 2007; J. C. P. Kingdom & Kaufmann, 1997; Lackman, Capewell, Gagnon, et al., 2001; Lumey, 1998; Redmer et al., 2004). Fetal crown-to-rump lengths were also decreased in MNR pregnancies, but again less than the corresponding decrease in fetal weights resulting in a ~15% decrease in fetal weight-to-length ratio and indicating leaner animals which was also noted by Kind *et al.* with moderate MNR in guinea pigs (Kind et al., 2005). Likewise, leanness is often a characteristic in human infants with moderate growth restriction whether resulting from maternal undernourishment or idiopathic placental insufficiency (K. Godfrey & Robinson, 1998; Kramer et al., 1990).

We set a threshold of ≥ 80 g or < 80 g for categorizing AGA-control and FGR-MNR fetal cohorts, respectively, which is in accord with the criteria we (Piorkowska et al., 2014) and others (T Jansson & Persson, 1990) have used for categorizing AGA and FGR fetal weights in guinea pigs near-term. Of note, this threshold of 80 g was close to the 10th percentile for the population weight distribution of the liveborn control fetuses

at ~78 g further justifying its use. We did not include the requirement for an increased brain-to-liver weight ratio for the FGR cohort as in other studies with uterine artery ligation/ablation (Detmer & Carter, 1992; Piorkowska et al., 2014), since we did not want to presume how this would be impacted by MNR induced FGR. This establishment of AGA-control and FGR-MNR cohort groups has the advantage of avoiding any confounding effects of tissue/metabolite study in AGA fetuses from MNR pregnancies and FGR fetuses from control pregnancies. Furthermore, it reduces the fetal weight variation with the CV in these cohort groups being decreased to ~9% and 16%, respectively; which better reflects the human situation with AGA and FGR birth weight distributions being separate and often delineated by the 10th percentile adjusted for gestational age (Lackman, Capewell, Richardson, et al., 2001). As expected, the decrease in FGR-MNR fetal weights vs. the AGA-controls at ~37% was considerably more than the decrease in overall MNR fetal weights compared to the overall controls at ~28%. Moreover, growth restriction in MNR pregnancies was asymmetrical with the mean decrease in liver weights at ~40%, which is much higher than that of the brain and heart at ~12% and 23%, respectively. Accordingly, the brain-to-liver weight ratio was increased by almost 50% in FGR-MNR fetuses which was similar to that noted by Kind *et al.* in their MNR fetuses, while the heart-to-fetal weight ratio was increased by almost 25% in FGR-MNR fetuses (Kind et al., 2005). This asymmetrical growth restriction is likely due in part to chronic blood flow redistribution favouring the vital organs, including the brain and heart at the expense of the liver and carcass tissues (Richardson, 1989), and altered gluconeogenic capacity and/or protein synthesis with a greater impact on the liver and muscle than other tissues (Thorn et al., 2009). Of note, asymmetrical FGR is also seen in guinea pigs with mid-gestation uterine artery

ligation/ablation (Lafeber HN, Rolph TP, 1984; Piorkowska et al., 2014; a. J. Turner & Trudinger, 2009), and in humans with placental insufficiency leading to growth restriction (Abrams & Newman, 1991; Kramer, 1987), with both of these likely to involve chronic fetal hypoxemia as a primary signaling mechanism (Lackman, Capewell, Richardson, et al., 2001; Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009). It is also of note that MNR induced FGR in sheep leads to ventricular hypertrophy, which is thought to reflect increased afterload due to increased placental vascular resistance (Vonnahme et al., 2003). Accordingly, the present increase in heart-to-fetal weight ratio in FGR-MNR fetuses may also indicate a degree of ventricular hypertrophy secondary to increased placental vascular resistance. This is in fact likely with the altered vascular development and structural changes reported in the placenta of guinea pigs subjected to moderate MNR (C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001).

Blood sampling at necropsy revealed FGR-MNR fetuses to be relatively polycythemic and hypoglycemic compared to AGA-control fetuses. Likewise, an increase in hemoglobin and decrease in glucose are well associated with FGR in several animal models and human clinical studies. These include those in guinea pigs after uterine artery ligation and sheep after MNR or carunclectomy, and in human pregnancies with suspected placental insufficiency subjected to cordocentesis (W. Cox et al., 1988; Economides & Nicolaides, 1989; Harding et al., 1985; T Jansson & Persson, 1990; Lafeber HN, Rolph TP, 1984; Soothill, Nicolaides, & Campbell, 1987; Vonnahme et al., 2003). These studies indicate that the basis for the hypoglycemia with FGR is likely multifactorial including lowered maternal glucose (Vonnahme et al., 2003), reduced placental glucose transport and/or fetal glucose delivery (Economides & Nicolaides, 1989; Harding et al., 1985; Lafeber HN, Rolph TP, 1984), and reduced fetal

gluconeogenesis (T Jansson & Persson, 1990), and dependent on the underlying etiology with maternal hypoglycemia likely to play a greater role with undernourishment. However, the basis for the polycythemia with FGR is likely singularly due to stimulated erythropoiesis attempting to maintain oxygen carrying capacity in response to chronic hypoxemia as variably shown in these studies (W. Cox et al., 1988; Economides & Nicolaides, 1989; Harding et al., 1985; Lafeber HN, Rolph TP, 1984; Soothill et al., 1987).

Moderate MNR in guinea pigs has been well studied for modeling human FGR including maternal, placental, and fetal growth characteristics, associated IGF and IGFBP alterations, and mechanisms for programming longer-term adverse outcomes in offspring (Kind et al., 2003, 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998). We now add to these findings by further characterizing breeding and pregnancy success in MNR animals and showing low fetal demise rates in contrast to that seen with uterine artery ligation/ablation models (Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009), albeit with increased preterm delivery as is seen with FGR in humans (Lackman, Capewell, Richardson, et al., 2001). Similar studies using the same moderate MNR dietary regime and impact on maternal/fetal weights and litter size (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998) highlight the importance of maternal pre-pregnancy weight as a measure of nutritional availability for fetal/placental growth when nutrient intake is compromised during pregnancy (Abrams & Newman, 1991; Belkacemi et al., 2010; Clarke et al., 1998; K. Godfrey & Robinson, 1998; Kramer, 1987; Redmer et al., 2004). As previously shown (Kind et al., 2005; Sohlstrom et al., 1998), we confirm that MNR fetuses are leaner and have increased placental-to-fetal weight ratios as is often seen in human infants with

moderate growth restriction whether resulting from maternal undernourishment or placental insufficiency (Belkacemi et al., 2010; K. Godfrey & Robinson, 1998; Kramer et al., 1990; Lackman, Capewell, Richardson, et al., 2001; Lumey, 1998). We also provide justification for using a fetal weight threshold for categorizing AGA-control and FGR-MNR cohorts which approximates the 10th percentile in our *ad libitum* fed animals, and serves to reduce the population variance in these groups. Of note, these FGR-MNR fetuses show asymmetrical growth restriction, and are polycythemic and hypoglycemic which are well associated with moderate growth restriction during human pregnancy (Abrams & Newman, 1991; W. Cox et al., 1988; Economides & Nicolaides, 1989; Kramer et al., 1990; Soothill et al., 1987). These findings along with the altered vascular development and structural changes reported in the placenta of guinea pigs subjected to moderate MNR (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001) also raise the possibility of chronic hypoxemia as a primary signaling mechanism for the decreased fetal growth in these pregnancies which requires further study. As such, the present and past studies of moderate MNR in guinea pigs (Kind et al., 2003, 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998) support the utility of this model for inducing FGR with many similarities to that in humans with moderate growth restriction whether resulting from maternal undernourishment or placental insufficiency. While there will be differences in FGR outcomes resulting from maternal undernourishment vs. placental insufficiency including the impact on fetal gene expression (Nüsken et al., 2011), it is likely that these will also depend on the timing, severity and duration of the nutrient deprivation as much as the cause (Clarke et al., 1998; McMillen et al., 2001).

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CHAPTER 4
MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS WITH FETAL GROWTH RESTRICTION LEADS TO ALTERED BRAIN DEVELOPMENT

4.1 INTRODUCTION

Fetal growth restriction (FGR) is associated with a number of neurological disorders and cognitive deficits. In children, FGR leads to reduced cognitive skills including impaired memory and learning, inattention, reduced psychosocial function and lower mathematical ability and intelligence quotient (IQ) scores (Geva et al., 2008; Indredavik et al., 2010; Pallotto & Kilbride, 2006; Rodrigues et al., 2006; Synnes et al., 2010; D.-M. Walker & Marlow, 2008). Studies of the brain in FGR have shown changes in neurogenesis, with animal models of FGR having demonstrated reduced number and length of neurons (Mallard et al., 2000; Tolcos & Rees, 1997), reduced size of the dendritic tree, and a decreased density of dendritic spines (Dieni & Rees, 2003). These changes in neuronal connectivity could underlie many of the cognitive deficiencies observed in early life of FGR-born children, particularly changes in the hippocampus may underlie deficiencies in memory and cognition.

Development of the fetal brain is an intricate process that involves successful completion of many processes, including neurogenesis, axon and dendrite migration, and pre-and postsynaptic element coupling. An interruption in these processes could lead to aberrant neuronal communication between brain regions. It has been demonstrated in a previous study that moderate maternal nutrient restriction (MNR) in guinea pigs leading to FGR involves aberrant placental development with chronic hypoxia (Elias et al., 2013), as such this disruption in oxygen and nutrient flow, as well as increased oxidative/endoplasmic reticulum (ER) stress markers may provide mechanistic pathways for adverse fetal development. The human brain, as well as the precocious guinea pig brain, undergoes neuronal differentiation at its highest absolute

rate of synaptic formation during fetal gestation (Dobbing & Sand, 1970). As such, FGR with nutrient transport impairment may disrupt synapse formation, as it is a growth process with high energy demand (Jiang & Schuman, 2002) and requires precise presynaptic and postsynaptic element coupling, and adequate neuronal growth, migration, and branching to achieve proper localization and function (Scheiffele, 2003). The exact mechanism underlying synaptic development is not well understood, however, presynaptic maturation is evident with an increase in the number of vesicles in developing synapses. The amount of vesicle production in the synapse increases with its maturation and decreases steadily in the case of synapse degeneration (Ruthazer, Li, & Cline, 2006). Studies have shown a presynaptic active zone at the synaptic cleft to which vesicles adhere prior to exocytosis; additionally, there is a region of postsynaptic density on the dendritic spine that anchors receptors and scaffolding proteins. These presynaptic and postsynaptic areas mature to form a functioning synapse and establish neuronal communication (Bourne & Harris, 2008; Garner, Kindler, & Gundelfinger, 2000). As such, proteins present in the vesicle, active zone, and postsynaptic density may be used as markers of synaptic maturation, development and degeneration.

Synaptophysin (SYN) is a common 38 kDa presynaptic protein marker that is present in the presynaptic bouton and on the membrane of presynaptic vesicles in the central nervous system (Calhoun et al., 1996; Jahn et al., 1985). SYN shows punctate staining along a neuron localized to the presynaptic bouton (Calhoun et al., 1996; Fletcher et al., 1991; Mundel et al., 1997); it is found in the vesicles of mature synapses as well as in immature synapses prior to vesicle development (Daly & Ziff, 1997; Fletcher, De Camilli, & Banker, 1994). Thus, an increase in SYN protein levels is indicative of synapse formation and maturation (Daly & Ziff, 1997; Fletcher et al., 1991).

It has been found in excitatory and inhibitory synapses and is involved in competitive strengthening of synapses as well as activity dependent synapse maturation (Tarsa & Goda, 2002). SYN is consequently often used as a marker of the presence of synapses and overall changes in synaptic numbers.

