6-28-2012 12:00 AM

Placental insufficiency resulting in fetal growth restriction alters synaptic development and neuronal myelination in guinea pigs at term

Karolina Piorkowska
The University of Western Ontario

Supervisor
Bryan Richardson
The University of Western Ontario

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

PLACENTAL INSUFFICIENCY RESULTING IN FETAL GROWTH RESTRICTION ALTERS SYNAPTIC DEVELOPMENT AND NEURONAL MYELINATION IN GUINEA PIGS AT TERM

(SPINE TITLE: FETAL GROWTH RESTRICTION AND GUINEA PIG BRAIN DEVELOPMENT)

(THESIS FORMAT: INTEGRATED-ARTICLE)

by

Karolina Piorkowska BMSc

Graduate Program in Physiology- Developmental Biology

Submitted in partial fulfillment

of the requirements for the degree

Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Bryan Richardson

Supervisory Committee

Dr. Marco Prado

Dr. Robert Hammond

Dr. John Ciriello

Examiners

Dr. David Lee

Dr. Stan Leung

Dr. Daniel Hardy

The thesis by

Karolina Piorkowska

entitled:

Placental Insufficiency resulting in Fetal Growth Restriction Alters Synaptic Development and Neuronal Myelination in Guinea Pigs at Term

is accepted in partial fulfillment of the
requirements for the degree of
Masters of Science

Date

Chair of the Thesis Examination Board

ABSTRACT

Aberrant neuronal connectivity *in utero* may underlie the association between fetal growth restriction (FGR) and increased risk for later cognitive disorders and encephalopathy. This study examines changes in synaptic development and myelination focussing on the hippocampus using a guinea pig model of placental insufficiency. Placental insufficiency was induced at mid-gestation by uterine artery ligation or cauterization which produced fetuses with a range of body weight and proportion at term. Synaptic markers, synaptophysin and synaptopodin, were decreased in FGR animals suggesting fewer synapses were formed and furthermore that fewer synapses matured with symmetrical growth restriction when compared to appropriate for gestational age guinea pigs. Myelination, measured using myelin basic protein and luxol fast blue, was also reduced in FGR animals and increased in large animals compared to appropriate size for gestational age guinea pigs. Therefore, neuronal connections altered with FGR and may spur difficulties in early childhood neurodevelopment and various neurological sequellae.

KEYWORDS

Fetal growth restriction, brain development, guinea pig, synaptogenesis, myelination, synaptophysin, synaptopodin, myelin basic protein, luxol fast blue, hippocampus

CO-AUTHORSHIP

The following people contributed to the manuscripts contained within this thesis in the following ways:

Dr. B Richardson:	Supervisor throughout all projects, provided grant funding to complete manuscripts, edited manuscript
J Thompson:	Performed animal surgery and necropsy, edited manuscripts
K Nygard:	Provided technical support for histology and image analysis, input into data analysis
B Matushewski:	Performed animal surgery and necropsy
Dr. M Prado:	Graduate Studies representative and member of advisory committee, provided support and advice throughout the project
Dr. J Ciriello:	Member of advisory committee, provided support and advice throughout the project
Dr. R Hammond:	Member of advisory committee, provided neuroanatomy and technical support for histology and image analysis, provided support and advice throughout the project

Don’t be afraid to take a big step if one is indicated. You can’t cross a chiasm in two small jumps

(…without extensive planning, creativity, and building materials -Karolina Piorkowska)

-David Lloyd George

To everyone who has supported me

TABLE OF CONTENTS

	Page
CERTIFICATE OF EXAMINATION	ii
ABSTRACT AND KEYWORDS	iii
CO-AUTHORSHIP	iv
EPIGRAPH	v
DEDICATION	vi
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS AND SYMBOLS	xiii
CHAPTER 1 - LITERATURE REVIEW	1
1.1 Clinical Relevance	2
1.1.1 Incidence and classification of FGR	2
1.1.2 Etiology of FGR	5
1.1.2.1 Placental insufficiency	6
1.1.2.2 Developmental origin of symmetrical FGR	8
1.1.2.3 Developmental origin of asymmetrical FGR	9
1.1.3 FGR and life-long health	10
1.1.4 FGR and neurological outcome	13
1.2 Fetal Brain Growth and Development	15
1.2.1 Synaptogenesis	16
1.2.1.1 Protein markers of synapse development	19
1.2.2 Myelination	21
1.2.2.1 Markers for myelin development	23
1.2.3 Development and function of the hippocampus	24
1.3 Animal Models of FGR and Adverse Development	25
1.3.1 Guinea pig brain development	25
1.3.2 Animal studies of FGR with placental insufficiency	26
1.3.3 Animal studies of FGR and structural brain changes	28
1.3.4 Animal studies of FGR and neurobehavioural outcomes	29
1.4 Summary	30
1.5 References	32
CHAPTER 2 - RATIONALE, HYPOTHESIS AND RESEARCH OBJECTIVES	46
2.1 Rationale	47
2.2 Hypotheses	49
2.3 Objectives	50
2.4 References	52

CHAPTER 3 FETAL GUINEA PIGS WITH ALTERED GROWTH AT TERM AFTER MID-GESTATION LIGATION OR ABLATION OF UTERINE VESSELS 55

3.1 Introduction 56
3.2 Materials and Methods 61
3.2.1 Surgical preparation 61
3.2.2 Surgical procedure and post-operative care 61
3.2.3 Necropsy and organ collection 62
3.2.4 Data acquisition and statistical analysis 63

3.3 Results 67
3.3.1 Generating fetal growth restriction 67
3.3.2 Fetal cohort characteristic data 68
3.3.3 Fetal cohort organ weights 69

3.4 Discussion 89
3.5 Conclusion 94
3.6 References 96

CHAPTER 4 THE IMPACT OF FETAL GROWTH RESTRICTION ON SYNPASE FORMATION AND MATURATION IN THE GUINEA PIG BRAIN AT TERM 100

4.1 Introduction 101
4.2 Materials and Methods 105
4.2.1 Surgical preparation and necropsy 105
4.2.2 Fetal guinea pig cohorts and brain processing for histology 105
4.2.3 Fetal guinea pig brain processing for Western blots 106
4.2.4 Immunohistochemistry of synaptophysin 107
4.2.5 Immunofluorescence of synaptopodin 108
4.2.6 Imaging and statistical analysis of synaptophysin and synaptopodin 109
4.2.7 Western blot protocol for synaptophysin and synaptopodin 112

4.3 Results 126
4.3.1 Synaptophysin quantification 126
4.3.2 Synaptopodin quantification 126
4.3.3 Western blot analysis for synaptophysin and synaptopodin 127

4.4 Discussion 138
4.5 Conclusion 142
4.6 References 143

CHAPTER 5 EFFECT OF FETAL GROWTH RESTRICTION ON MYELIN FORMATION IN THE GUINEA PIG BRAIN AT TERM 150

5.1 Introduction 151
5.2 Materials and Methods 154
5.2.1 Fetal guinea pig collection and brain processing for histology and Western blots 154
5.2.2 Immunohistochemistry of myelin basic protein 155
5.2.3 Histochemical stain for myelin: luxol fast blue 155
5.2.4 Imaging and statistical analysis for myelin basic protein and luxol fast blue 156
5.2.5 Western blot protocol for myelin basic protein 158

5.3 Results 164
5.3.1 Myelin basic protein quantification 164
5.3.2 Luxol fast blue quantification 164
5.3.3 Western blot analysis for myelin basic protein 165

5.4 Discussion 176
5.5 Conclusion 180
5.6 References 181

CHAPTER 6 GENERAL DISCUSSION 185

6.1. General discussion 186
6.2. Future studies 192
6.3. Conclusions 193
6.4. References 195

CURRICULUM VITAE 200

LIST OF TABLES

Table	Description	Page
Chapter 3		
3.1	Survival rate of fetal guinea pigs to term based on surgical treatment	71
3.2	Rate of FGR in fetuses at term based on surgical treatment	75

LIST OF FIGURES

Figure	Description	Page
Chapter 3		
3.1	Guinea pig uterus depicting UAL and UABD	59
3.2	Guinea pig fetuses *in utero* during surgery	65
3.3	Litter size and number of pups in a horn and fetal body weights at term	73
3.4	Surgical treatment and fetal body weights at term	77
3.5	Body weights of fetal cohorts at term	79
3.6	Brain-to-liver weight ratios of fetal cohorts at term	81
3.7	Placental weights and placenta-to-body weight ratios of fetal cohorts at term	83
3.8	Liver weights of fetal cohorts at term	85
3.9	Brain weights and brain-to-body weight ratios of fetal cohorts at term	87
Chapter 4		
4.1	Body weights and brain-to-liver weight ratios of fetal cohorts at term	114
4.2	Coronal section level #860 in the guinea pig brain	116
4.3	Body weights and brain-to-liver weight ratios of fetuses used for Western blot analysis	118
4.4	Gross pictures of brain levels sectioned during fetal brain microdissection for Western blot analysis	120
4.5	Synaptophysin stained coronal brain with analyzed brain regions labelled	122
4.6	Representations of synaptophysin and synaptopodin immunoreactivity and the threshold applied to indicate percent area positive staining	124

4.7	Synaptophysin immunoreactivity in the hippocampal sub-areas of fetal cohorts	128
4.8	Synaptophysin immunoreactivity in the CC of fetal cohorts	130
4.9	Synaptopodin immunoreactivity in the hippocampal sub-areas of fetal cohorts	132
4.10	Synaptopodin immunoreactivity in the CC of fetal cohorts	134
4.11	Western blots of synaptophysin and synaptopodin in the dorsal and ventral hippocampus	136

Chapter 5

5.1	Thionen stained coronal brain with analyzed brain regions labelled	160
5.2	Representations of myelin basic protein immunoreactivity and luxol fast blue staining and the threshold applied to indicate percent area positive staining	162
5.3	Myelin basic protein immunoreactivity in the hippocampal efferent tracts of fetal cohorts	166
5.4	Myelin basic protein immunoreactivity in the CC, OT, PSW and PVW of fetal cohorts	168
5.5	Luxol fast blue staining in the hippocampal efferent tracts of fetal cohorts	170
5.6	Luxol fast blue staining in the CC, OT, PSW and PVW of fetal cohorts	172
5.7	Western blot of Myelin basic protein in the dorsal and ventral hippocampus	174

LIST OF ABBREVIATIONS AND SYMBOLS

~	approximately
±	plus or minus
+	plus
<	less than
>	greater than
≥	greater than or equal to
%	percent
^{3}	cubed
β	beta
µm	microns
aFGR	asymmetrical fetal growth restriction
AGA	appropriate size for gestational age
CC	corpus callosum
D	dorsal
DG	dentate gyrus
et al.	et alia (and others)
FGR	fetal growth restriction
g	grams
HPF	high power field
IR	immunoreactivity
IQ	intelligence quotient
kDa	kilodalton
LFB	luxol fast blue
LGA	large for gestational age

MBP	myelin basic protein
MBP IR	myelin basic protein immunoreactivity
OT	optic tract
PSG	parasagittal grey matter
PSW	parasagittal white matter
PVW	periventricular white matter
r_s	Spearman's correlation coefficient
sFGR	symmetrical fetal growth restriction
SYN	synaptophysin
SYN IR	synaptophysin immunoreactivity
SYNPO	synaptopodin
SYNPO IR	synaptopodin immunoreactivity
UAL	uterine artery ligation
UABD	uterine artery branch diathermy
V	ventral

CHAPTER 1

LITERATURE REVIEW

1.1 CLINICAL RELEVANCE

1.1.1 Incidence and classification of FGR

Pathological restriction of growth *in utero* is an expanding area of research aimed at understanding both the etiology of growth restriction and its continued impact on health. The Canadian Perinatal Health Report of 2004 stated that the nation-wide occurrence of low weight at birth (below 2500 g) has been steady for fifteen years with a rate of 5.9%. The reason for this statistic is multi-faceted, encompassing the decreasing rate of small-for-gestational age term births (those falling in the smallest 10^{th} percentile based on birth weight) and an increasing rate of preterm birth prior to 37 weeks gestation (Public 2008). Growth restriction is defined clinically and in the research setting as below the 10^{th}, 5^{th}, or 3^{rd} percentile for a given gestational age or below one or two standard deviations from a control group. In Canada, following the World Health Organization standards, fetal growth restriction (FGR) is below the tenth percentile for gestational age and less than 2500 g at term. (Ghidini 1996; Kramer 2003; Lackman 2001; Pollack 1992; Salafia 2006). These definitions are not always able to identify genuine FGR which is a failure to attain genetic growth potential during fetal life. Specificity of terminology across clinicians, researchers, and the public would improve information exchange.

Early discrimination identifying pathological FGR from inherently small fetuses *in utero* is important, as misdiagnosed small and healthy fetuses as FGR dissipates resources. Applying ethnicity-specific growth curves is important in correctly characterizing FGR (Gardosi 2009, George 2009; Kierans 2008). A Canadian standard growth curve applied to a Kaniyambadi population identified a greater number of FGR babies than a standard South Asian growth curve or a decade old Indian growth curve (George 2009). Customized growth curves accounting for maternal, environmental and socioeconomic factors strongly correlated with FGR may be

beneficial for identifying pathological FGR (Arbuckle 1993; Figueras 2007; Gardosi 2009; Hutcheon 2010; Kierans 2008; Kramer 2001). A child of a mother 10 cm taller and 10 kg heavier than an average sized mother would have an expected mean body weight at term 140 g heavier than a child born to an averaged sized mother (Gardosi 2009). Thus, identification of FGR would require a higher threshold based on this maternal size to avoid misdiagnosis of FGR. Customised growth curves result in a reclassification of 2.7-4.3% of babies (Figueras 2007). Early and correct classification of FGR through serial ultrasounds would allow for proper intervention and treatment through gestation.

There are other prognosticators of fetal growth used as adjuncts to fetal growth curves. Ponderal index is the ratio of weight-to-height^3 and is positively correlated with fetal weight and nutritional status at term (Brandi 2003). Also, amniotic fluid total protein concentration is negatively correlated with fetal weight (Tisi 2004). A study from London, Ontario at St Joseph's Health Centre found placental weight to have a positive relationship with birth weight, but other studies find this relationship variable unless examining large fetuses (Lackman 2001; Pollack 1992). It is a functional reduction in the placenta rather than a decrease in placental size that is an important indicator of fetal growth. Markers for fetal size and shape at a particular gestational age are useful as they can predict the health of a fetus and postnatal outcome.

FGR phenotypically presents as symmetrical (sFGR) or asymmetrical (aFGR) growth restriction, based on body proportion. The sFGR fetus has reduced growth measurements earlier in pregnancy, wherein brain growth inhibition remains proportional to reductions in weight, length and head circumference with a normal ponderal index at birth (al Riyami 2011; Halliday 2009; Kramer 1990; Pallotto 2006; Pollack 1992). The aFGR fetus has late-onsetting growth restriction in the latter half of gestation (al Riyami 2011; Halliday 2009), accompanied by 'brain

sparing' where brain size and head circumference is relatively maintained through to term in order to maximize fetal survival (al Riyami 2011; Campbell 1977; Jones 1983; Halliday 2009; Laferber 1984; and Pallotto 2006), whereas skeletal muscle is wasted and there is a reduction in soft tissue mass (Pollack 1992, Villar 1990). aFGR phenotypically presents at term as a low ponderal index reflective of disproportionate body dimensions. Additional indices *in utero* are used to infer proportionality. Head-to-abdomen circumference ratio, as measured by ultrasound, reflects brain sparing, skeletal wasting and reduction in soft tissue mass (al Riyami 2011). Campbell *et al.* differentiated between aFGR and sFGR with echograms of head and abdomen circumference showing aFGR fetuses have ratios above the 95th percentile, while sFGR fetuses are within the normal distribution (Campbell 1977). Unlike aFGR, sFGR can be difficult to distinguish from constitutively small fetuses because they are proportionally similar, but extreme early disparity from a growth curve or evidence of a severe antenatal condition infers pathological sFGR.

The most reliable method for distinguishing aFGR and sFGR is the brain-to-liver weight ratio; with aFGR disproportional with a decrease in liver weight, but a relatively normal head circumference and brain size, while sFGR have small livers and brains but proportionally similar to appropriate for gestational age (AGA) fetuses (Cox 2009). Accordingly, brain-to-liver weight ratio is often used in animal studies to accurately identify aFGR at necropsy, whereas the head circumference-to-abdominal circumference ratio measurements serves as a proxy for the brain sparing process and disproportional phenotype in human fetuses and newborns. A low brain-to-liver weight ratio occurs in large for gestation (LGA) newborns who often are born from mothers with gestational diabetes (Cox 2009). The higher the brain-to-liver weight ratio the more sensitive it is for detecting aFGR; a ratio of 4 or more has a sensitivity of 85%, a ratio of 5 or

more has a sensitivity of 93%, and a ratio of 6 or more has a sensitivity of 97% (Cox 2009). More accurate identification of growth restriction and symmetry of growth could be obtained with serial *in utero* measurements of body, brain and liver size throughout gestation using ultrasound or more elegant imaging means.

aFGR is reported to occur in 70% of growth restricted fetuses (Campbell 1977). Interestingly, Villar *et al.* examined the proportion of aFGR and sFGR (defined by low and appropriate ponderal index, respectively) across the world and found that asymmetrical growth restriction occurs in 60-80% of growth restricted babies in developed countries whereas symmetrical growth restriction occurs more frequently in developing countries constituting 69-79% of growth restricted babies (Villar 1986). There is an abundance of literature regarding aFGR as a pathological condition; however sFGR is less studied because of the difficulties in early and accurate diagnosis. A sub-analysis comparing sFGR and aFGR to AGA fetuses at the same gestational age is rarely reported and difficulties in such an analysis arise because of the overlap of etiology.

1.1.2 Etiology of FGR

The greatest percent increase in body weight occurs early in pregnancy during embryogenesis due to the rapid replication of cells, organogenesis and cellular hyperplasia. In the third trimester, the highest absolute weight gain coincides with cellular hypertrophy and differentiation while tissues mature (Cox 2009; Pollack 1992). Normal fetal growth depends on the genetic growth potential of the fetus, the efficiency of the placenta in transporting nutrients and oxygen and the state of the maternal environment. FGR is induced when these essential

factors create a sub-optimal intrauterine environment that is incapable of sustaining normal growth and development. Fetal causes of FGR include abnormal karyotype, infections, errors of metabolism, multiple fetuses, or malformations (Cetin 2004; Han 1993; Pollock 1992). In cases of abnormal cord insertion, multiple placental infarcts, placenta previa, abrupto placenta, or multiple gestations, the placenta is the primary cause of FGR (Pollack 1992). Maternal causes include malnutrition, hypertension, hypoxic conditions, vascular disease or environmental choices such as substance abuse (Feng 2005; Pollock 1992). Additionally, epidemiological studies show FGR to be associated with lower socio-economic status, parental education, maternal age, maternal height and previous delivery history (Gardosi 2009; Pallotto 2006). However, sixty percent of FGR is idiopathic deriving from a combination of factors, the majority of which are related to some form of abnormal placental development deemed placental insufficiency (Ghidini 1996; Pallotto 2006; Pollack 1992).

1.1.2.1 Placental insufficiency

The human placenta is hemochorial wherein maternal blood is in direct contact with fetal tissue. It has a complex branching system of fetal villi that constitute the main area of fetal-maternal exchange. At 21 days post-conception, the secondary villi undergo vasculogenesis, forming the fetoplacental blood vessels (Kingdom 2000). Thereafter, placental nutrient transfer increases throughout gestation transforming the initially hypoxic *in utero* environment to one that is able to sustain a surge in fetal weight gain in the latter half of gestation. This is achieved by a 10-fold increase in volume of the vasculature within the villi, an increase in the surface area of the villi and a decrease in trophoblast thickness thereby reducing the materno-fetal diffusion distance from 55.9 µm to 4.8 µm (Myatt 2006). The basic function of the placenta is to transfer

blood and within it, the essential substrates, nutrients and oxygen required to support fetal development.

Placental insufficiency is a general term used to describe any complication in placental function that results in an inefficiency in the transfer of sufficient oxygen and nutrients for fetal growth. This inefficiency or dysfunction can arise from poor extravillous cytotrophoblast invasion, increased umbilical cord length, reduced density of stem arteries, tertiary villi maldevelopment, thrombosis, or abnormal patterning of the chorionic plate (Chaddha 2004; Kingdom 2000). These alterations may result in FGR due to inefficient blood flow and thereby inadequate oxygen, glucose and essential amino acid transfer to the fetus (Jansson 1990; Mehendran 1993; Jones 1983; Sparks 1985; Myatt 2006). Under such conditions, the fetus adapts its metabolic and developmental processes in order to ensure survival at the expense of appropriate growth. Ultrasound, Doppler measurements and MRI can be used in an attempt to identify uteroplacental blood flow changes and fetal vascular redistribution that are associated with early or late-onset FGR.

Ninety percent of early-onset FGR is associated with abnormal umbilical Doppler ultrasound. The blood flow abnormality that typically presents in sFGR is a reduced, absent or reversed end-diastolic flow velocity in the umbilical artery as measured by Doppler ultrasound (Kingdom 2000; Krebs 1996). Using electron microscopy, Krebs *et al.* discovered fewer terminal villi in placenta of second trimester sFGR fetuses compared to age matched controls (Krebs 1996). It was also found that capillary looping which functions to increase fetoplacental surface area was diminished in number and volume in sFGR placenta consequently impairing oxygen

extraction (Jackson 1994; Krebs 1996). Under-endowment in gas-exchanging villi and related placental insufficiency seem to be a common underlying pathology in sFGR fetuses.

aFGR presents with normal Doppler waveforms in the umbilical artery in the majority of cases or else abnormal Doppler waveforms occur later during gestation but usually are not severe or associated with absent or reversed end-diastolic flow. Hershkovitz *et al.* used Doppler ultrasound and found abnormal middle cerebral artery waveforms but normal umbilical waveforms to be present in a FGR fetus with head circumference-to-abdominal circumference in the 95th percentile (Hershkovitz 2000). Also, frequently encountered in aFGR is a mature placenta at term, placental thrombosis, avascular villi, infarcts, terminal villi hypoplasia and a thin umbilical cord (Cox 2009; Feng 2005). The normal response of the placenta in a hypoxic or mal-nourished environment is to increase villous branching and capillary growth in order to increase surface area for gas exchange (Kingdom 2000). This response leads to accelerated placental maturation and a normal to large placental weight in relation to fetal weight which becomes superfluous when uptake efficiency of the placenta if reduced. Placental transport is impaired in aFGR because amino acid transporters have a reduced rate of activity on both the maternal and fetal membranes (Mehendran 1993). This placental dysfunction is particularly detrimental in late gestation during the surge in fetal growth that occurs prior to term, thereby leading to the brain sparing response and morphologic phenotype of aFGR.

1.1.2.2 Developmental origin of symmetrical FGR

In an American population, 8.6% of all live births fall two standard deviations below normal birth weight, usually coinciding with the 3rd percentile of body weight, and 30% of those are sFGR, with normal ponderal index or body proportions (Feng 2005). sFGR onsets earlier in

pregnancy resulting from an interruption in cell proliferation that affects all organs equally and often can be diagnosed when the size of the organ does not reflect its maturity (Cox 2009). Impairments in cellular hyperplasia cause a proportional decrease in size of all the fetal organs (Pollack 1992). Skin fold thickness measurements in sFGR neonates indicate reduced fat deposition compared to normally grown neonates but more than aFGR neonates (Rodriguez 2011). As such, the sFGR fetus develops in parallel with growth curves, following the shape of normal growth patterns but delayed and below the 5th percentile (Cox 2009). sFGR is more often due to a fetal cause, with either congenital abnormalities including trisomies 13,18, and 21 or infections (Feng 2005; Cox 2009). Often genetic disorders coincide with other phenotypic maladies such as dismorphic facial appearance or skeletal abnormalities. Earlier-onsetting sFGR is more difficult to diagnose with increased risk for fetal/neonatal mortality or preterm birth.

1.1.2.3 Developmental origin of asymmetrical FGR

aFGR onsets later in pregnancy, when growth is dependent on adequate placental blood flow, fat deposition, and nutrition. During this time, growth is less dependent on cell division and more on cellular hypertrophy and therefore this suboptimal environment leading to growth restriction will affect organ maturity, depending on its timing, and may lead to relatively atrophic organs (Cox 2009; Feng 2005). Examination of skin fold thickness in Caucasian newborns with aFGR, demonstrated reduced subcutaneous and central fat deposition relative to normally grown and sFGR babies (Rodriguez 2011). As well, aFGR is associated with reduced amniotic fluid volume and essential amino acids near term thus indicating reduced oxygenation and substrate availability (Hershkovitz 2000; Jansson 1990). aFGR development follows normal fetal growth curves until crossing the curve with diminishing estimated weight in the last half of gestation; timing that coincides with fat deposition and an increased requirement for amino acids during the

exponential growth spurt in the third trimester of pregnancy (Cox 2009). Under these conditions, the aFGR fetus adapts by means of redistributing cardiac output in order to protect essential organs.

'Brain sparing' is the maintenance of brain growth and other vital organs such as the adrenals and heart by redistributing cardiac output both centrally and peripherally at the expense of other organs (Barker 1998). Late gestation fetuses have mature mechanisms of cardiovascular regulation which redirect circulating oxygen and nutrients in favour of the brain at the expense of the viscera, in order to maximize fetal survival (al Riyami 2011; Cox 2009; Laferber 1984). This redistribution of blood flow is apparent in Doppler assessment of the middle cerebral artery showing an increase in blood flow associated with aFGR (Hershkovitz 2000). Changes in brain growth with aFGR that might be occurring would not necessarily be reflected by head circumference, which is often a measure used to identify aFGR, as cranial growth is subsequent to altered brain growth (Duncan 2005). Therein, brain and head size may be reasonably well maintained relative to body size in aFGR, but there could still be subtle alterations in brain development affecting neurological health.

1.1.3 FGR and life-long health

FGR is the second most common complication in newborns behind preterm birth. Al Riyami *et al.* examined human early-onset sFGR in pregnant humans and found that only 50% of these fetuses survived to term (al Riyami 2011). A group in British Colombia reported a gender effect where females have a better chance of surviving FGR compared to males (Synnes 2010).

A study of Mexican newborns reported 7% to be born preterm, with 1% sFGR and 3% aFGR (Haas 1987). A study in London, Ontario showed that the risk for spontane

remaining at a lower weight compared to AGA babies up to 3 years old (Strauss 1997). Indredavik *et al.* examined growth of sFGR-born at term and preterm and found that weight, height and head circumference was still reduced in sFGR-born 14 year olds (Indredavik 2010). Within 2 years, most FGR children do experience catch up growth, whereby their body and various organs have gained sufficient weight that matches those of children born appropriately sized (Pollotto 2006.) However, sFGR infants are likely to remain short statured even through 4 years of age, but both sFGR and aFGR generally achieve catch-up head circumference (Jelliffe 2004). Post-natal treatment is not pre-emptive; it does not diminish the long term morbidity that arises from alterations in development of organ growth *in utero*.

Fetal programming is the theory that organ development during critical periods, in which the system is sensitive to environmental cues, sets the platform for health in later life (Barker 1998). Fetal programming can occur in three ways: 1) direct damage, such as early loss of a limb 2) induction, deletion, or impaired development of a somatic structure resulting from a stimulus or insult during a critical period 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 popularized the idea of fetal programming in 1998, with seminal evidence supporting that undernutrition *in utero* resulting in FGR increases the risk for cardiovascular disease as an adult (Barker 1998). Other studies suggest that the etiology of neurological disorders can also be traced to the effect of the suboptimal environment leading to FGR.

1.1.4 FGR and neurological outcome

The association between FGR and neurological delays and cognitive deficits is apparent in every age group and has been reported in studies all around the world. FGR is associated with an increased risk of developing a number of severe neurological disorders that manifest in childhood including cerebral palsy, attention deficit hyperactivity disorder, and autism spectrum disorder (de Rodrigues 2006; Halliday 2009; Indredavik 2010; Walker 2010). More subtle effects of FGR are evident in childhood as reduced cognitive skills, impaired memory, learning difficulties, poor academic performance including difficulties with reading, writing, and adaptive skills, inattention, reduced psychosocial function, behavioural problems, sensorineural deterioration, reduced mathematics abilities and reduced intelligence quotient (IQ) scores (de Rodrigues 2006; Geva 2008; Indredavik 2010; Pallotto 2006; Synnes 2010; Walker 2010). Altered brain development due to disturbed intrauterine environment may not be evident until later life as FGR-born adults are also at increased risk for schizophrenia, epilepsy, and psychiatric hospitalization (Cannon 2003). Most of these studies also show that the severity of these childhood and adult neurological disabilities depends on the severity of FGR.

Several studies in developed countries conducted over the past 2 decades have assessed school-age children for behaviour and cognitive function with standardized tests, academic performance reports, parental behavioural reports, and controlled observation. For instance, a California study found FGR term infants to be at increased risk for neurodevelopmental difficulties that persisted from 8 months to 4 years of age, including deficits in intellectual functioning, both mental and psychomotor delay, and even borderline to profound mental retardation that was more severe in aFGR, but also apparent in sFGR, compared to AGA infants

(Jelliffe 2004). A British Colombia study also looked at the intellectual performance of FGR infants from 4 months to 4.5 years of age and found these children had cognitive impairment and delays in psychomotor and mental development at all ages measured, the severity of which increased with age and therefore the FGR children remained delayed compared to AGA infants (Synnes 2010). Furthermore, a Belarusian study of 6.5 year old children showed similar patterns wherein body weight at term was positively associated with IQ scores and equated FGR with having the lowest IQ scores compared to AGA and LGA-born children (Yang 2010). They also found IQ to be increased in children born closer to term (Yang 2010). Overall, these studies highlight the consistency of the association between neurological deficits and FGR-born children.

Studies have shown the neurological difficulties of children born FGR to continue into adolescence and young-adulthood. One such study by Isaacs *et al.*, found poor everyday memory, mathematical reasoning and numerical operations skills in FGR-born 13 year olds compared to age matched controls even after learning-skills interventions (Isaacs 2000). Geva *et al.* specifically examined late-onset aFGR and found that at 10 years of age these children continued to have deficiencies in verbal short term memory of auditory or visiospatially presented information and reduced IQ scores, while controlling for gender and general cognitive ability (Geva 2008). Furthermore, Indredavik *et al.* followed the progress of Norwegian sFGR neonates born at term and preterm through to adolescence and reported head circumference was still reduced at 14 years of age and these adolescents to have increased hyperactivity, inattention, psychiatric diagnosis, autism spectrum disorder scores, and reduced psychosocial function (Indredavik 2010). As such, behavioural and cognitive difficulties appear to become more complicated and severe with age for offspring born FGR.

The hippocampus is the least genetically regulated brain area and thereby more vulnerable to environmental and developmental influence (Lodygensky 2008). Lodygensky *et al.* examined preterm FGR and preterm control infants at 2 years of age and showed reductions in grey matter volume in the hippocampus in the FGR infants with a significant correlation between total hippocampal volume and size at birth (Lodygensky 2008). This group also found FGR to be highly correlated with poor performance in motor, attention-interaction, and self –regulation behavioural function maturation assessments (Lodygensky 2008). Other studies also report changes in the hippocampus, for instance, MRI scans show bilaterally reduced hippocampal volume and enlarged lateral ventricles in FGR infants compared to age matched controls (Isaacs 2000). Perturbations in development of the hippocampus including neuronal connectivity may, at least in part, explain the basis for of the neurological sequellae associated with FGR.

1.2 FETAL BRAIN GROWTH AND DEVELOPMENT

Brain growth is a complex and dynamic process that occurs at an exponential rate through gestation and continues to be refined after birth. The developing human fetal brain at term constitutes one fifth of the fetus's body mass and expends four fifths of all energy (Gilles 2011). At the end of the 2nd trimester until term, the rate of brain growth is at its peak. Once neurons are formed and have reached their final migration point, myelination begins and there is a marked decrease in brain growth rate reaching a plateau at two years of age (Gilles 2011). At this time, the brain alterations occur according to experience and the environment.

Essentially, brain growth requires the generation and differentiation of neurons, navigation and organization of the axonal projections between neurons and the formation and maturation of synaptic contacts (Bourgeois 1997). The selective stabilization hypothesis proposes that the initial formation of large amounts of synaptic contacts is dominated by intrinsic mechanisms of growth and that subsequent maturation of selective neuronal connections is due to evoked potentials and usage (Bourgeois 1997). As such, environmental influences may interfere with these processes and thereby alter neurological health in later life.

1.2.1 Synaptogenesis

There are five phases of synaptogenesis that occur during gestation and through to about puberty in humans. Phases 1 and 2 occur prior to the 3rd trimester and involve neurogenesis, neuronal migration, individualization of cortical layers and synapse formation that are controlled by intrinsic mechanisms termed "experience-independent" (Bourgeois 1997). Relative to later stages of pregnancy, limited synaptogenesis takes place during these earlier phases. In phase 3, synapses are formed at a rate of 40,000 synapses per minute driven by an "experience expectant" mechanism (Bourgeois 1997). This process creates an abundance of synapses in brain areas therefore pre-adapting the brain for "experience dependent" phases and individual customization by experience and environmental cues (Bourgeois 1997). At term, there are approximately 600-900 million synapses per mm^3 neuropil (Bourgeouis 1997). The final phases of synaptogenesis are phase 4 and 5, which occur between birth and puberty in humans. The Plateau phase involves the elimination of certain synapses and the selective maturation of others, in order to fine tune and establish certain neuronal circuits. This phase is dominated by 'experience-expectant' and

'experience dependent' mechanisms (Bourgeois 1997). Phase 5 is solely 'experience-dependent' in which synapse architecture changes relative to the experience of the individual (Bourgeois 1997). Gestational environment may affect the breadth of earlier phases priming deficiencies in later phases that reorganize throughout life.

The specialized mechanisms underlying synaptic development are poorly understood but essentially involve the contact of a presynaptic element and a postsynaptic element in the formation of a synapse. These elements are typically an axonal growth cone and dendritic growth cone contacting to become an immature synapse. Contact between these elements is dependent on attractive and repulsive cues, on filopodia extending from axons and dendrites, families of growth factors promoting growth cone migration, polysialic acid inhibiting cell-to-cell interaction, semaphorins and ephrins as repulsive axon guidance factors, and extracellular glutamate concentrations inhibiting spine motility (Scheiffele 2003). Axonal growth cones, the initial presynaptic site that store synaptic proteins, are involved with axonal branching and migration and can at any point become a branch or a presynaptic bouton (Ruthazer 2006). The main postsynaptic site on a dendrite is the highly motile dendritic spine from which filopodia can create new synapses or a new spine can form on the dendritic shaft that has made a synaptic connection (Majewska 2000; Okabe 2001). Upon contact of an axon and dendrite, it takes approximately 4 days for synaptic markers to become present at the contact site and they are abundant by 7 days after initial contact, as shown by *in vitro* studies (Fletcher 1994). Multiple immature connections can form between axons and dendrites but only select connections will form functional synapses.

Synapses are very asymmetric in that the presynaptic end contains neurotransmitter synthesizing and releasing machinery and the postsynaptic end contains protein machinery that create specialized structures to receive those transmitters. Bidirectional signalling between the pre and postsynaptic elements function to differentiate the appropriate neurotransmitter-receptor match and put them on the appropriate end of the synapse (Scheiffele 2003). Major players in forming a synapse include neuroligans and neurexins that may be involved in differentiating the presynaptic end of the synapse, recruiting scaffolding proteins to stabilize synaptic contact and recruitment of calcium channels to the presynaptic terminal (Scheiffele 2003). Cadherins may function to sort appropriate pairs of pre and postsynaptic partners (Scheiffele 2003). Ephrins and ephrin receptors may regulate synaptic recruitment and receptor specialization with their retrograde signalling ability from the postsynaptic dendrite (Scheiffele 2003). Immature synapses establish connection, differentiate into the presynaptic and the postsynaptic ends of the synapse, and begin to function as they mature.

In order to mature, a synapse requires an abundance of energy and specialized protein structures on either end of the synapse. Areas with newly forming synapses have high levels of cytochrome C complex and mitochondria to supply the large amount of energy required for protein synthesis and synapse formation (Mjaatvedt 1988). Presynaptic maturation is evident with an increase in the number of vesicles as well as an increase in reliability of neurotransmitter release (Ruthazer 2006). The amount of vesicle production in the synapse increases with its maturation and decreases steadily if the synapse is degenerating (Ruthazer 2006). At the synaptic cleft there is a presynaptic active zone, to which vesicles adhere to prior to undergoing exocytosis, and a postsynaptic density on the dendritic spine becomes juxtaposed to it, which functions to anchor receptors and scaffolding proteins (Garner 2000; Bourne 2008). Structure

does not guarantee function, as activity at the synapse is necessary for maturation and stabilization.

Function and stabilization of a synapse requires activity. In a neurotransmitter knockout study, synaptic development occurred normally in terms of structure, but without activity at the synapse it later degenerated (Verhage 2000). Axonal branches stabilize when synaptic vesicles accumulate and retract when immature synapses fail to mature (Ruthazer 2006). Activity not only stabilizes the synaptic connection, but also perpetuates neuronal arborisation. A stable synapse initiates the formation of more dendritic spines, subsequently developing into more synapses, on the same presynaptic bouton and perpetuates their maturity after 16 hours, strengthening a neuronal connection, as demonstrated with time-lapse two-photon microscopy (Nagerl 2007). Also in culture, mature axons can perpetuate synaptic maturation earlier than immature axons can (Fletcher 1994). Activity matures and stabilizes synaptic connections while maturation perpetuates the formation of new synapses and instigates the exponential growth of synapses during brain development.

1.2.1.1 Protein markers of synapse development

Synaptophysin (SYN) is a 38 kDa presynaptic protein marker present in all presynaptic boutons on the membrane of presynaptic vesicles in the CNS (Calhoun 1996; Jahn 1985). Histological staining using SYN antibodies shows punctate staining throughout the hippocampus localized to presynaptic boutons (Calhoun 1996; Fletcher 1991; Mundel 1997). This presynaptic protein marker has been used in animal studies of induced FGR as a measure of changes in synaptic numbers in the brainstem, cortical areas of memory consolidation, thalamus, white matter, cortical layers and the hippocampus (Camm 2005; Tolcos 2003). It is suggested that SYN

may also be located in neuronal growth cones in immature axons that lead axonal migration; and therefore may also be found in small quantities in white matter (Calhoun 1996; Fletcher 1991; Leclerc 2010). It is also found in immature synapses and small synaptic vesicles, although these vesicles do not yet exocytose (Daly 1997; Fletcher 1994.) SYN protein levels directly increase with synapse formation and development (Daly 1997; Fletcher 1991). It is a protein expressed in both inhibitory and excitatory synapses and is involved in competitive strengthening of synapses as well as activity dependent synapse maturation (Tarsa 2002). Using immunohistochemistry, the immunoreactivity of SYN has been shown to be an accurate presynaptic marker for the detection of synapse formation.

The functional protein machinery that creates neurotransmitter receptors and a mature synapse is located in the postsynaptic dendritic spine. One such protein that is critical for synaptic maturation is synaptopodin (SYNPO), a proline rich 100 kDa protein found in dendritic spines of telencephalic neurons (Mundel 1997; Deller 2000). SYNPO's function is not entirely known, but it is an actin-binding protein thought to function in the storage of calcium, local regulation of calcium dependent mechanisms and is closely associated with the spine apparatus that plays a critical role in learning and memory (Deller 2000b; Deller 2003; Majewska 2000). In the developing rat brain, SYNPO is expressed only during the last 1/3 of gestation appearing on gestational day 15 and is primarily associated with the spine apparatus which is only found in mature synapses (Deller 2000b). The critical role of this protein in spine elongation and morphology is evident in SYNPO knockout mice in which no spine apparatuses are formed (Deller 2003). It is notably located at synapses in areas particularly important for synaptic plasticity, memory, and learning such as the hippocampus, amygdala and olfactory bulb (Deller

2000b; Mundel 1997). As such, SYNPO appears to be an accurate marker for mature excitatory synapses.

1.2.2 Myelination

Myelin is essential for insulating the electrochemical signal sent along axons of neurons; proper myelination throughout development ensures neuronal connectivity and communication between brain areas. While *in vitro* studies in mice have shown the ability of oligodendrocytes to form myelin sheath-like substances in the absence of neurons (Temple 1986), the presence of neurons and specifically the electrical activity in the axons of neurons perpetuates myelination in that axon (Barres 1993). A study in fetal guinea pigs during fetal development has shown that axonal length of 0.5 μm is the threshold for myelination to occur (Nitsos 1990). Conversely, myelin sheath thickness dictates conduction velocity of an axonal signal and is instrumental in synchronous firing of action potentials which strengthens neuronal connections (Fields 2008.) As such, the increasing electrical activity in the maturing neuron maintains myelination which in turn establishes saltatory conduction and coordinates synaptogenesis between neuronal connections.

In humans, the rate of myelination of the brain increases towards the end of gestation and continues into the 2nd year of life. The myelin phospholipids appear in CNS white matter prior to initiation of active myelin synthesis and increase in expression throughout gestation (Kinney 1994). Myelin sheath proteins are seen in the brain as early as 5 gestational weeks and become more abundant with advancing gestational age (Jakovcevski 2007). The earliest myelin sheath is seen in the thalamus at ~18 weeks gestation and in the internal capsule at 21 weeks gestation, but

only develops in telencephalic neurons after mid-gestation (Jakovcevski 2005). Peak myelination occurs at different time points either during gestation or after-birth depending on the brain area.

Myelin is produced by glia cells, particularly oligodendrocytes in the CNS. Oligodendrocyte precursors are prevalent in the human fetus at ~30 weeks gestation and increase in number thereafter with increasing myelination (Back 2001). At midgestation, oligodendrocyte precursor cells are present in a gradient fashion from the subventricular zone to the cerebral cortex of the telencephalon indicating a sequence for myelination (Jakocevski 2005). Precursor oligodendrocyte cells must differentiate into myelinating oligodendrocytes containing myelin proteins before they are able to myelinate axons (Jakocevski 2005). Myelination is dependent on the differentiation and expression of oligodendrocytes. Delay in this differentiation will delay myelination.

Oligodendrocytes and neurons signal each other to ensheath the axon. Pre-myelinating oligodendrocytes and oligodendrocytes contain the major myelin proteins needed for myelination (Jakcevski 2005). Oligodendrocytes ensheath axons in a multilayer concentric pattern of myelin leaving un-myelinated nodes at regular intervals along the axon. Precise spacing and location of oligodendrocytes along the axon is achieved with continuous extension and retraction of filopodia-like processes which migrate along the axon until they reach the end localization where the filopodia wrap the axon (Kirby 2006). Axons and oligodendrocytes signal to each other to coordinate the exact amount of myelin to be layed down along the axon (Trajkovic 2006). Therefore, the coordinated effort of the neuron and oligodendrocyte function to correctly space and lay down myelin in sufficient thickness and compaction to facilitate adequate conduction velocity and preservation of the signal from origin to target.

Myelin is made up of 70-85% lipid and 15-30% protein which make it electrically insulating; lipids include cholesterol, phospholipids and glycosphingolipids (Simons 2007). These lipids, along with essential proteins, are arranged in perfect proportion along the axon. Proteolipid protein regulates cholesterol and myelin basic protein release from oligodendrocytes onto the axon (Simons 2007). When myelin basic protein is released, it brings along preassembled lipid clusters and condenses them on the axon (Simons 2007). Myelin basic protein then binds to itself creating striations of lipids and protein in a concentric pattern along the intermodal myelin sheath.

1.2.2.1 Markers for myelin development

Myelin Basic Protein (MBP) comprises 35% of the protein in the myelin sheath and is often used as a marker for myelination. There is no MBP immunostaining in the human brain until after 27 weeks gestation, appearing in minimal amounts at ~30 weeks and then more so at 40 weeks gestation, but mostly in superficial white matter areas (Back 2001). Prior to myelination, MBP is found in precursor oligodendrocytes and is transported onto the axon upon initiation of myelin formation (Pedraza 1997). There are several isoforms of MBP; in guinea pigs they range in size from 14 kDa,16kDa, 17.2kDa, 18.5kDa, 20.2kDa, and 21.5 kDa (Maatta 1997). The 14 and 18.5 kDa isoforms participate in membrane compaction and the 17.2 and 21.5 kDa MBP isoforms are expressed earliest in development and may play a role in oligodendrocyte maturation and myelination initiation (Pedraza 1997). MBP is essential for both formation and maturation of myelin. A common histological stain used for myelin is Luxol fast blue (LFB). LFB is an arylguanidinium salt of anionic chromagens that stains proteolipids and hydrophobic domains of myelin using a lithium carbonate differentiation (Clasen 1967; Kiernan 1990). The

base of the dye substitutes for a base of the target lipoprotein creating a blue precipitate. LBF staining has also been used as a marker of myelination throughout gestation (Brody 1987; Kinney 1988). These myelin markers have been shown to accurately identify myelin in the brain.

1.2.3 Development and function of the hippocampus

The hippocampus is comprised of specific subareas that work together for cognition; 1) the dentate gyrus (DG), 2) three sub-fields of the hippocampus proper being the CA1, CA3, and CA4, 3) the subicular complex, and 4) the entorhinal cortex (Anderson 2007). As shown by both golgi stain and cellular recordings, the entorhinal cortex feeds into the DG granule cells, which project to the CA3 pyramidal sub-area, which in turn projects to the CA1 pyramidal sub-area of the hippocampus, which projects back to the entorhinal cortex (Anderson 2007; Gnatkovsky 2006). This 'Perforant Pathway' is unidirectional with modulatory interneurons that feed-forward and feed-back to create a regulated system for learning and memory. It undergoes peak neurogenesis around mid-gestation in guinea pigs (Clancy 2009). An overabundant production of synapses is critical for the function of the hippocampus in preparation for 'experience dependent' individualization during learning post-natally.

The hippocampus is an area critical for learning and memory. Studies of human patients with hippocampal damage show difficulties with both word and faces recognition tests (Reed 1997). This makes it inherently difficult to learn and remember lessons in school and social interaction would be challenging. A study in mice, found that the hippocampus had a particular role in consolidation of memory and that deficiencies in memory tasks were correlated with a decrease

in probability of neuronal firing from CA3 to CA1 (Nakashiba 2009). Intact neuronal networks in the hippocampus are therefore essential for proper cognitive functioning.

1.3 ANIMAL MODELS OF FGR AND ADVERSE DEVELOPMENT

1.3.1 Guinea pig brain development

The guinea pig is an excellent animal model for the study of FGR and neurologic developmental programming. Unlike other rodents but like humans, guinea pigs are precocious developers and undergo rapid brain growth in the latter half of gestation (Dobbing 1970). Both humans and guinea pigs are thought to be born with the full complement of synaptic and neuronal numbers (Lennon 1980). Therefore, intrauterine insults will coincide with critical periods of development and the associated long-term outcomes are more accurately extrapolated to humans.

Relative to the human, the guinea pig brain is larger and at a more advanced stage of functionality at term. In humans, brain growth begins to plateau around 2 years of age whereas in guinea pigs this plateau occurs just prior to birth at term (Dobbing 1970). In guinea pigs most brain areas, including the hippocampus, undergo peak neurogenesis much earlier than in humans (Clancy 2007). At birth, the functionality of the guinea pig brain including motor, cognitive and regulatory processes compares to that of a human toddler. Therefore the impact of impaired development from *in utero* events may be magnified in the guinea pig brain in comparison to humans. As well, the variable influence of post natal environmental factors that can play a significant role in ongoing brain development will be lessened in the more mature guinea pig brain. The precocious functionality and comparable neuroanatomy of the guinea pig brain

compared to the human brain is an asset in determining neurodevelopmental changes and their extrapolation to human conditions.

1.3.2 Animal studies of FGR with placental insufficiency

FGR occurs spontaneously in 2-4% of guinea pig pups (Ibsen 1928). FGR has been further induced in animals using models of maternal undernutrition, chronic hypoxia, reduction in uterine blood flow, reduction in placental size and endocrine alterations (Han 1993). These models mimic the variety of human conditions that may contribute to FGR. A common technique used to simulate placental insufficiency and induce FGR is uterine artery ligation (UAL). UAL has been used to induce FGR in rats (Olivier 2007), guinea pigs (Laferber 1984), and sheep (Rees 1999). In the guinea pig the technique involves ligation of the main uterine artery leading to one horn of the bicornuate uterus at approximately mid-gestation. UAL reduces blood flow, nutrients, and ions to the fetus and creates a hypoxic intrauterine environment during gestation (Jansson 1990; Jones 1983). Its effect on organogenesis occurs within hours (Jones 1983; Laferber 1984). This technique induces FGR in at least one guinea pig fetus in 14%-30% of experimental litters (Jansson 1990; Laferber 1984). Taken as a whole, UAL leads to FGR in approximately 14% of pups from litters undergoing UAL which equates to about one growth restricted pup per litter (Tolcos 1997). Generally, UAL causes growth restriction on a graded scale depending on the proximity of the fetus to the ligated artery, the duration of the insult through gestation and the number of fetuses in the horn (Turner 2009; Wigglesworth 1964). Furthermore, the UAL technique in guinea pigs successfully mimics human placental insufficiency in that it reduces oxygen and nutrient delivery (Jansson 1990; Jones 1983; Laferber

1984). Furthermore, the wide distribution of birth weights in UAL litters allows for the examination of relationships between birth weight and various developmental outcomes.

Turner and Trudinger (2009) introduced a cauterizing technique in an attempt to increase the incidence of FGR and reduce the rate of fetal demise that occurs with UAL. The surgical technique involves cauterizing one of every three or two of every four uterine artery branches along one horn of the bicornate uterus leading specifically to an individual fetal placenta again at mid-gestation (Turner 2009). In a preliminary study, this model was shown to have a better distribution of FGR across positions in the horn, a higher survival rate, and a balanced reduction of blood flow along the horn (Turner 2009; Camprubi 2009). The success rate in producing FGR by cauterization was reported to be 23% albeit with a 47% pup mortality rate (Turner 2009). Although relatively novel, the cauterization technique is similar to the UAL technique in that it mimics placental insufficiency by ablating blood flow.

Animal models whereby FGR is induced by reduction in uterine or umbilical blood flow lead to various changes in the intrauterine environment. UAL results in decreases in essential amino acid transfer, insufficient caloric intake, altered hormone circulation, and down-regulated metabolism changing the balance of anabolic vs. catabolic processes and leading to reductions in fetal growth (Jones 1983; Jansson 1990; Sparks 1985). These changes are similar to those that can occur in the intrauterine environment with human placental insufficiency.

1.3.3 Animal studies of FGR and structural brain changes

Although brain weight is often maintained in animal studies leading to aFGR, volumetric measures have shown aFGR animals to exhibit maladaptive changes in brain areas including the hippocampus (Mallard 1999), retina (Rees 1992), ventricles (Mallard 1999; Rehn 2004), cerebellum (Mallard 2000; Rees 1999), brainstem (Tolcos 1997, 2003), and cortex (Rehn 2004). Rees *et al.* clamped the vascular blood flow to the placenta of sheep for 12 hours to cause aFGR and found reduced density of Purkinje cells, a 25% decrease in density of dendritic branching, a 46% decrease in branch length, a reduced number of dendritic spines and in some cases dendrites devoid of spines in the cerebellum (Rees 1999). However, there was no change in SYN or MBP as measured in the brainstem of animals similarly studied (Tolcos 2003). At 7 days of age, guinea pig offspring who were growth restricted by a UAL model of placental insufficiency had reduced neuronal numbers in the CA1 region and stratum oriens layer above the CA1 region of the hippocampus, a reduced number of Purkinje cells, and reduced grey and white matter volume in the cerebellum, as estimated using the Cavalieri Principle (Mallard 2000). These changes were significantly correlated with reductions in brain weight (Mallard 2000). Another study using an UAL model of placental insufficiency in guinea pigs found FGR-born animals at 8 weeks of age to have increased lateral ventricle size and reduced basal ganglia volume (Rehn 2004). The structural changes that occur with FGR vary between regions of the brain and also persist after birth.

Various studies have demonstrated that FGR is related to changes in white matter development and myelination. Nitsos and Rees (1990) used a guinea pig model of placental insufficiency and found reduced myelination and a marked reduction in the number of

myelinated fibers in FGR offspring compared to control animals in the corticospinal tract. This study also showed that the thickness of the myelin sheath was disproportionally thin in comparison to the axonal diameter in growth restricted fetuses (Nitsos 1990). Acute, intermittent hypoxia produced by umbilical cord occlusion in fetal sheep led to reduced MBP immunoreactivity in white matter areas of the brain and shorter fiber penetration into cortical grey matter (Rocha 2004; Mallard 1997). Likewise, a study of growth restriction induced by UAL in rats showed a delay in myelination with growth restriction of a moderate degree, and the severity and longevity of white matter damage to correlate with the severity of growth restriction (Olivier 2007). Together, the above studies make evident that there are white matter changes associated with FGR.

1.3.4 Animal studies of FGR and neurobehavioural outcomes

There are few studies that have explored the behavioural changes related to induced FGR in animals. Guinea pig offspring who were growth restricted by UAL did not show body weight catch up relative to control offspring and demonstrated a diminished prepulse inhibition of the startle response at 12 weeks of age (Rehn 2004). This is indicative of diminished sensorimotor gating. FGR guinea pig offspring from an undernutrition model remained smaller at 42 days but caught up in weight at 98 days, but still at 15 weeks, behaviour tests revealed heightened threatening behaviour, increased nosing, and decreased latency to interact (Byrne 1978). At 26 weeks of age, growth restricted offspring displayed blunted exploratory behaviour, reduced emotional behaviour as measured by defecation, and increased purposeless locomotion, compared to control animals (Simonson 1971). However, there are also studies that show no

differences in the behavioural responses of FGR animals or have debatable results. Behavioural testing to define cognitive differences between FGR and AGA animals needs to be rigorously organized and take advantage of the technological advances available today.

1.4 SUMMARY

Fetal growth restriction is a failure to attain genetic growth potential during fetal life. It can present as either asymmetrical or symmetrical growth restriction based on the occurrence of brain sparing. When a fetus develops in a sub-optimal intrauterine environment in the latter half of gestation, there is a redistribution of blood flow along with circulating nutrients and substrates, to the brain in an attempt to maintain brain weight, at the expense of visceral organs. This results in asymmetrical growth restriction defined by body weight at term below 2500 g and an elevated brain weight-to-liver weight ratio. aFGR at term in guinea pigs is defined as body weight below 80 g and a brain weight-to-liver weight ratio above 0.8 (Banks 1989; Dieni 2003; Laferber 1984; Mallard 1999; Nitsos 1990; Rees 1992; Tolcos 1997; Turner 2009). Conversely, sFGR is an early-onset growth failure that results in a small fetus proportionally similar to AGA fetuses. In guinea pigs, sFGR is characterized by weight at term below 80 g of weight at term with a brain-to-liver weight ratio below 0.8.

FGR leads to an increased risk for many cognitive deficiencies and neurological disorders throughout life. This includes an increased risk for autism spectrum disorder, attention deficit hyperactivity disorder and schizophrenia. Cognitive deficits present as poor academic performance, learning disabilities and memory deficiencies in childhood and continue through

life. These neurostructural phenotypes have been reproduced by FGR animal models. Such outcomes produced by various animal models include changes in neuronal number, dendritic arborisation and volumetrics of brain regions. Altered development of the hippocampus is particularly important as the early human deficiencies related to FGR revolve around learning and memory. Examining the intricate development of neuronal connectivity at term, in the precocious guinea pig, may give insight to the particularities of synaptogenesis and myelination that are amiss with FGR. Synaptophysin and synaptopodin are pre and postsynaptic markers, respectively, that are involved in synapse formation and maturation. Myelin basic protein is essential for myelin formation and maturation and luxol fast blue is a reliable marker for myelination. These markers may reveal alterations in neuronal connectivity that contribute to the increased risk of developing neurological sequellae in FGR fetus.

1.5 REFERENCES

al Riyami N, Walker M, Proctor LK, Yinon Y, Windrim RC, Kingdom JCP. (2011) Utility of head/abdomen circumference ratio in the evaluation of severe early- onset intrauterine growth restriction. *J Obstet Gynaecol Can* 33(7):715-719

Anderson P, Morris R, Amaral D, Bliss T, and O'Keefe J. (2007) *The hippocampus book.* Oxford University Press.

Arbuckle TE, Wilkins R, and Sherman GJ. (1993) Birth weight percentiles by gestational age in Canada. *Obstet Gynecol* 81(1):39-48

Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, and Kinney HC. (2001) Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci*. 21(4):1302-12

Banks R. (1989) The guinea pig: biology, care, identification, nomenclature, breeding, and genetics. *USAMRIND Sem Ser.*

Barker DJP. (1998) *In utero* programming of chronic disease. *Clin Sci*. 95:115-128

Barres BA and Raff MC (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature* 361, 258-260

Bourgeois JP. (1997) Synaptogenesis, heterochrony and epigenetics in the mammalian neocortex. *Acta Paediatr.* Suppl 422:27-33

Bourne JN and Harris KM. (2008) Balancing structure and function at hippocampal dendritic spines. *Annu Rev Neurosci* 31:47-67

Brandi I, Sticker EJ, and Lentze MJ. (2003) Catch-up growth of head circumference of very low birth weight, small for gestational age pre term infants and mental development to adulthood. *J Pediatics*. May:463-68

Brody BA, Kinney HC, Kloman AS, Gilles FH. (1987) Sequence of central nervous system myelinaiton in human infancy. I. An autopsy study of myelination. *J Neuropathol Exp Neurol*. 46(3):23-301

Byrne EA, Smart JL, Dobbing J, and Sands J. (1978) Behaviour, brain and body growth of guinea pigs after prenatal growth restriction. *Br J Nutr*. 40:543-51

Calhoun ME, Jucker M, Martin LJ, Thinakaran G, Price DL, and Mouton PR. (1996) Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J Neurocytol*. 25:821-28

Camm EJ, Gibbs ME, Harding R, Mulder T, and Rees SM. (2005) Prenatal hypoxia impairs memory function but does not result in overt structural alterations in the postnatal chick brain. *Brain Res Dev Brain Res.* 160(1):9-18

Campbell A and Thoms A. (1977) Ultrasound measurement of the fetal head to abdomen circumference ratio in the assessment of growth retardation. *Brit J Obstet Gynaecol*. March 84: 165-74

Camprubi M, Balaguer A, Iglesias I, Girabent M, and Callejo J. (2009) Cauterization of meso-ovarian vessels, a new model of intrauterine growth restriction in rats. *Placenta.* 30(9):761-766

Cannon TD, van Erp TGM, Bearden CE, Loewy R, Thompson P, Toga AW, Huttunen MO, Keshavan MS, Seidman LJ, and Tsuang MT. (2003) Early and late neurodevelopmental influences in the prodrome to schizophrenia: Contributions of genes, environment, and their interactions. *Schizophrenia Bull.* 29(4):653-69

Cetin I, Foidart JM, Miozzo M, Raun T, Jansson T, Tsatsaris V, Reik W, Cross J, Hauguel-de-Mouzon S, Ilsley N, Kingdom J, and Huppertz B. (2004) Fetal growth restriction: a workshop report. *Placenta*. 25:753-57

Chaddha V, Viero S, Huppertz B, and Kingdom J. (2004) Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med*. 9(5):357-69.

Clancy B, Kersh B, Hyde J, Darlington RB, An KJS and, Finlay BL (2007) Web-Based Method for translating Neurodevelopment from laboratory species to humans. *Neuroinformatics* 5-1:79-94. http://people.psych.cornell.edu/~blf2/pdfs/BCBKBLFNI07.pdf

Clasen RA, Simon GR, Ayer JP, Pandolfi S, and Laing IR. (1967) A chemical basis for the staining of myelin sheaths by luxol dye techniques; further observations (abstract and discussion). *J Neuropathol Exp Neurol*. 26:153-4

Cox P, and Marton T. (2009) Pathological assessment of intrauterine growth restriction. *Best Pract Res Clin Obstet Gynaecol*. Dec 23(6):751-64

Daly C and Ziff EB. (1997) Post-transcriptional regulation of synaptic vesicle protein expression and the developmental control of synaptic vesicle formation. *J Neurosci*. 17(7):2365-2375

Deller T, Merten T, Roth SU, Mundel P, Frotscher M. (2000) Actin-associated protein synaptopodin in the rat hippocampal formation: localization in the spine neck and close association with the spine apparatus of principal neurons. *J Comp Neurol* 418:164-181

b) Mundel P and Frotscher M (2000) Potential role of synaptopodin in spine motility by coupling actin to the spine apparatus. *Hippocampus*10:569-581

Deller T, Korte M, Chabanis S, Drakew A, Schwegler H, Stefani GG, Zunlga A, Schwarz K, Bonhoeffer T, Zeller R, Frotscher M, and Mundel P. (2003) Synaptopodin-deficient mice

lack a spine apparatus and show deficits in synaptic plasticity. *Proc Natl Acad Sci USA*. 100(18): 10494-9

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

Dieni S and Rees S. (2003) Dendritic morphology is altered in hippocampal neurons following prenatal compromise. *J Neurobiol.* 55(1): 41-52

Dobbing J and Sands J. (1970) Growth and development of the brain and spinal cord of the guinea pig. *Brain Res*. 17:115-123

Duncan KR, Issa B, Moore R, Baker PN, Johnson IR, and Gowland PA. (2005) A comparison of fetal organ measurements by echo-planar magnetic resonance imaging and ultrasound. *BJOG* 112:43-9

Feng S. (2005) Management of preterm infants with intrauterine growth restriction. *Early Hum Dev.* 81:9-900

Fields RD. (2008) White matter in learning, cognition, and psychiatric disorders. *Trends Neurosci.* 31,361-370.

Fletcher TL, Cameron P, De Camilli P and Banker G. (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J Neurosci.* 11(6) 1617-26

Fletcher T, de Camilli P, and Banker G. (1994) Synaptogenesis in hippocampal cultures: Evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. *J Neurosci*. 14(11):6695-706

Figueras F, Figueras J, Meler E, Eixarch E, Coll O, Gratacos E, Gardosi J, Carbonell X. (2012) Customized birthweight standards accurately predict perinatal morbidity. *Arch Dis Child Fetal Neonatol.* 92:F277-F280

Gardosi J and Francis A. (2009) A customized standard to assess fetal growth in a US population. *Am J Obstet Gynecol*. 201(1):25.e1-7

Garner CC, Kindler S, and Gundelfinger ED. (2000) Molecular determinants of presynaptic active zones. *Cur Opin Neurobiol* 10:3321-327

George K, Prasad J, Singh D, Minz S, Albert DS, Muliyil J, Joseph KS, Jayaraman J, and Kramer MS. (2009) Perinatal outcomes in a South Asian setting with high rates of low birth weight. *BMC Pregnancy Childbirth* 9:5

Geva R, Eshel R, Leitner Y, Fattal-Valevski A, and Harel S. (2008) Verbal short-term memory span in children: long-term modality dependent effects of intrauterine growth restriction. *J Pediatr*. 49(12):1321-30

Ghidini A. (1996) Idiopathic fetal growth restriction, a pathophysiologic approach. *Obs Gynecol Sur*. 51(6): 376-382

Gilles FH. (2011) The developing human brain: What the emerging pediatric neurologist needs to know. *Sem Pediatr Neurol.* 124-27

Gnatkovsky V and de Curtis M. (2006) Hippocampus-mediated activation of the superficial and deep layer neurons in the medial entorhinal cortex of the isolated guinea pig brain. *J Neurosci*. 26(3):873-81

Haas JD, Balcazer H, and Caulfield L. (1987) Variation in early neonatal mortality for different types of fetal growth restriction. *Am J Physical Anthropol*. 73:467-73

Halliday HL. (2009) Neonatal management and long-term sequelae. *Best Prac Clin Obstet Gynaecol.* 23:871-880

Han VK. (1993) Pathophysiology, cellular and molecular mechanisms of foetal growth retardation. *Equine Vet J Suppl.* (14):12-6

Hershkovitz R, Kingdom JCP, Geary M, and Rodeck CH. (2000) Fetal cerebral blood flow redistribution in late gestation: identification of compromise in small fetuses with normal umbilical artery Doppler. *Ultrasound Obstet Gynecol.* 15:209-212

Hutcheon JA, Walker M, and Platt RW. (2010) Assessing value of customized birth weight potentials. *Prac Epidemiol*. 173(4):459-467

Ibsen HL. (1928) Prenatal growth in guinea-pig with special reference to environmental factors affecting weight at birth. *J Exp Zool.* 51:51

Indredavik MS, Vik T, Evensen KAI, Skranes J, Taraldsen G, and Brubakk AM. (2010) Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr* 31(4):286-94

Isaacs EB, Lucas A, Chong WK, Wood SJ, Johnson CL, Marshall C, Vargha-Khadem F, and Gadian DG (2000) Hippocampal volume and everyday memory in children of very low birthweight. *Pediatr Res*. 47:713-20

Jackson MR, Walsh AJ, Morrow RJ, Mullen JB, Lye SJ, and Ritchie JWK. (1994) Reduced placental villous tree elaboration in small-for-gestational-age pregnancies: relationship with umbilical artery Doppler waveforms. *Am J Obstet Gynecol*. 172(2) part1: 518-24

Jahn R, Schiebler W, Ouimet C, and Greengard P. (1985) A 38,00-dalton membrane protein (p38) present in synaptic vesicles. *Proc Natl Acad Sci USA* 82:4137-4141

Jakovcevski I, Mo Z, Zecevic N. (2007) Down-regulation of the axonal polysialic acid-neural cell adhesion molecule expression coincides with the onset of myelination in the human fetal forebrain. *Neurosci.* 149: 328-37

Jakovcevski I and Zecevic N. (2005) Sequence of oligodendrocyte development in the human fetal telencephalon *Glia* 49, 480-491

Jansson T and Persson E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Ped Res.* 28:203-208

Jelliffe-Pawlowski LL and Hansen RL. (2004) Neurodevelopmental outcome at 8 months and 4 years among infants born full-term small-for-gestational age. *J Perinatol*. 24:505-514

Jones CT and Parer JT. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *J Physiol.* 343:525-537

Kierans WJ, Joseph KS, Luo ZC, Platt R, Wilkins R, and Kramer MS. (2008) Does one size fit all? The case for ethnic-specific standards of fetal growth. *BMC Pregnancy Childbirth*. 8:1-9

Kiernan JA. (1990) *Histological and Histochemical methods: Theory and practice second edition*. Pergamon press, Great Britain Exeter by BPCC Wheatons Ltd. p320

Kingdom J, Huppertz B, Seaward G, and Kaufman P. (2000) Development of the placental villous tree and its consequences for fetal growth. *Eur J Obstet Gynecol*. 92:35-43

Kinney HC, Brody BA, Kloman AS, Gilles FH. (1988) Sequence of central nervous system myelination in human infancy. II. Patterns of myelination in autopsied infants. *J Neuropathol Exp Neurol*. 47(3):217-34

Kinney HC, Karthigasan, Borenshteyn NI, Flax JD, and Kirschner DA. (1994) Myelination in the developing human brain: Biochemical correlates*. *Neurochem Res* 19(8):983-996

Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, Kelsh RN, and Appel B. (2006) *In vivo* time-lapse imaging shows dynamic oligodendrocyte progenitor behaviour during zebrafish development. *Nature Neuroscience.* 9(12):1506-11

Kramer MS, Platt RW, Wen SW, Joseph KS, Allen A, Abrahamowicz M, Blondel B, and Breart G. (2001) A new and improved population-based Canadian reference for birth weight. *Pediatrics* 108(2):E35

Kramer MS. (2003) The epidemiology of adverse pregnancy outcomes: An overview. *J Nutr.* 133(5 Suppl 2): 1592S-1596S

Krebs C, Macara LM, Leiser R, Bowman AW, Greer IA, and Kingdom JCP. (1996) Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. *Am J Obstet Gynecol*. 175(6):1534-42

Lackman F, Campbell V, Richardson B, DaSilva O, and Gangon, R. (2001) The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol*. 184(5):946-53

Laferber HN, Rolph TP, and Jones CT. (1984) Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol.* 6:441-459

Leclerc N, Beesley PW, Brown I, Colonnier M, Gurd JW, Paladino T, and Hawkes R. (2010) Synaptophysin expression during synaptogenesis in the rat cerebellar cortex. *J Comp Neurol.* 280(2):197-212

Lennon AM, Francon J, Fellous A, and Nunez J. (1980) Rat, mouse, and guinea pig brain development and microtubule assembly. *J neurochem.* 35(4):804-813

Lodygensky GA, Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F, and Huppi PS. (2000) Intrauterine growth restriction affects the preterm infants hippocampus. *Pediatr Res*. 63(4):438-42

Lucas A (1994) Role of nutritional programming in determining adult morbidity. *Arch Dis Child*. 71:288-290

Maatta JA, Coffey ET, Hermonen JA, Salmi AA, and Hinkkanen AE. (1997) Detection of myelin basic protein isoforms by organic concentration. *Biochem Biophys Res Comm*. 238: 498-502

Majewska A, Tashiro A, and Yuste R. (2000) Regulation of spine calcium dynamics by rapid spine motility. *J Neurosci*. 20(22):8262-68

Mallard C, Loeliger M, Copolov D, and Rees S. (2000) Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neurosci.* 100(2): 327-333

Mallard CE and Rees S. (1997) Effects of chronic placental insufficiency on brain development in fetal sheep. *Pediatr Res.* 43(2):262-70

Mallard C, Rehn A, Rees S, Tolcos M, and Copolov D. (1999)Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: implications for the aetiology of schizophrenia. *Schizop Res.* 40:11-21

Matthews Stephen. Oral talk: Fetal Programming of brain hormones and behaviour across generations. University of Western Ontario. Monday April 30th 2012.

Mehendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RDH, and Sibley CP. (1993) Amino acid (system A) transporter activity in microvillous membrane vesicles from placentas of appropriate and small for gestational age babies. *Pediatric Res*. 34(5):661

Mjaatvedt AE and Wong-Riley TT. (1988) Relationship between synaptogenesis and cytochrome oxidase activity in Purkinje cells of the developing rat cerebellum. *J comp neurol*. 277(2):155-18

Mundel P, Heid HW, Mundel TM, Kruger M, Reiser J, and Kriz W. (1997) Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J Cell Biol* 139:193-204

Myatt L. (2006) Placental adaptive responses and fetal programming. *J Physiol*. 572(1):25-30

Nagerl UV, Kostinger G, Anderson JC, Martin KAC, and Bonhoeffer T. (2007) Protracted synaptogenesis after activity-dependent spinogenesis in hippocampal neurons. *J Neurosci.* 27(30):8149-56

Nakashiba T, Buhl DL, McHugh TJ, and Tonegawa S. (2009) Hippocampal CA3 output is crucial for ripple-associated reactivation and consolidation of memory. *Neuron*. 62(6):781-7

Nitsos I and Rees S. (1990) The effects of intrauterine growth retardation on the development of neuroglia in fetal guinea pigs. An immunohistochemical and an ultrastructural study. *Int J Devl Neurosci.* 8(3): 233-244

Okabe S, Miwa A, and Okado H (2001) Spine formation and correlated assembly of presyanptic and postsynaptic molecules. *J Neurosci*. 21(16)6105-6114

Olivier P, Baud O, Bouslama M, Evrard P, Gressens P and Verney C. (2007) Moderate growth restriction: deleterious and protective effects on white matter damage. *Neurobiol Dis* 26(1):253-263

Pallotto EK and Kilbride HW. (2006) Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynaecol*. 49(2):257-269

Pedraza L, Fidler L, Staugaitis SM, and Colman DR. (1997) The active transport of myelin basic protein into the nucleus suggests a regulatory role in myelination. *Neuron*. 18:579-589

Pollack RN and Divon MY. (1992) Intrauterine growth retardation: Definition, classification, and etiology. *Clin Obstet Gyne*. 35:99-107

Public Health Agency of Canada. (2008) Canadian Perinatal Health Report, 2008 Edition. Ottawa, ON, Canada. Published by the authority of the Minister of Health.

Reed JM and Squire LR. (1997) Impaired recognition memory in patients with lesions limited to the hippocampal formation. *Behav Neurosci*. 111(4):667-75

Rees S and Bainbridge A. (1992) The structural and neurochemical development of the fetal guinea pig retina and optic nerve in experimental growth retardation. *Int J Devl Neuroscience* 10(1):93-108

Rees S, Breen Sm Loelinger M, McCrabb G, and Harding R. (1999) Hypoxia near mid-gestation has long term effects on fetal brain development. *J Neuropathol Exp Neurol*. 58(9): 932-45

Rehn AE, Van Den Buuse M, Copolov D, Briscoe T, Lambert G, and Rees S. (2004) An animal model of chronic placental insufficiency: relevance to neurodevelopmental disorders including schizophrenia. *Neurosci.* 129: 381-391

Rocha E, Totten S, Han V, Richardson B. (2004) Structural proteins during brain development in the preterm and near term ovine fetus and the effect of intermittent umbilical cord occlusion. *Am J Obstet Gynecol* **191**: 497-505, 2004.

Rodriguez G, Collado MP, Samper MP, Biosca M, Bucno O, Valle S, Ventura P, and Garagorri JM. (2011) Subcutaneous fat distribution in small for gestational age newborns. *J Perinatol.* 39:355-57

Ruthazer ES, Li J, and Cline HT (2006) Stabilization of axon branch dynamics by synaptic maturation. *J Neurosci.* 26(13):3594-3603

Salafia CM, Charles AK and Maas EM. (2006) Placenta and growth restriction. *Clin Obstet Gynecol*. 49(2):236-256

Scheiffele P. (2003) Cell-cell signaling during synapse formation in the CNS. *Annu Rev Neurosci*. 26:485-508

Simons M and Trotter J. (2007) Wrapping it up: the cell biology of myelination. *Curr Opin Neurobiol*. 17 (5):533-540

Simonson M, Stephan JK, Hanson HM, and Chow BF. (1971) Open field studies in offspring of underfed mother rats. *J Nutr.* 101(3):331-5

Sparks JW, Girard JR, Callikan S, and Battaglia FC (1985) Growth of fetal guinea pig: physical and chemical characteristics. *Am Physiol Soc*. E132-39

Strauss RS and Dietz WHD. (1997) Effects of intrauterine growth retardation in premature infants on early childhood growth. *J Pediatrics. 130(1):*95-102.

Synnes AR, Anson S, Arkesteijn A, Butt A, Grunau RE, Rogers M, and Whitfield MF. (2010) School entry age outcomes for infants with birth weight ≤800 grams. *J Pediatr*. 157(6):989-94

Tarsa L and Goda Y. (2002) Synaptophysin regulates acitivity-dependent synapse formation in cultured hippocampal neurons. *PNAS* 99:1012-16

Temple S and Raff MC. (1986) Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions *Cell* 44, 773-779

Tisi DK, Emard JJ, and Koski KG. (2004) Total protein concentration in human amniotic fluid is negatively associated with infant birth weight. *J Nutri*. 134(7):1754-8

Tolcos M, Harding R, Loeliger M, Breen S, Cock M, Duncan J, and Rees S. (2003) The fetal brainstem is relatively spared from injury following intrauterine hypoxemia. *Dev Brain Res*. 143:73-81

Tolcos M and Rees S. (1997) Chronic placental insufficiency in the fetal guinea pig affects neurochemical and neuroglial development but not neuronal numbers in the brainstem: A new method for combined stereology and immunohistochemistry. *J comp neurol.* 379:99-112

Trajkovic K, Dhaunchak AS, Goncalves JT, Wenzel D, Schneider A, Bunt G, Nave KA, and Simons M. (2006) Neuron to glia signaling triggers myelin membrane exoycytosis from endosomal storage sites. *J Cell Biol.* 172(6):937-4

Turner AJ and Trudinger BJ. (2009) A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta.* 30:236-240

Verhage M, Maja AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, Van der Berg TK, Missler M, Geuze HJ, Sudhof TC. (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287(4): 864-67

Villar J, Altobelli L, Kestler E, and Belizan J. (1986) A healthy priority for developing countries: the prevention of chronic fetal malnutrition. *Bull WHO.* 64:47-51

Villar J, de Onis M, Kestler E, Bolanos F, Cerezo R, and Bernedes H. (1990) The differential neonatal morbidity of the intrauterine growth retardation syndrome. *Am J Obstet Gynecol*. 163(1 Pt 1):151-7

Walker DM and Marlow N. (2010) Neurocognitive outcome following fetal growth restriction. *Arch Dis Child Fetal Neonatal Ed.* 93:F322-25

Wigglesworth JS. (1964) Experimental growth retardation in the fetal rat. *J Pathol Bacteriol.* 88:1-13

Yang S, Platt RW, Kramer MS (2010) Variation in child cognitive ability by week of gestation among healthy term births. *Am J Epidemiol*. 171(4):399-406

CHAPTER 2

RATIONALE, HYPOTHESIS, AND RESEARCH OBJECTIVES

2.1 RATIONALE

Fetal growth restriction (FGR) is the second most common antenatal complication in developed countries after prematurity and increases the risk for morbidity and mortality in newborns (Ghidini 1996; Lackman 2001). It is also increasingly an issue in developed countries where limitations with diagnostic modalities and inconsistent literature make it difficult to consolidate treatment regiments. Sixty percent of FGR is idiopathic, deriving from an unknown combination of factors, but the majority of FGR is associated with placental insufficiency (Ghidini 1996; Pallotto 2006; Pollack 1992). Placental insufficiency is inefficiency at the maternal-placental-fetal border in the transport of oxygen and substrates resulting in a sub-optimal intrauterine environment for fetal growth. The guinea pig is an excellent model to use for study of human placental insufficiency because of the similarities in their placental structures. Both guinea pigs and humans have a discoid haemochorial placenta in which fetal vessels and maternal blood are in direct contact. Using a guinea pig model of placental insufficiency with decreased placental blood flow, one can induce FGR and study changes that have occurred to organs *in utero*. Existing techniques include uterine artery ligation (UAL), shown to induce FGR in at least one guinea pig fetus in 14%-30% of experimental litters (Jansson 1990; Laferber 1984). An alternate model, uterine artery branch diathermy (UABD) has been reported by Turner and Trudinger to induce FGR at a rate of 23% but has not been as thoroughly investigated unlike the more commonly used ligation model (Turner 2009). Thus, utilising these techniques should induce FGR in pregnant guinea pigs allowing for examination of alterations in fetal development.