Previous studies have shown that compensatory mechanisms in the brain, which are usually protective in times of low energy and oxygen supply, may become limited and brain energy levels may be sufficiently impacted, leading to membrane failure with an increase in necrotic cell injury and/or changes in apoptotic regulators (Rocha et al., 2004). While these mechanisms have yet to be elucidated, regional differences in the balance of pro-apoptotic and anti-apoptotic gene expression and activity-dependent changes in this balance with the strengthening of incoming afferent activity are likely to be involved (Anand & Scalzo, 2000). Some of these pro- and anti-apoptotic factors include Bcl-2 associated X protein (Bax), B-cell Lymphoma 2 (Bcl-2) and cleaved caspase 3. Pro-apoptotic Bax and anti-apoptotic Bcl-2 are known to synergistically regulate apoptosis (D. Liu et al., 2013; McCullough et al., 2001). Extrinsic and intrinsic apoptotic pathways both converge on caspase-3, with the downstream event consisting of substrate cleavage. There is abundant evidence that pathways leading to caspase-3 cleavage and activation are engaged following neonatal hypoxia-ischemia (Blomgren et al., 2001; Felderhoff-Mueser et al., 2002; Hu et al., 2000; Northington et al., 2001).

A previous study on apoptotic pathways after neonatal cerebral ischemia found that a sex difference exists in both the mechanism and the degree of apoptotic injury (Renolleau, Fau, & Charriaut-Marlangue, 2008). This study reported findings of an apoptosis-inducing factor (AIF) dependent pathway in males and a cytochrome c/caspase dependent pathway in females (Du et al., 2004). The male pathway included

activation of poly ADP ribose polymerase 1 (PARP1) which is required for translocation of AIF from the mitochondria into the nucleus, where it induces large scale DNA fragmentation (Daugas et al., 2000). The female pathway, however, had a release of cytochrome c, which activated caspase-3 and led to a downstream caspase-dependent cell death (Renolleau et al., 2008) Therefore, sex is an additional variable that needs to be accounted for in study design, this does however present another possible avenue by which to better understand the occurrence and mechanism of brain injury in the FGR fetus.

Another means by which we can study mechanisms of brain injury as a result of hypoxia and low amino acid supply is via investigation of ER stress. ER stress arises due to improper protein maturation and folding in the ER lumen, and in cases where it is not alleviated, leads to activation of downstream apoptotic pathways (D. Liu et al., 2013; Matsumoto et al., 1996). G-protein coupled receptor 78 (Grp78) is an essential component of the ER translocation machinery and plays a key role in degradation of aberrant proteins, as such, synthesis of Grp78 is induced under conditions of ER stress (Hendershot et al., 1994). Therefore, Grp78 can be utilized as an early marker of ER stress. Hypoxia and low amino acid supply, as seen in the MNR guinea pig model (Elias et al., 2013; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001) have been demonstrated to hinder disulfide bond formation, which is essential to protein maturation and folding in the ER lumen (Benham et al., 2013; Frand & Kaiser, 1999; Yu et al., 2012; Zhang et al., 2014). Prolonged ER stress during critical time points in development of the fetal brain may have negative impacts on essential signaling and transport functions and give rise to aberrant development

(Braakman et al., 1991; Frand & Kaiser, 1999; Kawakami et al., 2014; Red-Horse et al., 2004).

In the present study we sought to determine the extent to which moderate MNR in guinea pigs as a causative factor for FGR also impacts markers for brain necrosis and apoptosis and ER stress as measured by Western blot as well as differences in structural cell damage and SYN expression as measured by histology, thereby indicating threshold effects on brain growth and development, ranging from no measurable effect, to occult injury with altered synapse formation to overt injury with cellular necrosis/apoptosis.

4.2 MATERIALS AND METHODS

4.2.1 Animal Cohorts and Tissue Collection

A previously established model of moderate MNR in guinea pigs (Kind et al., 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998) was used, with all experimental protocols approved by The University of Western Ontario Animal Use Subcommittee and following the Canadian Council on Animal Care. Animal feeding, breeding and pregnancy outcomes have been reported in Chapter 3. Briefly, nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked.

Guinea pig sows were assigned to either the control group, which was fed *ad libitum*, or the MNR group, which was fed 70% of the average food intake per kilogram of body weight of the control animals. After 4 weeks on their respective feeding

regimes, animals were mated. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the control animals per kilogram body weight.

On day 60-61 of pregnancy (term = ~68 days), animals were sedated followed by laparotomy and delivery of each of the fetuses. Body and placental weights were obtained from all live-born fetuses along with crown-rump length measurements. Fetuses were considered to be appropriate for gestational age (AGA) if ≥ 80 g and FGR if < 80 g, which is in accord with the criteria we (Piorkowska et al., 2014) and others (T Jansson & Persson, 1990) have used for categorizing AGA and FGR fetal weights in the near-term guinea pig. Moreover, this threshold of 80 g is close to the 10th percentile for the population weight distribution of the live-born control fetuses at ~78 g and thereby in accord with the FGR definition often used for human pregnancies (Lackman, Capewell, Gagnon, et al., 2001). Litter size, number of fetuses per uterine horn, and fetal position within the horn are all variables known to impact fetal growth (Piorkowska et al., 2014; A. J. Turner & Trudinger, 2000), therefore we chose to establish a cohort of AGA fetuses from the control group pregnancies and a cohort of FGR fetuses from the MNR group pregnancies to allow for more in-depth comparative study of growth related parameters. Subsequently, only AGA fetuses from control group litters and FGR fetuses from MNR group litters were subjected to full necropsy which consisted of an initial cardiac puncture to obtain ~1cc of blood for glucose and hemoglobin analysis using an ABL 725 Blood Gas Analyzer (Radiometer, Copenhagen, Denmark). This was followed by dissection and weighing of the brain, heart, liver and kidneys, extraction of the gonads for determining fetal sex, and extraction of skeletal muscle and peri-renal

adipose tissue. The brain was dissected coronally between the cerebral peduncle and the mammillary body as well as through the caudal limit of the optic chiasm. The caudal section was fast frozen in liquid nitrogen and stored at -80°C for later molecular analysis by Western Blot. The rostral and middle sections which were used for later immunohistochemistry analysis, were immersion fixed in 4% paraformaldehyde for 72 hours, then washed in phosphate buffered saline (PBS) daily for 3 days before being placed in 70% ethanol for 7-14 days; they were then blocked in paraffin wax and cut at a thickness of 5μm on a rotary microtome and mounted on superfrost Plus slides (VWR Scientific, Westchester, PA).

Eighteen AGA-control fetuses (9 male and 9 female) and 18 FGR-MNR fetuses (9 male and 9 female) were selected for brain tissue analysis. These animals were representative of the mean fetal weights for their respective cohort groups and were either the first or second medial position in the uterine horn, and with no more than one male and one female fetus from each litter.

4.2.2 Necrosis Analysis with Haematoxylin and Eosin (H&E) Stain

Necrotic cell injury in the brain was studied by staining with H&E. Tissue sections were deparaffinized with three 5-minute washes in xylene and then rehydrated in a series of ethanol baths (100%, 100%, 90%, 90% and 70%) lasting 2 minutes each. Tissue sections were then rinsed once in deionized water for 5 minutes before being immersed in Harris modified haematoxlyn stain (Fisher Scientific) for 10 seconds. The stain was differentiated in 1% acid ethanol (2 mL HCl in 198 mL 70% ethanol) for

approximately 1 second and then flushed with running water for 1 minute. Next, sections were stained with eosin (1% eosin Y in 95% ethanol and 0.5% acetic acid; Fisher Scientific) for approximately 1 second. Tissue sections were then dehydrated in a series of ethanol baths (70%, 70%, 90%, 100% and 100%) followed by three 5-minute washes in xylene and coverslipped using Permount (Fisher Scientific). Slides were left for 24 hours to dry in a fume hood before being visualized. All slides were stained on the same day using the same solutions to minimize variation in intensity of stain.

Analyses were carried out in coronal sections at the level of the mammillary bodies and at the level of the optic chiasm. Brain regions analyzed included the parasagittal gray matter, convexity gray matter, periventricular white matter, thalamus, and hippocampus regions CA1, CA4, and dentate gyrus (Figure 4.1). Grey matter regions were further divided into layers 1-3 and layers 4-6 for analysis and thalamus was divided into medial and lateral thalamus, but results were combined for all grey matter and thalamic subregions from both coronal sections when no significant differences were found. Images were captured using a Zeiss upright light microscope (Carl Zeiss Microimaging, Thornwood, NY) with a 40x objective. Identical illumination settings were used for all brain regions to allow for comparison between regions. For all experiments, a minimum of 6 randomly selected 40x high-power fields (HPFs) per region were captured, with the goal being 4 HPFs per region per side of the brain. Necrotic-appearing cells were identified by the characteristic hallmarks of cellular necrosis in H&E staining, including eosinophilic cytoplasm, concave/elongated cell bodies and a loss of nuclear detail (Figure 4.2) and were scored manually. Each HPF was scored on a 5-point scale based on the estimated percentage of necrotic-appearing cells, with 0 = 0% necrotic cells, 5 = 1%-10% necrotic cells, 30 = 11%-50% necrotic cells, 70 =

51%-90% necrotic cells and 95 = 91%-100% necrotic cells, as reported previously (Rocha et al., 2004). In order to eliminate experimenter bias, all tissue slides were coded with the experimenter blinded to the animal treatment group.

4.2.3 Apoptosis Analysis with ApopTag

Apoptotic cell injury in the brain was measured by the presence of DNA fragmentation within cells using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay method (Apoptag Peroxidase In Situ Appoptosis Detection kit; Millipore, Billerica, Massachusetts). To reduce variability, all staining was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinized as described in H&E, they were then equilibrated in PBS for 5 minutes. Tissues were then incubated with proteinase K (20 ug/mL) for 20 minutes at room temperature and then rinsed 4 times in deionized water for 1 minute each. Positive control slides were generated by equilibrating sections in DN buffer for 5 minutes and then removing excess water and applying 1:25 DNAse 1:DN Buffer (Sigma D7291 Sigma-Aldrich; Oakville, Ontario). A parafilm coverslip was applied and the positive control slides were incubated at 37°C for 10 minutes in a humidifying chamber and then rinsed 4 times in deionized water for 1 minute each. All slides were quenched with 3% hydrogen peroxide in methanol for 10 minutes at room temperature and then washed in running tap water for 5 minutes and then rinsed in deionized water for 1-2 minutes. Tissue sections were covered with the provided equilibration buffer for 30 minutes in a humidifying chamber at room temperature. They were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 hour in a

humidifying chamber. Negative control slides were generated via omission of TdT enzyme with and without DNAse 1. Sections were then placed into coplin jars containing 1:34 mix of the provided Stop/Wash buffer and deionized water, they were agitated then incubated for 10 minutes. Slides were then washed in PBS 3 times and then incubated with rhodamine-conjugated antidigoxigenin antibody for 30 minutes in a humidifying chamber at room temperature. Excess antibody was removed by four 2-minute washes in PBS. A peroxidase substrate (Cardassian Diaminobenzidine (DAB), Biocare Medical; Concord, California) was prepared using 1 mL of the provided DAB substrate, and 1 drop of the DAB enchancer and DAB chromogen for every 5 slides. The substrate was applied, one slide at a time, for exactly 2 minutes before being rinsed in running tap water for 5 minutes and then placed in deionized water. Tissues were counterstained in 50% Harris modified Haematoxylin (Fisher Scientific) for 5 seconds, rinsed in running tap water, dehydrated as described in H&E, and then coverslipped using Permount (Fisher Scientific) and stored at 4°C.