Infants born growth restricted are at an increased risk of developing both short and long term neurological sequellae. FGR-born children have an increased risk of developing cerebral

palsy, attention deficit hyperactivity disorder, and autism spectrum disorder (de Rodrigues 2006; Halliday 2009; Indredavik 2010; Walker 2010.) As adults, the risk for schizophrenia, epilepsy, and for psychiatric hospitalization is increased in FGR offspring than those of normal size (Cannon 2003). More subtle deficits in cognitive function associated with FGR manifest at a young age. Children born growth restricted are associated with reduced cognitive skill, impaired memory and learning, poor academic performance, inattention, reduced psychosocial function, sensorineural deterioration, reduced mathematics abilities, and reduced IQ scores (de Rodrigues 2006; Geva 2008; Indredavik 2010; Pallotto 2006; Synnes 2010; Walker 2010). These neurological skills are functions of the hippocampus and associated afferent pathways such as the entorhinal cortex (Anderson 2007). These areas are endowed with the prenatal development of an abundance of neuronal connections in order to equip the brain with the capacity for learning postnatally (Bourgeois 1997). Changes to neuronal connectivity *in utero* could therefore contribute to the encephalopathies noted for infants born growth restricted. Few studies have examined changes in neuronal connections in large for gestational age (LGA) humans, although the paradigm of developmental programming associates many adverse postnatal outcomes with altered *in utero* development across spectrum including FGR and LGA. Thus, examining markers of neuronal connectivity at term in the precocious guinea pig would give insight to particular abnormalities in neurodevelopment that could contribute to early neurological deficits associated with altered birth weight.

A neuronal connection is dependent on proper migration of an axon and dendritic spine to their destined final area, followed by cell-to-cell contact in creation of an immature synapse, selective maturation of the synapse at the synaptic cleft and proper myelination of the axon in order to strengthen the synapse between axon and dendritic spine (Bourgeois 1997).

Synaptophysin (SYN) is a presynaptic vesicle membrane protein that is expressed in migrating axonal growth cones that later become immature synaptic connections upon contact with a dendritic spine (Calhoun 1996). Expression levels of SYN represent the extent of synapse formation. Synaptopodin (SYNPO) is a postsynaptic dendritic spine protein present only in mature synapses of telecephalic neurons (Mundel 1997). Therefore the degree of synapse maturation may be inferred by expression levels of SYNPO. Myelination is equally essential for neuronal communication as it controls conductance velocity needed for signal transduction. Proper conduction velocity, in turn, is a factor in synaptic maturation as activity is required to strengthen a synapse (Verhage 2000). Myelin basic protein (MBP) is essential for myelin formation and the extent of myelination may be determined by expression levels of MBP (Pedraza 1997). Together, examination of the expression of these synaptic and myelin markers in the hippocampus of guinea pigs in relation to size at birth may provide insight into *in utero* changes in neuronal connectivity associated with FGR and the mechanisms underlying the risk for later development of cognitive disorders in humans. This study will look at changes in protein expression of markers for synaptogenesis and myelination in the guinea pig brain at term focusing within the hippocampus.

2.2 HYPOTHESES

1. UAL and UABD at mid-gestation in guinea pig sows will induce fetal growth restriction in pups at term; with bilateral UABD optimizing fetal survival and the breadth of inducing altered growth more so than unilateral UAL.

2. Guinea pig pups at term, separated into cohorts based on body weight and proportion after UAL or UABD at mid-gestation will show differences in synaptogenesis in the brain. Compared to appropriate for gestational age (AGA) animals, FGR animals will have reduced synaptogenesis.
3. Guinea pig pups at term, separated into cohorts based on body weight and proportion after UAL or UABD at mid-gestation, will show differences in myelination in the brain. Compared to AGA animals, FGR animals will have reduced myelination.

2.3 OBJECTIVES

1. To apply either UAL or bilateral UABD techniques to pregnant guinea pig sows at mid-gestation in order to create a sub-optimal intrauterine environment for fetal development, that will lead to FGR in guinea pig fetuses; compare the survival rate and rate of inducing FGR using either technique; and study organ system development in relation to altered growth patterns.
2. To determine differences in synaptic protein expression between AGA guinea pigs and animals with altered growth at term. Specifically, examining SYN and SYNPO expression using immunohistochemisty and immunofluorescent techniques, respectively, in various sub-areas of the hippocampus at the coronal level of the entorhinal cortex. Also, to substantiate the histology using Western blots on the dorsal and ventral halves of the hippocampus at the same coronal level to determine changes in total SYN and SYNPO protein expression.
3. To determine differences in myelination between AGA guinea pigs and animals with altered growth at term. Specifically, examining MBP expression using immunohistochemistry and

differences in myelin formation using a luxol fast blue staining technique in various sub-areas of the hippocampus at the coronal level of the entorhinal cortex. Also, to substantiate the histology using Western blots on the dorsal and ventral halves of the hippocampus at the same coronal level to determine changes in total MBP protein expression.

2.4 REFERENCES

Anderson P, Morris R, Amaral D, Bliss T, and O'Keefe J. (2007) *The hippocampus book*. Oxford University Press.

Bourgeois JP. (1997) Synaptogenesis, heterochrony and epigenetics in the mammalian neocortex. *Acta Paediatr.* Suppl 422:27-33

Calhoun ME, Jucker M, Martin LJ, Thinakaran G, Price DL, and Mouton PR. (1996) Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J Neurocytol*. 25:821-28

Cannon TD, Van Erp TG, Bearden CE, Loewy R, Thompson P, Toga AW, Hutttunen MO, Keshaven MS, Seidman LJ, and Tsuang MT. (2003) Early and late neurodevelopmental influences in the prodrome to schizophrenia: contributions of genes, environment and their interactions. *Schizophr Bull.* 29(4):653-669

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

Geva R, Eshel R, Leitner Y, Fattal-Valevski A, and Harel S. (2008) Verbal short-term memory span in children: long-term modality dependent effects of intrauterine growth restriction. *J Pediatr*. 49(12):1321-30

Ghidini A. (1996) Idiopathic fetal growth restriction, a pathophysiologic approach. *Obs Gynecol Sur*. 51(6): 376-382

Halliday HL. (2009) Neonatal management and long-term sequelae. *Best Prac Clin Obstet Gynaecol.* 23:871-880

Indredavik MS, Vik T, Evensen KAI, Skranes J, Taraldsen G, and Brubakk AM. (2010) Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr* 31(4):286-94

Jansson T and Persson E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Ped Res.* 28:203-208

Lackman F, Campbell V, Richardson B, DaSilva O, and Gangon, R. (2001) The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol*. 184(5):946-53

Laferber HN, Rolph TP, and Jones CT. (1984) Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol.* 6:441-459

Mundel P, Heid HW, Mundel TM, Kruger M, Reiser J, and Kriz W. (1997) Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J Cell Biol* 139:193-204

Pallotto EK and Kilbride HW. (2006) Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynaecol*. 49(2):257-269

Pedraza L, Fidler L, Staugaitis SM, and Colman DR. (1997) The active transport of myelin basic protein into the nucleus suggests a regulatory role in myelination. *Neuron*. 18:579-589

Pollack RN and Divon MY. (1992) Intrauterine growth retardation: Definition, classification, and etiology. *Clin Obstet Gyne*. 35:99-107

Synnes AR, Anson S, Arkesteijn A, Butt A, Grunau RE, Rogers M, and Whitfield MF. (2010) School entry age outcomes for infants with birth weight ≤800 grams. *J Pediatr*. 157(6):989-94

Turner AJ and Trudinger BJ. (2009) A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta.* 30:236-240.

Verhage M, Maja AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, Van der Berg TK, Missler M, Geuze HJ, Sudhof TC. (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287(4): 864-67

Walker DM and Marlow N. (2010) Neurocognitive outcome following fetal growth restriction. *Arch Dis Child Fetal Neonatal Ed.* 93:F322-25

CHAPTER 3

FETAL GUINEA PIGS WITH ALTERED GROWTH AT TERM AFTER MID-GESTATION LIGATION OR ABLATION OF UTERINE VESSELS

3.1 INTRODUCTION

The guinea pig is an excellent animal model for the study of fetal growth restriction (FGR) and developmental programming. Unlike other rodents and more like humans, guinea pigs are precocious developers and undergo rapid brain growth in the latter half of gestation (Dobbing 1970). Therefore, intrauterine insults will coincide with critical periods of brain development and the associated long-term outcomes are more accurately extrapolated to humans. Guinea pigs also have a comparatively long gestation of approximately 65 days that allows sufficient time for manipulation and study (Banks 1989). Another similarity between the human and guinea pig fetus is in the structure and function of the placenta, whose transport mechanisms for substrates sustain fetal growth. They both have a discoid haemochorial placenta in which fetal vessels and maternal blood are in direct contact (Myers 1982). In sum, the guinea pig model is feasible for generational and long-term studies, similar in size to other rodents, and at the same time offers benefits in terms of comparative physiology to that of humans.

Reduction in blood flow between the mother, placenta and fetus is a common feature of placental insufficiency leading to FGR in human pregnancy. Placental insufficiency is a dysfunction of the placenta related to the inability of the placenta to transfer adequate nutrients and oxygen necessary to sustain growth of the fetus. Blood flow is a critical factor in guinea pig fetal growth since placental blood flow is directly related to fetal body weight and placental weight (Myers 1982; Peeters 1982; Saintonge 1981). There is extensive guinea pig literature regarding the anastamotic uterine-ovarian blood supply to the fetal-placental unit during pregnancy that describes the elaborate labyrinth of maternal-fetal vasculature supporting fetal growth. A study by Myers *et al.* reported that the largest fetus in the litter receives a surplus of placental blood flow, to the detriment of the runt of the litter (Myers 1982). Placental blood flow

also increases with increasing litter size and to the uterine horn with the greater number of fetal pups (Peeters 1982; Chiachareon 1976). Eckstein *et al.* showed that placental weight and subsequently fetal size decreased when there were an increased number of fetal pups in the contralateral horn (Eckstein 1955). As well, the position of the pup within the horn affects the growth of that fetus (Figure 3.1). The pups in the medial 1/3 end of the uterine horn closest to the uterine artery and the pups in the lateral 1/3 end of the uterine horn closest to the ovarian artery, tend to be larger than those in the middle 1/3 of the uterine horn (Chaichareon 1976; Ibsen 1928; Peeters 1982). Therefore adequate maternal blood flow to the fetus via the placenta is critical to achieve healthy fetal weight. With their relatively large average fetal size and a bicornate uterus, there are various techniques to abate uterine blood flow thereby inducing FGR.

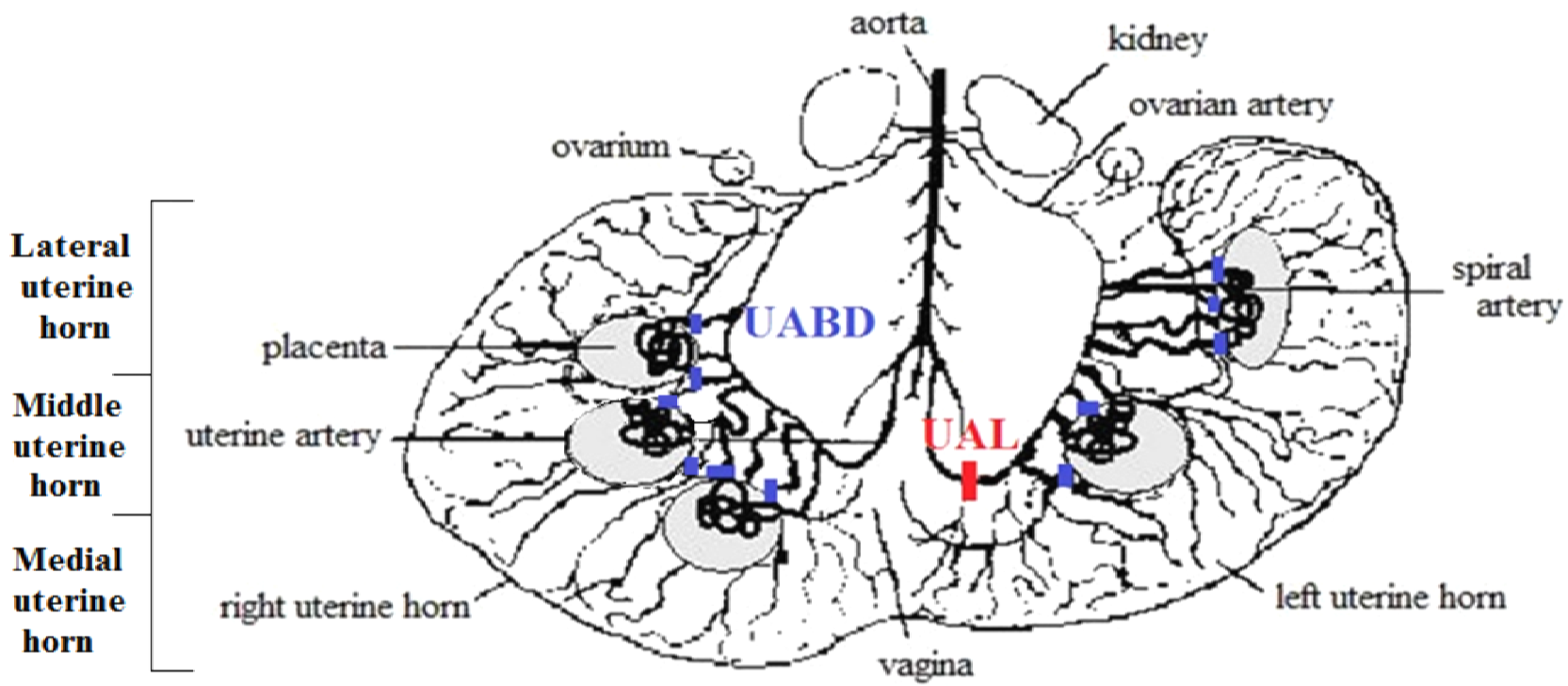
The unilateral uterine artery ligation (UAL) technique has been used as a model of placental insufficiency to induce FGR in rats (Olivier 2007), guinea pigs (Laferber 1984), and sheep (Rees 1988). Unilateral UAL is a ligation of the main uterine artery leading to one uterine branch of the bicornate uterus in guinea pigs (Figure 3.1). Applying UAL at the medial end of the uterine horn reduces blood flow, nutrients, amino acids and oxygen to the fetus during gestation (Jansson 1990; Jones 1983; Laferber 1984; Sparks 1985). Its effect on organogenesis occurs within hours (Jones 1983). Specifically, there is a large decrease in size of all organs except the heart, adrenal glands, and brain that show more subtle changes (Jones 1983; Laferber 1984). This technique induces varying degrees of FGR and mortality. Tolcos *et al.* induced FGR at a rate of 14% with the UAL technique which equalled, on average, to one growth restricted pup per litter (Tolcos 1997). Other studies reported one or more FGR guinea pig fetuses in 14%-30% of experimental litters (Jansson 1990; Laferber 1984). Turner and Trudinger reported a 78% rate of fetal demise in the ligated horn (Turner 2009). Generally, UAL causes growth restriction

on a graded scale depending on the proximity of the fetus to the ligated uterine artery, the duration of the insult throughout gestation and the number of remaining viable fetuses in the horn (Turner 2009; Wigglesworth 1964). The wide distribution of birth weight in UAL litters allows for the examination of relationships between birth weight and various developmental outcomes.

Turner and Trudinger (2009) introduced a cauterizing technique to increase the incidence of FGR in guinea pig litters and reduce the amount of fetal demise that occurs with UAL. Instead of ligating the main uterine artery leading to a horn in the bicornate uterus, this surgical technique involves cauterizing one of every three or two of every four uterine artery branches leading from the uterine artery to an individual fetal placenta (Turner 2009) (Figure 3.1). This technique results in 30% of guinea pig pups with body weight below 75g, a better distribution of FGR across positions in the horn, an increased survival rate at 53% suggesting a more balanced reduction of blood flow along the horn (Turner 2009; Camprubi 2009).

In humans, FGR is a risk factor for fetal mortality and morbidity leading to a variety of adult diseases including neurologic sequellae. It is important to establish a good animal model for FGR in order to gain insight into the mechanisms of the developmental origins of these diseases. This study uses UAL or uterine artery branch diathermy (UABD) in pregnant guinea pigs to reduce placental blood flow and induce FGR. Placental blood flow is a critical factor in human pregnancies as alterations are often the cause of placental insufficiency leading to FGR. This study will take a comparative look at the UAL and UABD techniques on the effectiveness of producing fetal growth restriction.

Figure 3.1 The guinea pig bicornate uterus (adapted from Laferber 1984). There is a medial and lateral end of the uterine horn depending on its proximity to the uterine artery or the ovarian artery, respectively. There are two depictions of abating uterine blood flow, uterine artery ligation (UAL) (red) is a ligation of the main uterine artery leading to one horn of the bicornate uterus using a silk thread. Uterine artery branch diathermy (UABD) (blue) is a selective cauterization of one of every three or two of every four uterine artery branches leading from the uterine artery to an individual fetal placenta.


aorta
kidney
ovarian artery
ovarium
Lateral uterine horn
Middle uterine horn
Medial uterine horn
spiral artery
placenta
UABD
uterine artery
UAL
right uterine horn
left uterine horn
vagina

3.2 MATERIALS AND METHODS

3.2.1 Surgical preparation

Thirty-five time-mated pregnant Dunkin-Hartly guinea pigs were purchased from Charles River Laboratories (Wilmington, Massachusetts) at 21 days gestation and housed in Animal Care and Veterinary Services at the University of Western Ontario according to guidelines set out by the Animal Ethics Committee. All of the sows were over 600g at the time of conception and placed in housing with a 12 hour light/dark cycle and constant access to food. At mid-gestation (~30 days gestation) the sows were anesthetised in an induction chamber at 2 L/min O_2 and 5% isofluorane and maintained under anesthesia with 1-1.5 L/min O_2 and 2-3% isofluorane. The sows were given a subcutaneous injection of glycopyrrolate (0.01 mg/kg.) The abdomen was shaved and cleaned with alcohol, toluene, and betadine and aseptic conditions were maintained. A 3 cm midline incision below the umbilicus exposed the bicornate uterus and the number of fetuses in each horn was noted visually (Figure 3.2).

Foremost, one sham control sow was selected when 2 or more sows were prepared for surgery on a given day (n=8). When possible, pregnant sows with large litter size $\geq$ 5 pups were deemed sham controls because of the increased risk for fetal demise (n=2). UAL sows (n=19) and UABD sows (n=8) were selected arbitrarily from sows, in most cases from sows with smaller litter sizes to increase the chance of survival of the pups and induce FGR.

3.2.2 Surgical procedure and post-operative care

Sham control sows had their incisions sutured closed shortly after the number of fetuses were counted, and were given 0.025 mg/kg Buprenorphine subcutaneously to aide with recovery.

The procedure usually took ~10 minutes to complete. One sow gave birth prior to experimental completion and was not included in further data analysis.

In the UAL sows, the uterine artery leading to the horn containing fewer pups was ligated with silk thread at the cervical end of the arterial anastamosis just caudal to the first branched labyrinth leading to the placentas (Figure 3.1). The sows subsequently had their incision sutured closed and recovered. The procedure usually took ~15 minutes to complete. One sow aspirated after surgery and died and three sows gave birth prior to experimental completion and were not used in further data analysis.

In the UABD sows, uterine arterial branches in both horns of the uterus were cauterized (Figure 3.1). Every second branch uterine branch artery leading to an individual placenta was cauterized using an Aaron 2250 electrosurgical generator (Bovine Medical, Clearwater Florida), effectively reducing the number of irrigating arteries by either one third (with 3 branch arteries) or one half (with 4 branch arteries). The incisions were subsequently sutured closed and animals recovered. The procedure usually took ~15 minutes to complete. One sow died prior to experimental completion and was not included in further data analysis.

3.2.2 Necropsy and organ collection

At term (~65 days gestation) the twenty-nine remaining sows were sacrificed using an intramuscular injection of 5 mg/kg Midazolam followed by 50 mg/kg Ketamine and 3 mg/kg Xylazine. The fetuses were immediately delivered via hysterotomy and any reabsorbed or demised fetuses were noted. Body weight and placental weights were obtained for all fetuses. A full necropsy was performed on the most medial two or three fetuses in each horn to ensure rapid

tissue collection and preserve tissue integrity. Brains were fixed by intracardial left ventricle perfusion with isotonic 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4 for 10 minutes each. Major fetal organs including: brain, heart, liver, and kidneys were weighed and gender was determined. These organs were then either fixed with 4% paraformaldehyde or fast frozen in liquid nitrogen and stored at -80ºC for later analysis.

3.2.3 Data acquisition and statistical analysis

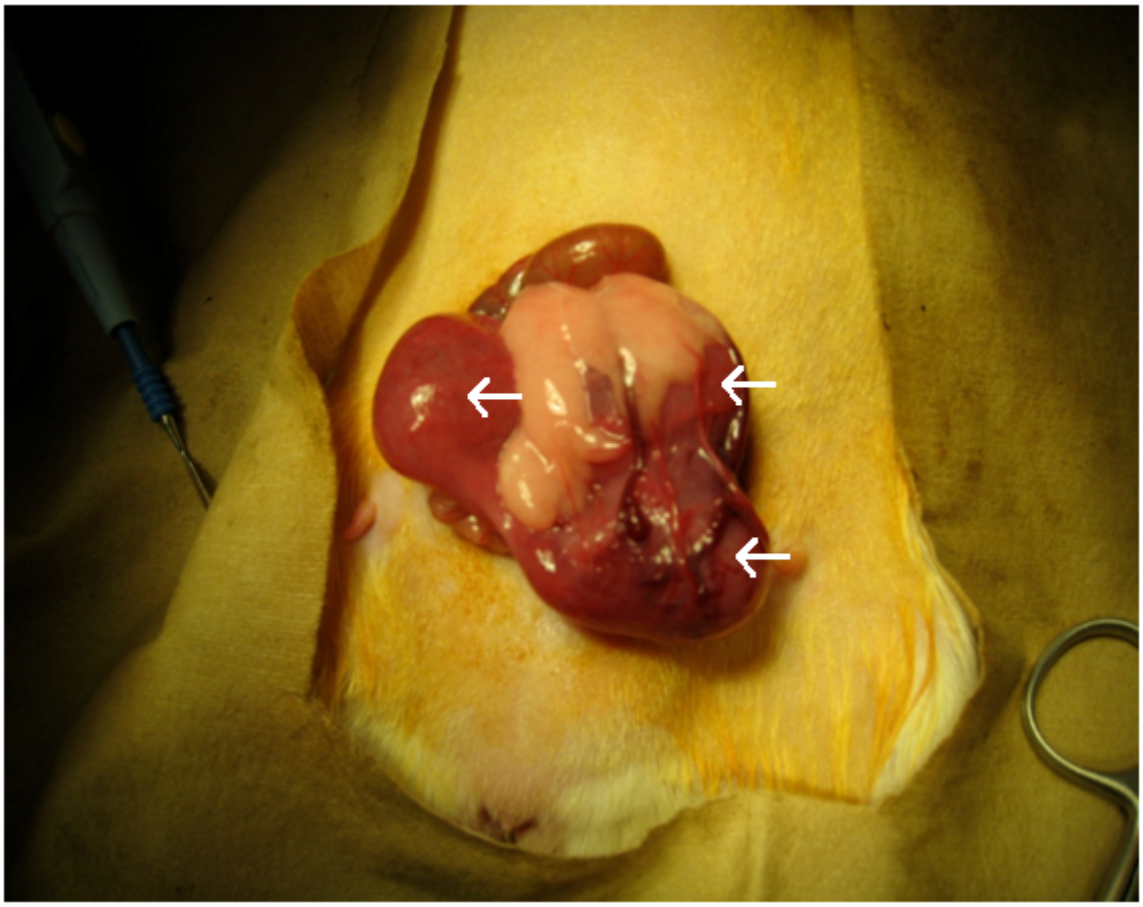
All fetuses were counted at put down of the pregnant sow. Litter size was based on the number of pups noted at the time of surgery at mid-gestation. Reabsorbed pups were counted as demised pups. Pups that were counted at the time of surgery at mid-gestation and still alive at the time of necropsy near term were counted as survivors. The number of fetuses per horn was also noted at the time of surgery and necropsy.

The fetal guinea pigs collected at term were divided into 4 cohort groupings that aligned with previously defined term guinea pig weight criteria. The sixty-three pups in the present study undergoing full necropsy were separated into quartiles based on body weight and brain to liver weight ratio. Those >75th percentile in body weight were called large for gestation age (LGA), the middle 25-75th percentile in body weight were called appropriate for gestational age (AGA) and the smallest 25th percentile in body weight were FGR. Thirty-six of the pups that had full necropsy data were selected to represent LGA animals whose weights were over 100 g and ± one standard deviation from the mean weight of all LGA animals (n=8), AGA pups who weighed between 80 and 100g and ± one standard deviation from the mean weight of all AGA animals (n=12), and FGR animals that weighed below 75g (n=16). The FGR animals were further subdivided on the basis of brain-sparing as indicated by brain-to-liver weight ratios. aFGR or

brain-sparing growth restriction animals were deemed to be those with brain-to-liver weight ratios above 0.85 (n=8), and symmetrical FGR (sFGR) or non-brain-sparing growth restricted animals were deemed to be those with brain weight-to-liver weight ratios below 0.85 (n=8), which was the same as for the AGA and LGA animals. According to literature, appropriate guinea pig term body weight at birth was defined as 80g-110g (Banks 1989), whereas FGR was indicated by fetal body weight <75g, and with asymmetrical FGR (aFGR) and brain-sparing indicated by brain weight to liver weight ratio >0.8 (Dieni 2003; Laferber 1984; Mallard 1999; Nitsos 1990; Rees 1992; Tolcos 1997; Turner 2009).

Fetal body weight and organ weights collected are presented in grams (g) as mean ± standard error of the mean (SEM). The correlation between litter size at mid-gestation or number of pups in a horn at term and fetal body weight at term was determined with Spearman's rank correlation coefficient analyses (r_s). The relationship between surgical treatment or position of the fetus in the horn and fetal body weight was examined using a one-way ANOVA with Tukey correction for multiple comparisons. The effect of gender on fetal body weight was determined using a student's t-test. Relationships between the cohort groupings and fetal body weight, brain weight, liver weight, brain-to-liver weight ratio, brain weight-to-body weight ratio or placental weight were determined using a one-way AVOVA comparing each cohort to the AGA cohort of animals. Comparisons of ratios were first transformed from a Cauchy distribution to create Normal distribution and then analyzed with the appropriate statistical test. All graphs present untransformed data. Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, California). For all analyzes, statistical significance was assumed for $p<0.05$.

Figure 3.2 Surgery at mid-gestation (~30 days gestation) showing the abdominal incision and revealing one uterine horn of the pregnant guinea pig bicornate uterus and three fetuses within (white arrows).



3.3 RESULTS

3.3.1 Generating fetal growth restriction

Twenty-nine guinea pig sows carried pups to term with necropsy at ~65 days, although one sow had no viable pups. The survival rates of the pups under different treatments are shown in Table 3.1. In sham control animals, 19 of 24 pups survived to term giving a survival rate of 79%. In the UAL sows, 18 of the 37 pups in the ligated horn survived to term giving a survival rate of 49%. In the non-ligated horn, 33 pups of the 53 pups noted at mid-gestation survived to term giving a survival rate of 62%. Overall in UAL sows, including fetuses from both ligated and non-ligated horns, the survival rate was 57%. Often it was the most medial pup, closest to the ligation that was resorbed. In UABD sows, 15 of 22 pups noted at surgery survived to term, giving a survival rate of 68%. The survival rates were not influenced by litter size or number of pups in a horn.

The weights for these surviving pups (n=85) ranged from 42 g to 129 g. There was a strong negative correlation where increasing litter size at mid-gestation was associated with decreasing fetal body weight at term (r_s= -0.5, $p<0.0001$) (Figure 3.3a). As shown in Figure 3.3b, there was also a strong negative correlation where increasing number of pups in the horn at term was associated with decreasing fetal body weight at term (r_s= -0.5, $p<0.0001$). Neither fetal gender nor position in the uterine horn showed a relationship with fetal weight at necropsy. The effect of litter size and number of fetal pups in a horn on body weight was evident for all three treatment groups.

The success in inducing FGR varied across treatment groups (Table 3.2). Twenty percent of the eighty-five pups that survived to term were deemed FGR: defined as the lowest quartile by

body weight and below 75 g in this study. In Sham control sows, 2 of 7 litters or 26% had one or more FGR pups. In UAL sows, 9 of 15 litters or 60% had one or more FGR pups; in the ligated horn 27% had one or more FGR pups while in the non-ligated horn 47% had one or more FGR pups. In UABD sows, 1 of 7 litters or 14% had one or more FGR pups. Figure 3.4 shows the mean fetal body weight at term for those pups subjected to full necropsy organized according to treatment group. There were no differences in mean fetal body weight at term in pups from control litters, either the ligated horn or non-ligated horn from UAL litters, or from UABD litters ($p>0.05$). Each treatment resulted in a range of fetal body weights that were not significantly different from each other. Compiling the pups from all three treatment groups allowed for analysis of fetal characteristics based on fetal weight and phenotype at term.

3.3.2 Fetal cohort characteristic data

Since it was evident multiple factors affected fetal body weight at necropsy, including litter size, number of fetuses per horn and likely the presence of demised pups within a horn, thirty-six of the sixty-three fetal guinea pigs collected at term were selectively organized into four cohorts for a step-wise analysis based on fetal body weight and body proportion as previously outlined. Figure 3.5 shows the body weight of the fetal cohorts. LGA pups (n=8) had larger mean body weights of 117 ± 3g than AGA pups (n=12) with mean body weights of 89 ± 1g ($p<0.05$). The aFGR pups (n=8) and sFGR pups (n=8) were the smallest animals with mean body weights of 53 ± 4g and 67 ± 3 g respectively. AGA pups were larger than aFGR and sFGR animals (both $p<0.05$), and aFGR and sFGR animals were not significantly different in body

weight from each other. LGA, AGA, aFGR and sFGR animals were further characterized on the basis of their brain weight-to-liver weight ratio.

For the four fetal cohorts, brain weight-to-liver weight ratios are depicted in Figure 3.6. The LGA animals had a brain-to-liver weight ratio of 0.42 ± 0.01 which is reduced than that of the AGA, aFGR and sFGR animals ($p<0.0001$). The sixteen FGR animals, comprising the aFGR and sFGR animal cohorts, were differentiated on the basis of in their brain-to-liver weight ratios with sFGR animals having brain-to-liver weight ratios below the median for all 16 animals (brain-to-liver weight ratio <0.82) and aFGR animals having a brain-to-liver weight ratio above the median for all 16 animals (brain to liver weight ratio >0.85). Upon comparing the four fetal cohorts (Figure 3.6), the brain-to-liver weight ratio of the aFGR animals at 1.07 ± 0.08 was larger than either the sFGR animals at 0.71 ± 0.03 or the AGA animals at 0.67 ± 0.02 (both $p<0.0001$). However, the sFGR cohort did not differ in brain-to-liver weight ratio from the AGA cohort ($p>0.05$).

3.3.3 Fetal cohort organ weights

Figure 3.7A and B show fetal placenta weights and fetal placenta-to-body weight ratios for the four fetal cohorts. The mean placenta weight for LGA animals was larger at 7.5 ± 0.3g than any of the other cohorts ($p<0.05$). The placenta weights for the AGA cohort (mean placenta weight of 5.5 ± 0.3g) were larger than the aFGR cohort (mean placental weight of 4.3 ± 0.5g, $p<0.05$). The placenta weights of the sFGR cohort (mean placenta weight of 4.7 ± 0.3g) did not differ from either the aFGR animals or the AGA animals. Mean fetal placenta-to-body weight ratios were not significantly different between any of the cohorts.

Liver size was decreased with decreasing body weight of the fetal cohorts (Figure 3.8). The LGA animals mean fetal liver weight of 6.4 ± 0.2g was increased than that of the AGA animals at 3.9 ± 0.1g, the aFGR animals at 2.3 ± 0.2g, and the sFGR animals at 3.2 ± 0.2g (all $p<0.0001$). AGA animal liver weights were larger than that of sFGR ($p<0.05$) and aFGR animals ($p<0.0001$), while sFGR animals had larger livers than aFGR animals ($p<0.0001$).

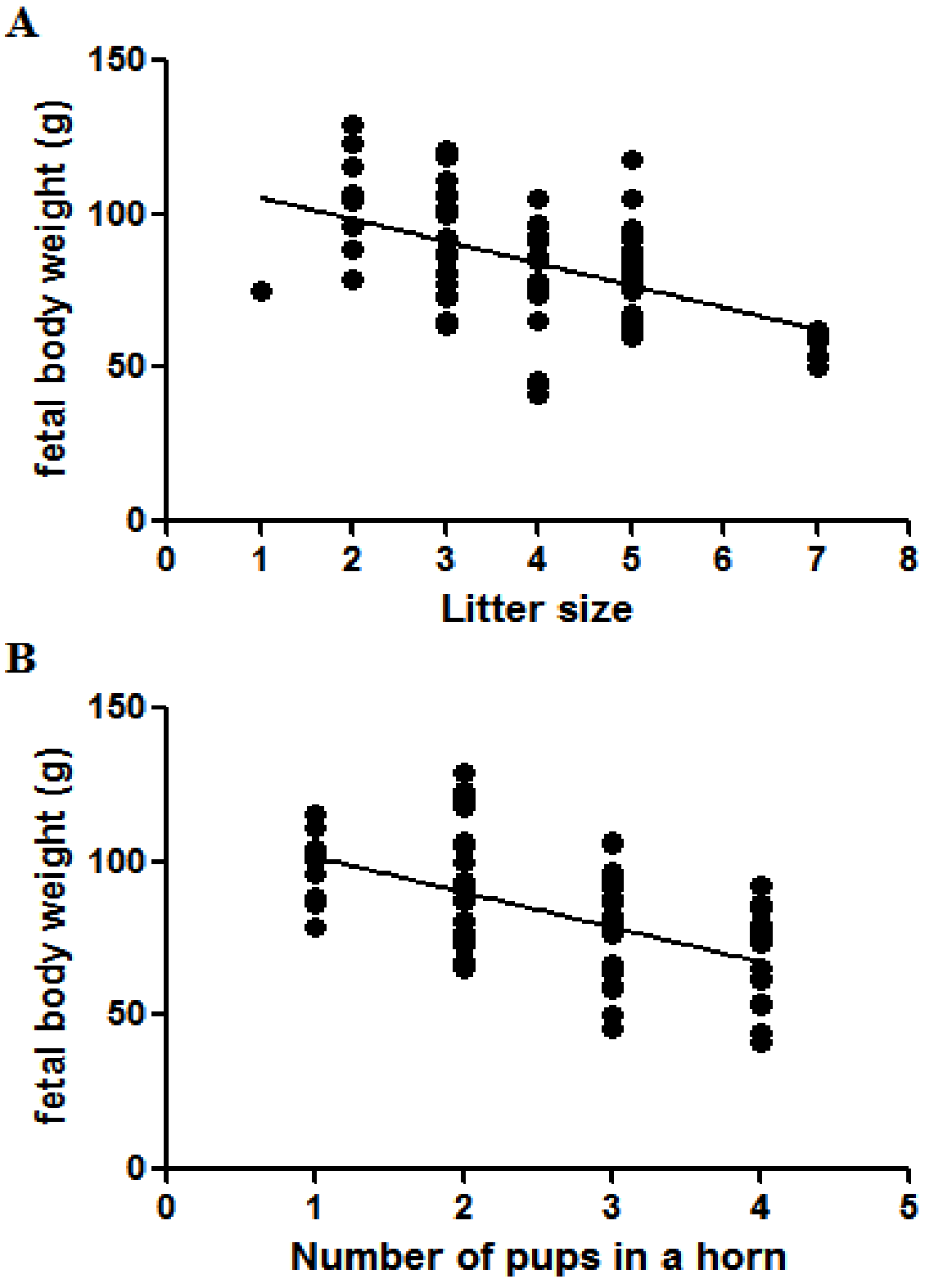
Brain weights did not follow the reduction in size as seen for the placenta and liver weights (Figure 3.9A). The mean fetal brain weights for LGA animals at 2.7 ± 0.1g and AGA animals at 2.6 ± 0.1g were not significantly different. For both of these cohorts, their brain weights were increased than that of the sFGR animals ($p<0.0001$) and aFGR animals ($p<0.05$). The sFGR animals mean fetal brain weight at 2.2 ± 0.1g and aFGR brain weights at 2.3 ± 0.1g were also not significantly different from each other. The brain-to-body weight ratios, as depicted in Figure 3.9B, shows the AGA animals had an increased ratio of 2.9 ± 0.1 than LGA animals with a ratio of 2.3 ± 0.1 ($p<0.05$). Both sFGR animals at 3.4 ± 0.1, and aFGR animals at 4.2 ± 0.2 had increased brain-to-body weight ratios than AGA animals (both $p<0.0001$). sFGR animals were also noted to have reduced brain-to-body weight ratios than aFGR animals ($p<0.05$).

Table 3.1 The survival rate of guinea pig fetuses from uterine artery treatment based on the number of pups noted at the time of surgery (~30 days gestation) and the number of viable pups upon collection at term (~65 days gestation). Survival rates presented as percent of total observed at surgery.

Surgical treatment	Fetal survival rate
Sham control (n=7)	79%
UAL (n=15)	57%
ligated horn	49%
non-ligated horn	62%
UABD (n=7)	68%

Figure 3.3 A The number of pups in the litter at the time of surgery (~30 days gestation) and the fetal body weight at term (~65 days gestation). There is a strong negative correlation between the number of pups in a litter and fetal body weight at term (r_s= -0.5, $p<0.0001$).

B The number of pups in horn at the time of surgery (~30 days gestation) and fetal body weight at term (~65 days gestation). There is a strong negative correlation between the number of pups in a litter and fetal body weight at term (r_s= -0.5, $p<0.0001$).


A
150
100
50
0
fetal body weight (g)
0
1
2
3
4
5
6
7
8
Litter size
B
150
100
50
0
fetal body weight (g)
0
1
2
3
4
5
Number of pups in a horn

\

Table 3.2 The rate of fetal growth restriction (FGR) based on treatment applied at time of surgery (~ 30 days gestation). FGR was a pup in the lowest quartile of body weight for the pups collected and thus under 75 g. Successful induction of FGR was the occurrence of one or more FGR pups within a litter of pups.

Surgical treatment	Litters with ≥1 FGR pup
Sham control (n=7)	26%
UAL (n=15)	60%
ligated horn	27%
non-ligated horn	47%
UABD (n=7)	14%

Figure 3.4 Uterine artery surgical treatment at mid-gestation (~30 days gestation) and fetal weight in grams at term (~65 days gestation). The fetuses were collected from sows that underwent different surgical procedures at mid-gestation, either from control sham (n=13), the ligated horn of uterine artery ligated (UAL) sows (LigH, n=13), the non-ligated horn of UAL sows (nonLigH, n=22), or uterine artery branch diathermy (UABD) sows (n=15), No surgical treatment caused a significant difference in fetal body weight.

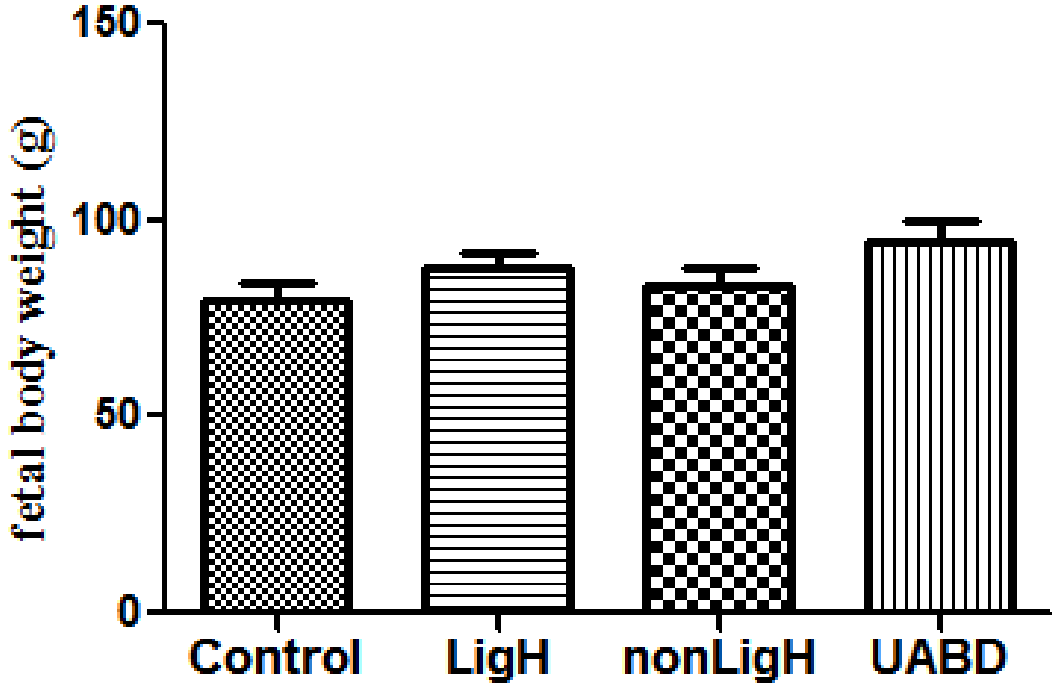

fetal body weight (g)
150
100
50
0
Control
LigH
nonLigH
UABD

Figure 3.5 Body weight at term of fetal cohorts showed that large for gestational age (LGA) animals (n=8, mean body weight 117 ± 3g) were larger ($p<0.05$) than appropriate size for gestational age (AGA) animals (n=12, mean body weight = 89 ± 1g.) Both AGA and LGA animals were larger than asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animals (n=8, mean body weight 53 ± 4g and n=8, mean body weight 67 ± 3g, respectively, both $p<0.05$) although aFGR and sFGR animals were not different in body weight from each other ($p>0.05$). Different letters indicates significantly different means = $p<0.05$.

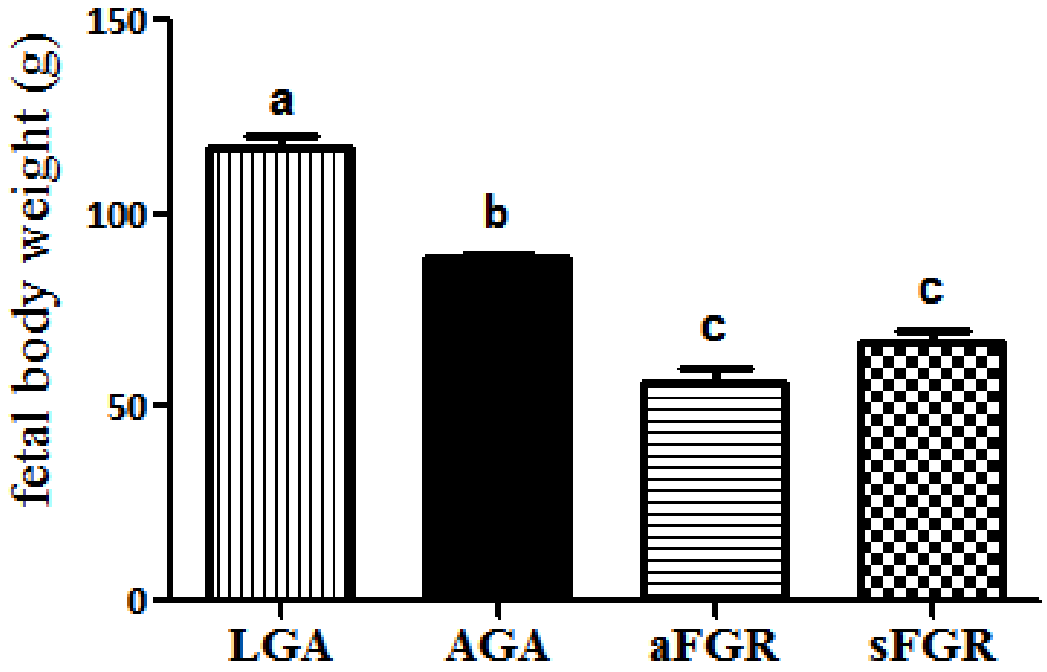

fetal body weight (g)
150
100
50
0
a
b
c
c
LGA
AGA
aFGR
sFGR

Figure 3.6 Brain-to-liver weight ratio of fetal cohorts showed that large for gestational age (LGA) animals had a significantly reduced ratio at 0.42 ± 0.1 than appropriate size for gestational age (AGA), asymmetrical fetal growth restricted (aFGR), and symmetrical fetal growth restricted (sFGR) animals ($p<0.0001$). The aFGR animals had an increased ratio at 1.07 ± 0.1 than LGA, AGA and sFGR cohorts ($p<0.0001$). The brain-to-liver weight ratios of AGA animals (0.67 ± 0.1) and sFGR animals (0.71 ± 0.1) were not significantly different. Different letters indicates significantly different means = $p<0.05$.

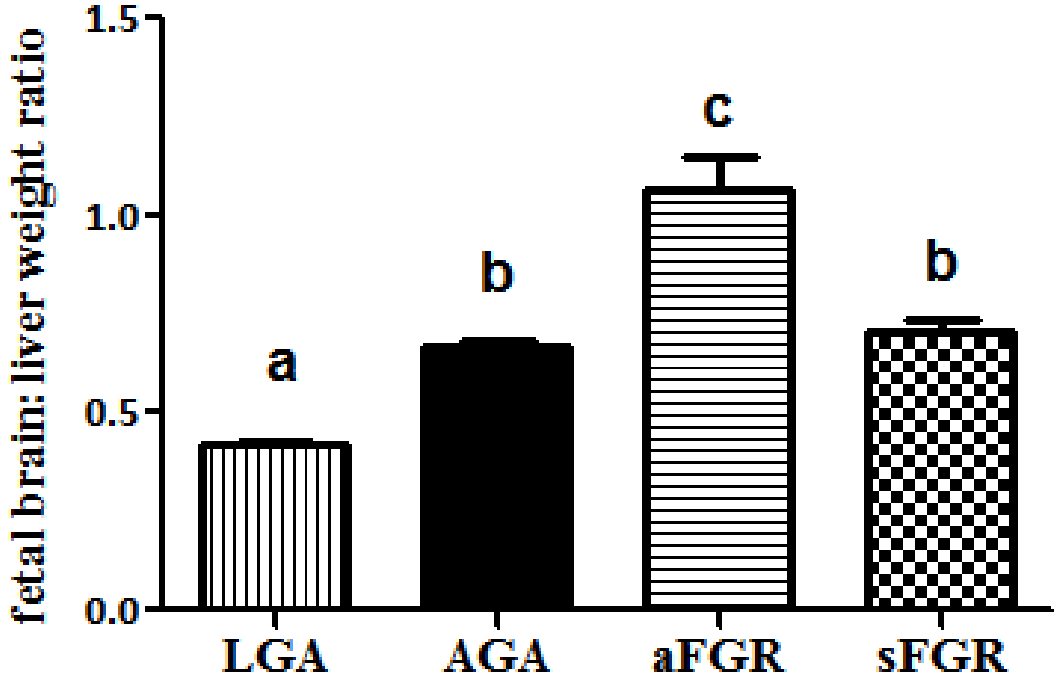

fetal brain: liver weight ratio
1.5
1.0
0.5
0.0
a
b
c
b
LGA
AGA
aFGR
sFGR

Figure 3.7 A Placental weight of fetal cohorts showed large for gestational age (LGA) animals had an increased mean placental weight at 7.5 ± 0.3g than appropriate size for gestational age (AGA), asymmetrical fetal growth restricted (aFGR), and symmetrical fetal growth restricted (sFGR) animals (all $p<0.0001$). The aFGR animals had a reduced placental weight than that of AGA animals ($p<0.05$). AGA and sFGR animals did not have a significant difference in placenta weight. Different letters indicates significantly different means = $p<0.05$.

B Placenta-to-body weight ratios of fetal cohorts did not show significant differences between any of the cohorts.

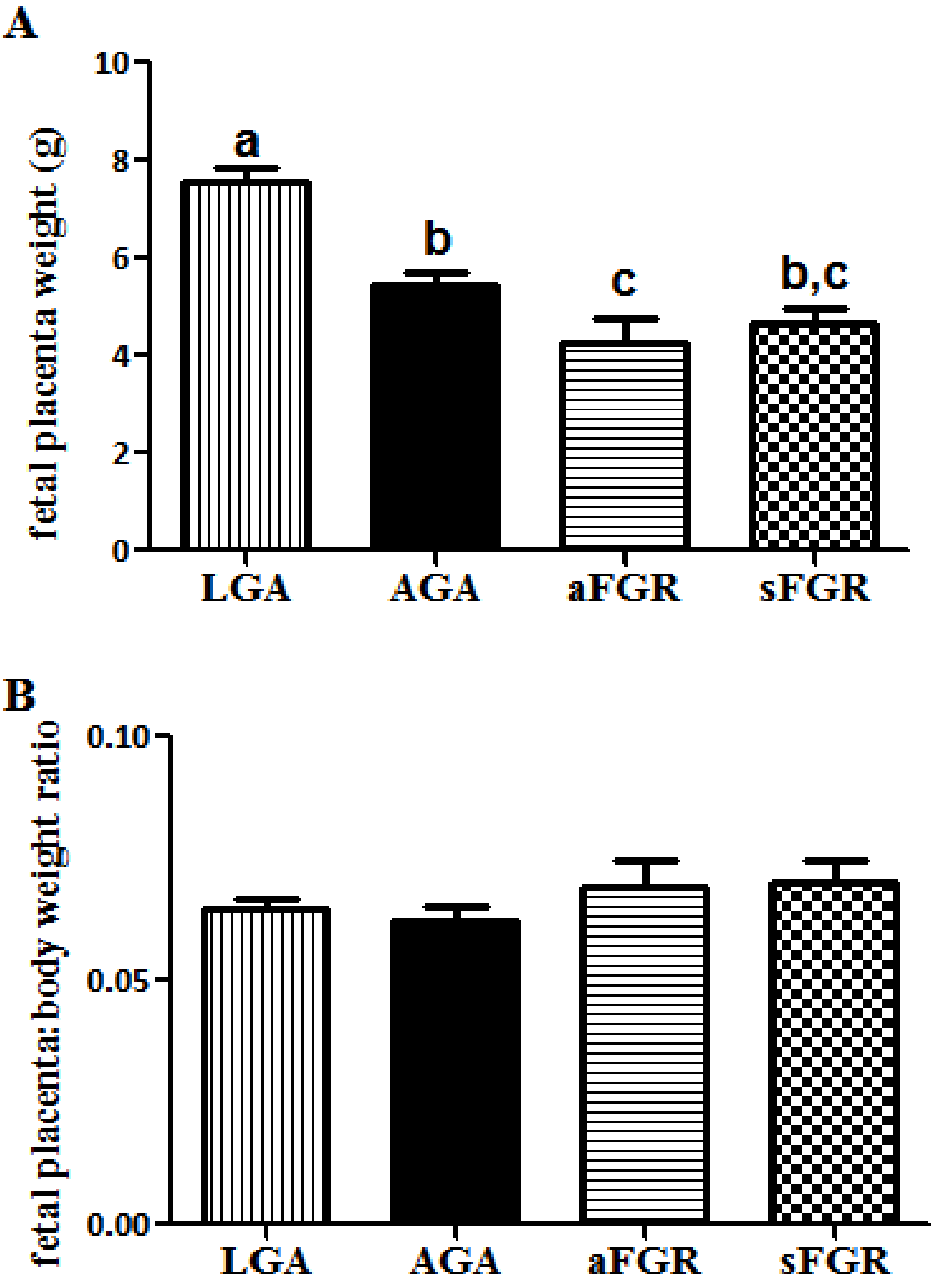

A
fetal placenta weight (g)
10
8
6
4
2
0
a
b
c
b,c
LGA
AGA
aFGR
sFGR
B
fetal placenta: body weight ratio
0.10
0.05
0.00
LGA
AGA
aFGR
sFGR

Figure 3.8 Liver weights of fetal cohorts showed that large for gestational age (LGA) animals at 6.4 ± 0.2g had an increased mean liver weight than appropriate size for gestational age (AGA) animals at 3.9 ± 0.1g, asymmetrical fetal growth restricted (aFGR) animals at 2.3 ± 0.2g, and symmetrical fetal growth restricted (sFGR) animals at 3.2 ± 0.2g (all $p<0.0001$). The AGA cohort had an increased mean liver weight than the aFGR animals ($p<0.0001$) and sFGR cohort ($p<0.05$). sFGR animals had increased mean liver weights than aFGR animals ($p<0.0001$). Different letters indicates significantly different means = $p<0.05$.

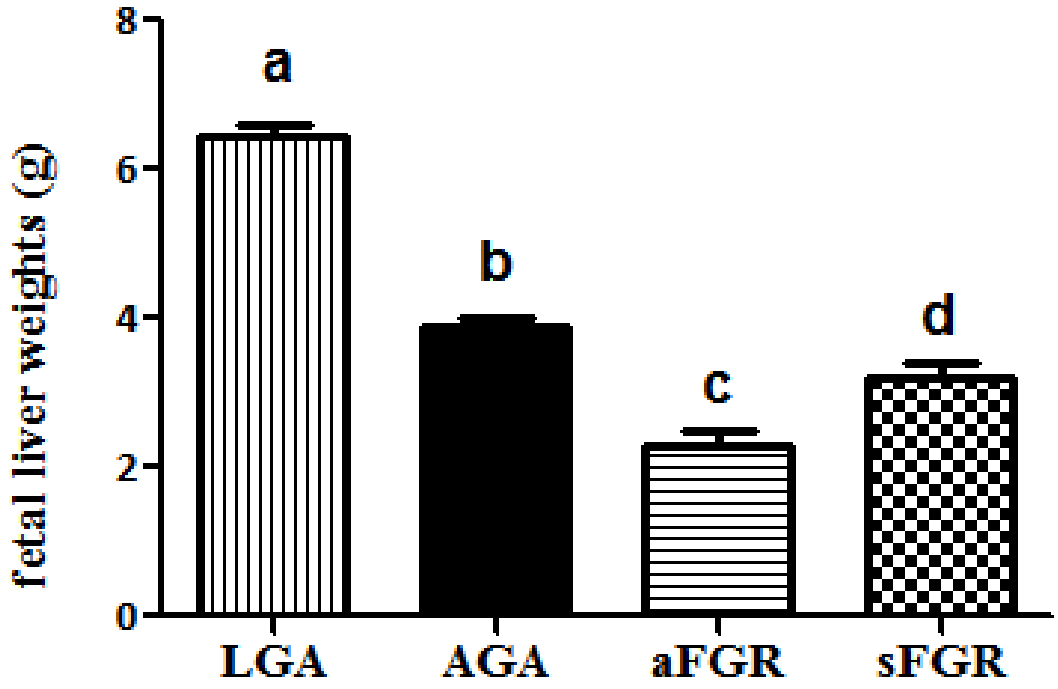
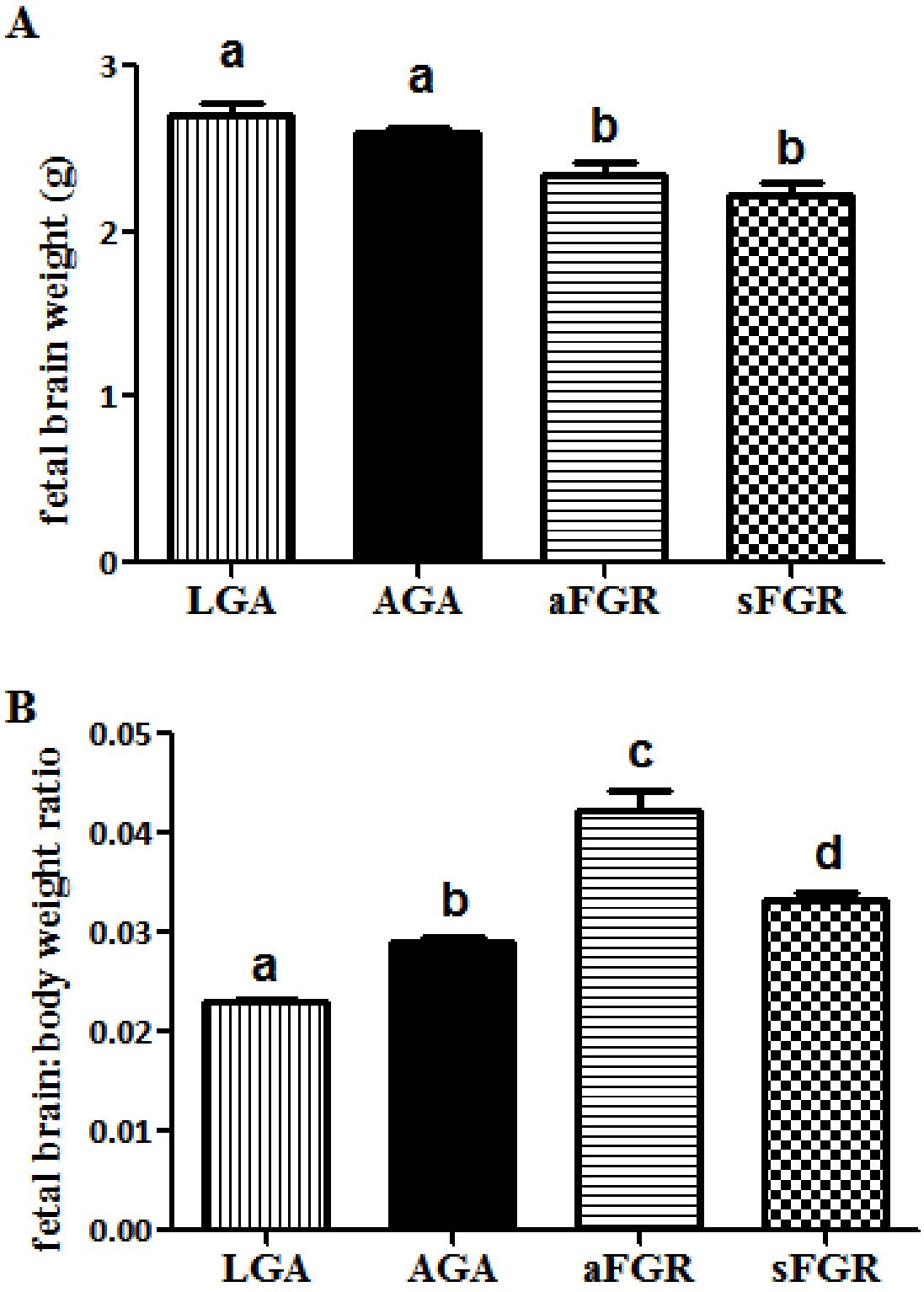

fetal liver weights (g)
8
6
4
2
0
a
b
c
d
LGA
AGA
aFGR
sFGR

Figure 3.9 A Brain weight of fetal cohorts showed the mean brain weights for large for gestational age (LGA) animals (2.7 ± 0.1g) and appropriate size for gestational age (AGA) animals (2.6 ± 0g) were not significantly different. Both these cohorts had increased brain weights than asymmetrical fetal growth restricted (aFGR) ($p<0.05$) and symmetrical fetal growth restricted (sFGR) animals ($p<0.0001$). aFGR brain weights at 2.3 ± 0.1g and sFGR brain weights at 2.2 ± 0.1g were also not significantly different. Different letters indicates significantly different means = $p<0.05$.

B Brain-to-body weight ratio of fetal cohorts showed the LGA animals had a reduced ratio at 2.3 ± 0.1 than the AGA animals at 2.9 ± 0.1 ($p<0.0001$). The sFGR animals (3.4 ± 0.1) and aFGR animals (4.2 ± 0.2) had increased weight ratios than AGA animals ($p<0.0001$) and were different from each other ($p<0.001$). Different letters indicates significantly different means = $p<0.05$.


A
fetal brain weight (g)
3
2
1
0
a
a
b
b
LGA
AGA
aFGR
sFGR
B
fetal brain:body weight ratio
0.05
0.04
0.03
0.02
0.01
0.00
a
b
c
d
LGA
AGA
aFGR
sFGR

3.4 DISCUSSION

In the present study, interference in maternal-placental blood flow was induced throughout the latter half of gestation. This stage of development corresponds to a time of rapid fetal growth in guinea pigs and a critical period of organ maturation (Ibsen 1928; Peeters 1982). In humans, impairment of oxygen and nutrient exchange between mother and fetus due to abnormal placental function is one of the primary causes of FGR in developed countries. The guinea pig is similar to the human in that considerable neurodevelopment occurs prenatally and it is an ideal animal model for long-term study of *in utero* insults. The goal of this study was to utilize this animal model for inducing FGR using traditional UAL and the more recently reported UABD and compare success rates along with survival rate.

Although, there was no significant relationship of fetal survival rate with treatment group, litter size or number of pups in a horn, this may have been impacted by the study design. The distribution of treatment based on litter size is unbalanced, with twice as many sows undergoing UAL than UABD or control sham surgery. The survival rate of our fetuses in the ligated horn of a UAL sow at 49% is increased compared to 28% reported by Turner and Trudinger (2009). This could be due to our horn selection for ligation, always the horn with the fewer number of pups. As well, the survival rates of fetuses subjected to UABD at mid-gestation at 68% is increased compared to the 50% reported in other studies (Camprubi 2009, Turner 2009). Of note, Turner and Trudinger (2009) found cauterization of the uterine artery branches caused placental infarcts further reducing blood flow and causing inflammation. The sham control animals had a 78% survival rate with the loss of pups occurring in horns carrying increased number of fetuses; large litter sizes are well known to have a higher incidence of still birth or spontaneous resorption of

pups during gestation (Banks 1989). A larger experimental number applying treatment to varying litter sizes would give better insight into the impact of treatment on survival rate.

The present study found the number of fetuses in a horn to be a significant factor affecting fetal body weight at necropsy. This may be due to limited ability of the uterine-ovarian artery anastamosis to provide sufficient blood flow to each fetus in a uterine horn with a large number of fetuses (Chaichareon 1976). Litter size, position in the horn, and number of pups in a horn and in the contralateral horn have all been previously shown to affect fetal body weight (Detmer 1992; Eckstein 1955; Chaichareon 1976). As well, redistribution of blood flow and increased vascularisation to the more populated horn occurs at the expense of the contralateral horn, when the number of fetuses in the more populated horn exceeds three (Peeters 1982; Eckstein 1955; Ibsen 1928). Blood flow increases in litters and horns that contain more pups (Detmer 1992). Limiting blood flow with UAL and UABD may make it difficult for this to occur, perpetuating FGR in litters and horns with more pups.

With the UAL technique in particular, the medial most fetuses were often resorbed allowing the more lateral fetuses to survive, those furthest from the ligation. Resorbtion of pups often occurs to increase the chance of survival of remaining pups in a horn by shunting available nutrients to certain pups at the expense of others, just as there is a redistribution of blood flow to the horn with the greater number of pups (Detmer 1992). Also, LGA pups came from litters with 1 or more resorbed pups at term and the majority of FGR pups came from litters without resorbed pups. Uterine artery blood flow to a fetus is proportional with body weight at term (Myers 1982; Peeters 1982; Saintonge 1981). Partitioning a finite amount of blood flow, limited by UAL or UABD, amongst pups in a sub-optimal intrauterine environment will result in each

pup receiving a reduced amount of blood flow resulting in FGR. This is similar to what occurs in a graded fashion with increasing litter size, wherein increasing vascularisation and blood flow with larger litter sizes is insufficient and leads to FGR. Upon resorbtion of a pup under such conditions, blood flow will increase to a surviving pup, potentially in excess, resulting in a LGA animal at term. Essentially, this alters Normal distribution of weights in pups to resemble a Student's t distribution that has emphasis on the tails taking into account the more often occurrence of values that would fall far outside of the mean. Accordingly, fetal pups were analyzed based on body weight at term and not mid-gestation treatment due to the various factors contributing to fetal size at term.

Term guinea pigs have previously established criteria for assigning growth weight categories with appropriate guinea pig term body weight between 80g-110g and FGR indicated by fetal body weight below 80g (Banks 1989; Mallard 1999). Furthermore, guinea pig models with placental insufficiency often seek to induce brain-sparing and leading to aFGR designated by brain weight-to-liver weight ratio >0.8 (Dieni 2003; Laferber 1984; Mallard 1999; Nitsos 1990; Rees 1992; Tolcos 1997; Turner 2009). However, few studies have examined sFGR as a pathological reduction in fetal growth, but maintaining the proportions of AGA fetuses which may involve earlier on-setting growth restriction and/or failure or brain sparing mechanisms (Pallotto 2006, Pollack 1992). Quartiles were chosen to examine changes in fetuses based on body weight and proportion, with growth characteristics consistent with that used by others. The four cohorts of fetal animals in the present study, LGA, AGA, aFGR and sFGR were delineated based on birth weight quartiles and the brain-to-liver weight ratio. The LGA animals in the present study were greater than 100g in weight, while the FGR animals were below 75g in weight. The brain-to-liver ratio was used to further differentiate the aFGR and sFGR term

fetuses. The aFGR animals had brain to liver weight ratios above 0.82 and previous studies have used a brain to liver weight ratio above 0.8 as indicative of aFGR in term guinea pigs (Banks 1989; Dieni 2003; Laferber 1984; Mallard 1999; Nitsos 1990; Rees 1992; Tolcos 1997; Turner 2009). While some sFGR fetuses may be constitutionally small and thereby their 'symmetrical' growth, sFGR fetuses may also result for earlier onsetting of growth restriction or failure of 'brain sparing; mechanisms. Therefore, as occurs in the clinically in humans alterations in fetal growth was deemed herein using body weight and body proportion cut-off standards.

LGA can be a healthy condition, or else low brain-to-liver weight ratio may indicate disease from otherwise healthy AGA fetuses. LGA babies come from mothers with high triglyceride levels and gain excessive weight through pregnancy (Mattos 2011). With certain disorders, there may be excess nutrients that bloat visceral organs but with little effect on brain weight, resulting in a low brain-to-liver ratio. Rodriguez *et* al. (2011) found in humans increasing fat deposition with LGA compared to AGA which would impact liver size as well as reductions in visceral fat deposition in aFGR compared to sFGR and AGA. Storage in fat and desensitization to excess fat could lead to programming of metabolic diseases. It can be indicative of an adapting HPA axis and an increased risk for cardiovascular disease, obesity, and diabetes in later life for LGA animals (Barker 1999). In terms of fetal programming, risk increases in a 'U'-shaped or curvilinear pattern with highest risk for programming occurring in FGR and LGA fetuses. Thus although not the focus, LGA animals are included in analysis to see if this patterning occurs in our study.

Not surprisingly, liver size in the present study was progressively smaller with decreasing fetal weight; with the smallest liver weights in the aFGR animals. The liver is the first organ affected by *in utero* insults leading to growth restriction and has the most pronounced changes

with the severity of changes in the liver proportional to the severity of aFGR (Carter 1993; Laferber 1983). This involves a reduction in hepatocyte cell volume, higher glycogen concentration, decrease in hepatic triacylglycerol, reduced protein concentration, and reduced haematopoietic cells all suggestive of a delay in the differentiation of liver cells as well as reduced fat storage (Laferber 1983). Liver size is a crude indicator of fetal development along the curvilinear spectrum of risk relating to programming of disease and life-long health.

Brain weight is relatively maintained across the fetal cohorts as it is an organ that receives increased blood flow with blood flow redistribution upon uterine artery manipulation (Detmer 1992; Hershkovitz 2000). However, aFGR and sFGR animals had significantly reduced brain weight compared to AGA and LGA animals. This is consistent with Turner and Trudinger's findings (2009) where altering uterine artery irrigation with UABD and UAL decreased brain weights. Brain-to-body weight ratio of these pups is more indicative of appropriate relative brain size. Each cohort had a significantly different brain-to-body weight showing the proportions of their development. aFGR was characterized by a large head and small body, and thereby the largest brain-to-body ratio. LGA had a normal head and large body reflected as the smallest brain-to-body weight ratio due to the large liver and presumed excess fat deposits. While AGA and sFGR cohorts had similar brain-to-liver weight ratios, they have different brain-to-body weight ratios. This is indicative of the smaller body weight of the sFGR animals while the brain weight was relatively maintained.

Maternal-placental blood flow is positively related to placenta size which is proportional to fetal size especially in large fetuses (Jansson 1990; Jones 1983; Myers 1982; Peeters 1982). This study reflects this, in that the FGR animals had the lowest placental size and the LGA animals had the highest placental size possibly reflecting the amount of blood flow to the

placenta in either situation. The placenta-to-fetal body weight ratio decreases dramatically throughout gestation as the placenta undergoes a 40 fold increase in size in guinea pigs reflecting an enhanced efficiency of transfer through the placenta supporting exponential fetal growth (Myatt 2006). No significant differences in placenta-to-body weight ratios were found across the cohorts in the present study which suggests placental efficiency remained unaltered across the study groups and did not impact birth weight outcomes.

3.5 CONCLUSION

This study examined the growth changes with chronic UAL and UABD in guinea pigs. The procedure to induce FGR involved manipulating uterine artery blood flow to the fetus using a UAL or UABD technique. It is well documented that many factors affect fetal size including litter size, crowding in the uterine horn, maternal metabolism, placental blood flow, and fetal position. This study attempted to take advantage of many factors that reduce fetal weight by altering blood flow in litters of various sizes at mid-gestation ~30 days, and prior to major organ differentiation and exponential fetal body growth. This led to a spectrum of fetal body weights at term (~65 days gestation) creating LGA, AGA, aFGR and sFGR cohorts of fetuses to study the relationship of body weight with various developmental outcomes. Liver, placental, and brain weights were compared between the cohorts to identify changes in organ growth that can be associated with FGR. In conclusion, this study identified limitations of using UAL or UABD in a guinea pig model of placental insufficiency in terms of survival rate and inducing a variety of body weights in litters at term. Furthermore, fetal guinea pig cohorts were delineated and chabased on body weight and proportion at term and characterized by differences in organ size

and proportions, will allow for analysis of developmental changes in the brain according to body weight at term.

3.6 REFERENCES

Banks R. (1989) The guinea pig: biology, care, identification, nomenclature, breeding, and genetics. *USAMRIND Sem Ser*.

Barker DJP. (1998) *In utero* programming of chronic disease. *Clin Sci*. 95:115-128

Carter A. 1993. Current topic: restriction of placental and fetal growth in the guinea pig. *Placenta* **14**: 125-135.

Camprubi M, Balaguer A, Iglesias I, Girabent M, and Callejo J. (2009) Cauterization of meso-ovarian vessels, a new model of intrauterine growth restriction in rats. *Placenta.* 30(9):761-766

Chaichareon DP, Rankin JH, and Ginther OJ. (1976) Factors which affect the relative contributions of ovarian and uterine arteries to the blood supply of reproductive organs in guinea pigs. *Biol Repro.* 15:281-290

Detmer A and Carter AM. (1992) Factors influencing the outcome of ligating the uterine artery and vein in a guinea pig model of intrauterine growth retardation. *Scand J Lab Anim Sci.* 19:9-15

Dieni S and Rees S. (2003) Dendritic morphology is altered in hippocampal neurons following prenatal compromise. *J Neurobiol.* 55(1): 41-52

Dobbing J and Sands J. (1970) Growth and development of the brain and spinal cord of the guinea pig. *Brain Res*. 17:115-123

Eckstein P, McKeown, T, and Record RG. (1955) Variations in placental weight according to litter size in the guinea pig. *J Endocrin.* 12:108-114

Hershkovitz R, Kingdom JCP, Geary M, and Rodeck CH. (2000) Fetal cerebral blood flow redistribution in late gestation: identification of compromise in small fetuses with normal umbilical artery Doppler. *Ultrasound Obstet Gynecol.* 15:209-212

Ibsen HL. (1928) Prenatal growth in guinea-pig with special reference to environmental factors affecting weight at birth. *J Exp Zool.* 51:51

Jansson T and Persson E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Ped Res.* 28:203-208

Jones CT and Parer JT. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *J Physiol.* 343:525-537

Laferber HN, Rolph TP, and Jones CT. (1984) Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol.* 6:441-459

Mallard C, Rehn A, Rees S, Tolcos M, and Copolov D. (1999)Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: implications for the aetiology of schizophrenia. *Schizop Res.* 40:11-21

Mattos SS, Chaves ME, Costa SM, Ishigami AC, Rego SB Souto MV, Severi R, and de Lima Filho JL. (2011) Which growth criteria better predict fetal programming? *Arch Dis Child Fetal Neonatal Ed.* Mar 27 [Epub ahead of print]

Myers SA, Sparks JW, Makowski EL, Meschia G, and Battaglia FC. (1982) Relationshipp between placental blood flow and placental and fetal size in guinea pig. *Am J Physiol.* 243(3):H404-

Myatt L. (2006) Placental adaptive responses and fetal programming. *J Physiol*. 572(1):25-30

Nitsos I and Rees S. (1990) The effects of intrauterine growth retardation on the development of neuroglia in fetal guinea pigs. An immunohistochemical and an ultrastructural study. *Int J Devl Neurosci.* 8(3): 233-244

Olivier P, Baud O, Bouslama M, Evrard P, Gressens P and Verney C. (2007) Moderate growth restriction: deleterious and protective effects on white matter damage. *Neurobiol Dis* 26(1):253-263

Pallotto EK and Kilbride HW. (2006) Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynaecol*. 49(2):257-269

Peeters LLH, Sparks JW, Grutters G, Girard J, and Battaglia FC. (1982) Uteroplacental blood flow during pregnancy in chronically catheterized guinea pigs. *Pediatr Res.* 16:716-720

Pollack RN and Divon MY. (1992) Intrauterine growth retardation: Definition, classification, and etiology. *Clin Obstet Gyne*. 35:99-107

Rees S and Harding R. (1988) The effects of intrauterine growth retardation on the development of the purkinje cell dendritic tree in the cerebellar cortex of the fetal sheep: a note on the ontogeny of the purkinje cell. *Int J Dev Neurosci.* 6(5):461-669

Rees S and Bainbridge A. (1992) The structure and neurochemical development of the fetal guinea pig retina and optic nerve in experimental growth retardation. *Int J Neurosci.* 10(1):93-108

Rodriguez G, Collado MP, Samper MP, Biosca M, Bucno O, Valle S, Ventura P, and Garagorri JM. (2011) Subcutaneous fat distribution in small for gestational age newborns. *J Perinatol.* 39:355-57

Saintonge J and Rosso P. (1981) Placental blood flow and transfer of nutrient analogs in Large, Average, and guinea pig littermates. *Pediatr Res.* 15:152-156

Sparks JW, Girard JR, Callikan S, and Battaglia F. (1985) Growth of fetal guinea pig: physical and chemical characteristics. *Am J Physiol.* 248:E132-9

Tolcos M and Rees S. (1997) Chronic placental insufficiency in the fetal guinea pig affects neurochemical and neuroglial development but not neuronal numbers in the brainstem: A new method for combined stereology and immunohistochemistry. *J comp neurol.* 379:99-112

Turner AJ and Trudinger BJ. (2009) A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta.* 30:236-240.

Wigglesworth JS. (1964) Experimental growth retardation in the fetal rat. *J Pathol Bacteriol.* 88:1-13

CHAPTER 4

THE IMPACT OF FETAL GROWTH RESTRICTION ON SYNPASE FORMATION AND MATURATION IN THE GUINEA PIG BRAIN AT TERM

4.1 INTRODUCTION

Fetal growth restriction (FGR) is associated with a number of cognitive deficiencies. In children, FGR leads to reduced cognitive skills including impaired memory and learning, inattention, reduced psychosocial function and lower mathematics abilities and intelligence quotient scores (de Rodrigues 2006; Geva 2008; Indredavik 2010; Pallotto 2006; Synnes 2010; Walker 2010). Although many adult disorders are associated with altered neuronal connectivity and signal transduction in the brain, most early studies of cognitive ability only infer aberrant neuronal connectivity in their etiology. Studies examining FGR have shown changes in neurogenesis, FGR in animal models gives rise to reduced number and length of neurons (Mallard 2000; Tolcos 1997), size of the dendritic tree (Dieni 2003), and a decreased dendritic spine density (Dieni 2003). Changes in neuronal connectivity associated with FGR could underlie early cognitive deficiencies in life.

Examining neurodevelopmental outcome based on body weight alone is insufficient, since FGR can present as asymmetrical (aFGR) or symmetrical (sFGR). The sFGR fetus has an earlier onsetting growth restriction that affects the brain and other tissues similarly while aFGR fetuses with later onsetting growth restricted have more mature adaptive mechanisms that spare relative brain growth at the expense of other tissues (Halliday 2009; Pallotto 2006; Walker 2010). Few studies have examined changes in neuronal connections in large for gestational age (LGA) humans, although the paradigm of developmental programming associates many adverse postnatal outcomes with altered *in utero* development across spectrum including FGR and LGA. Most neurodevelopmental outcomes are based on aFGR findings with few studies examining sFGR and associated cognitive deficiencies, although both phenotypes are indicated to have altered brain development. There is MRI evidence that fetal brain growth slows prior to growth

of the cranial vault and in one human study relating head circumference with cognitive ability, cognitive deficiencies are more severe with early-onset (prior to mid-gestation) reduced head circumference *in utero* (Duncan 2005; Harvey 1982). Thus, examining aFGR and sFGR fetuses and changes in neuronal connectivity could give insight to life-long neurological health.