Analyses were carried out in coronal sections at the level of the mammillary bodies and at the level of the optic chiasm in a similar manner to that described for the necrosis analyses (Figure 4.1). Grey matter regions were again divided into layers 1-3 and layers 4-6 for analysis and thalamus was divided into medial and lateral thalamus, but results were combined for all grey matter and thalamic subregions when no significant differences were found. Images were captured using a Zeiss upright light microscope (Carl Zeiss Microimaging, Thornwood, NY) with a 40x objective. Identical illumination settings were used for all brain regions to allow for comparison between regions. For all experiments, a minimum of 6 randomly selected 40x HPFs per region were captured, with the goal being 4 HPFs per region per side of the brain. Apoptotic-

appearing cells were identified as all cells with 3,3'-diaminobenzidine-stained cytoplasm (Figure 4.3). Thresholds were set to count positively stained cells in the positive control slides, while at the same time ensuring no signal was scored as positive within the negative control slides. Image analysis software used was Image Pro Premier 9.2 software (Meyer Instruments), the software was calibrated for magnification.

4.2.4 Markers of ER stress and Apoptosis via Western Blot

Fast frozen caudal sections of the brain were put on dry ice and a cortical portion was removed and homogenized in RIPA buffer (50mM Tris-HCl, pH 7.4, 150 mM NaCL, 1mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$), supplemented with phosphatase inhibitors (20 mM NaF, 40 mM Na-pyrophosphate, 40mM Na_3Vo4_4, 200 mM β-glycerophosphate disodium salt hydrate), and a protease inhibitor cocktail (Roche). The solution was sonicated at 30% amplitude for 5 seconds total, 1 second per pulse. It was then mixed in a rotator for 10 minutes at 4°C and centrifuged at 300g for 15 minutes at 4°C. The supernatant was collected and centrifuged at 16000g for 20 minutes at 4°C. The supernatant was collected as the total cellular protein extract and quantified by colorimetric DC protein assay (BioRad).

Loading samples were prepared with fresh total cellular protein extract (avoiding repeated freeze-thaw cycles), NuPAGE LDS Sample Buffer (4X) (Invitrogen), NuPAGE Reducing Agent (10X) (Invitrogen), and deionized water. Loading samples were heated at 70°C for 10 minutes to denature the proteins. Proteins (20 ug/well) were separated by size via electrophoresis at a voltage of 180V in gradient

polyacrylamide gels (Invitrogen Life Technologies Inc.). AGA-control and FGR-MNR male samples were loaded and run together on one gel, while AGA-control and FGR-MNR female samples were run together on a separate gel. Each gel included a positive control consisting of a rat placenta that had shown reactivity in a previous study using the same antibodies (Wong, Nicholson, Holloway, & Hardy, 2015). Electrophoresis was followed by a transfer onto polyvinylidene difluoride membrane (Millipore) at 100V for two hours. Protein transfer was confirmed and visualized with Amido Black (Sigma-Aldrich). Membranes were blocked in 1X Tris-buffered saline-Tween 20 (TBST) buffer with 5% non-fat milk (blocking solution) for two hours at room temperature, and then probed using primary antibodies of the protein targets of interest, all diluted in the blocking solution (Table 4.1), overnight at 4°C. Secondary antibodies were used to detect the species-specific portion of the primary antibody, all diluted in the blocking solution (Table 4.2).

Immuno-reactive bands were visualized using a Luminata Forte Western HRP enhanced chemiluminescence detection system (Thermo Scientific) and imaged using VersaDoc Imaging System (BioRad Laboratories). Protein bands underwent densitometry analysis using Image Lab 4.0 Software (Bio-Rad). β-actin is ubiquitously expressed, and so probing for it allowed for the normalization of blot densities to correct for loading variances.

4.2.5 Synaptic Development with Synaptophysin Immunohistochemistry

SYN immunoreactivity (IR) was assessed using ImmPRESS Polymerized Reporter Enzyme Staining System (Vector Laboratories). To reduce variability, all

immunohistochemistry for SYN was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinized as described in H&E, then endogenous peroxidase was quenched by a 10-minute bath in 3% hydrogen peroxide. Slides were rinsed with tap water for 5 minutes and then placed in a humidity chamber and rinsed with PBS. Slides were then blocked for 30 minutes using ImmPRESS 2.5% Normal Horse Serum and then rinsed with PBS. Sections were then incubated with primary antibody (mouse anti-rat monoclonal SYN, 1:400, Sigma-Aldrich, Oakville Ontario) overnight at 4°C. Slides were then rinsed with PBS 3 times at room temperature. Sections were then incubated with secondary antibody (ImmPRESS (Peroxidase) Polymer Anti-Mouse IgG Reagent) for 40 minutes at room temperature. Slides were rinsed 3 times with PBS and then a peroxidase substrate DAB kit was used as described in ApopTag. Tissues were counterstained in Carazzi's Haematoxylin for 90 seconds, rinsed in running tap water, rinsed in deionized water, dehydrated as described in H&E and then coverslipped as described for ApopTag. Negative control slides were generated by the substitution of the primary antibody with pure diluent.

Analyses were carried out in coronal sections at the level of the mammillary bodies. Slides were coded prior to image analysis to ensure this was done blinded to the animal cohort. Brain regions analyzed included the parasagittal gray matter, thalamus, and hippocampus regions CA1, CA4, and dentate gyrus (Figure 4.1). Images were captured using a Zeiss upright light microscope (Carl Zeiss Microimaging, Thornwood, NY) with a 63x objective with immersion oil. For all experiments, a minimum of 6 randomly selected 40x HPFs per region were captured, with the goal being 4 HPFs per region per side of the brain. Figure 4.4 illustrates appropriate SYN IR in the hippocampal regions of the brain, as clustered, punctate staining throughout the

terminal end of the axon surrounding neurons, indicating the presence of synapses. Identical illumination and exposure settings were used for all SYN slides to allow for comparison between animals and regions. With each new slide, the field diaphragm was centered and re-focused to maximize Kohler illumination.

Percent area positive staining, as well as intensity of stain, were measured using ImagePro Premier 9.2 software (Meyer Instruments, Houston Texas). The software was calibrated for magnification. SYN IR was identified as DAB-stained cytoplasm. Thresholds were set using several images to count positively stained cells, while at the same time ensuring no signal was scored as positive within the negative control slides.

4.2.6 Data Acquisition and Statistical Analysis

Overall control and MNR population characteristics included data from all control sows and their liveborn fetuses and all MNR sows and their liveborn fetuses undergoing necropsy at 60/61 days gestation (Figure 4.5). Select AGA-control and FGR-MNR population characteristics included data from 18 (9 male and 9 female) control animals and 18 (9 male and 9 female) MNR animals who were liveborn, underwent full necropsy, and met the weight criteria noted, thus making them representative of their respective groups (Figure 4.6). Outliers were statistically identified using the Grubbs' test (Grubbs, 1969). Maternal and fetal characteristic findings are presented as group means ± SEM. Overall control and MNR population characteristics and select AGA-control and FGR-MNR population characteristics were compared using ANOVA and a non-paired student`s t-test which were also nested for litter size (Graphpad Software, San Diego, CA). Comparison for apoptosis and necrosis findings were done using a one-

way ANOVA followed by unpaired t tests. Comparison for SYN IR was performed using a non-paired student's t-test. Western Blots were evaluated using a non-paired student's t-test on the difference between normalized mean values. All statistical analyses were performed using GraphPad Prism 6 software. All results were expressed as means of normalized values ± SEM and for all analyses statistical significance was assumed at $p<0.05$ compared to the AGA-control cohort.

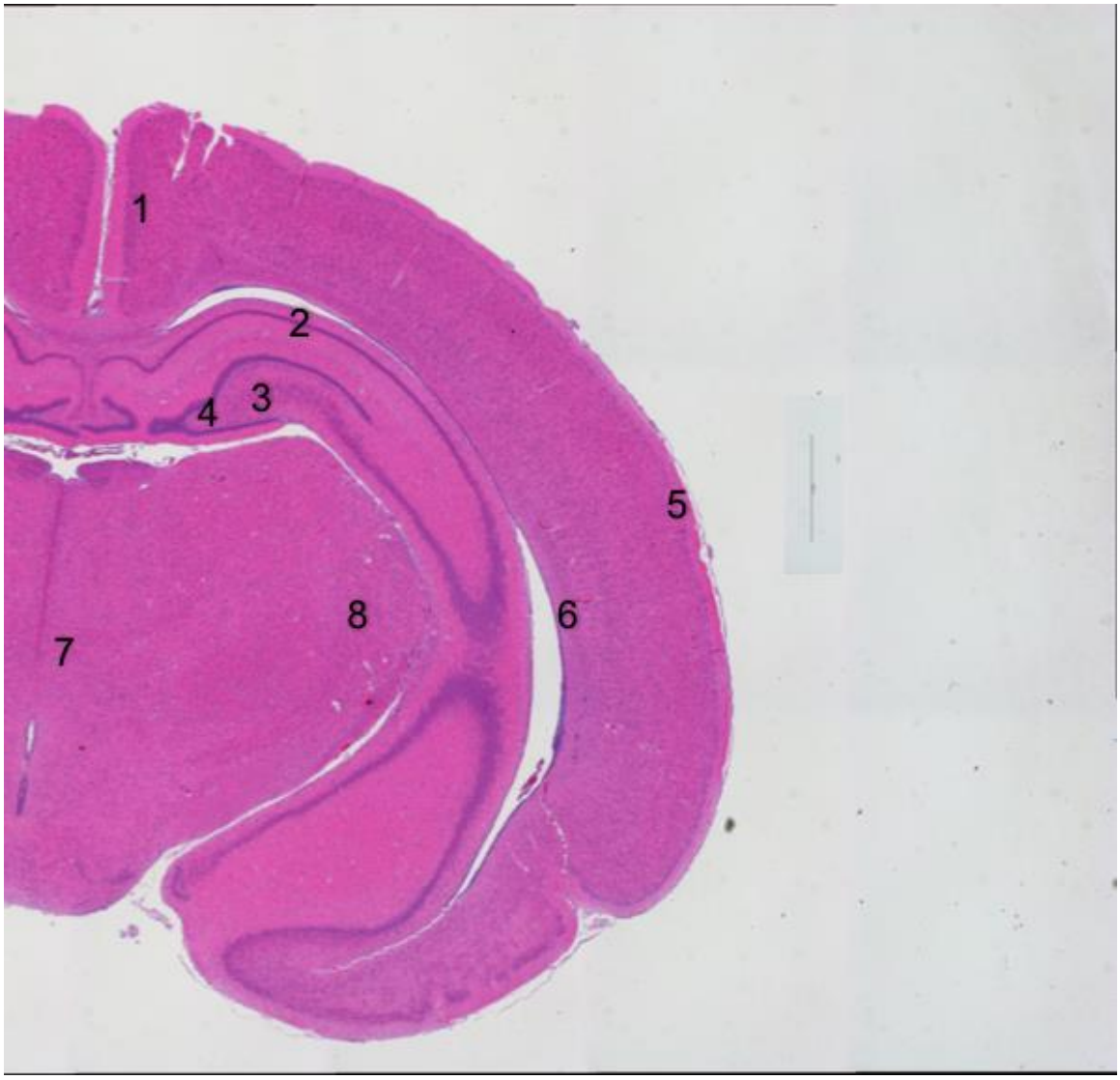

Figure 4.1 Gross picture of the fetal guinea pig brain microdissection. Areas of interest were labelled 1) parasagittal grey matter, 2) CA1, 3) CA4, 4) dentate gyrus, 5) convexity grey matter, 6) periventricular white matter, 7) medial thalamus, and 8) lateral thalamus. Scale bar is 1,000 μm