Cognitive ability correlates to endowment of neuronal networks and synapses, as they are the major area of communication between neurons. The hippocampus is an area critical for learning and memory. Studies of human patients with hippocampal damage show difficulties with both word and face recognition tests (Reed 1997). This would make it inherently difficult to learn and remember lessons in school and social interaction would be challenging. A study in mice, found the hippocampus has a particular role in consolidation of memory and that deficiencies in memory related tasks were correlated with a decrease in probability of neuronal firing from CA3 to CA1 (Nakashiba 2009). Intact neuronal networks in the hippocampus are essential for proper cognitive functioning.

Precocious guinea pigs like humans, undergo neuronal differentiation and the highest absolute rate of synaptic formation during fetal gestation (Dobbing 1971). This results in the culmination of number of synapses in the brain to occur around term. Synapse formation is complex requiring presynaptic and postsynaptic element coupling, and adequate neuronal growth, migration, and branching to achieve proper localization of a synapse (Scheiffele 2003). Generally, this begins with a presynaptic axon and postsynaptic dendrite spine coalescing to form an immature synapse (Scheiffele 2003). The specialized mechanism underlying synaptic development are poorly understood but presynaptic maturation is evident with an increase in the number of vesicles as well as increase in reliability of neurotransmitter release (Ruthazer 2006). The amount of vesicle production in the synapse increases with its maturation and decreases

steadily if the synapse is degenerating (Ruthazer 2006). At the synaptic cleft there is a presynaptic active zone, to which vesicles adhere to prior to being exocytosis, and juxtaposed to it is a postsynaptic density on the dendritic spine that anchors receptors and scaffolding proteins (Garner 2000; Bourne 2008). Together they mature to form a functioning synapse and establish neuronal communication.

Synaptophysin (SYN) is a common 38 kDa presynaptic protein marker present in the presynaptic bouton and on the membrane of presynaptic vesicles in the central nervous system (Calhoun 1996; Jahn 1985). SYN shows punctate staining along a neuron localized to the presynaptic boutons (Calhoun 1996; Fletcher 1991; Mundel 1997). Additionally, it is reported that SYN is localized in neuronal growth cones in immature axons that lead axonal migration therefore it may be found in small quantities in white matter (Calhoun 1996; Fletcher 1991; Leclerc 2010). It is also found in very immature synapses prior to vesicle development and in synaptic vesicles when the synapse matures (Daly 1997; Fletcher 1994). Thus, SYN protein levels increase directly with synapse formation and maturation (Daly 1997; Fletcher 1991). It has been found in both inhibitory and excitatory synapses and is involved in competitive strengthening of synapses as well as activity dependent synapse maturation (Tarsa 2002). SYN is often used as a protein marker to reflect the presence of synapse and changes overall in synaptic number.

The protein machinery that creates neurotransmitter receptors and a mature synapse is located in the postsynaptic dendritic spine. Synaptopodin (SYNPO) is a 100 kDa protein found in dendritic spines of telencephalic neurons. It is closely associated with the spine apparatus that plays a critical role in learning and memory (Mundel 1997; Deller 2000; Deller 2003). SYNPO is an actin-binding protein thought to store calcium and locally regulate calcium dependant

mechanisms and be involved in spine elongation and morphology (Deller 2000b; Deller 2003; Majewska 2000). As well, SYNPO knockout mice do not form spine apparatuses (Deller 2003). In the developing rat brain, SYNPO is present only during the last third of gestation and is only in mature synapses in the brain (Deller 2000b). Expression of SYNPO protein in the hippocampus would indicate changes in number of mature synapses.

Synapse formation is intricate and involves successful completion of many processes in order to occur, 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. *In utero* changes in synapse formation and consequent deficiencies in neuronal communication within the hippocampus could underlie the cognitive delays seen in children born small. Although, the guinea pig brain is relatively larger and at a more advanced stage of functionality than a human brain at term, both humans and guinea pigs are said to have full synaptic and neuronal numbers at term (Lennon 1980). Therefore the size of the guinea pig brain is thought to be an advantage for analyses, as the impact of impaired development may be magnified in the guinea pig brain in comparison to humans. The present study tested the hypothesis that induced FGR in guinea pig fetuses at term will result in reduced SYN and SYNPO expression as measured by histology and total protein levels as measured by Western blot analysis in the hippocampus and thereby indicating altered synapse formation and maturation compared to appropriate for gestational age (AGA) animals.

4.2 MATERIALS AND METHODS

4.2.1 Surgical preparation and necropsy

At ~30 days gestation pregnant guinea pigs underwent uterine artery ligation (UAL), uterine artery branch diathermy (UABD), or sham control surgery as previously described (Chapter 3). At term (~65 days gestation), sows (n=29) were anesthetized using an intramuscular injection of 5mg/kg Midazolam followed by 50 mg/kg Ketamine and 3 mg/kg Xylazine. The fetuses were delivered via hysterotomy and body weight and placental weights were obtained for all fetuses. A full necropsy was performed on the most medial two or three fetuses in each horn.

4.2.2 Fetal guinea pig cohorts and brain processing for histology

Thirty-six fetal guinea pigs that underwent full necropsy were organized into 4 cohorts; large for gestational age (LGA), AGA, aFGR, and sFGR as previously described (Chapter 3). Figure 4.1 shows the mean fetal body weights for LGA pups (n=8) was 117 ± 3g and for AGA pups (n=12) was 89 ± 1g. sFGR pups (n=8) and aFGR pups (n=8) were the smallest with mean fetal body weights of 67 ± 3g and 53 ± 4g, respectively (Figure 4.1). The FGR animals were further subdivided on the basis of brain sparing as indicated by brain-to-liver weight ratios. Brain-to-liver weight ratio of >0.8 is reflective of asymmetrical growth restriction in fetal guinea pigs which is due to the 'brain sparing effect' that is involved in the adaptive response to an insult applied in the second half of gestation (Dieni 2003; Laferber 1984; Mallard 1999; Nitsos 1990; Rees 1992; Tolcos 1997; Turner 2009). aFGR animals were deemed to be those with brain-to-liver weight ratios above 0.85 (n=8), while sFGR animals were deemed to be those with brain weight-to-liver weight ratios below 0.85 (n=8), which was the same as for the AGA and LGA animals (Figure 4.1).

Brains were fixed by intracardial left ventricle perfusion with isotonic 0.9% saline followed by 4% paraformaldehyde in 0.1 M PB, pH 7.4 for ~10 minutes each and subsequently weighed. Brains were post fixed in the 4% paraformaldehyde for 72 hours and then washed three times with phosphate buffered saline (PBS) and placed in 70% ethanol. Brains were cut into two coronal sections with the first cut caudal to the olfactory bulbs and through the mammilary bodies and the second cut at the rostral pons and paraffin embedded. Figure 4.2 shows that coronal section, at the 860 coronal level of the guinea pig brain according to the Wisconsin guinea pig brain atlas (Welker 2010). Serial coronal sections of the forebrain including the hippocampus were cut at 5 μm on a rotary microtome and mounted on Superfrost Plus slides (VWR Scientific, West Chester, Pa) by Victoria Research labs as previously described (Rocha 2004).

4.2.3 Fetal guinea pig brain processing for Western blots

Fourteen additional fetal guinea pigs from UABD sows, with body weights ranging from 51 g to 142 g, created a gamut of fetal guinea pigs to analyze for changes in SYN and SYNPO total protein levels using Western blots (Figure 4.3). Upon hysterotomy at term (~65 days gestation), fetal pups were decapitated and their brains were weighed, then subsequently slow frozen dorsal side down on dry ice for 15 minutes. The brains were then stored at -80º.

For microdissection, fetal brains were placed on dry ice then at room temperature to thaw sufficiently for sectioning. Using a razor blade, the brain was cut coronally just caudal to the optic chiasm and again at the rostral pons giving a section approximately 2 mm thick. Figure 4.4 shows the coronal sections were approximately level 760 and 920, respectively, from the

Wisconsin guinea pig brain atlas (Welker 2010). The section was covered in RIPA + protease and phosphatase inhibitor to prevent protein degradation. Areas of interest microdissected were the dorsal hippocampus that includes the dentate gyrus (DG) and CA4 subareas, and the ventral hippocampus that includes the CA1 area, CA3 area and fimbria (Figure 4.4). The microdissections were done with a scalpel whereby the cortex and thalamus were dissected away, the CC was excised with two diagonal slices, and the whole hippocampus was then sliced at a 45° angle to separate the dorsal from the ventral hippocampus. The samples were placed in eppendorf tubes with RIPA + protease and phosphatase inhibitor.

4.2.4 Immunohistochemistry of synaptophysin

SYN immunoreactivity (SYN IR) was assessed by avidin-biotin complex-enhanced immunohistochemistry (Vectastain; Vector Laboratories, Burlington Ontario). To reduce staining variability, all immunohistochemistry for SYN was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinized in 3 sequential xylene baths for 3 minutes and subsequently rehydrated in baths of 100% (2×2 min), 90% (2×2 min), and 70% (1×2 min) of ethanol followed by running tap water for 5 minutes. The sections were then subjected to post-fixation with AAF fixative which is composed of 95% alcohol, 0.1% acetic acid and 40% formaldehyde. After rinsing in PBS, the coronal brain sections underwent an antigen-retrieval procedure, which involved incubating them in 10-mM citrate buffer (pH 6.0) in a steam-set rice cooker for 25 minutes and then cooling for 25 minutes. Endogenous peroxidase was then quenched by a 10-min bath in 3% hydrogen peroxide. Endogenous biotin activity was blocked using an avidin and biotin kit (Vector Laboratories) for 40 minutes followed by

Background SNIPER (Biocare Medical, Brampton Ontario) and for 6 minutes to reduce non-specific background staining.

Sections were then incubated with primary antibody (mouse anti-rat monoclonal SYN, 1:1500, Lifespan Biosciences, Seattle Washington) overnight at 4°C in a covered humidity chamber. Antibodies were prepared using Universal Antibody Diluting Solution (Dako Canada, Burlington Ontario). Sections were subsequently rinsed and then incubated with secondary antibody (biotinylated goat anti-mouse immunoglobulin G, 1:500, Vector Laboratories) at room temperature for 30 minutes and again rinsed as described earlier. Sections were then incubated with an avidin and biotin complex (ABC) solution (avidin-biotin peroxidase complex, 1:50; Vector Laboratories) at room temperature for 30 minutes. The bound antibody was visualized with Cardassian D.A.B (3',3'-diaminobenzadine) chromagen (Biocare) at room temperature for 90 seconds. Sections were rinsed with PBS and then dehydrated in alcohol baths of increasing concentration (70% 2×30 sec; 90% 1×1 min; 100% 2×3 min) followed by three 5-minute rinses in xylene before being cover slipped using Permount. To demonstrate nonspecific binding, the primary antibody was substituted with pure DAKO diluant. The positive control for SYN immunohistochemistry was an adult guinea pig brain run concurrently with fetal brains.

4.2.5 Immunofluorescence of synaptopodin

SYNPO-IR was assessed using immunofluorescence. To reduce staining variability, all immunofluorescence for SYNPO was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinised, subjected to post-fixation with AAF fixative, underwent an antigen-retrieval protocol, and blocked with Background SNIPER

(Biocare) as described for SYN immunohistochemistry. Sections were then incubated with primary antibody (mouse anti-rat monoclonal SYNPO, undiluted, Novus Biologicals, Ontario Canada) overnight at 4°C in a light-proof covered humidity chamber. Sections were subsequently rinsed with PBS and then incubated with secondary antibody (goat anti-mouse Alexafluor 546, 1:200, Invitrogen Life Technologies, Burlington Ontario) at room temperature for 30 minutes and again rinsed. Sections were then counterstained with HOESCHT nuclear counterstain (1:10000, Invitrogen) for 4 minutes at room temperature. After rinsing, slides were dipped in 0.3% Sudan black in 70% EtOH for 30 seconds then quickly rinsed with PBS thoroughly and coverslipped with Pro-long Gold Anti Fade mountant (Invitrogen). Slides were stored at 4°C in the dark. To demonstrate nonspecific binding, the primary antibody was substituted with pure Universal Antibody Diluting Solution. The positive control for SYNPO immunofluorescence was an adult guinea pig brain run concurrently with fetal brains.

4.2.6 Imaging and statistical analysis of synaptophysin and synaptopodin

The slides were coded prior to image analysis to ensure this was done blinded to the animal cohort. For SYN (Figure 4.5), the corpus callosum (CC), parasagittal grey matter (PSG) layers 1-3 and 4-6, and the CA1, CA3, CA4, and DG sub-areas of the hippocampus were imaged, while for SYNPO only the CC, and CA1, CA3 and DG sub-areas of the hippocampus were examined.

SYN IR was imaged as 3-6 high power fields (HPF, images >400x magnification) in each brain area with a 63x objective using a Leica DM RB light microscope (Leica Microsystems, Buffalo Grove Illinois). Figure 4.6A depicts appropriate SYN IR in the PSG area of the brain, as

clustered, punctate staining throughout the axonal arborisation surrounding neurons indicating the presence of a synapse. SYNPO IR was imaged as 3-6 HPF images and six Z-stacks 0.8µm apart each with a 63x oil objective on a Zeiss upright fluorescent compound microscope (Carl Zeiss Microimaging, Thornwood, NY) with apotome camera. Figure 4.6B depicts appropriate SYNPO IR in the CA1 sub-area of the hippocampus, as small punctate staining along dendrites indicative of dendritic spines.

Constant white balance and exposure settings were used for all SYN slides in the same brain region as determined by optimal settings for imaging the negative control slide, as well as for the particular brain area being imaged. With each new slide or change in objective, the field diaphragm was centered and focused to maximize Kohler illumination. HPF images were taken manually over the course of one sitting without altering platform position or macro focus in order to ensure consistent imaging between slides.

Percent area positive staining was measured of protein-IR using ImagePro Plus 6.0 software (Meyer Instruments, Houston Texas). ImagePro Plus was calibrated for magnification. Immunohistochemistry colour images of SYN IR were background corrected and then equalized individually; the 256 bit range was shortened to 200 bits incorporating all the data on the slide. The shortened range was spread over 256 bits in order to better define colour differences. This was done blinded to treatment groups and the range controlled to a whiteness level that still maintained definition of intracellular components but defined a true white. An automatic standard brown threshold was then applied to the HPF image from the 3 colours; red – 215, green – 175, and blue – 142 to count DAB visualized protein-IR (Figure 4.6A). The threshold for defining positive staining for SYN was then further adjusted on an individual image basis to define percent area positive stain for SYN IR per HPF.

SYNPO IR HPF images were taken using Axiovision 4.0 software (Carl Zeiss Microimaging, Thornwood, NY) with a 660 wavelength fluorescent cube. HOESCHT staining was only used for localization of brain areas. Individualized exposure settings and focus were used for each image to increase sensitivity for SYNPO fluorescence. The SYNPO images were converted to 16-bit .tiff images and quantified using Image pro plus software as described above. Images were equalized to a 20pt range and an automatic threshold of 56-256 was applied (Figure 4.6B). The threshold for defining positive staining was adjusted on an individual image basis to define percent area stained positive for SYNPO IR per HPF.

The mean fractional area positively stained for SYN or SYNPO was calculated for each brain area of each animal, based on the average in all the HPF images taken for that area. Percent area positively stained did not reflect Normal distribution so data was transformed to allow for comparison using parametric tests. In cohorts with percent area staining in the range of 0-30% or 70-100% a square root transformation was applied. In cohorts with data that did not constitute a square root transformation or data that did not lie in the 30-70% range, an arc sine transformation was applied for analysis. All statistical procedures were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, California). All graphs presented depict untransformed data but significant differences were based on transformed data. Comparison of cohorts to AGA animals was done using an unpaired *t*-test. All results are presented as mean percent ± standard error of the mean (SEM) and for all analyses, a statistical significance was assumed for $p<0.05$.

4.2.7 Western Blot protocol for synaptophysin and synaptopodin

Each sample was homogenized into RIPA + protease and phosphatase inhibitor with an electric IKA and then sonicated with an amplitude of 20 for 10 seconds (approximately 8 joules) to further shear the tissue. The samples were then placed on ice before being centrifuged at low speed, 1500rpm for 10 minutes at 4°C. The supernatant was quantified for total protein concentration using colorimetric Pierce® BCA Protein Assay kit (Pierce Corp., Madison Wisconsin) and Spectramax pro software (Molecular Devices, Sunnyvale California).

15µg of protein was fractionated in a 4-12% gradient polyacrylamide gel (Invitrogen)for SYN analysis, while 30µg of protein was fractionated in a 3-8% gradient polyacrylamide gel (Invitrogen) for SYNPO analysis, and transferred onto polyvinyllidenedifluoride membrane (Millipore, Etobicoke Ontario). Protein transfer was visualized with Amido Black (Sigma-Aldrich, Oakville Ontairo). Blots were then blocked with 5% milk+Tris buffered saline-Tween 20 buffer for 1 hour at room temperature and probed using mouse anti-rat monoclonal SYN (1:8000, Lifespan) or mouse anti-rat monoclonal SYNPO (1:250, Novus) overnight at 4ºC. After rinsing, Blots were incubated with horseradish peroxidise conjugated donkey anti-mouse IgG (1:10 000, Jackson ImmunoResearch Laboratories, West Grove Pennsylvania) as the secondary antibody. Immunoreactive bands, 38kDa and44kDA for SYN and SYNPO, respectively, were visualized using an Luminata^{TM} Forte Western HRP enhanced chemiluminescence detection system (Thermo Scientific, Waltham Maryland) and imaged using Quantity One 1-D Analysis Software (Bio-Rad laboratories, Mississauga Ontario) The SYNPO primary antibody reacts with a 44kDa degredation fragment of the 100kDa SYNPO protein molecule.

Membranes were stripped with Mild Stripping buffer for 15 minutes at room temperature and reblocked with 7.5% milk+Tris buffered saline-Tween 20 buffer for 1 hour at room temperature and incubated with mouse anti-β tubulin monoclonal antibody (1:1500, EMD Millipore, Billerica, Massachusetts) for 2 hours at room temperature then with the secondary antibody, horseradish peroxidise conjugated donkey anti-mouse IgG (1:10 000, Jackson), for 2 hours at room temperature. Immunoreactive bands for β-tubulin at 50kDa was visualized and imaged as described above.

SYN IR and SYNPO IR protein bands underwent densitometry analysis using Image Lab 3.0 Software (Bio-Rad) for the dorsal and ventral hippocampus for each animal. β-tubulin is ubiquitously expressed in the immature brain and so reprobing allowed for normalization of blot densities to correct for loading variances. Spearman's rank correlation coefficient analyses were performed using GraphPad Prism 5.0 (GraphPad) to determine if there was a monotonic relationship between total protein changed with guinea pig body weight at term in either half of the hippocampus. All results are presented as mean of arbitrary values and a weak correlation was deemed for Spearman's coefficient (r_s) >0.2, and a relationship was deemed for Spearman's coefficient (r_s) >0.4 and a statistical significance was assumed for $p<0.05$.

Figure 4.1 Body weights and brain-to-liver weight ratios of fetal cohorts forming large for gestational age (LGA), appropriate size for gestational age (AGA), asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) cohorts for analysis of changes in synaptic proteins using histology. Their body weights at term range from 41 g to 129 g and a brain-to-liver weight ratio <0.82 denotes sFGR fetuses while a ratio >0.85 denotes aFGR fetuses.

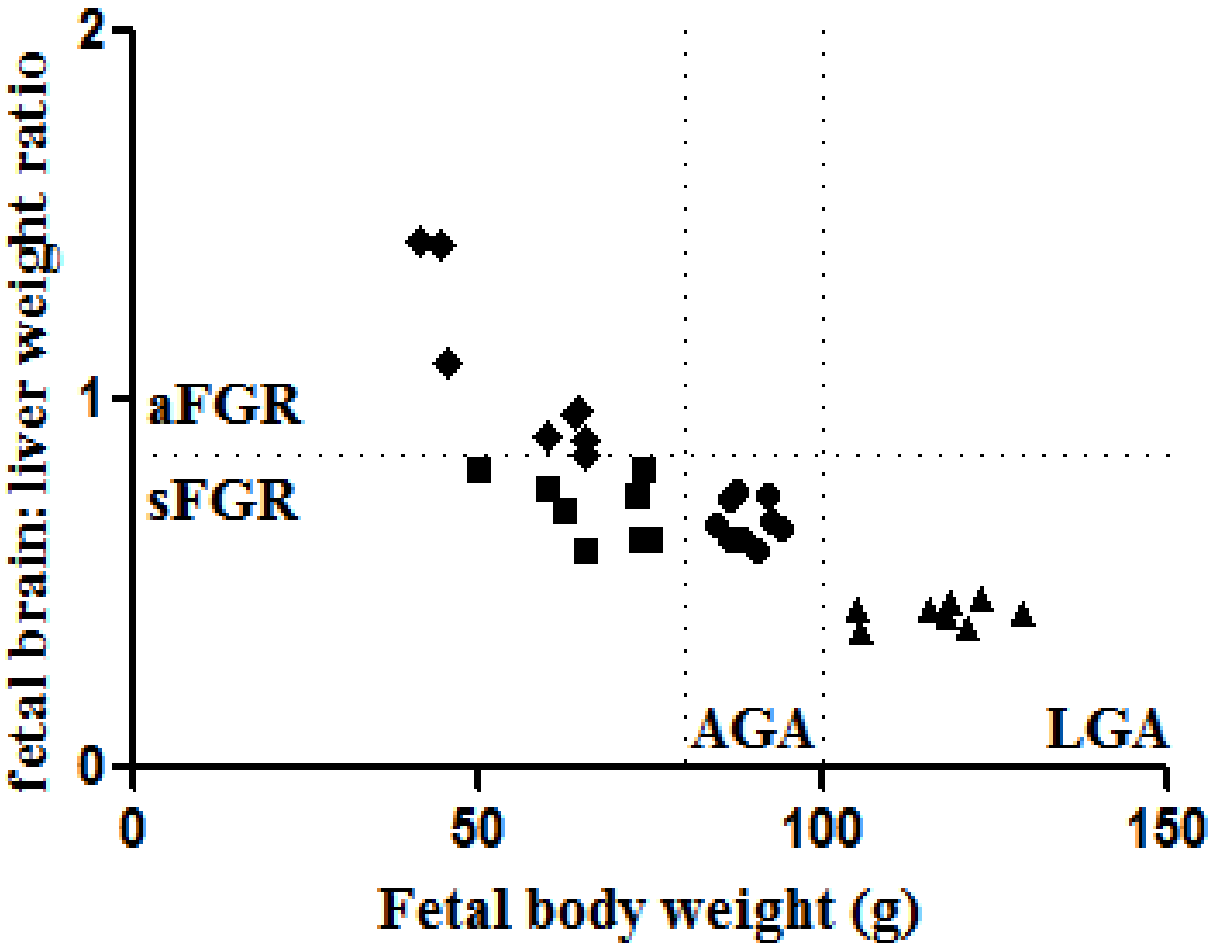
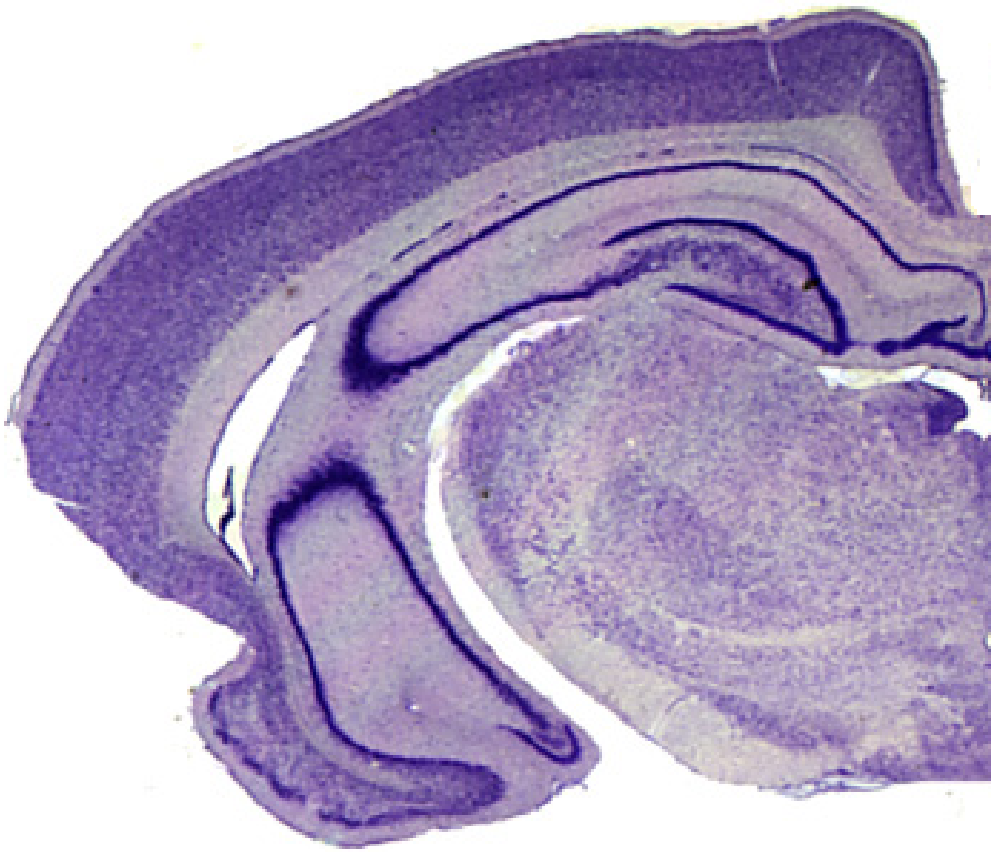

fetal brain: liver weight ratio
2
1
0
aFGR
sFGR
AGA
LGA
0
50
100
150
Fetal body weight (g)

Figure 4.2 Thionin stained coronal section 860 through the hippocampus at the level of the entorhinal cortex of an adult guinea pig (Welker 2010).

60-1 #860



University of Wisconsin - Madison Brain Collection

5 mm

Figure 4.3 Body weight and brain-to-liver weight ratio of fetal guinea pigs (n=14) used for analysis of changes in synaptic proteins using Western blots. Their body weights at term range from 51 g to 142 g and their brain-to-liver weight ratios increased with decreasing body weight.

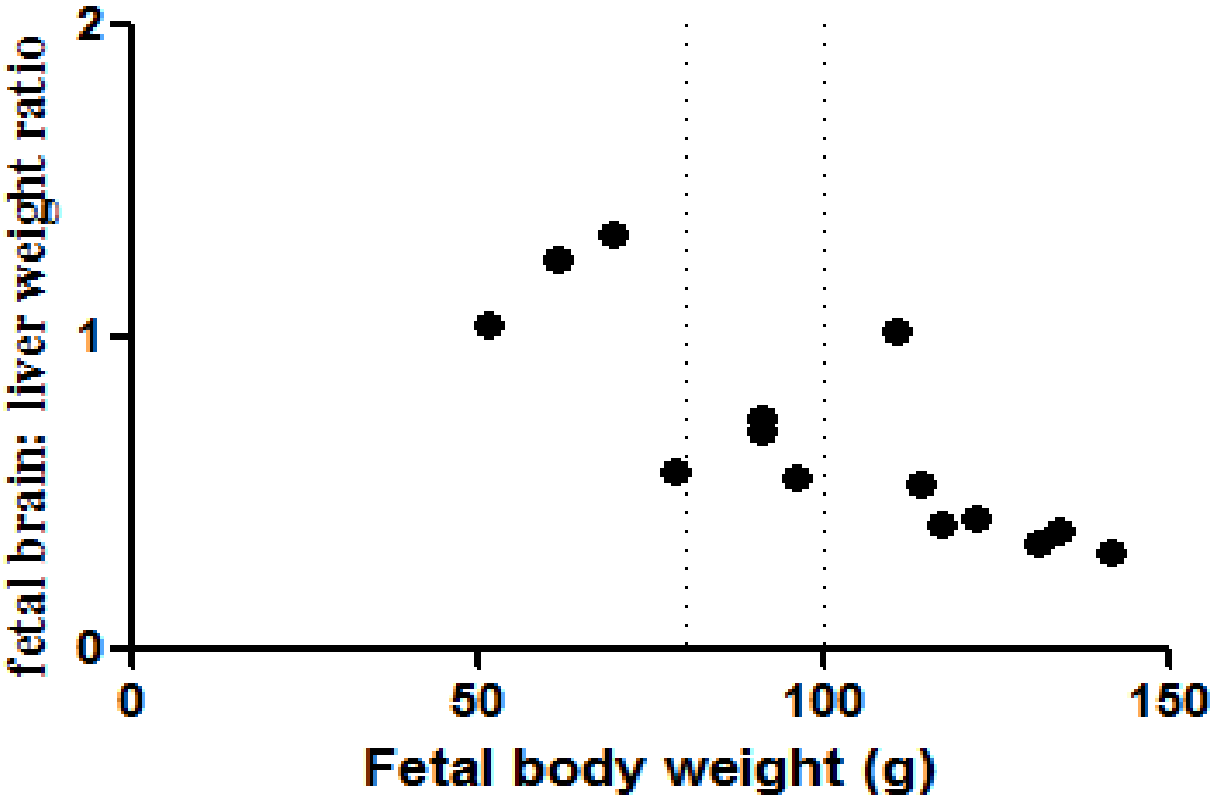
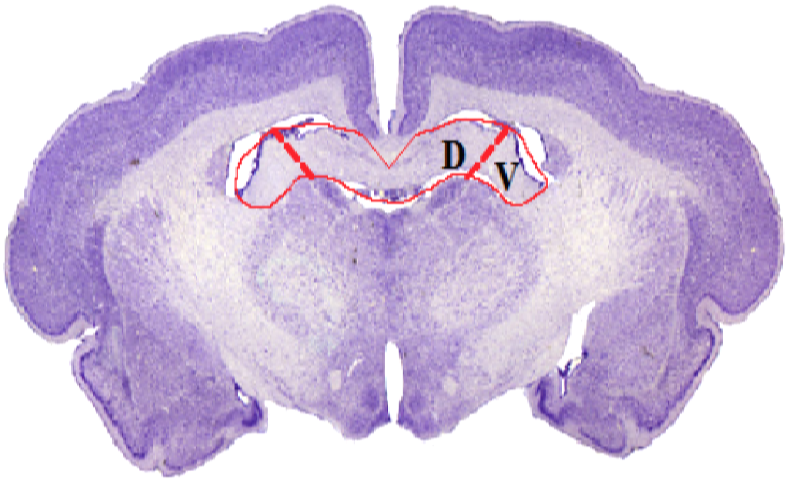

fetal brain: liver weight ratio
2
1
0
0
50
100
150
Fetal body weight (g)

Figure 4.4 Gross pictures of the fetal guinea pig brain microdissection. The rostral side is equivalent to level 760 by the Welker Guinea Pig brain atlas (on the left) and the caudal side of the section is equivalent to level 920 (on the right) (Welker 2010). Dissected out were the dorsal and ventral hippocampus. The dashed line indicates a 45° angle slice to microdissect out the dorsal (D) from the ventral (V) hippocampus.

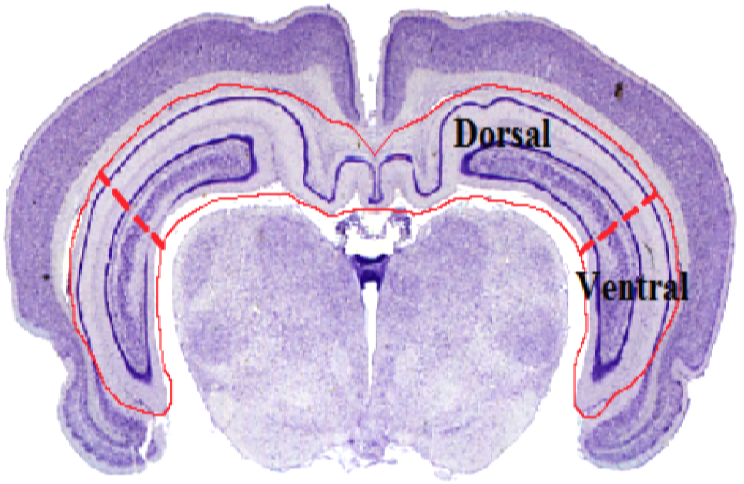
#760



5 mm

Rostral side

#920



Caudal side

Figure 4.5 Synaptophysin (SYN) stained coronal brain section of a fetal guinea pig brain indicating brain regions examined for SYN and synaptopodin (SYNPO) expression. Areas of interest were the corpus callosum (CC), parasagittal grey matter (PSG), and CA1, CA3, CA4 and dentate gyrus (DG) sub-areas of the hippocampus. Rectangles represent high power field (HPF) images.

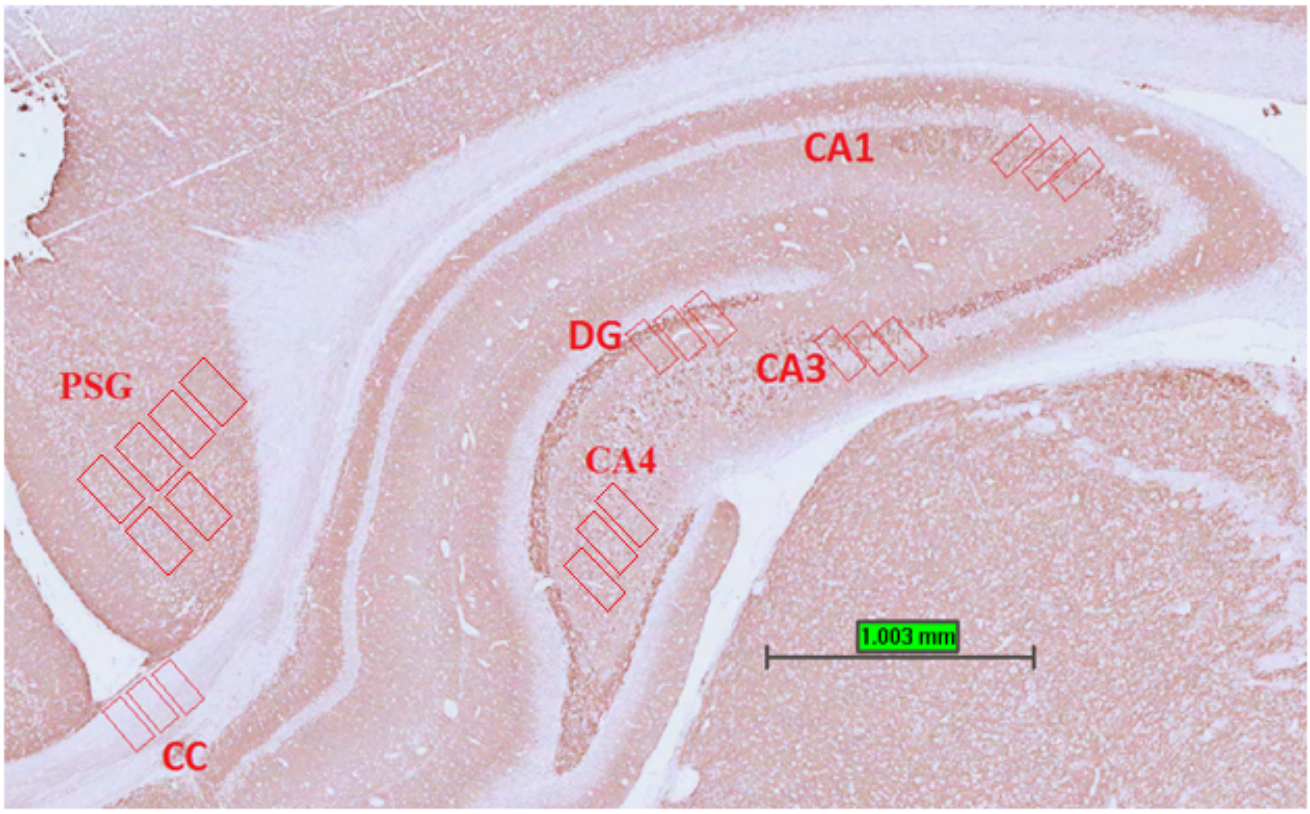
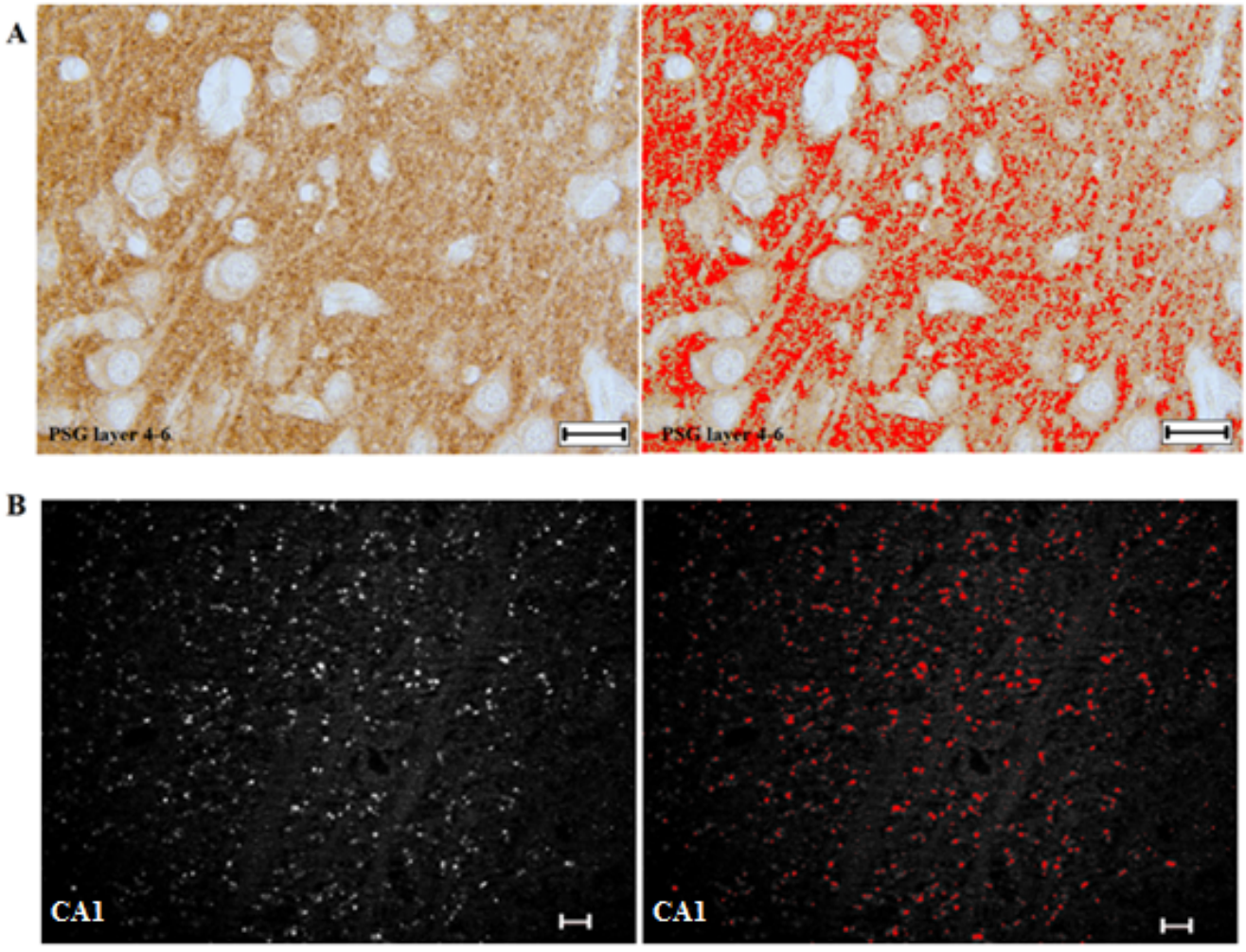

CA1
DG
CA3
CA4
PSG
CC
1.003 mm

Figure 4.6 A Synaptophysin immunoreactivity (SYN IR) in the parasagittal grey matter (PSG) layers 4-6 showing appropriate SYN IR punctate staining in the original high power field (HPF) image for analysis (left) and the threshold applied (red) indicating positive stain (right). The scale bar indicates 10 µm

B Synaptopodin immunoreactivity (SYNPO IR) in the CA1 subarea of the hippocampus showing appropriate SYNPO IR punctate staining in the original HPF image for analysis (left) and the threshold applied (red) indicating positive stain. The scale bar indicates 5 µm.