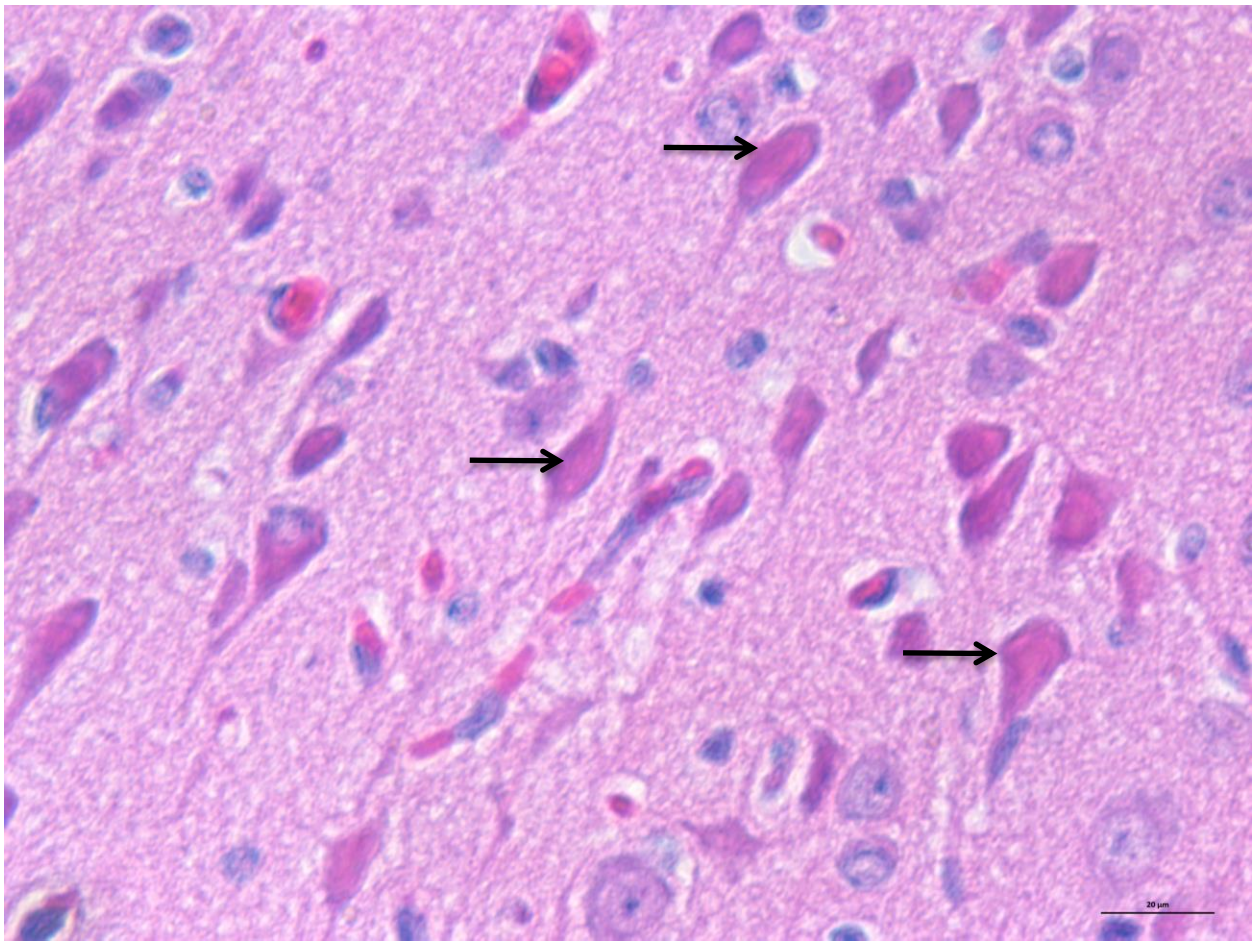

Figure 4.2 High power section from FGR-MNR fetus of necrotic-appearing cells in the parasagittal grey matter displaying eosinophilic cytoplasm with concave and elongated cell bodies and a loss of nuclear detail indicated by arrow (Image at 40x magnification, scale bar is 20μm).

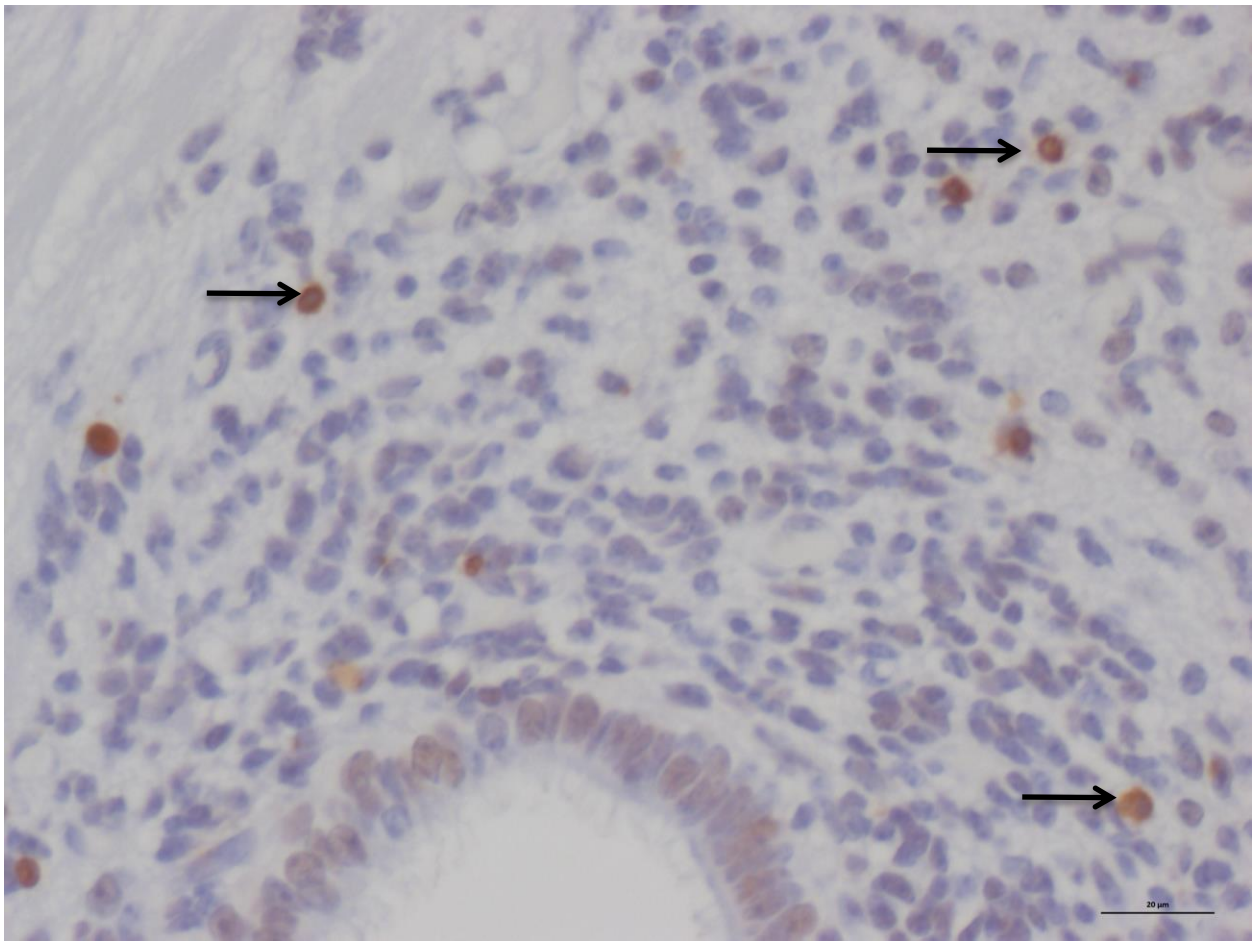

Figure 4.3 High power section from FGR-MNR fetus of a TUNEL positive cell in the periventricular white matter. Identified as dark brown stained nuclei indicated by arrow (Image at 40x magnification, scale bar is 20μm).

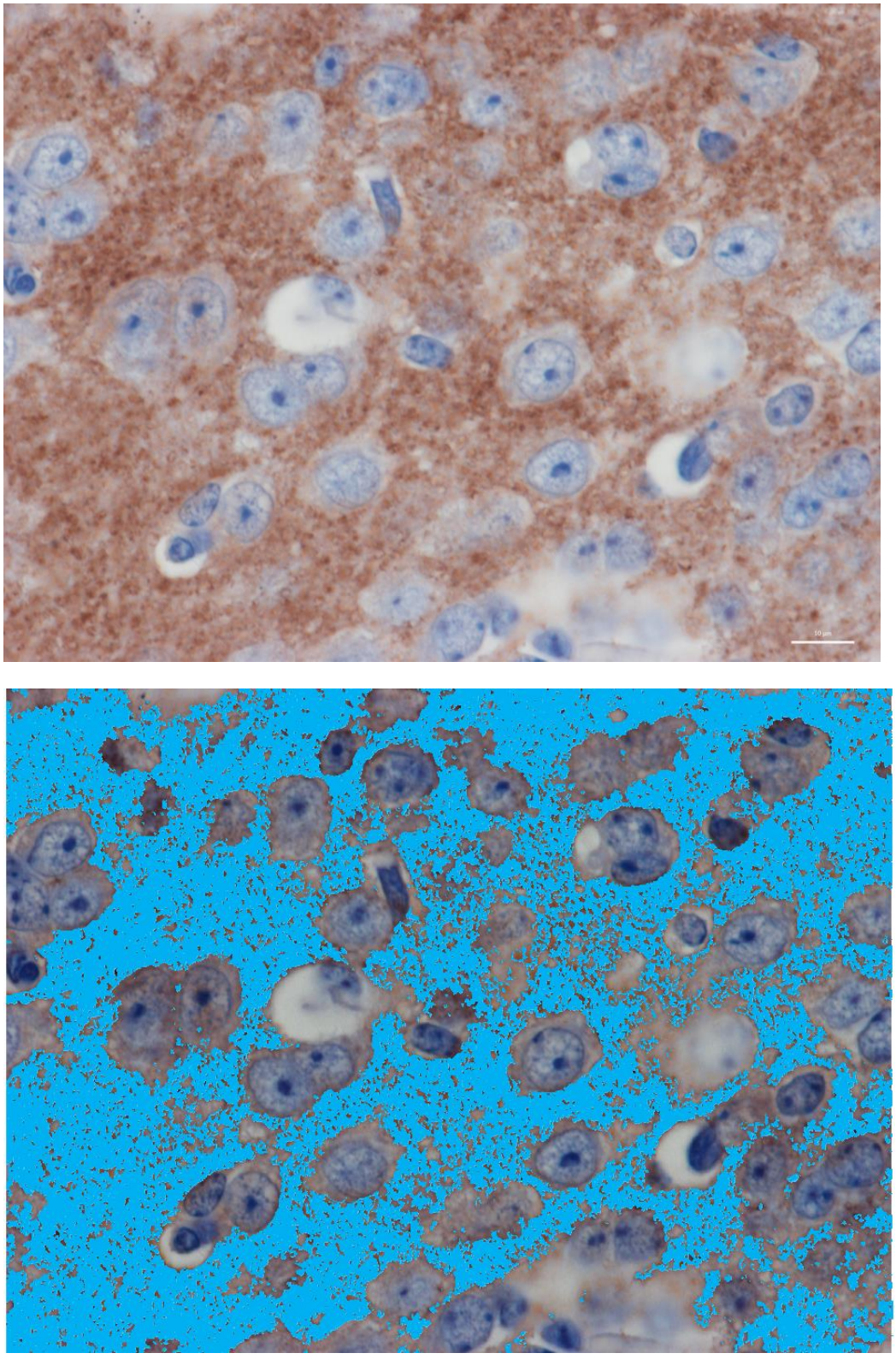

Figure 4.4 High power section from AGA-control fetus of SYN IR in the thalamus. Showing SYN IR punctate staining in the original HPF for analysis (top) and the threshold applied (blue) indicating positive stain (bottom) (image at 63x magnification, scale bar is 10μm).

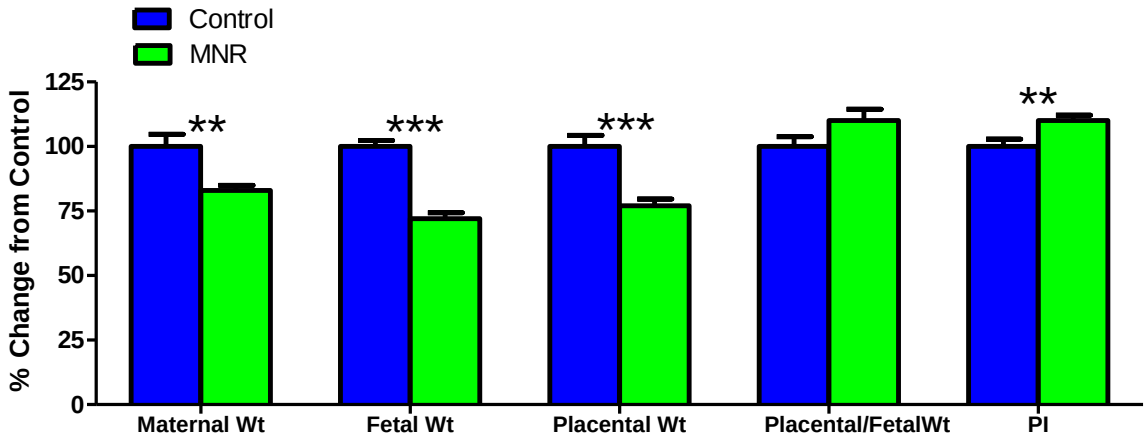

Figure 4.5 Growth measures for all control (9 sows/31 fetuses) and MNR (12 sows/42 fetuses) animals. Data presented as mean % change from control values with SEM; **p<0.01, ***p<0.001

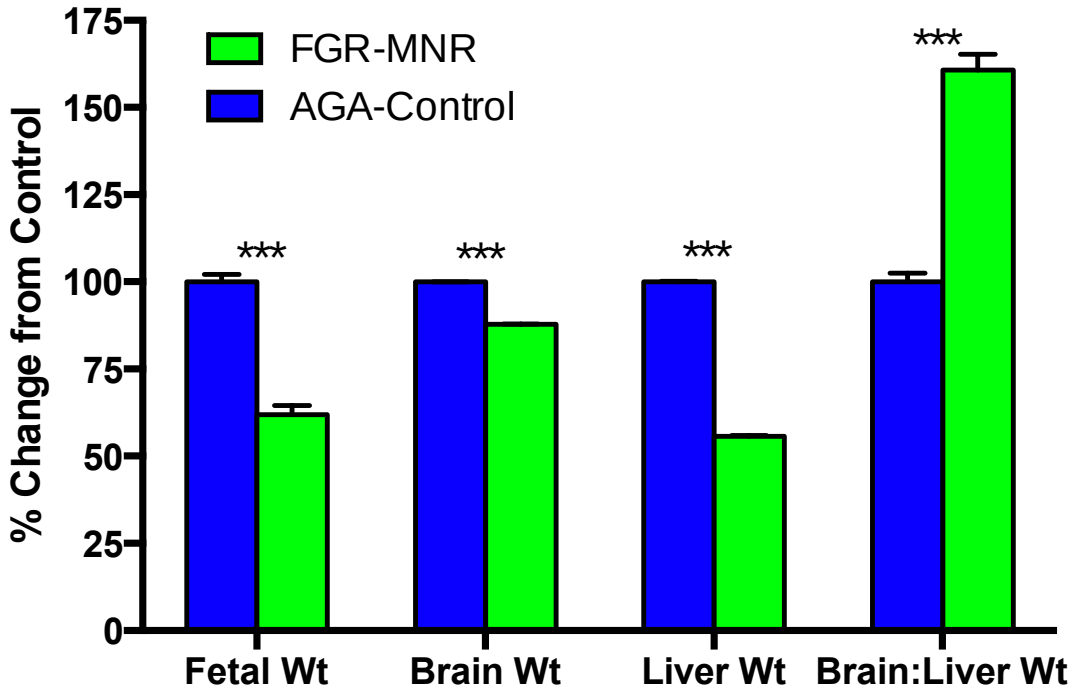

Figure 4.6 Growth measures from select AGA-control (18 fetuses) and FGR-MNR (18 fetuses) animals undergoing full necropsy. Data presented as % change from control values with SEM; ***p<0.001

Table 4.1 Western Blot primary antibodies, dilutions used in experiments and company and catalogue information.

Antibody name	Source	Dilution	Company (#Catalogue)
Grp 78	Mouse monoclonal	1:300	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-58774)
Bax	Rabbit monoclonal	1:2000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-493)
PARP1	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9542)
Capasase-3	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9665)
β-Actin	Mouse monoclonal	1:50000	Sigma-Aldrich Co., St. Louis, MO, USA (#A3854)

Table 4.2 Western Blot secondary antibodies, dilutions used in experiments and company and catalogue information.

Antibody name	Dilution	Company (#Catalogue)
Donkey Anti-Rabbit IgG (H+L)	1:10000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-011-033)
Donkey Anti-Mouse IgG (H+L)	1:5000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#715-011-033)

4.3 RESULTS

4.3.1 Fetal Population Characteristics

As stated in Chapter 3, nine control animals and 12 MNR animals had continuing pregnancies out to necropsy at 60/61 days gestation with 31 and 42 liveborn fetuses respectively, which formed the overall control and MNR populations. The fetal growth characteristics from these animals are shown in Table 4.3 and have previously been reported in Chapter 3. In summary, fetal weights were ~28% lower in the MNR pregnancies than in the control pregnancies ($p<0.001$). Placental weights were also ~23% lower in MNR pregnancies ($p<0.001$), resulting in placental/fetal weight ratios being increased by ~12 % (p0.05). Fetal crown rump lengths were also decreased in the MNR pregnancies by ~15% ($p<0.01$), but again less than the decrease in fetal weights such that the fetal weight/length as a measure of leanness was also decreased by ~15% ($p<0.001$).

As noted, 80 g was used as the threshold for categorizing AGA and FGR fetal weights and determining which control and MNR fetuses were to be subjected to full necropsy. This resulted in 20 AGA-control fetuses and 25 FGR-MNR fetuses, which have also been outlined in the results section of Chapter 3. From each of these groups 9 male and 9 female AGA-control and FGR-MNR fetuses were selected for full necropsy and further analysis. These fetuses were representative of the mean fetal weights from these select cohort groups with their population characteristics shown in Table 4.4. Changes in these population characteristics in the FGR-MNR fetuses from that of the AGA-control fetuses were similar for both males and females with no sex differences noted. In summary, FGR-MNR fetal weights were decreased by ~37% compared to that of AGA-

control fetuses ($p<0.001$). FGR-MNR brain weights were also decreased, but only by ~12% ($p<0.001$) while FGR-MNR liver weights were markedly deceased by ~40% ($p<0.001$). Consequently, the brain/liver weight ratio as a measure of asymmetrical growth was increased almost 50% in the FGR-MNR fetuses compared to that of the AGA-control fetuses ($p<0.001$).

4.3.2 Quantification of Necrotic-Appearing Cells

A total of 36 late-gestation fetal brains were examined by means of H&E staining for quantification of necrotic-appearing cells. As previously described, characteristic morphologic features were used to distinguish necrotic-appearing cells (Figure 4.2). The mean percentage of necrotic-appearing cells per HPF for each examined brain region is shown in Figure 4.7. Overall, low levels of necrotic appearing cells were observed in studied brain regions, averaging 0.45/HPF and 0.59/HPF (40x) for AGA-control and FGR-MNR animals, respectively. Most values were not significantly different from zero and no differences were observed between AGA-control and FGR-MNR animals for any of the brain regions studied.

4.3.3 Quantification of Apoptotic-Appearing Cells

A total of 36 late-gestation fetal brains were examined for the appearance of apoptotic-appearing cells by means of TUNEL assay. The mean count of apoptotic-appearing cells per HPF for each examined brain region is shown in Figure 4.8. While low levels of apoptotic appearing-cells were observed, FGR-MNR levels were higher in the periventricular white matter at 3.83 ± 0.66 cells/HPF (2x) ($p<0.05$), the hippocampus CA1 at 0.26 ± 0.06 cells/HPF (3x) ($p<0.05$), CA4 at 1.19 ± 0.15 cells/HPF

(1.7x) ($p<0.05$) and dentate gyrus at 0.34 ± 0.10 cells/HPF(3x) ($p<0.05$) compared to their respective AGA-control levels. Additionally, apoptotic indices were significantly increased in males compared to females with AGA-control levels being higher in all areas but of particular note the periventricular white matter at 3.09 ± 0.55 cells/HPF (3.7x) ($p<0.05$), the hippocampus CA1 at 0.17 ± 0.05 cells/HPF (8.5x) ($p<0.01$) and CA4 at 1.05 ± 0.22 cells/HPF (2x) ($p<0.01$) and dentate gyrus at 0.17 ± 0.05 cells/HPF (2.8x) ($p<0.01$). The same trend held in FGR-MNR animals with apoptotic indices being significantly increased in males compared to females in all areas, but of note being the periventricular white matter at 5.11 ± 0.91 cells/HPF (1.9x) ($p<0.05$), the hippocampus CA1 at 0.45 ± 0.08 cells/HPF (4x) ($p<0.01$) and CA4 at 1.55 ± 0.17 cells/HPF (1.7x) ($p<0.01$) (Figure 4.9).

4.3.4 Western Blot Analysis

A total of 15 male and 15 female late-gestational fetal brains were examined for each Western Blot that was performed for measurement of markers of ER stress and apoptosis. Grp78 was significantly increased in the female FGR-MNR cohort with a 224 ± 31% increase relative to female AGA-control animals. Otherwise there were no significant changes in male Grp78, male Bax, female Bax, male PARP1, female PARP1, or female cleaved caspase-3 content between groups (Figure 4.10).

4.3.5 Quantification of SYN IR

A total of 36 late-gestation fetal brains were examined for SYN-IR using the ImmPRESS Polymerized Reporter Enzyme Staining System (Vector Laboratories). As previously described, SYN IR was observed as clustered, punctate staining throughout

the terminal end of the axon surrounding neurons, indicating the presence of synapses (Figure 4.4). The mean percent area stained per HPF for each examined brain region is shown in Figure 4.11. Results showed no significant differences between AGA-control and FGR-MNR groups and no significant differences between male and female AGA-controls or male and female FGR-MNR cohorts.

Table 4.3 Overall Fetal Population Characteristics

	Ad Libitum Control	Maternal Nutrient Restricted
Fetal weight (gms)	96±2	69±2***
Placental weight (gms)	6.6±0.3	5.1±0.2**
Placental/fetal weight ratio	0.068±0.003	0.076±0.003*
Crown rump length (cm)	12.3±0.2	10.6±0.1***
Fetal weight/length (gm/cm)	7.8±0.1	6.4±0.1***

Data presented as means ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control group value; n values were 31 and 42 for fetal control and MNR characteristics respectively.

Table 4.4 Select Fetal Population Characteristics

	Male		Female	
	AGA-Control	FGR-MNR	AGA-Control	FGR-MNR
Fetal weight (gms)	100±3	63±3***	101±3	61±4***
Brain weight (gms)	2.72±0.08	2.36±0.07*	2.75±0.07	2.45±.09*
Liver weight (gms)	4.7±0.4	2.7±0.2***	4.7±0.2	2.6±0.3**
Brain/liver weight ratio	0.58±0.05	0.87±0.05**	0.58±0.02	.095±0.08**

Data presented as means ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control AGA-control group value; n values were 18 and 18 for fetal AGA-control and FGR-MNR characteristics, respectively.