A
PSG layer 4-6
PSG layer 4-6
B
CA1
CA1

4.3 RESULTS

4.3.1 Synaptophysin quantification

In the CA1 sub-area of the hippocampus, the percent area SYN IR in aFGR animals at 19 ± 2% and sFGR animals at 13 ± 2% were reduced compared to AGA animals at 26 ± 3% (both $p<0.05$) (Figure 4.7A). In the CA3 sub-area of the hippocampus, the percent area SYN IR in aFGR at 14 ± 2% and sFGR at 8 ± 2% cohorts were also reduced compared to the AGA cohort at 18 ± 1% (both $p<0.05$) (Figure 4.7B). In the CA4 sub-area of the hippocampus, there were no significant differences in percent area SYN IR between any of the cohorts compared to AGA animals (Figure 4.7C). In the DG sub-area of the hippocampus, the percent area SYN IR in the sFGR animals at 12 ± 2%) was reduced compared to AGA animals at 20 ± 1% ($p<0.05$) (Figure 4.7D).

SYN IR in the CC was minimal, with no significant differences in percent area SYN IR in any cohort compared to the AGA animals (Figure 4.8). There were also no significant differences in percent area SYN IR in the PSG layers 1-3 or PSG layers 4-6 between any of the cohorts and AGA animals (data not shown).

4.3.2 Synaptopodin quantification

In the CA1 sub-area of the hippocampus, the percent area SYNPO IR was reduced in the sFGR at 9 ± 1% compared to the AGA animals at 26 ± 4% ($p<0.03$), but there were no differences in the aFGR or LGA animals (Figure 4.9A). In the CA3 sub-area of the hippocampus, the percent area SYNPO IR showed no significant differences between any of the fetal cohorts and AGA animals (Figure 4.9B). In the DG subarea of the hippocampus, the percent area SYNPO IR was decreased in sFGR at 24 ± 5% compared to AGA animals at 42 ± 11%

($p<0.05$), but again there were no differences in the aFGR or LGA animals (Figure 4.9C). The percent area SYNPO IR in the CC was also found to be minimal as seen for SYN IR and again with no significant differences between any of the fetal cohorts and the AGA animals (Figure 4.10).

4.3.3 Western blot analysis for synaptophysin and synaptopodin

The total SYN protein in the ventral hippocampus had a strong negative correlation with fetal body weight ($r_s = -0.8$, $p<0.0006$) but the total SYN protein in the dorsal hippocampus had no significant correlation with fetal body weight ($r_s = 0.1$, $p>0.05$) (Figure 4.11A). The total SYNPO protein in the dorsal hippocampus had non-significant negative correlation with fetal body weight ($r_s = -0.3$, $p>0.05$) and the total SYNPO protein in the ventral hippocampus had a non-significant positive correlation with fetal body weight ($r_s=0.3$, $p>0.05$) (Figure 4.11B). Interestingly, all tests suggested an approximated Gaussian distribution of the data points.

Figure 4.7 Representation of synaptophysin immunoreactivity (SYN IR) amidst the Pyramidal cells in the CA3 sub-area of the hippocampus of a large for gestational age animal (LGA), appropriate size for gestational age (AGA) animal, asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animal.

A Percent area SYN IR per high power field (HPF) in the CA1 sub-area of the hippocampus of fetal cohorts showing that asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animals had reduced SYN IR than the AGA animals. *=$p<0.05$ compared to the AGA cohort.

B Percent area SYN IR per HPF of the CA3 sub-area of the hippocampus of fetal cohorts showing aFGR and sFGR animals had reduced SYN IR than the AGA animals. *=$p<0.05$ compared to the AGA cohort.

C Percent area SYN IR per HPF of the CA4 sub-area of the hippocampus of fetal cohorts showing no significant difference between any of the cohorts compared to AGA animals.

D Percent area SYN IR per HPF of the dentate gyrus (DG) sub-area of the hippocampus of fetal cohorts showing sFGR animals had reduced SYN IR that the AGA animals. *=$p<0.05$ compared to the AGA cohort.

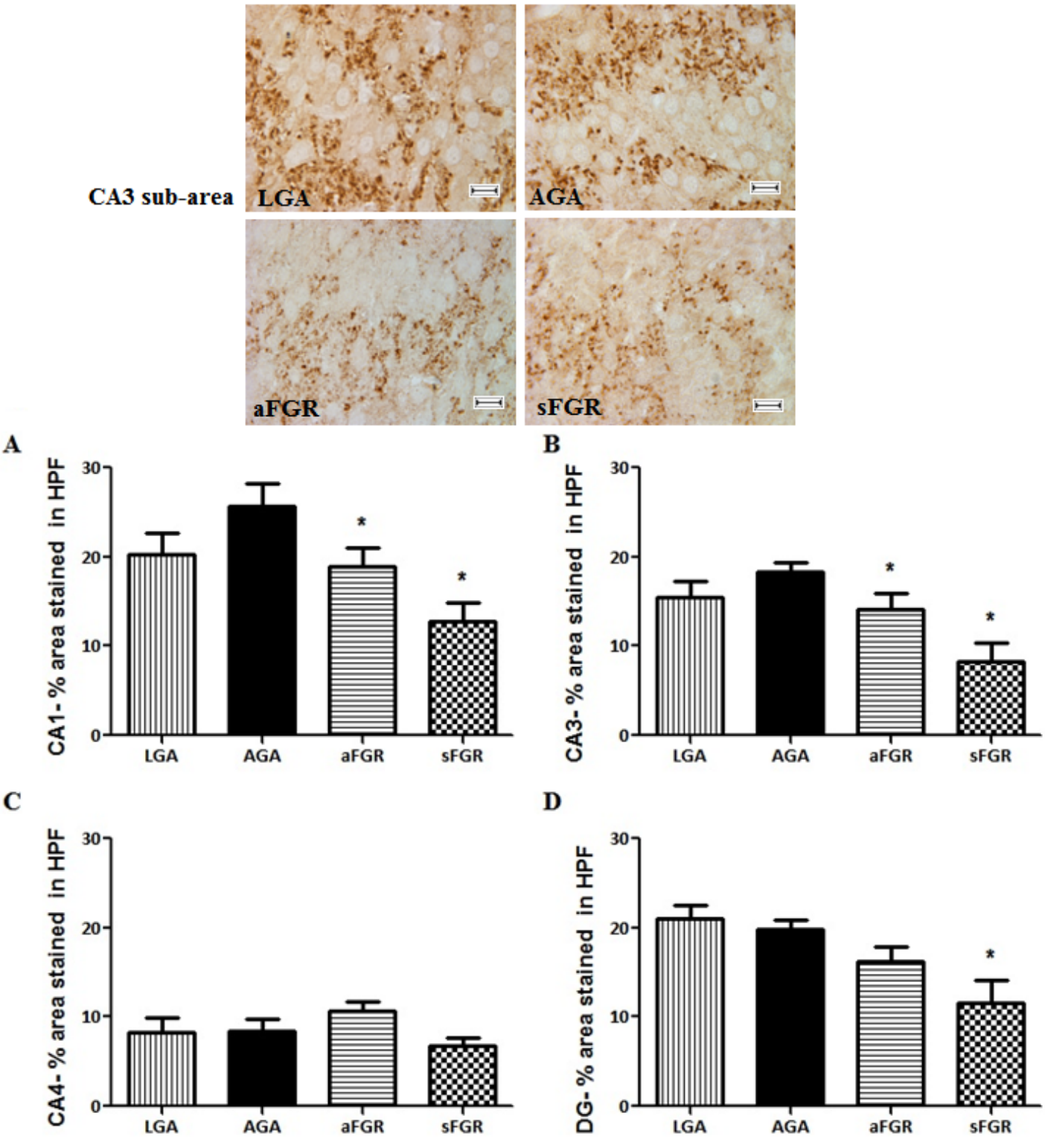

CA3 sub-area
LGA
AGA
aFGR
sFGR
A
CA1- % area stained in HPF
B
CA3- % area stained in HPF
C
CA4- % area stained in HPF
D
DG- % area stained in HPF
0
10
20
30
*
LGA
AGA
aFGR
sFGR

Figure 4.8 Percent area synaptophysin immunoreactivity (SYN IR) per high power field (HPF) in the corpus callosum (CC) of fetal cohorts showing that there was minimal staining and no significant differences between any of the fetal cohorts and the appropriate size for gestational age (AGA) animals

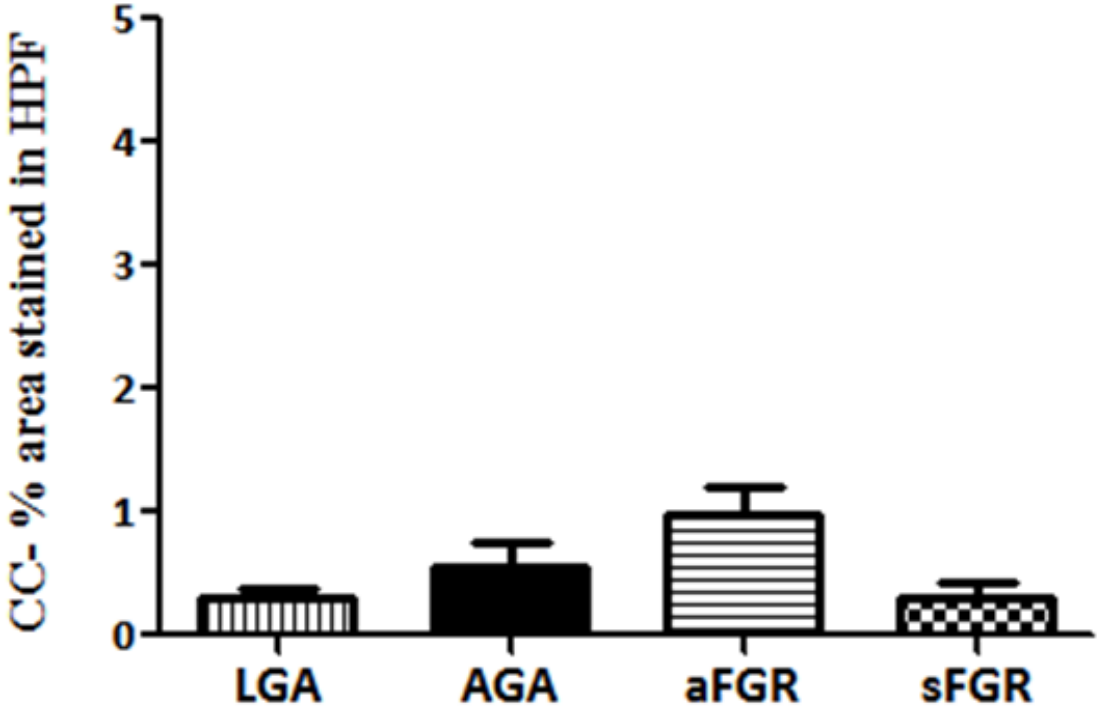

CC- % area stained in HPF
5
4
3
2
1
0
LGA
AGA
aFGR
sFGR

Figure 4.9 Representation of synaptopodin immunoreactivity (SYNPO IR) in the CA1 sub-area of the hippocampus of a large for gestational age animal (LGA), appropriate size for gestational age (AGA) animal, asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animal.

A Percent area SYNPO IR per high power field (HPF) in the CA1 sub-area of the hippocampus of fetal cohorts showing that sFGR animals had reduced SYNPO IR than the AGA animals. *=$p<0.05$ compared to the AGA cohort.

B Percent area SYNPO IR per HPF of the CA3 sub-area of the hippocampus of fetal cohorts showing no significant difference between any of the cohorts compared to AGA animals.

C Percent area SYNPO IR per HPF in the dentate gyrus (DG) sub-area of the hippocampus of fetal cohorts showing that sFGR animals had reduced SYNPO IR than the AGA animals. *=$p<0.05$ compared to the AGA cohort.

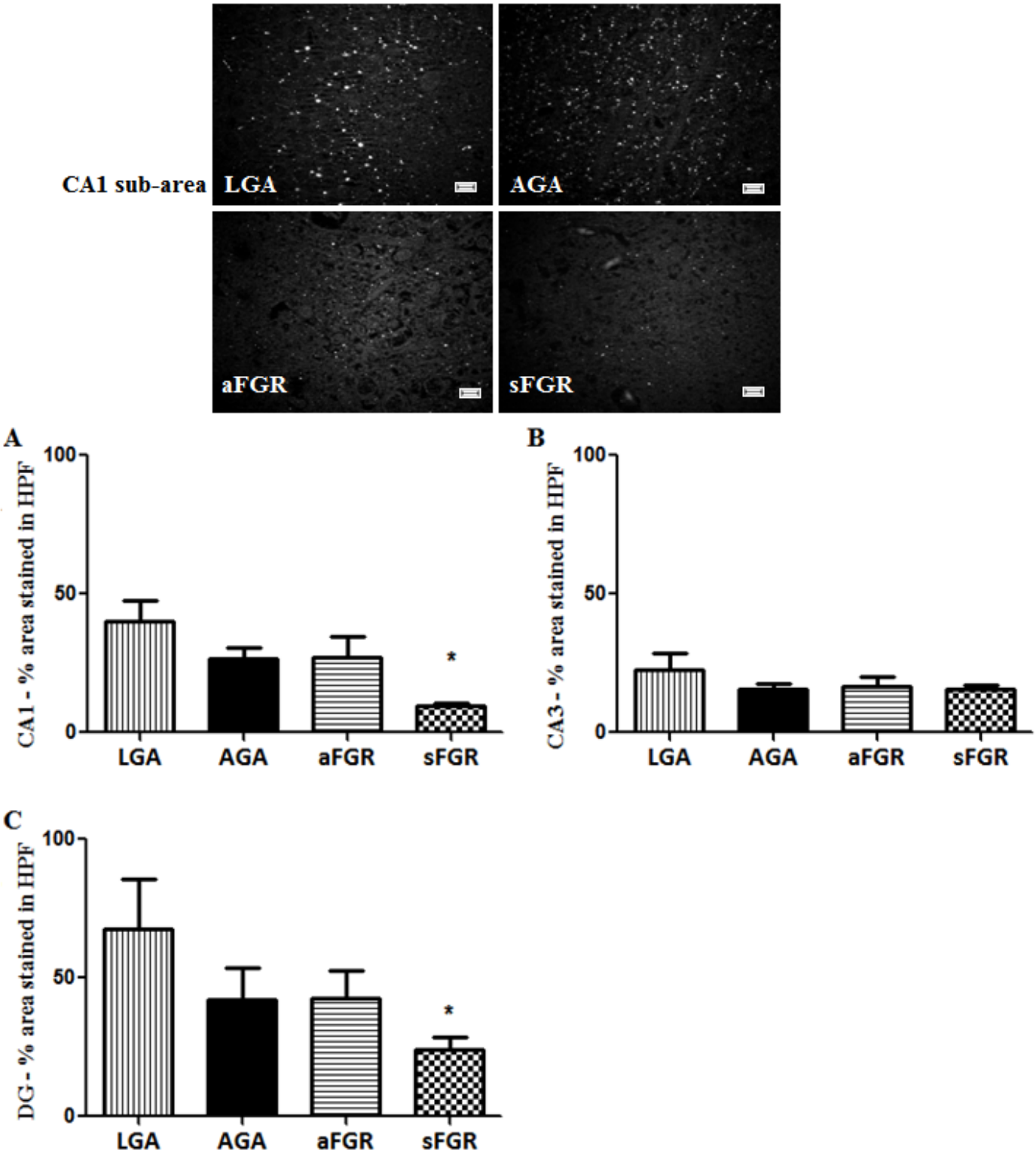

CA1 sub-area
LGA
AGA
aFGR
sFGR
A
CA1 - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR
B
CA3 - % area stained in HPF
100
50
0
LGA
AGA
aFGR
sFGR
C
DG - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR

Figure 4.10 Percent area synaptopodin immunoreactivity (SYNPO IR) per high power field (HPF) in the corpus callosum (CC) of fetal cohorts showing that there was minimal staining and no significant differences between any of the fetal cohorts and the AGA animals

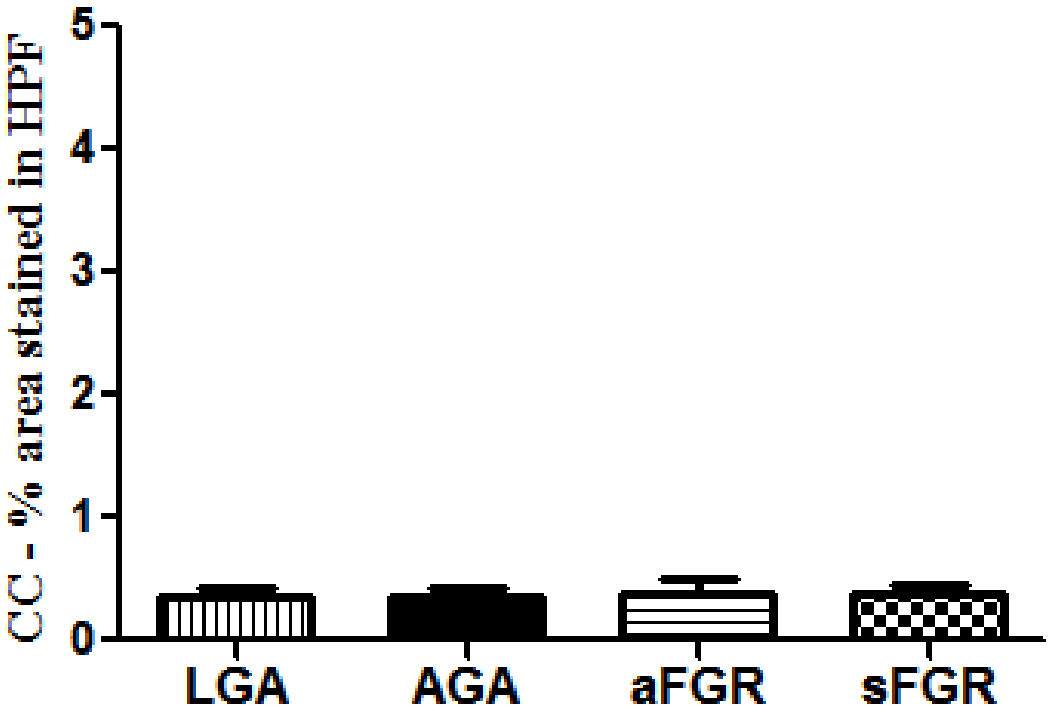
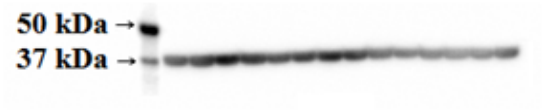
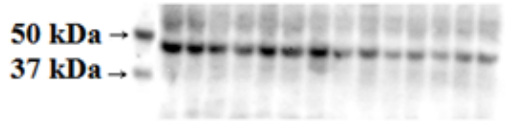

CC - % area stained in HPF
5
4
3
2
1
0
LGA
AGA
aFGR
sFGR

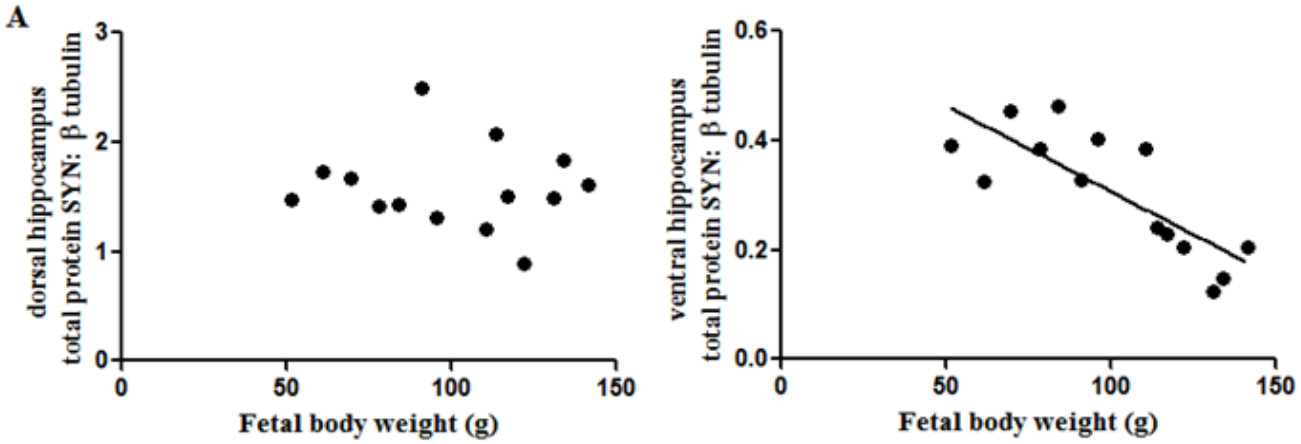
Figure 4.11 Representation of Western blot for total synaptophysin (SYN) and synaptopodin (SYNPO) protein where SYN protein bands are at 38kDa (left) and SYNPO bands are at 44kDa (right). Lane 1 is the Precision Plus protein standards dual colour marker. Lanes 2-14 are dorsal hippocampal protein samples.

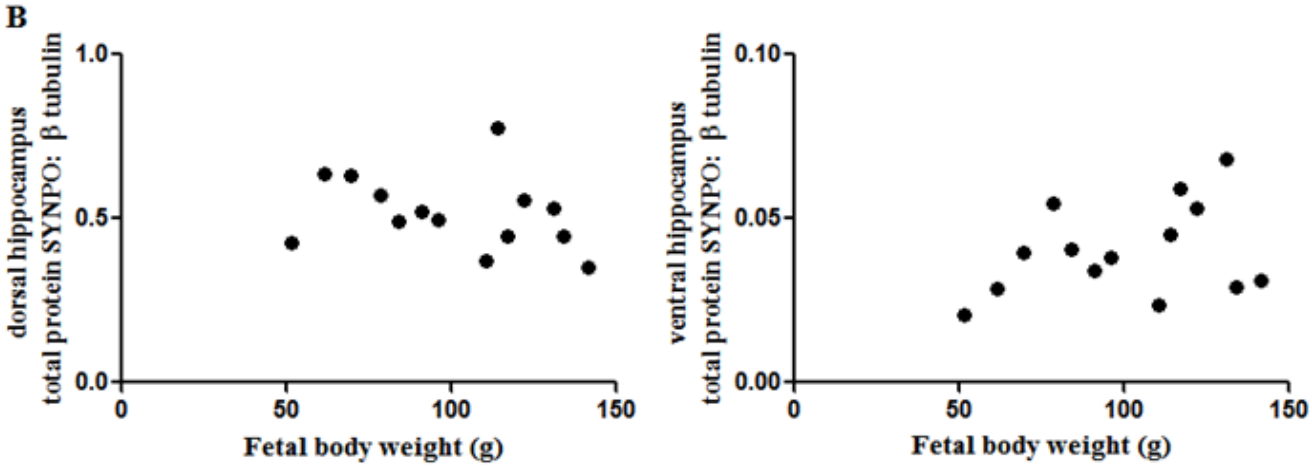
A Total SYN protein had no correlation with fetal body weight in the dorsal hippocampus (left) but showed a strong negative correlation ($r_s = -0.8$, $p<0.0006$) in the ventral hippocampus (right).

B Total SYNPO protein had a non-significant negative correlation with fetal body weight in the dorsal hippocampus (left) and a non-significant positive correlation in the ventral hippocampus (right).


50 kDa →
37 kDa →


50 kDa →
37 kDa →


A
dorsal hippocampus
total protein SYN: β tubulin
Fetal body weight (g)
ventral hippocampus
total protein SYN: β tubulin
Fetal body weight (g)


B
dorsal hippocampus
total protein SYNPO: β tubulin
Fetal body weight (g)
ventral hippocampus
total protein SYNPO: β tubulin
Fetal body weight (g)

4.4 DISCUSSION

During synaptic development there is an initial abundance of immature synapses formed followed by selective deletion or strengthening and stabilizing of the synapses with the input of stimulating activity (Dobbing 1971; Leclerc 2010). Thus, while the complement of synapses is set at term in both humans and guinea pigs, synapses are very dynamic with continual turnover. The most pertinent finding of the study is the reduced expression of SYN observed in the FGR animal cohorts in the hippocampal sub-areas as it may be the result of *in utero* delays in proliferation of synapses (Pallotto 2006). In culture, mature synapses can perpetuate presynaptic specializations and synaptic maturation earlier than immature synapses can (Fletcher 1994). The reductions in SYN IR in the FGR animals would suggest fewer mature synapses and thereby less perpetuation of forming new synapses. Fewer synapses formed or delays in maturation of synapses could then lead to the decreases in SYN IR in FGR cohorts as noted compared to AGA cohorts.

Another pertinent finding of this study is the reduced expression of SYNPO IR observed in the sFGR animals compared to AGA animals in the sub-areas of the hippocampus. Dendrites have protein synthesis machinery localized at synaptic sites which are abundant in areas at peak synaptogenesis. These areas with newly forming synapses have high levels of cytochrome C complex and mitochondria to supply the large amount of energy required for protein synthesis and synapse formation (Mjaatvedt 1988). As well, there is evidence of ongoing protein translation in synapses with the abundant presence of polyribosomes, Golgi apparatus, endoplasmic reticulum, translational factors, and rRNA that supports synaptic maturation (Jiang 2002). sFGR animals may have a pronounced and extended depletion of these essential substrates and a depression in metabolism hindering the maturation of synapses.

The reductions in SYN IR and SYNPO IR were more pronounced in the sFGR animals than in the aFGR animals when compared to AGA cohorts. It has long been held that hypoxic/nutritional insults later in gestation and during cellular hypertrophy results in the "brain sparing" response and aFGR phenotype, whereby redistribution of blood flow and available substrates to the brain supports brain growth and development to that of other organs and especially carcass and hepatic tissue (Chase 1971; Gagnon 2003; Gunn 2009; Jannson 1990; Jones 1983; Laferber 1984; Pallotto 2006; Pollack 1992). Human infants born small and proportional are thought to have experienced early-onset growth restriction in concert with organogenesis and cell proliferation and prior to the maturation of the 'brain-sparing' defense mechanisms (Pallotto 2006; Pollack 1992). The decrease in SYN IR in aFGR and sFGR cohorts compared to AGA animals could be baseline delays in forming new synapses when substrate and energy supply is reduced. The decrease in SYNPO IR in only sFGR animals and not aFGR animals compared to AGA animals could indicate the 'brain sparing' ensures selective maturation of synapses in the hippocampus at the expense of forming new immature synapses.

The most interesting finding from the Western blot analysis is the approximated Gaussian/Normal distribution pattern suggested for the data points. This means that the data points are curvilinear or upsidedown 'U' shaped where both FGR and LGA animals may have reduced total SYN and SYNPO protein compared to AGA animals in the hippocampus, although there were no differences in LGA animals in SYN IR and SYNPO IR compared to AGA animals using histological analysis. With greater sample numbers, this pattern may be visualized. There are only 3 fetuses in the Western blot analyses that would be considered FGR according to the criteria used to define the fetal cohorts, thus the Western blot analyses are largely biased toward AGA and LGA animals. The general risk of altered growth *in utero* in relation to morbidity

throughout life also follows a curvilinear relationship whereby both FGR and LGA fetuses are at an increased risk for diseases and metabolic disorders compared to AGA fetuses (Barker 1998). Although there is limited evidence for LGA-born fetuses and cognitive disorders, these Western blots suggest the potential for altered synaptogenesis in LGA fetuses.

In general, this study focused on the sub-areas of the hippocampus due to its role in memory and learning. Neurons of the DG, CA1, CA3 and CA4 synapse with each other in forming a functional hippocampus (Berger 2010), and accordingly similar patterns of protein expression for these sub-areas is to be expected. Hippocampal neurons of the CA1 and CA3 sub-areas are thought to be more sensitive to hypoxic or ischemic insults (Bickler 1998, Maitia 2007). Changes in synapse density in the hippocampus could be the result of chronic hypoxia associated with FGR. Interestingly, neurons of the DG are not particularly susceptible to hypoxia (Bickler 1998), but in the present study still showed a similar decrease in SYN expression in the FGR animals as CA1 and CA3 hippocampal sub-areas. The CA4 sub-area of the hippocampus did not show any difference in SYN IR in any of the fetal cohorts compared to AGA. This may suggest the CA4 sub-area shows a relatively high resistance to hypoxic changes. In summation, various sub-areas of the hippocampus appear to react differently based on their hypoxia-tolerance and adaptability during an insult.

There was no difference in staining in the CC or PSG cortical layers between any of the cohorts and AGA animals. The CC connects communication between the hemispheres of the brain; it does not contain any synapses and therefore SYN in this area would reflect its presence in the growth cones of axons as they migrate through to position (Calhoun 1996; Leclerk 2010). Additionally, there was no difference in SYN IR in the PSG layers 1-3 or layers 4-6 for any of the fetal cohorts compared to AGA. This could be due to the fact that peak neurogenesis occurs

in this area around mid-gestation (Clancy 2007), thereby lessening any effect growth restriction might have on brain development and allowing the full proliferation of synapses to form. The timing of peak synaptogenesis varies between brain areas thereby those brain areas that develop later in gestation are more susceptible to changes due to FGR.

Evidence of delayed neuronal growth due to FGR has been found in previous studies in various areas of the brain. Fletcher *et al* carried elegant *in vitro* analysis of hippocampal neural growth and found a direct decrease in density of synapses with a decrease in density of axons (Fletcher 1994). Conversely, increased dendritic density, branching and length directly increase synaptic number (Fletcher 1994). The growth, both in length and arborisation, of neurons affects synaptic development. Studies showing a reduction in dendritic arborisation in response to FGR would also be expected to be associated with a reduction of synapses. Studies have found there are reduced neuronal numbers in the hippocampus and cerebellum at term in FGR animals but not in the cortex or brainstem (Mallard 2000; Rees 1988; Tolcos 2003). These findings are keeping with the present results where there were a reduced number of synapses in various sub-areas of the hippocampus but not the cortex.

Synaptogenesis and synapse maturation coincide with experience (Bourgeois 1997). Neurons will down-regulate energy utilization under sub-optimal uterine environments by decreasing transmembrane ion fluxes through membrane ion channels or decreasing neuronal excitability (Bickler 1998). Synapse maturation is highly dependent on stimulatory activity which is altered in FGR animals with chronic hypoxemia (Keen 2011). FGR is associated with reduction in electrical activity (Nagerl 2007). Studies in our lab show that behavioural state activity is altered with FGR (Keen 2009). Electrical stimulation of synapses also increases translation in the dendrites in that area, inducing protein synthesis needed for synaptic maturation

(Jiang 2002). Depolarization at an immature synapse strengthens the stability of the synapse. With activity, there is also formation of new spines upon which synapses form, perpetuating synaptic maturation and proliferation in the hippocampus (Nagerl 2007). Accordingly, with altered behavioural state activity as seen in animal studies with FGR (Keen 2011), there may be altered stimulatory activity within the brain providing another mechanism for a reduction in synapse formation and maturation during development.

4.5 CONCLUSION

The significant reduction of expression of synaptic markers in the FGR animal cohorts could indicate impaired and/or delayed synaptogenesis. SYN is expressed throughout synaptic development and so the reduction in SYN IR in the hippocampus in both aFGR and aFGR animal cohorts could indicate either a delay in formation of synapses or a delay in the maturation of synapses. SYNPO is a protein restricted to mature synapses and the reduction of SYNPO IR could indicate impairment of or a delay in the maturation of synapses. Accordingly, aFGR animals showed decreases in synapses formed but no decreases in mature synapses, whereas sFGR animals showed decreases in both synapses formed and in mature synapses. These changes could be due to an interruption in energy and substrate availability for synapse formation and the neuroprotectve effect with 'brain sparing' preserving the maturation of certain synapses. This study gives insight into the effect of FGR on fetal synaptogenesis. The changes seen indicate altered synapse formation and potential deficiencies in neuronal communication that could give rise to cognitive deficiencies associated with FGR in humans.

4.6 REFERENCES

Barker DJP. (1998) *In utero* programming of chronic disease. *Clin Sci*. 95:115-128

Berger TW, Song D, Chan RH, Marmarelis VZ. (2010) The neurobiological basis of cognition: Identification by multi-input, multioutput nonlinear dynamic modeling: A method is proposed for measuring and modeling human long-term memory formation by mathematical analysis and computer simulation of nerve cell dynamics. *Proc IEEE Inst Electr Electron Eng.* 98(3):356-74

Bickler PE and Buck LT. 1998. Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical Ca^{2+} concentrations. *J Exp Biol* 201: 1141–1152

Bourgeois JP. (1997) Synaptogenesis, heterochrony and epigenetics in the mammalian neocortex. *Acta Paediatr.* Suppl 422:27-33

Bourne JN and Harris KM. (2008) Balancing structure and function at hippocampal dendritic spines. *Annu Rev Neurosci* 31:47-67

Buescher U, Hertwig K, Wolf C, and Ddenhausen JW. (1998) Erythropoietin in amniotic fluid as a marker of chronic fetal hypoxia. *Int J Gynecol Obstetr.* 60:257-263

Calhoun ME, Jucker M, Martin LJ, Thinakaran G, Price DL, and Mouton PR. (1996) Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J Neurocytol*. 25:821-28

Camm EJ, Gibbs ME, Harding R, Mulder T, and Rees SM. (2005) Prenatal hypoxia impairs memory function but does not result in overt structural alterations in the postnatal chick brain. *Brain Res Dev Brain Res.* 160(1):9-18

Chase PH, Dabiere CS, Welch NN, and O`Brien D. (1971) Intra-uterine undernutrition and brain development. *Ped* 47(3):491-500

Clancy B, Kersh B, Hyde J, Darlington RB, An KJS and, Finlay BL (2007) Web-Based Method for translating Neurodevelopment from laboratory species to humans. *Neuroinformatics* 5-1:79-94. http://people.psych.cornell.edu/~blf2/pdfs/BCBKBLFNI07.pdf

Daly C and Ziff EB. (1997) Post-transcriptional regulation of synaptic vesicle protein expression and the developmental control of synaptic vesicle formation. *J Neurosci*. 17(7):2365-2375

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

Deller T, Merten T, Roth SU, Mundel P, Frotscher M. (2000) Actin-associated protein synaptopodin in the rat hippocampal formation: localization in the spine neck and close association with the spine apparatus of principal neurons. *J Comp Neurol* 418:164-181

b) Mundel P and Frotscher M (2000) Potential role of synaptopodin in spine motility by coupling actin to the spine apparatus. *Hippocampus*10:569-581

Deller T, Korte M, Chabanis S, Drakew A, Schwegler H, Stefani GG, Zunlga A, Schwarz K, Bonhoeffer T, Zeller R, Frotscher M, and Mundel P. (2003) Synaptopodin-deficient mice lack a spine apparatus and show deficits in synaptic plasticity. *Proc Natl Acad Sci USA*. 100(18): 10494-9

Dieni S and Rees S. (2003) Dendritic morphology is altered in hippocampal neurons following prenatal compromise. *J Neurobiol.* 55(1): 41-52Jones CT and Parer JT. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *J Physiol.* 343:525-537

Dobbing J and Sands J. (1970) Growth and development of the brain and spinal cord of the guinea pig. *Brain Res*. 17:115-123

Duncan KR, Issa B, Moore R, Baker PN, Johnson IR, and Gowland PA. (2005) A comparison of fetal organ measurements by echo-planar magnetic resonance imaging and ultrasound. *BJOG* 112:43-9

Fletcher TL, Cameron P, De Camilli P and Banker G. (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J Neurosci.* 11(6) 1617-26

Fletcher TL, De Camilli P, and Banker G. (1994) Synaptogenesis in hippocampal culturesLevidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. *J Neurosci.* 14(11):6695-6706

Gagnon R. (2003) Placental insufficiency and its consequences. *Eur J Obstet Gynecol Reprod Biol.* Suppli 1:S99-107

Garner CC, Kindler S, and Gundelfinger ED. (2000) Molecular determinants of presynaptic active zones. *Cur Opin Neurobiol* 10:3321-327

Geva R, Eshel R, Leitner Y, Fattal-Valevski A, and Harel S. (2008) Verbal short-term memory span in children: long-term modality dependent effects of intrauterine growth restriction. *J Pediatr*. 49(12):1321-30

Gunn AJ and Bennet L. (2009) Fetal hypoxia insults and patterns of brain injury: insights from animal models. *Clin perinato.* 36(3): 579-93

Halliday HL. (2009) Neonatal management and long-term sequelae. *Best Prac Clin Obstet Gynaecol.* 23:871-880

Harvey D, Prince J, Bunton J, Parkinson C, and Campbell S. (1982) Abilities of children who were small-for-gestational-age babies. *Pediatrics*. 69(3):296-300

Indredavik MS, Vik T, Evensen KAI, Skranes J, Taraldsen G, and Brubakk AM. (2010) Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr* 31(4):286-94

Jahn R, Schiebler W, Ouimet C, and Greengard P. (1985) A 38,00-dalton membrane protein (p38) present in synaptic vesicles. *Proc Natl Acad Sci USA* 82:4137-4141

Jansson T and Persson E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Ped Res.* 28:203-208

Jiang C and Schuman EM. (2002) Regulation and function of local protein synthesis in neuronal dendrites. *Biochem Sci.* 27(10):506-513

Jones CT and Parer JT. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *J Physiol.* 343:525-537

Keen AE, Frasch MG, Sheehan MA, Matushewski B, and Richardson BS. (2011) Maturational changes and effects of chronic mypoxemia on electrocortical activity in the ovine fetus. *Brain Res.* 1402:38-45

Laferber HN, Rolph TP, and Jones CT. (1984) Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol.* 6:441-459

Leclerc N, Beesley PW, Brown I, Colonnier M, Gurd JW, Paladino T, and Hawkes R. (2010) Synaptophysin expression during synaptogenesis in the rat cerebellar cortex. *J Comp Neurol.* 280(2):197-212

Lennon AM, Francon J, Fellous A, and Nunez J. (1980) Rat, mouse, and guinea pig brain development and microtubule assembly. *J neurochem.* 35(4):804-813

Maitia P, Singh S, Muthurajua S, Veleria S, Ilavazhagana G. (2007) Hypobaric hypoxia damages the hippocampal pyramidal neurons in the rat brain. *Brain Res* **1175**: 1-9

Majewska A, Tashiro A, and Yuste R. (2000) Regulation of spine calcium dynamics by rapid spine motility. *J Neurosci*. 20(22):8262-68

Mallard C, Rehn A, Rees S, Tolcos M, and Copolov D. (1999)Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: implications for the aetiology of schizophrenia. *Schizop Res.* 40:11-21

Mallard C, Loeliger M, Copolov D, and Rees S. (2000) Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neurosci.* 100(2): 327-333

Mjaatvedt AE and Wong-Riley TT. (1988) Relationship between synaptogenesis and cytochrome oxidase activity in Purkinje cells of the developing rat cerebellum. *J comp neurol*. 277(2):155-18

Mundel P, Heid HW, Mundel TM, Kruger M, Reiser J, and Kriz W. (1997) Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J Cell Biol* 139:193-204

Nagerl UV, Kostinger G, Anderson JC, Martin KAC, and Bonhoeffer T. (2007) Protracted synaptogenesis after activity-dependent spinogenesis in hippocampal neurons. *J Neurosci.* 27(30):8149-56

Nakashiba T, Buhl DL, McHugh TJ, and Tonegawa S. (2009) Hippocampal CA3 output is crucial for ripple-associated reactivation and consolidation of memory. *Neuron*. 62(6):781-7

Nitsos I and Rees S. (1990) The effects of intrauterine growth retardation on the development of neuroglia in fetal guinea pigs. An immunohistochemical and an ultrastructural study. *Int J Devl Neurosci.* 8(3): 233-244

Pallotto EK and Kilbride HW. (2006) Perinatal outcomes and later implications of intrauterine growth restriction. *Clin Obstet Gynecol* 49(2):257-69

Pollack RN and Divon MY. (1992) Intrauterine growth retardation: Definition, classification, and etiology. *Clin Obstet Gyne*. 35:99-107

Reed JM and Squire LR. (1997) Impaired recognition memory in patients with lesions limited to the hippocampal formation. *Behav Neurosci*. 111(4):667-75

Rees S and Harding R. (1988) The effects of intrauterine growth retardation on the development of the purkinje cell dendritic tree in the cerebellar cortex of the fetal sheep: a note on the ontogeny of the purkinje cell. *Int J Dev Neurosci.* 6(5):461-669

Rocha E, Totten S, Han V, Richardson B. (2004) Structural proteins during brain development in the preterm and near term ovine fetus and the effect of intermittent umbilical cord occlusion. *Am J Obstet Gynecol* **191**: 497-505, 2004

Ruthazer ES, Li J, and Cline HT (2006) Stabilization of axon branch dynamics by synaptic maturation. *J Neurosci.* 26(13):3594-3603

Scheiffele P. (2003) Cell-cell signaling during synapse formation in the CNS. *Annu Rev Neurosci*. 26:485-508

Synnes AR, Anson S, Arkesteijn A, Butt A, Grunau RE, Rogers M, and Whitfield MF. (2010) School entry age outcomes for infants with birth weight ≤800 grams. *J Pediatr*. 157(6):989-94

Tarsa L and Goda Y. (2002) Synaptophysin regulates acitivity-dependent synapse formation in cultured hippocampal neurons. *PNAS* 99:1012-16

Tolcos M and Rees S. (1997) Chronic placental insufficiency in the fetal guinea pig affects neurochemical and neuroglial development but not neuronal numbers in the brainstem: A new method for combined stereology and immunohistochemistry. *J comp neurol.* 379:99-112

Tolcos M, Harding R, Loeliger M, Breen S, Cock M, Duncan J, and Rees S. (2003) The fetal brainstem is relatively spared from injury following intrauterine hypoxemia. *Dev Brain Res*. 143:73-81

Turner AJ and Trudinger BJ. (2009) A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta.* 30:236-240.