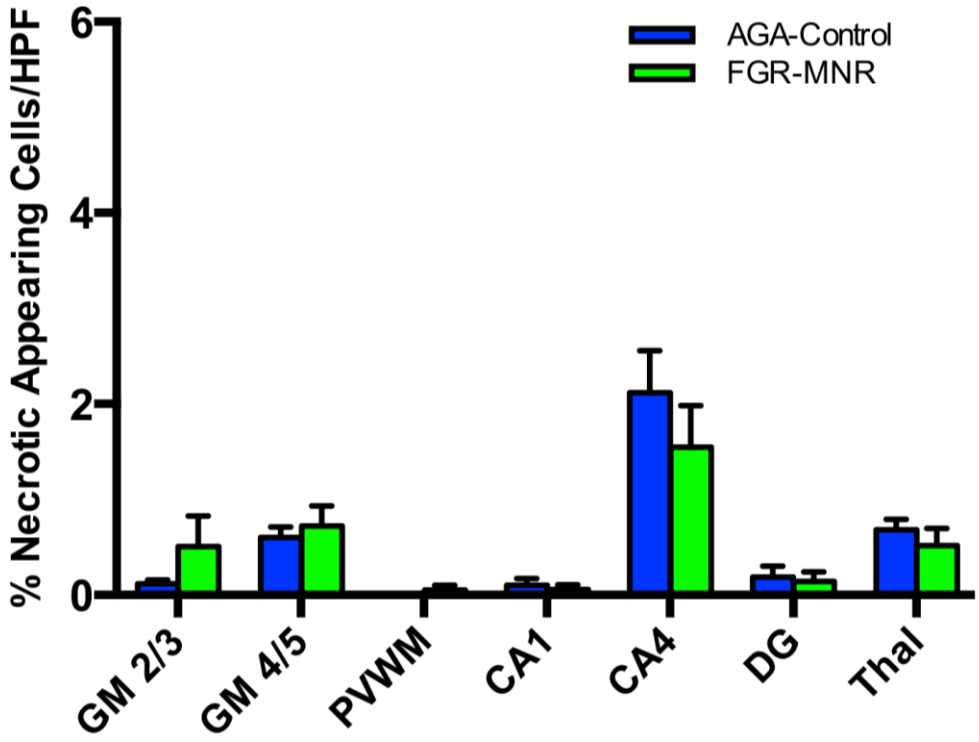

Figure 4.7 Bar chart of necrotic-appearing cells as assessed by morphologic change in H&E-stained sections for the brain regions shown of AGA-control (n=18) and FGR-MNR (n=18) fetuses. Data presented as mean % necrotic-appearing cells/HPF ± SEM.

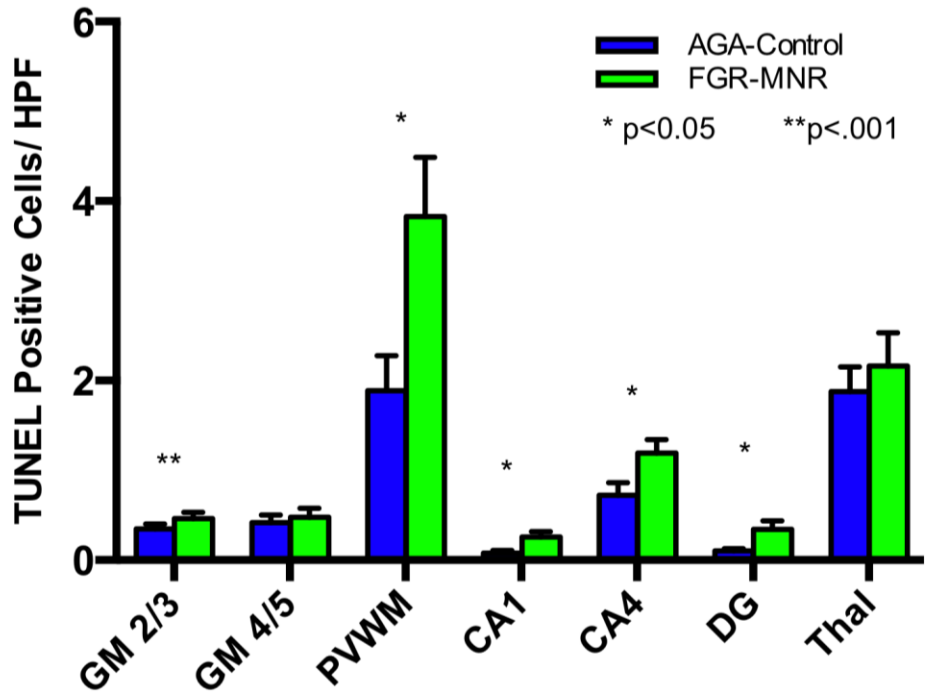

Figure 4.8 Bar chart of TUNEL positive cells as assessed by Apoptag® Peroxidase In Situ Appoptosis Detection kit (Millipore, Billerica, Massachusetts) for the brain regions shown of AGA-control (n=18) and FGR-MNR (n=18) fetuses. Data presented as mean TUNEL positive cells/HPF ± SEM.

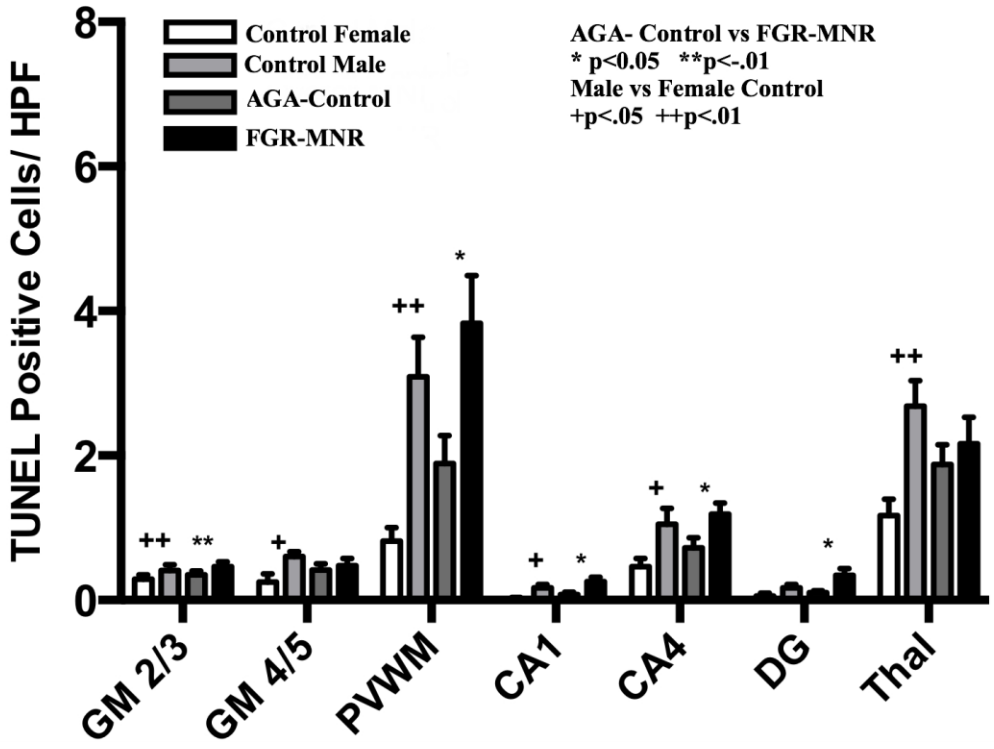

Figure 4.9 Bar chart of TUNEL positive cells as assessed by Apoptag® Peroxidase In Situ Appoptosis Detection kit (Millipore, Billerica, Massachusetts) for the brain regions shown of AGA-control (n=18) and FGR-MNR (n=18) fetuses as well as AGA-control male (n=9) and AGA-control female (n=9) fetuses. Data presented as mean TUNEL positive cells/HPF ± SEM.

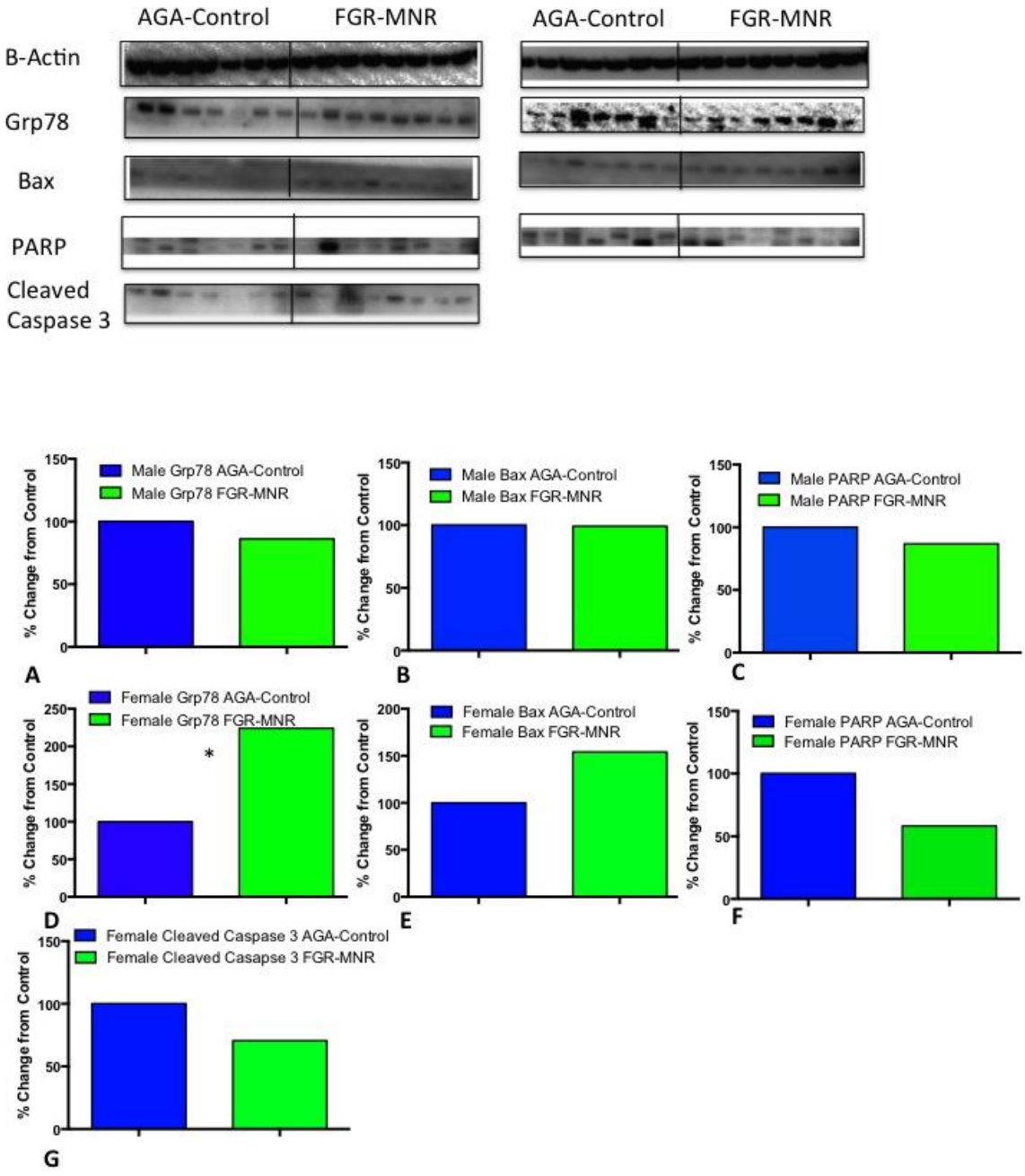

Figure 4.10 Representative immunoblots (top) of Grp78 (78kDa), Bax (23kDa), PARP1 (89kDa), cleaved caspase-3 (17,19kDa) and β-Actin (42kDa) from male and female AGA-control and FGR-MNR animals. Density of immunoblots (bottom) normalized to β-Actin and presented as the mean fold change ± SEM for the male and female FGR-MNR fetuses (green bars, n=8) from that of the male and female AGA-control fetuses (blue bars, n=7). *p<0.05.

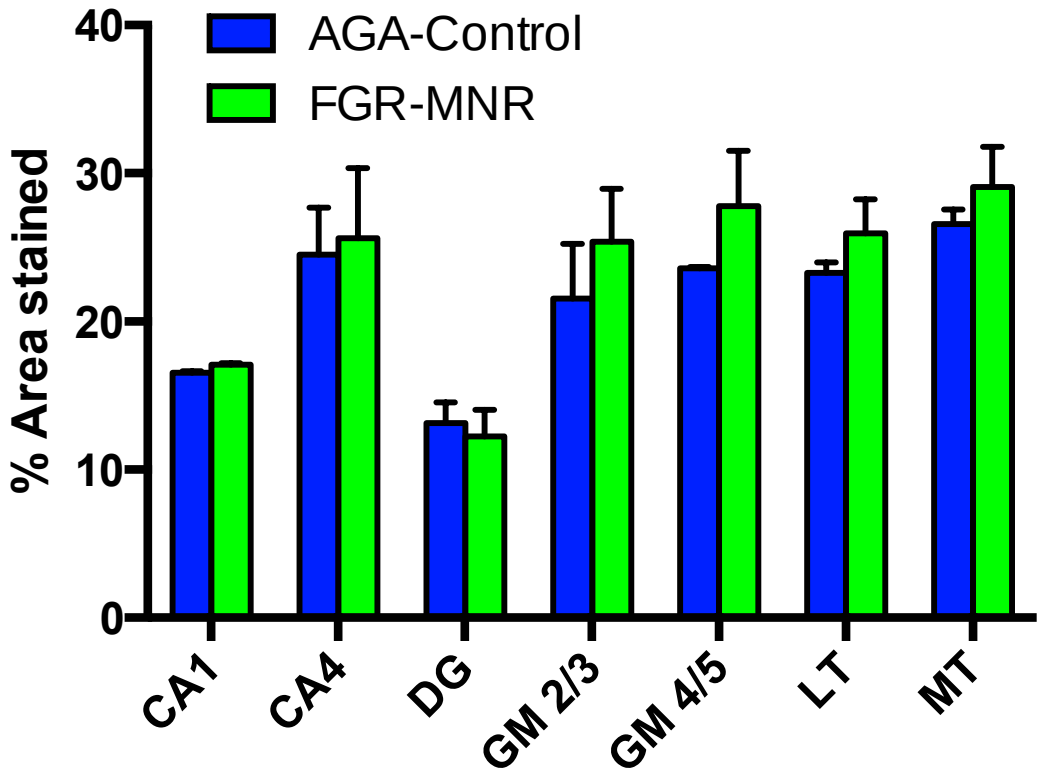

Figure 4.11 Bar chart of % area SYN-IR per HPF for the brain regions shown of AGA-control (n=18) and FGR-MNR (n=18). Data presented as mean % area stained/HPF ± SEM.

4.4 DISCUSSION

In the present study, we have examined markers of necrosis, apoptosis, ER stress, and synaptogenesis in fetal guinea pigs subjected to moderate MNR before and continuing throughout pregnancy as a model for inducing FGR with similarities to that seen in humans with maternal undernourishment and idiopathic placental insufficiency. As discussed in Chapter 3, and in other studies (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998), moderate MNR at 70% of the control ad-libitum diet beginning 4 weeks pre-pregnancy and increasing to 90% of the control ad-libitum diet at mid-gestation, results in a decrease in fetal weights by 30-40% on average for all MNR pregnancies necropsied near term. Increased placental/fetal weight ratios are observed in these pregnancies, which parallels what is seen in human FGR pregnancies with maternal undernourishment and placental insufficiency, and likely indicates a degree of adaptive compensatory growth by the placenta in an effort to minimize FGR (Abrams & Newman, 1991; Kramer, 1987). These placental changes make it likely that the FGR-MNR fetuses are likely to be hypoxic and therefore at risk of increased oxidative and ER stress, a possible mechanism for altered growth and development. These fetuses also have a decrease in brain weights and markedly decreased liver weights, giving rise to an increased brain/liver weight ratio which is characteristic of asymmetrical fetal growth restriction (aFGR) (Lackman, Capewell, Richardson, et al., 2001; Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009). Of note, aFGR is also seen in humans with placental insufficiency leading to growth restriction (W. Cox et al., 1988; Economides & Nicolaides, 1989; Soothill et al., 1987).

Development of the fetal brain requires the completion of several complex processes in order to develop in a complete and healthy manner. Adverse intrauterine

conditions may lead to prolonged episodes of lowered nutrients, including oxygen, and thus generate an unsuitable environment for the completion of necessary growth and development processes (Hochachka et al., 1996); this may result in a variety of consequences ranging from threshold effects on brain growth, to altered synapse formation and aberrant neuronal communication, all the way to alterations leading to cellular death in severe cases (Astrup, 1982). This is particularly true in perinatal brain developers such as humans, and guinea pigs as prenatal brain developers, as the most rapid phases of brain growth and development occur in the latter half of pregnancy (McIntosh et al., 1979), in these cases insults are occurring during critical time points in development and are significantly increasing the risk of potential derangements. The processes involved in brain growth and development are very dynamic with a very high energy demand. As such, FGR with oxygen and nutrient impairment may disrupt processes such as growth and synapse formation as energy demanding processes (Jiang & Schuman, 2002). Cellular death can occur in a physiological manner and likely also plays a central role during the fetal period of brain development that coincides with neuronal differentiation and synaptogenesis (Blaschke et al., 1996). Additionally, in cases of chronic hypoxia, reduced amino acid levels, and/or oxidative stress, it is possible to see an accumulation of misfolded or unfolded proteins in the ER, which if prolonged, can lead to the initiation of pro-apoptotic cascades (Koumenis et al., 2002; Marciniak & Ron, 2006; Szegezdi et al., 2006).

The most pertinent finding in this study was the increase in TUNEL positive staining in the FGR-MNR cohort when compared to the AGA-control cohort and more so in males than females, although this was also evident in the AGA-control group. This finding likely points to a sexual dimorphism in the mechanism and degree of apoptotic

injury, a finding that is consistent with previous studies of apoptosis in neonatal cerebral ischemia (Renolleau et al., 2008). The study by Renolleau et al. suggested that males and females undergo apoptosis at different levels and via different mechanisms. It is suggested that males may be more vulnerable to oxidative stress leading to production of reactive oxygen species in the mitochondria, large mitochondrial permeability with a subsequent release of pro-apoptotic proteins, such as AIF, leading to activation of PARP1 and subsequent translocation of mitochondrial AIF into the nucleus for DNA cleavage, resulting in a caspase-independent cell death (Renolleau et al., 2008). Females however, have an estrogen microenvironment which upregulates levels of anti-apoptotic Bcl-2 and may in part preserve the stability of the mitochondrial membrane and reduce the release of pro-apoptotic proteins; however in situations where cellular death pathways are sufficiently activated, females undergo a caspase dependent cell death involving the release of cytochrome c and activation of caspase-3 (Renolleau et al., 2008). This suggests there is a sexual dimorphism in apoptotic pathways and may implicate oxidative stress as a possible method by which damage is occurring in the fetal brain in the case of MNR induced FGR. The changes in apoptosis that were observed in our study were not accompanied by significant changes in the protein levels of pro-apoptotic factors such as Bax, PARP1, and cleaved casapse-3. This points to activity dependent changes in other apoptotic factors, such as AIF to explain the increases in apoptosis that are observed here.

In general, this study investigated sub-areas of the hippocampus due to its role in memory and learning. In normal development, neurons of the dentate gyrus and areas CA1 and CA4 synapse with each other in the formation of a functional hippocampus (Berger, Song, Chan, & Marmarelis, 2010). Hippocampal neurons of the

CA1 are thought to be more sensitive to hypoxic or ischemic insults (Bickler & Buck, 1998; Maiti, Singh, Muthuraju, Veleri, & Ilavazhagan, 2007). Considering that MNR-FGR fetuses have previously been found to be hypoxic (Elias et al., 2013), our findings would be consistent with these studies as these more susceptible regions did have increased evidence of apoptosis

Processes with high energy demand have been found to be highly sensitive as they are the most likely to be shut down in order to conserve energy necessary for cellular integrity and survival (Hochachka et al., 1996; Jiang & Schuman, 2002; Zhao & Flavin, 2000). On the basis of this and previous studies in FGR guinea pigs (Piorkowska et al., 2014), we hypothesized that MNR leading to FGR would have threshold effects on brain development processes, such as synaptogenesis, and thus we would see differences in SYN IR as a marker of the total number of synapses. Therefore, our results were unexpected. The dissimilarity in our results compared to previous studies may indicate a difference in the result of the long-term chronic insult that manifests with MNR compared to the more abrupt, acute insult in models such as uterine artery ligation/ablation. The chronic nature of nutrient restriction begins to impact the animal very early on in gestation and thus may create a larger window of opportunity for adaptations in the fetus and/or placenta. These adaptations may include mechanisms such as antioxidant release and result in better outcomes compared to animal models undergoing a one-time invasive procedure at mid-gestation. Additionally, studies of cerebral ischemia have demonstrated a reproducible sequence of changes, with an upper ischemic flow threshold of synaptic transmission failure but the maintenance of energy levels and a lower ischemic flow threshold of membrane failure with the development of structural cell damage (Astrup, 1982). With this study in mind, it is

possible that animal models of uterine artery ligation/ablation are below the upper ischemic flow threshold and above the lower ischemic flow threshold, whereas the MNR model is above both the upper and lower ischemic flow threshold, and therefore does not yield synaptic transmission failure or large amounts of structural cell damage.

In summary, there were overall low levels of necrosis and apoptosis, but with significant increases in apoptosis in FGR-MNR animals compared to AGA-controls, as well as in males compared to females. The lack of necrotic changes indicates that the threshold for membrane failure with energy depletion has likely not been reached; these findings paired with the increases in apoptosis are consistent with previous studies which state that milder insults are more likely to impact immature neurons and result in apoptotic death, while more severe insults are likely to affect terminally differentiated neurons and result in death by necrosis (Scott & Hegyi, 1997; Yue et al., 1997). The changes in apoptosis were however, not accompanied by any observable changes in protein markers of apoptosis or ER stress, which may indicate that other upstream pathways are responsible for the resultant apoptosis that is associated with MNR induced FGR. There was, unexpectedly, no changes in markers of synaptic numbers. Due to the constant state of nutrient restriction, it is likely that an adaptive response occurred in these animals at an earlier time point in gestation and therefore, by the time of the near-term necropsy, these FGR-MNR fetuses had better outcomes compared to those observed in previous studies with uterine artery ligation/ablation.