Walker DM and Marlow N. (2010) Neurocognitive outcome following fetal growth restriction. *Arch Dis Child Fetal Neonatal Ed.* 93:F322-25

Welker W, Johnson JI, and Noe A. (2010) Comparative mammalian brain collections: the domesticated guinea pig brain. http://www.brainmuseum.org/Specimens/rodentia/guineapig/sections/thumbnail.html

CHAPTER 5

EFFECT OF FETAL GROWTH RESTRICTION ON MYELIN FORMATION IN THE GUINEA PIG BRAIN AT TERM

5.1 INTRODUCTION

Myelin is essential for insulating the electrochemical signal sent along the axons of neurons. Proper myelination throughout development ensures neuronal connectivity and efficient communication between regions of the central nervous system. In humans, myelin sheath proteins are found in the brain as early as 5 weeks gestation and become more abundant with advancing gestational age (Jakovcevski 2005). While *in vitro* studies in mice have shown the ability of oligodendrocytes to form myelin sheath-like substances in the absence of neurons (Temple 1986), generally the presence of neurons and specifically the electrical activity in the axons of neurons stimulates myelination in that axon (Barres 1993). Myelin sheath thickness dictates conduction velocity of an axonal signal which is instrumental in synchronous firing of action potentials and thereby strengthens neuronal connections (Fields 2008). The increasing electrical activity in maturing neurons perpetuates myelination over the course of gestation which in turn ensures salutatory conduction and coordinated synaptogenesis between neuronal connections. Therefore this complex process of myelination is essential for neurological development and lifelong health of the brain.

Various studies in animals have shown that fetal growth restriction (FGR) is associated with changes in white matter development. The majority of studies examine changes in white matter associated with asymmetrical FGR (aFGR), while symmetrical FGR (sFGR) and alterations in myelination is largely unstudied. Nitsos *et al.* (1990) used a guinea pig model of placental insufficiency, similar to the present study, and found reduced myelination in the corticospinal tract and a reduced number of myelinated nerve fibers in aFGR compared to control animals. Additionally, aFGR fetuses exhibited myelin sheath thickness that was thin relative to the axonal diameter (Nitsos 1990). Another study reported white matter volume and

myelin proteins to have decreased in the cortex and cerebellum of aFGR guinea pig fetuses, although with no difference in mature axonal projections (Tolcos 2011). Furthermore, a study of FGR induced by uterine artery ligation in rats showed a delay in myelination with moderate growth restriction and that the severity and duration of white matter damage correlated with the severity of growth restriction (Olivier 2007). Since FGR is proportional to a deficiency in myelin, abnormal myelination in the hippocampus could indicate aberrant neuronal connectivity and subsequent cognitive deficits.

Myelin is produced by glial cells, specifically oligodendrocytes in the CNS. It is made up of 70-85% lipids and 15-30% protein that together make it electrically insulating. Oligodendrocytes precursors are highly expressed in the human fetus at ~30 weeks gestation and increase in number with increasing myelination (Back 2001). In humans, the myelin-phospholipids appear in CNS white matter prior to initiation of active myelin synthesis and increase in expression throughout gestation (Kinney 1994). Axonal growth must reach the critical amount of 0.5 μm in diameter to be able to myelinate (Nitsos 1990). The earliest myelin sheath is seen in the thalamus at ~18 weeks gestation, in the internal capsule at 21 weeks gestation, and develops in telencephalic areas after mid-gestation (Jakovcevski 2007). In humans major myelin proteins expression is temporally and spatially regulated depending on peak myelination in a brain region (Kinney 1994). The guinea pig undergoes precocious myelination (Booth 1980), and so delays in myelination are likely amplified making the guinea pig a good model to study the effect of FGR on myelination.

Myelin Basic Protein (MBP) comprises 35% of the protein in the myelin sheath and is often used as a marker for myelination. MBP is a common marker used for myelination as it is essential for both formation and maturation. MBP is not expressed in the human brain before 27

weeks gestation, thereafter appearing in minimal amounts at ~30 weeks and more so at 40 weeks gestation but mostly in superficial white matter areas (Back 2001). Prior to myelination, MBP is found in precursor oligodendrocytes and is later transported to the axon for myelination (Pedraza 1997). There are several isoforms of MBP; in guinea pigs they range in size from 14 kDA, 16 kDa, 17.2 kDa, 18.5 kDa, 20.2 kDa, and 21.5 kDa (Maatta 1997). The 14 and 18.5 kDA isoforms participate in membrane compaction and the 17.2 and 21.5 kDa MBP isoforms are expressed earliest in development and may play a role in oligodendrocyte maturation and myelination initiation (Pedraza 1997). Changes in MBP indicate alterations in myelination.

A common histological stain used for myelin is Luxol fast blue (LFB). LFB is an arylguanidinium salt of anionic chromagens which stains proteolipid protein, lipids and any hydrophobic domain of myelin using a lithium carbonate differentiation (Clasen 1967; Kiernan1990). The base of the dye substitutes the base of the target lipoprotein creating a blue precipitate. LBF staining has also been used as a marker of myelination during early development (Brody 1987; Kinney 1988). Changes in either of these myelin markers will indicate alterations in myelination in the fetal guinea pig brain.

Structural alterations in myelin are associated with an increased risk for a variety of disorders including attention deficit hyperactivity disorder, language disorder, autism spectrum disorder, cognitive decline, and schizophrenia (Fields 2008). Many of these disorders are also associated with FGR as previously discussed (de Rodriguez 2006; Synnes 2010; and Rehn 2004). Animal studies have shown induced FGR to affect myelination in areas that develop in the latter half of pregnancy such as the hippocampus more so than those areas that form and myelinate during early gestation such as the optic nerve (Schober 2009, Loeliger 2005). It has also been shown that oligodendrocyte precursors have delayed maturation with FGR and chronic hypoxia

potentially decreasing myelination (Segovia 2008). Few studies have examined changes in neuronal myelination in sFGR or in large for gestational age (LGA) animals, although the paradigm of developmental programming associates many adverse postnatal outcomes with altered *in utero* development across spectrum including FGR and LGA. This present study therefore tested the hypothesis that induced FGR in guinea pig fetuses at term will result in reduced MBP expression in the brain, as measured by immunohistochemistry, LFB staining and total MBP protein levels as measured by Western blot analysis in the hippocampus thereby indicating altered myelination compared to appropriate for gestational age (AGA) animals.

5.2 MATERIALS AND METHODS

5.2.1 Fetal guinea pig collection and brain processing for histology and Western blots

Fetal guinea pigs were collected as described in Chapter 3. Briefly explained, the cohorts were organized as LGA, AGA, aFGR and sFGR based on body weight at term and brain-to-liver weight ratio (Figure 4.1). Subsequent to fetal necropsy, brains were processed for histology and sectioned to the same coronal level described in Chapter 4 (guinea pig brain level 860, Figure 4.2), according to the brain museum guinea pig atlas (Welker 2010). In addition to 5 μm sections used for immunohistochemistry of MBP, 10 μm sections were cut for LFB staining. Fourteen additional fetal guinea pigs were collected at term for Western blot analysis of MBP with brains prepared and microdissected as previously described in Chapter 4 (Figure 4.3 and 4.4).

5.2.2 Immunohistochemistry of myelin basic protein

Immunohistochemistry for MBP was conducted with the same protocol as synaptophysin staining as described in Chapter 4. Briefly, MBP immunoreactivity (MBP-IR) was assessed by avidin-biotin complex-enhanced immunohistochemistry (Vectastain; Vector Laboratories, Burlingame, California). To reduce staining variability, all immunohistochemistry for MBP was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinized then subjected to post-fixation and antigen-retrieval. Endogenous peroxidase, biotin and avidin were then quenched followed by a SNIPER block. Sections were then incubated with primary antibody (mouse anti-human monoclonal MBP, 1:100, Lifespan Biosciences, Seattle Washington) overnight at 4°C then incubated with secondary antibody (biotinylated goat anti-mouse immunoglobulin G, 1:500; Vector) at room temperature for 30 minutes. Sections were then incubated with an avidin and biotin complex (ABC) solution (avidin-biotin peroxidase complex, 1:50; Vector Laboratories) and visualized with Cardassian D.A.B (3',3'-diaminobenzadine) chromagen (Biocare Medical, Brampton Ontario) at room temperature for 4 minutes and 30 seconds. Sections were then dehydrated before being cover slipped using permount.

5.2.3 Histochemical stain for myelin: luxol fast blue

The quantity of myelin was further assessed using LFB staining. To reduce staining variability, all the LFB staining was performed at the same time with the same reagents. In order to ensure adequate differentiation, adult human cerebellum sections were included with the fetal brain sections. These have distinct grey and white matter tracts that allow for a clear measure of

myelinated areas. Tissue sections were deparaffinised in 3 sequential xylene baths for 5 minutes each and subsequently rehydrated in baths of 100% (2x2minutes) and 95% (2x2minutes) ethanol. The slides were then placed in 0.1% LFB (Solvent blue 38 stock: A15395 from Alfa Aesar in 95% ethanol with 10% acetic acid to stabilize solution) overnight at room temperature in sealed container to prevent ethanol evaporation. After 24 hours of staining, sections were rinsed briefly in distilled water (1x2 minutes) then dipped ten times (~3 seconds) in 0.5% lithium carbonate solution (99% lithium carbonate stock:13418 from Alfa Aesar dissolved in distilled water) to differentiate and distinguish between grey and white matter. Tissue sections were then washed in distilled water (1 x 1 minute) and dehydrated in alcohol baths of increasing concentration (95% 2×2 min; 100% 2×2 min) followed by three 5-minute rinses in xylene before being cover slipped using permount.

5.2.4 Imaging and statistical analysis for myelin basic protein and luxol fast blue

The slides were coded prior to image analysis to ensure this was done blinded to the animal cohort. The white matter areas examined for MBP and LFB staining included hippocampal efferents: the dorsal fornix (FOR) and fimbria (FIM,) parasagittal white matter (PSW), periventricular white matter (PVW), optic tract (OT), and corpus callosum (CC) (Figure 5.1). Staining was found on appropriate white matter tracts as expected based on literature.

Each brain area was imaged as 3-6 high power fields (HPF: images >400x magnification) in each brain area with a 63x objecting using a Leica DM6000B light microscope (Leica Microsystems, Buffalo Grove Illinois). Figure 5.2A depicts appropriate MBP IR in the FOR area of the brain along white matter tracts while Figure 5.2B depicts appropriate LFB staining in the

OT area of the brain on all hydrophobic molecules including proteolipid protein and lipids. Brightfield imaging for MBP and LFB was performed with constant white balance and exposure settings for all slides in the same brain area for each respective stain as determined based on the negative control slide, as well as the particular brain area being imaged. With each new slide or change in objective, the field diaphragm was centered and focused to maximize Kohler illumination.

HPF images were scored for percent area staining of MBP immunoreactivity (IR) or histochemical staining of LFB using ImagePro Plus 6.0 software (Meyer Instruments, Houston Texas). ImagePro Plus was calibrated for magnification. Full colour images of MBP IR and LFB were background corrected to delete any debris from the Leica microscope camera that was imaged alongside the staining. If the HPF image included evident debris or tissue artifact then a polygon was drawn around the specific areas of interest in the HPF in order to count percent area positive stain in a particular brain area with confidence.

All MBP IR and LFB images were then equalized individually; the 256 bit display range was shortened to maximize the image data over the whole scale in order to better define colour differences. The display range was shortened to a colour range that maintained the integrity and definition of intracellular components and did not underestimate the intensity of staining. The display range was shortened for MBP IR to 200 bit, and for LFB to 150 bit that encompassed all the data and were then expanded back over the 256 bit range.

An automatic standard brown threshold was then applied to the MBP IR HPF image with DAB staining for the 3 colours; red – 215, green – 175, and blue – 142 to count DAB visualized protein-IR (Figure 5.2A). For LFB staining an automatic blue threshold was applied for the 3

colours; red- 85, green- 167 and blue- 255 (Figure 5.2B). The threshold to define positive staining for MBP IR and LFB was adjusted on an individual image basis to define positive stain and count percent area positive stain in a HPF.

The mean fractional area positively stained for MBP and LFB was calculated for each brain area based on the average in all the HPF images taken for that area. Percent area does not reflect normal distribution so data were transformed to allow for comparison using parametric tests. In cohorts with percent area staining in the range of 0-30% or 70-100% a square root transformation was applied. In cohorts with data that did not constitute a square root transformation or data that did not lie in the 30-70% range, an arc sine transformation was applied for analysis. All graphs presented depict untransformed data but significant differences were based on transformed data. Comparison to AGA animals was done using an unpaired t-test using GraphPad Prism 5.0 (GraphPad Software, San Diego, California). All results are presented as mean percent area ± standard error of the mean (SEM) and for all analyses, a statistical significance was assumed for $p<0.05$.

5.2.5 Western blot protocol for myelin basic protein

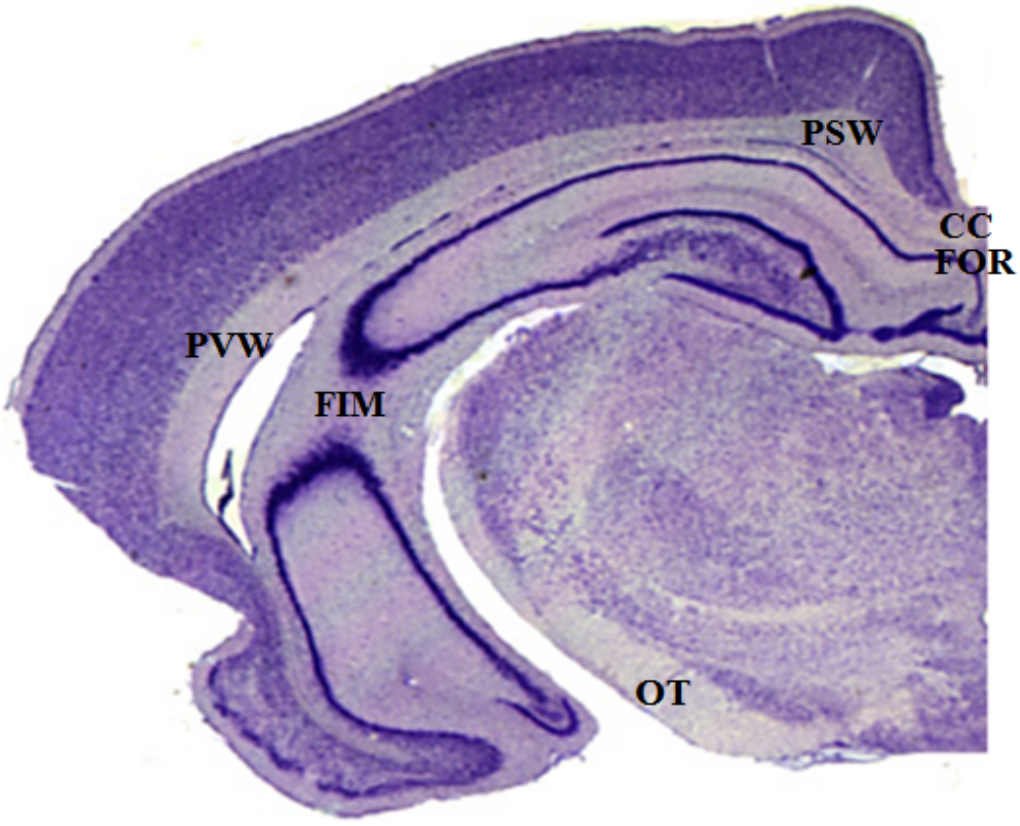
Western blot analysis for MBP was conducted with the same protocol as synaptophysin staining as described in Chapter 4. Briefly, 30 µg of protein was fractionated in a 13% polyacrylamide gel (Invitrogen Life Technologies, Burlington Ontario) for MBP analysis, and transferred onto polyvinyllidenedifluoride membrane (Millipore, Etobicoke Ontario). Protein transfer was visualized with Amido Black (Sigma-Aldrich, Oakville Ontairo). Blots were then blocked with 5% milk + Tris buffered saline-Tween 20 buffer for 1 hour at room temperature

and probed using mouse anti-human monoclonal MBP (1:2000, Lifespan) overnight at 4ºC. This was followed by horseradish peroxidise conjugated donkey anti-mouse IgG (1:10 000, Jackson ImmunoResearch Laboratories, West Grove Pennsylvania) as the secondary antibody. Membranes were stripped and reprobed with mouse anti-β tubulin monoclonal antibody (1:1500, EMD Millipore, Billerica, Massachusetts) for 2 hours at room temperature. Immunoreactive bands for MBP at 17.2 kDA and 21.5 kDa and β-tubulin at 50 kDa was visualized and imaged as described in Chapter 4.

MBP IR protein bands underwent densitometry analysis using Image Lab 3.0 Software (Bio-Rad) for the dorsal and ventral hippocampus of each animal. β-tubulin is ubiquitously expressed in the immature brain and so reprobing allowed for normalization of blot densities to correct for loading variances. Spearman's rank correlation coefficient analyses were performed using GraphPad Prism 5.0 (GraphPad) to determine if there was a monotonic relationship between total protein changed with guinea pig body weight at term in either half of the hippocampus. All results are presented as mean of arbitrary values and a weak correlation was deemed for Spearman's coefficient (r_s) >0.2, and a relationship was deemed for Spearman's coefficient (r_s) >0.4 and a statistical significance was assumed for $p<0.05$.

Figure 5.1 Thionin stained coronal section 860 through the hippocampus at the level of the entorhinal cortex of an adult guinea pig (Welker 2010), indicating brain regions examined by myelin basic protein (MBP) and luxol fast blue (LFB). Areas of interest were the hippocampal efferents fimbria (FIM) and fornix (FOR), the corpus callosum (CC), parasagittal white matter (PSW), periventricular white matter (PVW), and the optic tract (OT).

#860

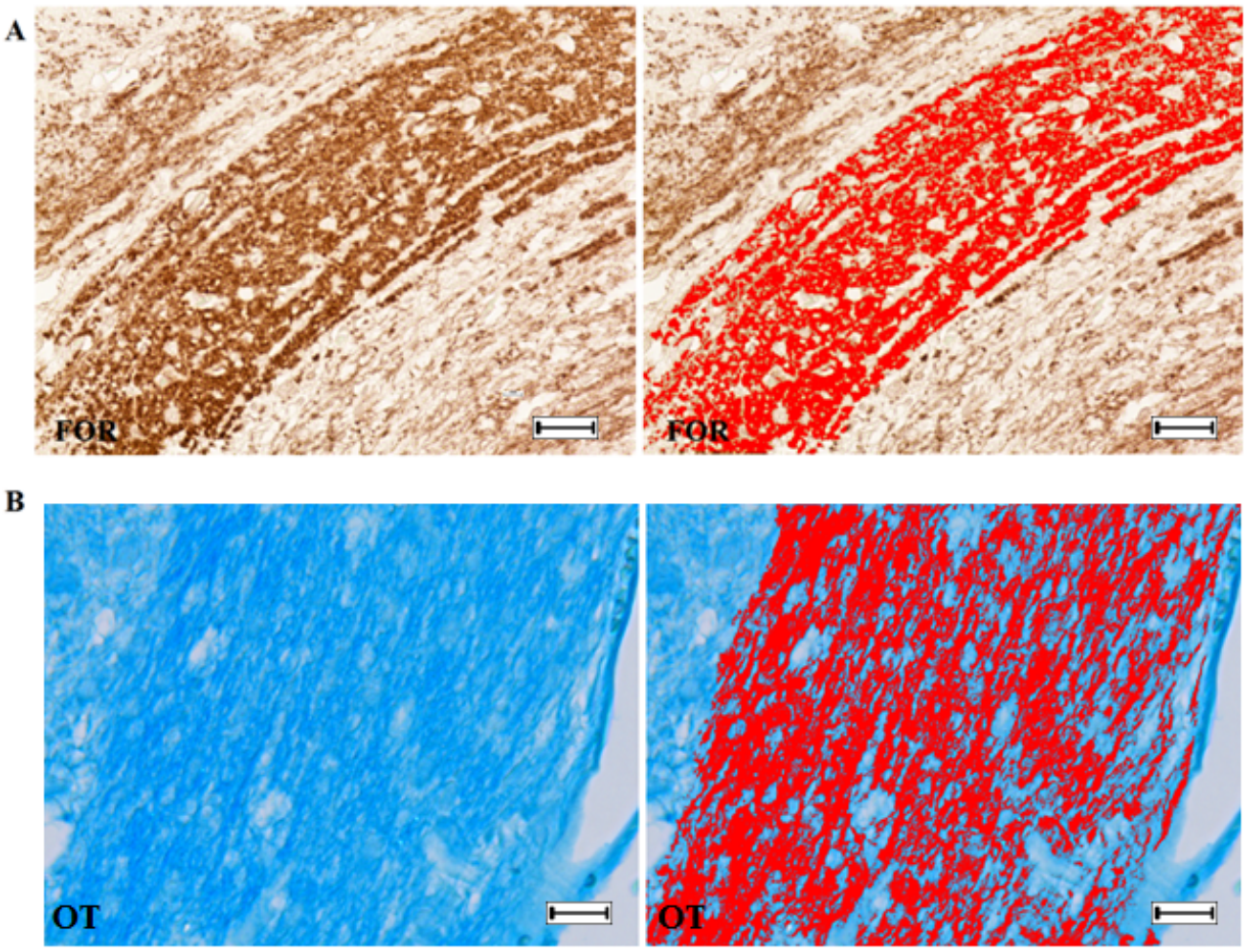


University of Wisconsin - Madison Brain Collection

5 mm

Figure 5.2 A Myelin basic protein immunoreactivity (MBP IR) in the fornix (FOR) showing appropriate MBP IR along white matter tracts in the original high power field (HPF) image for analysis (left) and the threshold applied (red) indicating positive stain.

B Luxol fast blue (LFB) staining in the optic tract (OT) showing appropriate LFB staining along white matter tracts in the original HPF image for analysis (left) and the threshold applied (red) indicating positive stain.


A
FOR
FOR
B
OT
OT

5.3 RESULTS

5.3.1 Myelin basic protein quantification

In the hippocampal efferent FOR, the percent area MBP IR was reduced in the aFGR at 38 ± 3% and the sFGR animals at 37 ± 4% compared to AGA animals at 49 ± 2% (both $p<0.005$) (Figure 5.3A). In the hippocampal efferent FIM, the percent area MBP IR in sFGR animals at 33 ± 2% was likewise reduced relative to AGA animals at 42 ± 2% ($p<0.02$), and while the percent area MBP IR in the aFGR animals at 36 ± 4% was not significantly different from that of AGA animals (Figure 5.3B).

The percent area MBP IR was reduced in the CC in aFGR at 6 ± 2% and sFGR animals at 3 ± 1% relative to AGA animals at 17 ± 3% (both $p<0.0001$) (Figure 5.4A). However, in the OT there were no significant differences in percent area MBP IR in any of the cohorts compared to the AGA animals (Figure 5.4B). In the PSW, percent area MBP IR in the aFGR at 17 ± 1% and sFGR animals at 14 ± 2% was reduced compared to AGA animals at 22 ± 2% (both $p<0.02$) (Figure 5.4C). In the PVW, the MBP IR in the sFGR animals at 10 ± 1% was likewise reduced compared to the AGA animals at 19 ± 2% ($p<0.0006$), while MBP IR in the aFGR animals at 16 ± 3% was not significantly different from that of AGA animals (Figure 5.4D).

5.3.2 Luxol fast blue quantification

In the hippocampal efferent FOR, percent area LFB staining in the LGA animals at 64 ± 7% was increased compared to the AGA animals at 38 ± 6% ($p<0.05$) and while the percent area LFB staining in aFGR animals at 25 ± 5% and sFGR animals at 26 ± 6% was reduced, they were

not significantly different from that of the AGA animals (Figure 5.5A). In the hippocampal efferent Fim, percent area LFB staining in the sFGR animals at 24 ± 10% was reduced compared to AGA animals at 54 ± 9% ($p<0.05$), but there were no other cohort group differences (Figure 5.5B).

In the CC and OT, there were no significant differences in percent area LFB staining for any of the cohort groups compared to the AGA animals (Figure 5.6A and B, respectively). In the PSW, the percent area LFB staining was again increased in the LGA animals at 55 ± 6% compared to the AGA animals at 35 ± 6% ($p<0.04$), and while the percent area LFB staining was reduced in the aFGR animals at 27 ± 12% and sFGR animals at 19 ± 8%, these were not significantly different from that of the AGA animals (Figure 5.6C). In the PVW, the percent area LFB staining was reduced in the sFGR animals at 12 ± 7% compared to AGA animals at 44 ± 5% ($p<0.003$), but there were no other cohort group differences (Figure 5.6D).

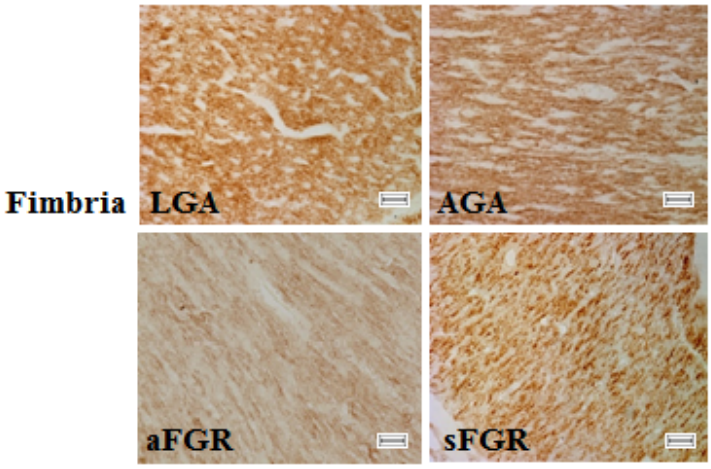
5.3.3 Western blot analysis for myelin basic protein

The total MBP 17.2 kDa protein had a weak positive relationship and a weak negative correlation with fetal body weight in the dorsal and ventral hippocampus (r_s=-0.2 and r_s=0.3, respectively, both $p>0.05$) (Figure 5.7a). The total MBP 21.5 kDa protein had no correlation with fetal body weight (r_s=-0.1, $p>0.05$) in the dorsal hippocampus and a weak positive correlation with fetal body weight (r_s=0.4, $p>0.05$) in the ventral hippocampus (Figure 5.7B). Interestingly, all tests suggested an approximated Gaussian distribution of the data points.

Figure 5.3 Representation of myelin basic protein immunoreactivity (MBP IR) in the fimbria (FIM) efferent tract of the hippocampus of a large for gestational age animal (LGA), appropriate size for gestational age (AGA) animal, asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animal.

A Percent area MBP IR per high power field (HPF) image in the fornix (FOR) a hippocampal efferent tract of fetal cohorts showing that aFGR and sFGR animals had reduced MBP IR than AGA animals. *=$p<0.05$ compared to the AGA cohort.

B Percent area MBP IR per HPF image in the FIM a hippocampal efferent tract of fetal cohorts showing that aFGR and sFGR animals had reduced MBP IR than AGA animals. *=$p<0.05$ compared to the AGA cohort.


Fimbria
LGA
AGA
aFGR
sFGR

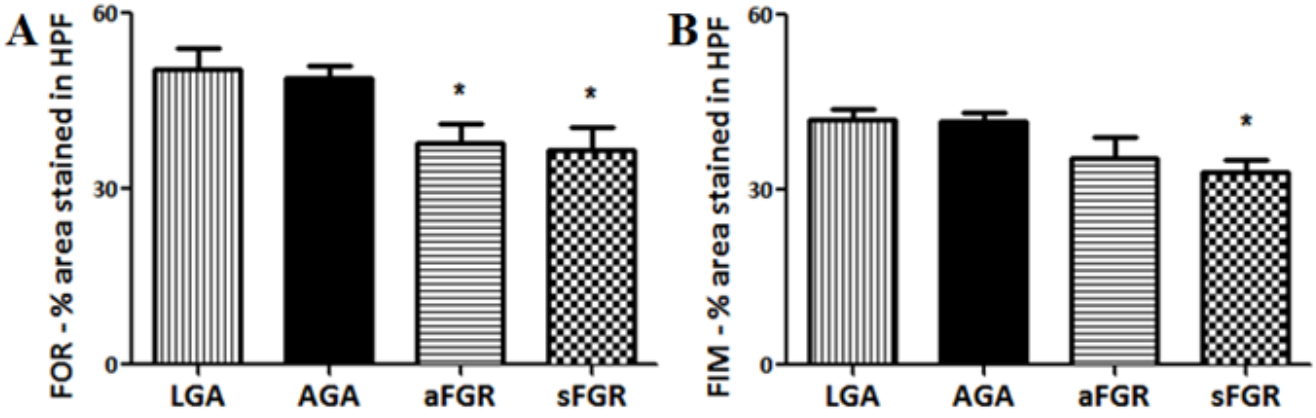

A
FOR - % area stained in HPF
60
30
0
*
*
LGA
AGA
aFGR
sFGR
B
FIM - % area stained in HPF
60
30
0
*
LGA
AGA
aFGR
sFGR

Figure 5.4 Representation of myelin basic protein immunoreactivity (MBP IR) in the parasagittal white matter (PSW) of a large for gestational age animal (LGA), appropriate size for gestational age (AGA) animal, asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animal.

A Percent area MBP IR per high power field (HPF) image in the corpus callosum (CC) of fetal cohorts showing that aFGR and sFGR animals had reduced MBP IR than AGA animals. *=$p<0.05$ compared to the AGA cohort.

B Percent area MBP IR per HPF image in the optic tract (OT) of fetal cohorts showing that aFGR and sFGR animals had reduced MBP IR than AGA animals., but this did not reach significance.

C Percent area MBP IR per HPF image in the PSW of fetal cohorts showing that aFGR and sFGR animals had reduced MBP IR than AGA animals. *=$p<0.05$ compared to the AGA cohort.

D Percent area MBP IR per HPF image in the periventricular white matter (PVW) of fetal cohorts showing that sFGR animals had reduced MBP IR than AGA animals. *=$p<0.05$ compared to the AGA cohort.

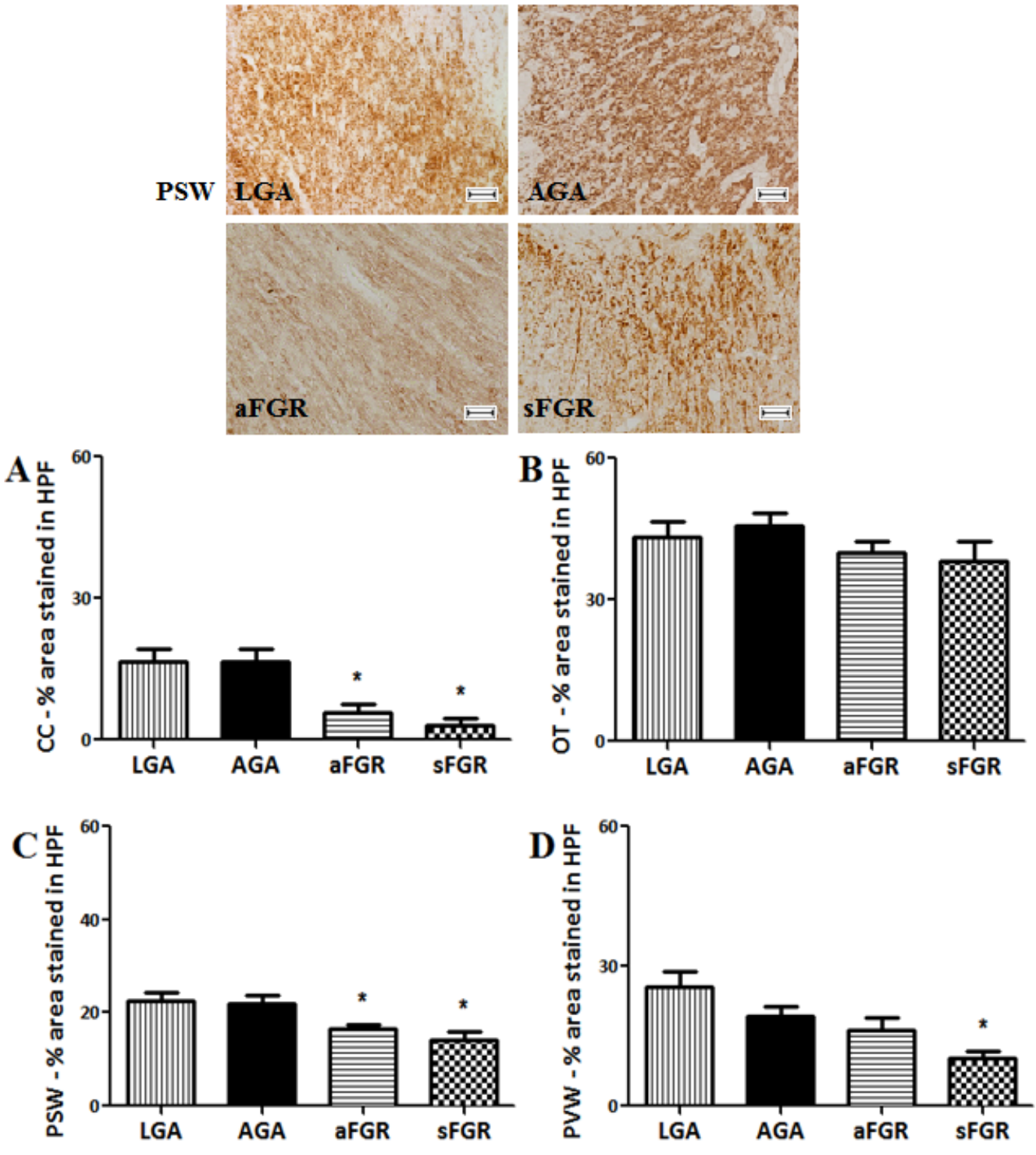

PSW
LGA
AGA
aFGR
sFGR
A
CC - % area stained in HPF
60
30
0
*
*
LGA
AGA
aFGR
sFGR
B
OT - % area stained in HPF
60
30
0
LGA
AGA
aFGR
sFGR
C
PSW - % area stained in HPF
60
40
20
0
*
*
LGA
AGA
aFGR
sFGR
D
PVW - % area stained in HPF
60
30
0
*
LGA
AGA
aFGR
sFGR

Figure 5.5 Representation of luxol fast blue (LFB) staining in the fimbria (FIM) efferent tract of the hippocampus of a large for gestational age animal (LGA), appropriate size for gestational age (AGA) animal, asymmetrical fetal growth restricted (aFGR) and symmetrical fetal growth restricted (sFGR) animal.