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CHAPTER 5
GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

It is now recognized that the condition of the intrauterine environment that supports the development of a fetus is a key determinant to the long term neurological health and cognitive capacity of that fetus. Several studies have demonstrated a link between low birth weight, suggestive of a sub-optimal intrauterine environment, and long-term adverse outcomes, including cardiovascular conditions, metabolic disabilities, cognitive deficits and neurological disorders. Conditions associated with fetal growth restriction (FGR) include common disorders such as cerebral palsy, attention deficit hyperactivity disorder, schizophrenia, epilepsy, and psychiatric hospitalization (M. Cannon et al., 2002; Halliday, 2009; Indredavik et al., 2010; Rodrigues et al., 2006; S. P. Walker et al., 2007b). The severity of the associated deficits and diseases correlates to the degree of FGR, often there is no recovery (Geva et al., 2008; Indredavik et al., 2010; Isaacs et al., 2000; Synnes et al., 2010). As such, altered development of the brain during critical periods may result in permanent impairments from which there is currently no course for recovery. Although many factors contribute to the incidence of FGR, improper placental growth and therefore impaired nutritional transport to the fetus, plays a major role in many human cases of FGR. As such, this study was designed to further characterize how a model of maternal nutrient restriction (MNR) may act as a representative model of human FGR, and to investigate alterations in the growth and development of the brain that arise from FGR.

Previous studies have utilized moderate MNR in guinea pigs to model the maternal, placental and fetal growth characteristics of human FGR (Kind et al., 2003, 2005; C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; Sohlstrom et al., 1998). This

study has further characterized the MNR guinea pig model via the addition of breeding success and pregnancy outcomes as outlined in Chapter 3. This study also demonstrated the utility of the MNR model to induce FGR, resulting in dramatically lower rates of fetal loss compared to those seen in uterine artery ligation/ablation models (Lafeber HN, Rolph TP, 1984; a. J. Turner & Trudinger, 2009), however an increase in preterm delivery was reported, as is seen in human cases of FGR (Lackman, Capewell, Richardson, et al., 2001). This study has confirmed the findings of previous studies, which have stated that MNR guinea pig fetuses have increased placental/fetal weight ratios; a finding that is often observed in human cases of FGR (Belkacemi et al., 2010; K. Godfrey & Robinson, 1998; Kramer et al., 1990; Lackman, Capewell, Gagnon, et al., 2001; Lumey, 1998). This study provided validation for the use of a fetal weight threshold of 80 g for categorizing average for gestational age (AGA)-control and FGR-MNR cohorts, with 80 g being representative for the 10^{th} percentile in the control animals. A key finding within the FGR-MNR cohort, is that the fetuses displayed asymmetrical fetal growth restriction (aFGR) and were polycythemic and hypoglycemic, all of which are characteristic of moderate growth restriction during human pregnancy (Abrams & Newman, 1991; W. Cox et al., 1988; Economides & Nicolaides, 1989; Kramer, 1987; Kramer et al., 1990; Soothill et al., 1987).

Moderate MNR alters the vasculature and structure of the placenta (C. T. Roberts, Sohlstrom, Kind, Earl, et al., 2001; C. T. Roberts, Sohlstrom, Kind, Grant, et al., 2001) and leads to reductions in blood and nutrient flow from maternal to fetal tissues, mimicking the human condition of placental insufficiency that often occurs in human FGR. This chronic insult has been shown to lead to a decrease in brain protein synthesis as a metabolic defense in hypoxia tolerant species, which was believed to have the

potential to impact brain development (Gagnon et al., 1997; Matthews, 2000).

The current study had the goal of investigating the consequences of MNR-induced FGR in the fetal brain and looking to gain a better understanding of the mechanisms at play. These fetuses have substantial "brain sparing", as their brain weights are reduced, but not nearly to the same degree as other organs such as the liver. There is also no increase in necrotic cell injury, which indicates that the threshold for membrane failure with energy depletion has likely not been reached. Apoptotic indices were found to be very low in both AGA-control and FGR-MNR cohorts but a statistically significant increase was observed in FGR-MNR animals compared to AGA-control animals, primarily in hippocampal regions This finding is consistent with previous studies of developing brain cells exposed to hypoxic/ischemic conditions; these studies state that milder insults are more likely to impact immature neurons and result in apoptotic death, while more severe insults are likely to affect terminally differentiated neurons and result in death by necrosis (Scott & Hegyi, 1997; Yue et al., 1997). Additionally, TUNEL-positive cells were observed to be significantly higher in males compared to females, and this was true for both AGA-control and FGR-MNR cohorts, which likely points to a sex difference in the mechanism and degree of apoptotic injury; which is consistent with findings from Renolleau et al. The study by Renolleau postulated a mechanism of apoptosis by which males were undergoing a caspase-independent cell death, via PARP1 initiating the translocation of apoptosis-inducing factor (AIF) to the nucleus for the induction of large scale DNA fragmentation (Daugas et al., 2000) and that this pathway was triggered by increases in oxidative stress. (Renolleau et al., 2008). These changes in apoptosis that were observed in our study were not accompanied by significant changes in the protein levels of pro-apoptotic

factors such as Bcl-2 associated X protein (Bax), poly ADP ribose polymerase 1 (PARP1), and cleaved casapse-3. This points to activity dependent changes in other apoptotic factors, such as AIF to explain the increases in apoptosis that are observed here.

Following investigations on necrosis and apoptosis, this study focused on determining if guinea pigs with MNR induced FGR exhibited changes in synaptogenesis. Synaptophysin (SYN) immunoreactivity was measured as a marker of the total number of synapses. No significant changes were observed in SYN immunoreactivity between AGA-control and FGR-MNR groups, which was unanticipated as a previous study measuring SYN immunoreactivity in FGR guinea pigs found significant decreases relative to fetuses of normal birth weight (Piorkowska et al., 2014). Synaptic formation and maturation are high-energy consuming processes and therefore the compromised supply of nutrients and oxygen observed in MNR induced FGR would be expected to result in reduced synapse formation and maturation. The maintenance of synaptic numbers possibly demonstrates the effectiveness of the brain-sparing response observed in aFGR fetuses in ensuring sufficient energy is preserved for the developmental processes occurring in the brain. Alternatively, it is possible that this may indicate a difference in the result of the long-term chronic insult observed in MNR compared to the abrupt/invasive nature of the insult in models such as uterine artery ligation/ablation. It is possible that the chronic nature of nutrient restriction begins to impact the animal early in gestation and as a result the placenta and/or fetus has a larger window of opportunity to adapt, with mechanisms such as increased release of antioxidants, and thus may yield better outcomes in comparison to animal models which involve a one-time invasive procedure performed at mid-gestation with less of an opportunity for adaptation and recovery. Additional studies of cerebral ischemia have

demonstrated a reproducible sequence of changes, with an upper ischemic flow threshold of synaptic transmission failure but the maintenance of energy levels and a lower ischemic flow threshold of membrane failure with the development of structural cell damage (Astrup, 1982). With this study in mind, it is possible that animal models of uterine artery ligation/ablation are below the upper ischemic flow threshold and above the lower ischemic flow threshold, whereas the MNR model is above both the upper and lower ischemic flow threshold, and therefore does not yield synaptic transmission failure or large amounts of structural cell damage.

This study as well as past studies of moderate MNR in guinea pigs support the utility of this model for the induction of FGR which parallels the condition of human FGR resulting from placental insufficiency or maternal undernourishment. This is the first study to date to investigate the impact of FGR in the brain of the MNR guinea pig model. This study has exhibited the merit of using a translatable chronic insult to induce growth restriction. The results provide insight into possible mechanisms by which MNR induced FGR is impacting the brain and also set the stage for future studies to further our understanding of the topic.

A major limitation of this study is that all investigations of the brain were carried out at a single time point in development. This raises the question of how certain markers, particularly apoptotic indices change over the course of development. Apoptosis is known have a physiological basis during fetal brain development that coincides with neuronal differentiation and synaptogenesis, therefore without measuring apoptosis across multiple time points we cannot know if the increase in apoptotic markers that we observe is a result of the MNR induced FGR, or an indicator

of a delayed developmental process. Additionally, the state of the caudal portion of the brain that was used for our Western Blots was less than ideal as the brains were damaged in the fast freezing process. This made it difficult to ensure which brain areas we were removing for our investigation and may have led to some of the high variability of our results here.

5.2 FUTURE STUDIES

Based on the current findings, future studies should focus on additional markers that may provide more insight in regards to the mechanisms by which neurological changes are occurring. Additionally, future studies should explore associated behavioural outcomes in the growth restricted MNR fetus. It would be worthwhile to further investigate markers of oxidative stress in the fetal guinea pig brain as they may elucidate more about mechanisms by which neurological changes are occurring. Examples of oxidative stress markers that can be investigated include the antioxidant glutathione, by-products of oxidative stress like malondialdehyde, and measures of DNA oxidative stress and cell damage like 8-hydroxyguanosine and 3-Nitrotyrosine.

Defining behavioural` changes in animal models can be done using various psychological tests that may suggest changes in learning, memory, cognition, and behavior. Tests such as the Morris water maze, T-arm maze, open field test, and forced swim test could provide information regarding the extent to which cognitive changes are occurring and the extent to which these changes can be associated with MNR and thus FGR. Correlating these behavioural changes with the severity of FGR resulting from MNR may provide useful information into the most beneficial forms of therapy,

depending on the duration, type, and severity of insult occurring during gestation.

Previous animal models of placental insufficiency, such as those using uterine artery ligation, have demonstrated neurological changes in the FGR fetus. Since this study has demonstrated that MNR leads to FGR, with an inclination towards aFGR it would be a valuable use of resources to further investigate additional markers of neurological changes and at multiple time points in the life of the animal. Initiation of myelination, axonal and dendritic growth and the proliferation of microglia and astrocytes are all examples of processes occurring during development that can be impacted if oxygen and nutrient supply are insufficient during critical period of development (Kind et al., 2005). A previous study, performed in a uterine artery ligation model, demonstrated reduced synaptophysin (SYN) immunoreactivity (IR) (Piorkowska et al., 2014). It would be worthwhile to test further markers from Piorkowska's study in the MNR model. Some of these markers include synaptopodin, which is found in dendritic spines of telencephalic neurons and is closely associated with the spine apparatus that plays a role in learning and memory (Deller, Merten, Roth, Mundel, & Frotscher, 2000; Deller, Mundel, & Frotscher, 2000; Mundel et al., 1997) and myelin basic protein, which comprises 35% of the protein in they myelin sheath, and is often used as a marker of myelination (Back et al., 2001). It would be useful to conduct studies of these markers in addition to those investigated in this study, and to look at them at the fetal stage, at the neonatal stage, and at the adult stage in order to investigate how MNR induced FGR affects these markers throughout life.

An additional area of future research that could be investigated once there is a better understanding of the type of injury and developmental change that occurs in the

brain as a result of MNR induced FGR, would be to determine the presence and extent of structural brain injury that could be detected using noninvasive approaches, such as magnetic resonance imaging (MRI) in the developing neonate. These measures could be used as a means for determining structural biomarkers for FGR adverse neurodevelopmental outcomes that could be correlated to microstructural changes in the brain found via accompanying histological analyses.

5.3 CONCLUSIONS

In conclusion, the focus of this thesis was on determining the relationship between MNR and the changes in fetal-placental growth, fetal-brain growth, neurodevelopment, and markers of cellular injury. The major findings of this study were:

1) MNR leads to aFGR fetuses with high brain-to-liver weight ratios.
2) Markers of necrosis do not change.
3) Markers of apoptosis are significantly increased in FGR-MNR cohort relative to the AGA-control cohort, however they do remain at generally low levels.
4) Markers of apoptosis are increased in males relative to females. This is true for both AGA-control and MNR-FGR cohorts.
5) Markers of synapse formation do not change.

These studies suggest that MNR induced FGR in fetal guinea pigs can have a significant role in future research. This model appropriately parallels the human condition as seen with placental insufficiency and under nutrition during pregnancy. These results suggest that there is a difference in the impact of MNR induced FGR on the brain

compared to more abrupt/invasive models such as uterine artery ligation with normal fetal growth then a sudden reduction in placental blood flow and a variable mismatch between metabolic needs and nutrient delivery. They also suggest that there may be differences in the incidence and mechanism of hypoxia-induced apoptosis in the brains of males and females.

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