A Percent area LFB per high power field (HPF) image in the fornix (FOR), a hippocampal efferent tract, of fetal cohorts showing that LGA animals had increased LFB than AGA animals. *=$p<0.05$ compared to the AGA cohort.

B Percent area LFB per HPF image in the FIM, a hippocampal efferent tract, of fetal cohorts showing that sFGR animals had reduced LFB than AGA animals *=$p<0.05$ compared to the AGA cohort.

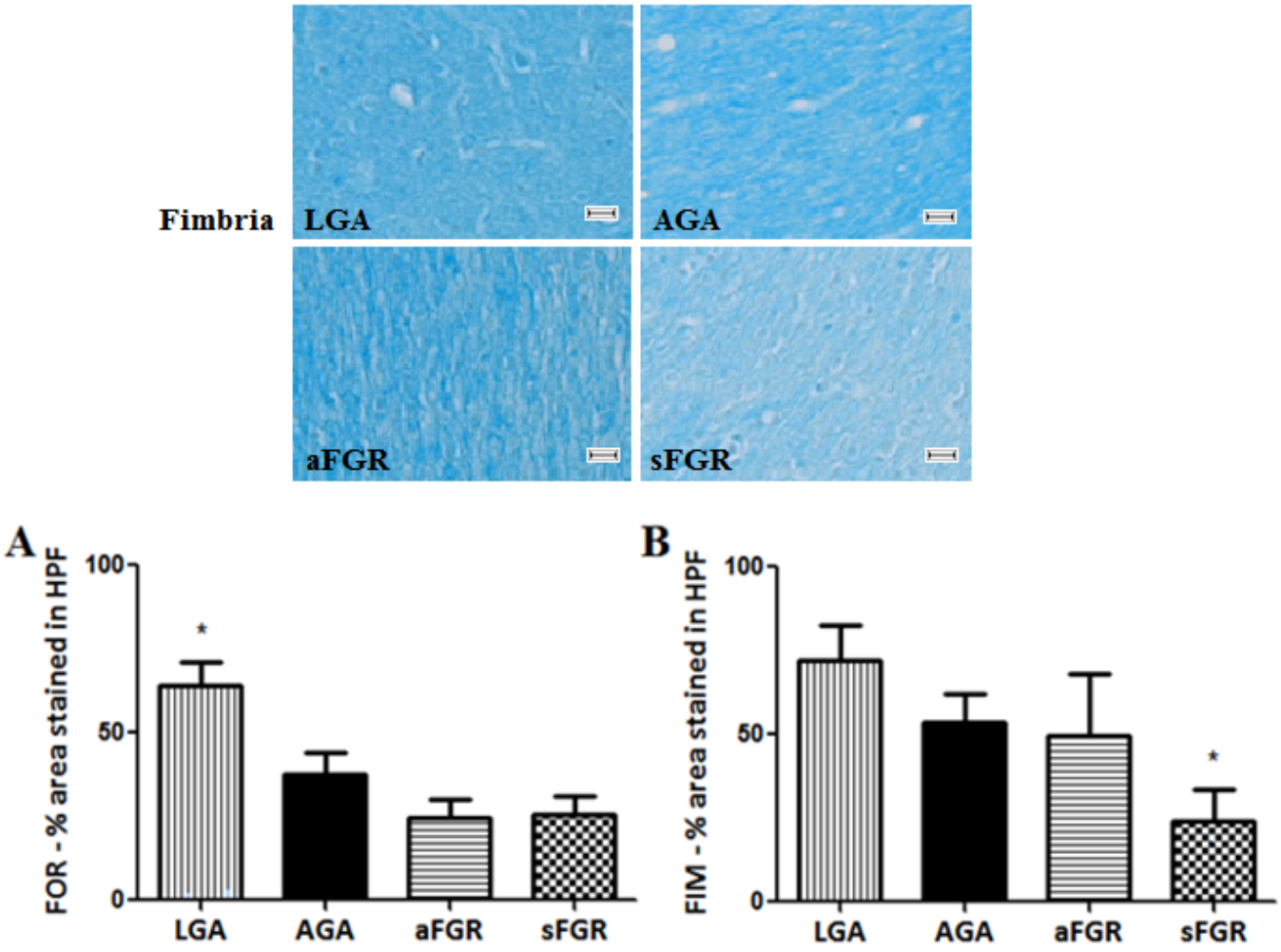

Fimbria
LGA
AGA
aFGR
sFGR
A
FOR - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR
B
FIM - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR

Figure 5.6 A Percent area luxol fast blue (LFB) per high power field (HPF) image in the corpus callosum (CC) of fetal cohorts showing no significant difference in staining in any cohort compared to appropriate size for gestational age (AGA) animals.

B Percent area LFB per HPF image in the optic tract (OT) of fetal cohorts showing no significant difference in staining in any cohort compared to AGA animals.

C Percent area LFB per HPF image in the parasagittal white matter (PSW) of fetal cohorts showing that large for gestational age (LGA) animals had increased LFB than AGA animals. *=$p<0.05$ compared to the AGA cohort.

D Percent area LFB per HPF image in the periventricular white matter (PVW) of fetal cohorts showing that symmetrical fetal growth restricted (sFGR) animals had reduced LFB than AGA animals *=$p<0.05$ compared to the AGA cohort.

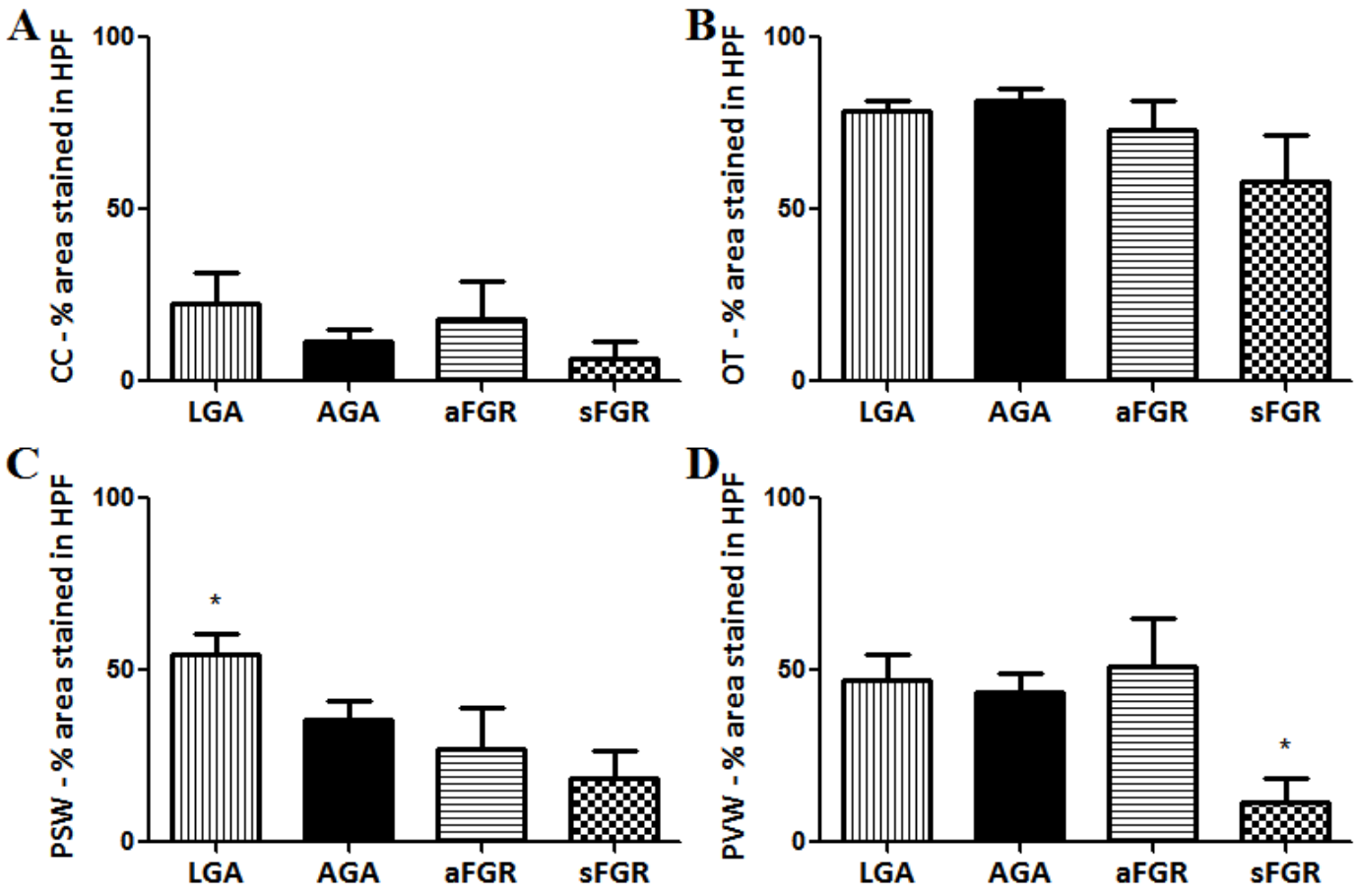
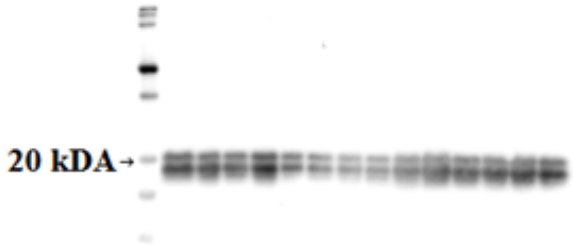
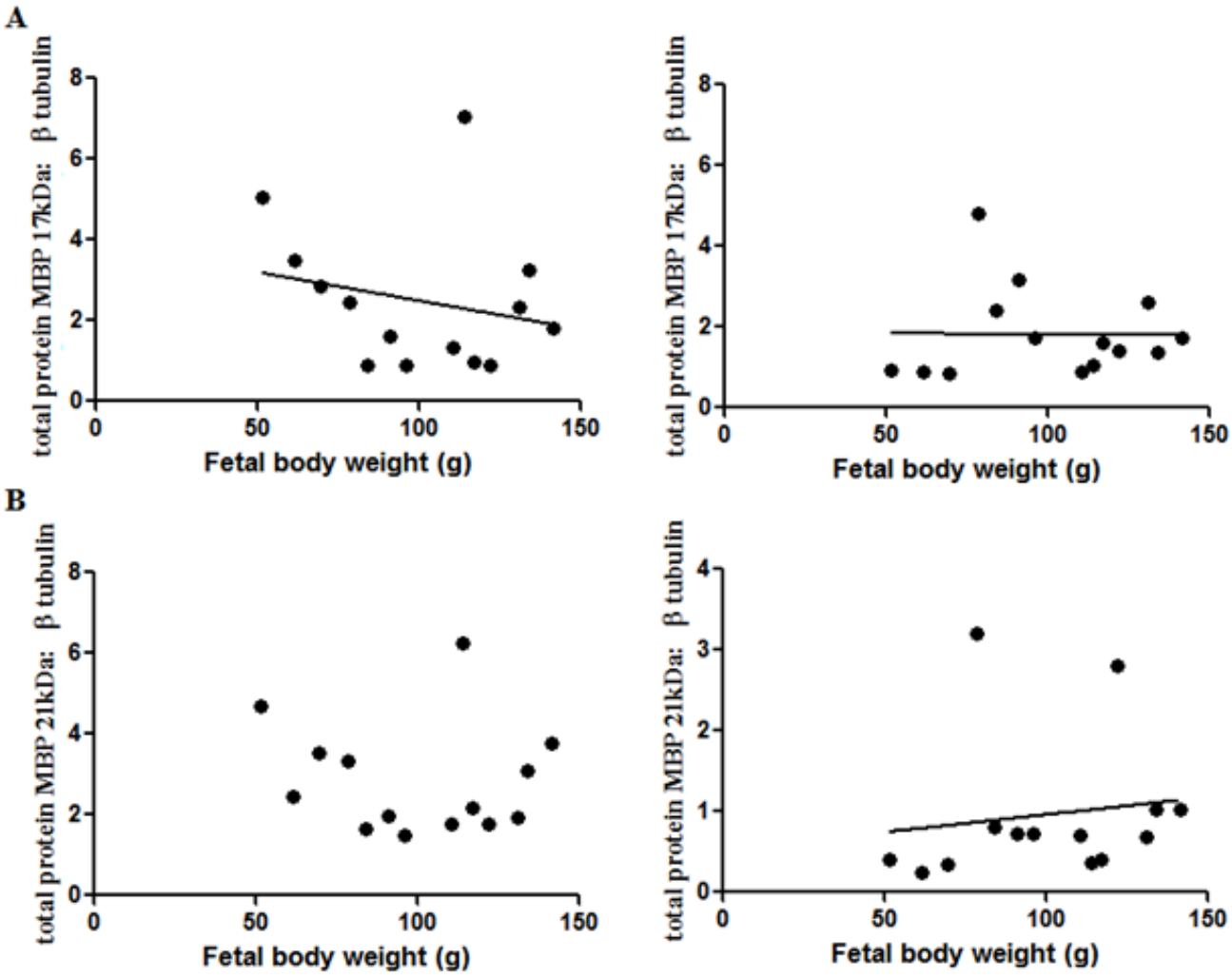

A
CC - % area stained in HPF
100
50
0
LGA
AGA
aFGR
sFGR
B
OT - % area stained in HPF
100
50
0
LGA
AGA
aFGR
sFGR
C
PSW - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR
D
PVW - % area stained in HPF
100
50
0
*
LGA
AGA
aFGR
sFGR

Figure 5.7A Representative Western blot for total myelin basic protein (MBP) protein where MBP protein bands are at 17.2 kDa (bottom bands) and at 21.5 kDa (top bands). Lane 1 is the Precision Plus protein standards dual colour marker. Lanes 2-15 are dorsal hippocampal protein samples (n=14).

A Fetal body weights and total MBP protein at 17.2 kDa had non-significant negative correlation in the dorsal hippocampus (left) and a non-significant positive correlation in the ventral hippocampus (right).

B Fetal body weights and total MBP protein at 21.5 kDa had no correlation with body weight in the dorsal hippocampus (left) and a weak positive correlation in the ventral hippocampus (right) but this did not reach significance.


20 kDA→


A
total protein MBP 17kDa: β tubulin
Fetal body weight (g)
total protein MBP 17kDa: β tubulin
Fetal body weight (g)
B
total protein MBP 21kDa: β tubulin
Fetal body weight (g)
total protein MBP 21kDa: β tubulin
Fetal body weight (g)

5.4 DISCUSSION

The precocious development of guinea pigs leads to a higher level of white matter development than humans at birth, accentuating changes in myelination due to FGR. There were reductions in MBP IR in both aFGR and sFGR animals compared to AGA animals in the FIM and FOR with similar patterns for LFB staining. The FIM and FOR are the areas containing the majority of efferent axons from the Perforant path of the hippocampus leading to many brain areas involved with learning and memory (Anderson 2007). Schober *et al* (2009) studied myelination in the hippocampi of rats with induced FGR and found similar decreases in MBP in both female and male rats. This suggests changes in myelination and thus neuroconnectivity involving the hippocampus with induced FGR in animal models as replicated in the present study. Interestingly, altered myelination in aFGR and sFGR fetuses was affected to similar degree in both fetal cohorts not reflecting the gradient change as in synaptogenesis as previously reported (Chapter 4). This could be a mechanism delaying intrinsic signalling between myelination and synaptogenesis to perpetuate synaptogenesis post-natally under better conditions. Proper myelination ensures the electrochemical signal that reaches the synaptic sites is at the proper frequency to create functional and stabilized connections (Fields 2008). As such, altered neuronal connections in the hippocampus could lead to the learning and memory difficulties associated with FGR in humans.

There was also decreased staining in the CC for MBP in the FGR animals compared to the AGA animals. However, there was no difference in staining in the OT between any of the groups. The OT is the fiber tract of the optic nerve caudal to the optic chiasm. Myelination has temporal and spatial specification and peak myelination in guinea pigs occurs in the OT during the first half of gestation and in the CC after mid-gestation (Clancy 2007). Accordingly, induced

FGR through reductions in placental blood flow and thereby in oxygen and nutrient delivery and operating mainly during the latter half of gestation, would be expected to impact much more on CC myelination than OT myelination as herein noted.

There was also a significant reduction of MBP IR in the FGR animals compared to AGA animals in the PSW area of the brain. This is not accompanied by a change SYN expression in the parasagittal grey matter (PSG) levels of the brain, as reported in Chapter 4, there appeared to be differences in myelination in the PSW area comprised of the afferent and efferent connections to and from the PSG area. At the coronal level measured, the PSG may correspond to the caudal portion of the entorhinal cortex which undergoes peak neurogenesis at ~24 days gestation in guinea pigs (Clancy 2009). Given the importance of the entorhinal cortex in memory function, changes in myelination *in utero* may describe a role for fetal programming for memory deficiency. Changes in structural integrity of MBP in various areas of the brain is associated with many psychiatric disorders including schizophrenia, obsessive-compulsive disorder, bipolar disease, depression, autism, dyslexia, and attention deficit hyperactivity disorder (Fields 2008). The mechanisms causing growth restriction in the present FGR animals seems to affect the cortex after synaptogenesis, but during myelination not allowing the neuronal connections to mature.

Changes to ventricles and PVW are often associated with FGR and schizophrenia. There is reduced ventricular size and also a reduction of white matter volume associated with aFGR and further supporting he causal relationship with schizophrenia (Rhen 2004). There was a significant decrease in MBP IR in the sFGR animals and a marginal decrease in aFGR animals compared to AGA animals in the PVW. Aberrant myelination could impact synaptogenesis as decreased myelination has been shown to inhibit axonal growth and prevents arborisation with

established neuronal connections (Fields 2008). Changes in myelination of various areas of the brain during early development could be associated with later neurological disorders seen in adult life.

LFB staining in the fetal cohort groupings showed a similar pattern to the MBP IR findings. LFB stains lipids in the myelin which is a large component of myelin, but the specificity of LFB will be different than MBP immunostaining since MBP immunohistochemistry targets specific myelin structural proteins, whereas LFB binds an assortment of hydrophobic molecules. Additionally, the proportion of lipid to protein in myelin is 80:20 and thereby with increased absolute lipid amounts more so than MBP in mature myelin. FGR animals in general showed a similar pattern of LFB staining as MBP staining but possibly indicating that decreases in myelination with FGR affected protein content more so than lipid content in myelin composition. Vallet *et al.* found myelin deposition decreased in FGR animals and increased in LGA animals at term (Vallet 2012). This could indicate thickness differences in mature and fully myelinated axons. There were increases in LFB staining in the LGA animals compared to the AGA animals in the FOR and PSW areas of the brain. LGA infants at birth are often associated with altered hypothalamis-pituitary axis disorder and risk for diabetes and cardiovascular disease, but have not been consistently shown to have cognitive difficulties (Yang 2011). Yang *et al.* did a study that included LGA, AGA and FGR children and found that IQ and cognitive scores increased with fetal size (Yang 2011). On the other hand, Heinonen *et al.* found a U-shaped or curvilinear relationship between body weight and later cognition showing that both small and large infants at term have lower scores on cognitive tests. (Heinonen 2008). This curvilinear pattern is similar to the pattern of the Western blot analysis, whereby none of the graphs showed significance but all were suggested to have a curvilinear pattern suggesting

altered myelination could occur in both FGR and LGA animals. With greater sample numbers, this pattern may be visualized. The size of the brain in the LGA animals was not different from the AGA animals in the present study, but it is probable that nutrient supply and availability differed accounting for the difference in body weight and possibly also impacting aspects of myelination. As LFB binds phospholipids, there may have been increased lipid availability and thereby accumulation and storage in the LGA animals accounting for the increased LFB staining seen in the FOR and PSW brain areas as large white tract areas.

Quite important for fetal development and often a treatment postnatally, are a number of growth factors necessary for optimizing fetal health. There are reductions in insulin and insulin-like growth factors (IGF) found in FGR animal studies (Barker 1998.) IGF plays an important role for increasing oligodendrocyte number and perpetuating myelination (Aberg 2010). In the cortex, there was a decrease in MBP IR expression but no change in synapse formation as measured by SYN IR in Chapter 4, which could be explained by decreased IGF-1 stimulus for oligodendrocyte and thereby myelin development, but with less impact on neurogenesis (Aberg 2010.) There is critical communication between axons, synapses and oligodendrocytes and specifically electrical activity in axons that can regulate myelination and synaptic maturation by signalling oligodendrocyte myelination to occur (Barres and Raff 1993, Demerens 1996; Ishibashi 2006; Stevens 1998). Accordingly, delays in myelination may interfere with synchrony of neuronal firing which is essential for synaptic maturation, by and large reducing competence of neuronal connectivity.

5.5 CONCLUSION

There was reduced myelination as measured by both MBP IR and LFB staining in FGR animals and increased myelination, but only as measured by LFB staining, in LGA animals when compared to that of AGA animals, albeit dependant on the brain area being studied. The decreases in myelination follow the patterns of reduced synaptic formation and maturation as previously reported (Chapter 4). The hippocampal efferent areas showed decreased myelination demonstrating delays in the maturation of neuronal connectivity compared to AGA animals. This delay if not corrected in early childhood could represent part of the substrate of cognitive deficits in affected children. Those structures that undergo myelination earlier, such as the OT, will be less affected in FGR animals than structures that undergo myelination later in gestation. As well, there were decreases in myelination in the PVW in the FGR animals which may contribute to the paradigm of developmental origin of schizophrenia which is also associated with reductions in PVW volume.

5.5 REFERENCES

Aberg D. (2010) Role of the growth hormone/insulin-like growth factor 1 axis in neurogenesis. *Endocr Dev.* 17:63-76

Anderson P, Morris R, Amaral D, Bliss T, and O'Keefe J. (2007) *The hippocampus book.* Oxford University Press.

Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, and Kinney HC. (2001) Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci*. 21(4):1302-12

Barker DJP. (1998) *In utero* programming of chronic disease. *Clin Sci*. 95:115-128

Barres BA and Raff MC (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature* 361, 258-260

Booth RFG, Patel TB, and Clark JB. (1980) The development of enzymes of energy metabolism in the brain of a precocial (guinea pig) and non-precocial (rat) species. *J* Neurochem. 34(1):17-25

Brody BA, Kinney HC, Kloman AS, Gilles FH. (1987) Sequence of central nervous system myelinaiton in human infancy. I. An autopsy study of myelination. *J Neuropathol Exp Neurol*. 46(3):23-301

Clancy B, Kersh B, Hyde J, Darlington RB, An KJS and, Finlay BL (2007) Web-Based Method for translating Neurodevelopment from laboratory species to humans. *Neuroinformatics* 5-1:79-94. http://people.psych.cornell.edu/~blf2/pdfs/BCBKBLFNI07.pdf

Clasen RA, Simon GR, Ayer JP, Pandolfi S, and Laing IR. (1967) A chemical basis for the staining of myelin sheaths by luxol dye techniques; further observations (abstract and discussion). *J Neuropathol Exp Neurol*. 26:153-4

Demerens C, Stankoff B, Logak M, Anglade P, Allinquant, Couraud F, Zalc B, and Lubetzki C. (1996) Induction of myelination in the central nervous system electrical activity. *Proc Natl Acad Sci USA.* 93(18):9887-92

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

Fields RD. (2008) White matter in learning, cognition, and psychiatric disorders. *Trends Neurosci.* 31,361-370.

Heinonen K, Rainkkonen K, Pesonen AK, Kajantie E, Andersson S, Eriksson JG, Niemela A, Vartia T, Peltola J, and Lano A. (2008) Prenatal and postnatal growth and cognitive abilities at 56 months of age: a longitudinal study of infants born at term. *Pediatr.* 121(5):e1325-33

Ishibashi T, Dakin KA, Stevens B, Lee PR, Kozlov SV, Stewart CL, Fields RD. (2006) Astrocytes promote myelination in response to electrical impulses. *Neuron.* 49(6):823-32

Jakovcevski I, Mo Z, Zecevic N. (2007) Down-regulation of the axonal polysialic acid-neural cell adhesion molecule expression coincides with the onset of myelination in the human fetal forebrain. *Neurosci.* 149: 328-37

Jakovcevski I and Zecevic N. (2005) Sequence of oligodendrocyte development in the human fetal telencephalon *Glia* 49, 480-491

Loeliger M, Duncan J, Louey S, Cock M, Harding R, and Rees S. (2005) Fetal growth restriction induced by chronic placental insufficiency has long term effects on the retina but not the optic nerve. *Invest Opthalmol Vis Sci* 46(9):3300-3308

Kiernan JA. (1990) *Histological and Histochemical methods: Theory and practice second edition*. Pergamon press, Great Britain Exeter by BPCC Wheatons Ltd. p320

Kinney HC, Brody BA, Kloman AS, Gilles FH. (1988) Sequence of central nervous system myelination in human infancy. II. Patterns of myelination in autopsied infants. *J Neuropathol Exp Neurol*. 47(3):217-34

Kinney HC, Karthigasan, Borenshteyn NI, Flax JD, and Kirschner DA. (1994) Myelination in the developing human brain: Biochemical correlates*. *Neurochem Res* 19(8):983-996

Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, Kelsh RN, and Appel B. (2006) *In vivo* time-lapse imaging shows dynamic oligodendrocyte progenitor behaviour during zebrafish development. *Nature Neuroscience.* 9(12):1506-11

Maatta JA, Coffey ET, Hermonen JA, Salmi AA, and Hinkkanen AE. (1997) Detection of myelin basic protein isoforms by organic concentration. *Biochem Biophys Res Comm*. 238: 498-502

Nitsos I and Rees S. (1990) The effects of intrauterine growth retardation on the development of neuroglia in fetal guinea pigs. An immunohistochemical and an ultrastructural study. *Int J Devl Neurosci.* 8(3): 233-244

Olivier P, Baud O, Bouslama M, Evrard P, Gressens P and Verney C. (2007) Moderate growth restriction: deleterious and protective effects on white matter damage. *Neurobiol Dis* 26(1):253-263

Pedraza L, Fidler L, Staugaitis SM, and Colman DR. (1997) The active transport of myelin basic protein into the nucleus suggests a regulatory role in myelination. *Neuron*. 18:579-589

Rehn AE, Van Den Buuse M, Copolov D, Briscoe T, Lambert G, and Rees S. (2004) An animal model of chronic placental insufficiency: relevance to neurodevelopmental disorders including schizophrenia. *Neurosci.* 124: 381-391

Schober ME, McKnight RA, Yu X, Callaway CW, Ke X and Lane RH. (2009) Intrauterine growth restriction due to uteroplacental insufficiency decreased white matter and altered NMDAR subunit composition in juvenile rat hippocampi. *Am J Physiol Regul Integr Comp Physiol* 296:R681-R692

Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, Riddle A, Craig A, Struve J, Sherman LS, Back SA. (2008) Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol*. 63(4):520-30

Stevens B, Tanner S, and Fields RD. (1998) Control of myelination by specific patterns of neural impulses. *J Neurosci.*18(22):9303-11.

Temple S and Raff MC. (1986) Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions *Cell* 44, 773-779

Tolcos M, Bateman E, O'Dowd R, Markwick R, Vrijsen K, Rehn A, and Rees S. (2011) Intrauterine growth restriction affects the maturation of myelin. *Exp Neurol*. 232:53-65

Vallet JL and Miles JR (2012) Comparison of myelination between large and small pig fetuses in late gestation. *Anim Repro Science.* Epub.

Yang S, Platt RW, and Kramer MS. (2011) Variation in child cognitive ability by week of gestation among healthy term births. *Am J Epidemiol*. 171(4):399-406

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The intrauterine milieu is now recognized as an important determinant of neurological health and cognitive capacity. Human studies have demonstrated a link between low birth weight, which suggests a sub-optimal intrauterine environment, and a number of cognitive deficits and neurological disorders in both childhood and adulthood. The various encephalopathies associated with fetal growth restriction (FGR) includes such prevalent disorders as cerebral palsy, attention deficit hyperactivity disorder, schizophrenia, epilepsy, and psychiatric hospitalization (Cannon 2003; de Rodrigues 2006; Halliday 2009; Indredavik 2010; Walker 2010). Cognitive limitations are also imposed. More subtle neurological deficiencies evident in FGR offspring throughout life include impaired memory and learning, poor academic performance, inattention, reduced psychosocial function, sensorineural deterioration, reduced mathematics abilities, and reduced intelligence quotient scores (de Rodrigues 2006; Geva 2008; Indredavik 2010; Pallotto 2006; Synnes 2010; Walker 2010). The severity and persistence of these deficits and diseases relates to the severity of FGR, often there is no recovery (Isaacs 2000; Geva 2008; Indredavik 2010; and Synnes 2010). Thus, altered neurodevelopment during critical periods may confer permanent neurological impairment from which there is no course for recovery. There are possible prenatal and postnatal interventions effective during the perinatal window of neurological plasticity that have not yet been realized. Currently, within the field of fetal programming, there is much emphasis on understanding how early environmental factors lead to diabetes and obesity. However, fetal programming with respect to neurodevelopment is understudied. The dangers of interference or potential for maximizing cognitive capacity during periods of neurological plasticity are intriguing areas of study.

This study utilised techniques that mimic placental insufficiency, a common cause of FGR in humans. The most pertinent findings revealed that growth restricted guinea pig fetuses exhibit changes in synaptogenesis and myelination that are dependent on the area of brain studied and the phenotype of FGR. This altered neurodevelopment was primarily seen in areas of the hippocampus, which are involved in memory and learning. Thus these findings provide evidence that deficient endowment and maturation of neuronal connections established *in utero* may underlie later cognitive impairment in FGR offspring. Further, this work demonstrates that the uterine artery branch diathermy (UABD) technique, which is used infrequently for induction of placental insufficiency, is comparable to the commonly used uterine artery ligation (UAL) technique in the success of producing FGR and superior in terms of survival rate.

UAL and bilateral UABD function to reduce fetal blood flow to the fetuses in order to mimic the human condition of placental insufficiency that so often occurs in human FGR. A limitation of any technique that involves abating the uterine arteries is the high degree of fetal demise but this study found bilateral UABD to have a lower incidence of fetal demise than unilateral The unilateral UABD technique introduced by Turner and Trudinger was reported to have a lower incidence of fetal demise compared to other techniques such as bilateral UAL, ligation of uterine and ovarian arteries and ligation of the uterine artery and vein (Chiacharion 1976). The current study used both the UAL and the unilateral UABD as well as tested an alternative technique, bilateral UABD. It was demonstrated that the bilateral UABD offers improved survival rates over what has been reported for the unilateral UABD technique

The current study helped define an overlooked phenotype, the symmetrically growth restricted (sFGR) fetus that displays more severe neurodevelopmental changes relative to the

typically studied asymmetrical FGR (aFGR) fetus. Human FGR infants present with low birth weight with and without altered body proportion. Typically, epidemiological data that has demonstrated the association between early environmental insults and later neurological health define FGR based solely on birth weight. To our knowledge, the differentiation of sFGR and aFGR in terms of neurodevelopment and long term outcomes has not been studied. sFGR according to the defined criteria, exhibit altered synaptogenesis, maturation and myelination in the brain and thus provides evidence that this group is pathologically growth restricted rather than naturally small. In actuality, guinea pigs have an inherent tendency toward FGR that increases with litter size and number of pups in a horn (Detmer 1992; Eckstein 1955; Chaichareon 1976). The UABD and UAL is considered a late-onset placental strain that leads to aFGR and may further curb the development of fetuses that have been relatively growth inhibited since early gestation prior to surgery, thereby leading to aFGR. This study is unable to confirm the specific cause of growth impairment in the sFGR group. Nevertheless, the human studies which originally brought to light the phenomenon of developmental programming show that the association between birth weight and long-term sequellae occurs along a continuum.

In areas of the hippocampus, the total number of synapses as reflected by synaptophysin immunoreactivity (SYN IR) was decreased in FGR relative to normal birth weight fetuses, an effect that was more pronounced in the sFGR compared to the aFGR group. These data suggest that the proliferation of synapses *in utero* is blunted in growth restricted fetuses. Early in gestation, an abundance of synapses is created with rapid proliferation followed by selective deletion and strengthening of the synapses throughout life (Bourgeois 1997). This early maturational period concurs with an exponential increase in brain growth since synaptic maturation perpetuates the formation and growth of new synapses (Fletcher 1994). Thus, it is

possible that the reduced number of synapses in FGR fetuses indirectly results from interference in maturation. Indeed, the number of mature synapses measured by synaptopodin immunoreactivity (SYNPO IR) was decreased in sFGR fetuses. This inhibition in synaptic maturation was found only in sFGR animals, further demonstrating that the small fetus without signs of brain sparing is more vulnerable to aberrant brain development. The maintenance of synaptic maturity together with a reduction in synaptogenesis observed in aFGR fetuses may reflect the effectiveness of the brain-sparing response in ensuring selective maturation of synapses at the expense of forming new immature synapses. Synaptic formation and maturation is a high-energy consuming process and thus a suboptimal supply of nutrients and oxygen may account for the blunted synapse formation and maturation and overall growth rate in aFGR and sFGR fetuses. Another possible mechanism of altered maturation in FGR fetuses is blunted electrical activity since this is the primary stimulus that drives synaptic strengthening and stabilization (Nagerl 2007). Previous studies have demonstrated reduced behavioural state activity in ovine fetuses growth restricted by placental insufficiency, however these fetuses were asymmetrically growth restricted. Behavioural state activity in sFGR is currently unknown. The decrease in SYNPO IR in only sFGR animals and not aFGR animals compared to AGA animals could indicate the 'brain sparing' ensures selective maturation of synapses in the hippocampus at the expense of forming new immature synapses.

While grey matter in the hippocampal regions show reductions in synaptic numbers, the white matter tracts containing efferent neurons that supply the hippocampus exhibit signs of decreased myelination in FGR fetuses. This reduced myelination was measured by myelin basic protein (MBP) expression and confirmed by luxol fast blue (LFB) staining. Similar to synaptic formation and maturation, MBP IR was more severely influenced in the sFGR group compared

to aFGR animals. Myelination occurs concurrently with synaptogenesis, in that it is focused on a particular neuron that is in the process of forming a synapse. Myelination determines conduction velocity of an axonal signal which in turn instrumental in synchronous firing of action potentials and thereby strengthening of neuronal connections (Fields 2008). Thus, it is not surprising that less myelination is evident in neurons leading to grey matter areas that have experienced blunted synaptogenesis. Further, reduced myelination can be a cause of reduced synaptogenesis since intrinsic myelin signals can induce or inhibit axonal growth and arborisation as neuronal connections become established. Reductions in either synaptogenesis or myelination cause delays in the other, together leading to deficient neuronal connectivity across brain regions. Interestingly, while FGR animals displayed decreased myelination, LGA animals showed an increase in myelination as shown by LFB staining. Since the LFB stain binds hydrophobic domains, primarily the proteolipid protein and lipids of myelin, these changes suggest altered proportion in lipid-to-protein accumulation in these animals. LGA animals have higher levels of visceral and subcutaneous fat and so the excess nutrients available may result in the accumulation of lipid in myelin. An abundance of myelin may alter conductance velocity suggesting that the LGA animal phenotype should be examined for aberrant neurological behaviour.

The timing of synaptogenesis and myelination in a particular brain area determines its sensitivity to FGR. This study found the effect of FGR to be spacially dependent, whereby there were no difference in SYN IR in the parasagittal grey matter (PSG) and but found myelination to be reduced in the adjacent white matter as well as in the corpus callosum but not in the optic tract. In the visual cortex, the rapid phase of synaptogenesis proceeds early in pregnancy and likewise the myelination of the optic tract is one of the earliest to complete in the brain of guinea

pigs (Bourgeois 1997; Clancy 2009). In the results of this study, the optic tract showed no difference in expression of myelination markers suggesting areas that undergo early development may be spared under conditions leading to FGR. This sparing of early developing areas is also reported in other studies, Tolcos found no difference in neurogenesis in the FGR guinea pigs in the brainstem of FGR guinea pigs, an area essential for life and one of the earliest to form *in utero* (Bourgeouis 1997; Clancy 2009; and Tolcos 2003). As well, the current study showed no changes in SYN IR in the PSG but did show reduced myelination in FGR fetuses suggesting that synapse formation is maintained in the cortex at the expense of completing neuronal myelination and establishing proper connectivity.

Hippocampal development occurs in the latter half of gestation coinciding with the induced placental insufficiency. There were reductions in synaptogenesis and myelination in the hippocampal subareas presumably priming cognitive difficulties in later life. The hippocampus is the least genetically regulated brain area and vulnerable to environmental and developmental influence (Lodygensky 2008). This suggests that intrauterine insults such as placental insufficiency will lead to permanent cognitive impairments but also that there is opportunity for intervention and therapies aimed to improve cognitive outcomes. In adults, changes in myelin in various areas of the brain are associated with many psychiatric disorders such as schizophrenia, obsessive-compulsive disorder, bipolar disease, depression, autism, dyslexia, and attention deficit hyperactivity disorder, diseases that have been associated with low birth weight (Fields 2008). There was a decrease in MBP IR and LFB in the sFGR and a modest decrease in aFGR animals compared to AGA animals in the periventricular white matter (PVW). Reduction of PVW volume is associated with aFGR humans and implicated in programming of schizophrenia (Rhen 2004). The etiology of neurological disorders is multifaceted but yet the present data

suggest that aberration in neuronal development *in utero* may be an initial trigger in escalating the risk for cognitive disorders and encephalopathies. This is the first study to report altered synaptogenesis and myelination in aFGR and sFGR guinea pig fetuses growth restricted by placental insufficiency.

6.2 FUTURE STUDIES

Based on the current findings, future studies should explore associated behavioural outcomes with neurological changes at term in both sFGR and aFGR to gain insight into the mechanisms of neurological programming. Specifically, defining behavioural changes in animal models should be done using various psychological tests that would indicate changes in cognition, learning, memory, and behaviour. Psychological test such as the Morris Water maze, T-arm maze, and open field tests could define the extent of cognitive changes that occur in the guinea pig due to FGR and thus associate the severity of the neurological changes at term with declining cognitive ability and behaviour. At the same time, environmental enrichment and therapeutics should be involved in order to examine the type of interventions that may be beneficial to FGR humans who are at increased risk of developing neurological morbidity. Some examples of influential factors could include amount of environmental enrichment such as toys and shelter, the affect of relaxation in early life, isolation, length of lactation and weaning, comorbidities, pharmaceutical therapy, and the changes that occur at the time of labour.

The differential responses and molecular mechanisms leading to aFGR have been well defined. Since our study demonstrates more severe neurological outcomes in sFGR fetuses, a phenotype that has to date been overlooked in terms of fetal programming, further study

elucidating the differential mechanisms involved in symmetrical growth restriction is warranted. Studies show amino acid transfer and total amniotic fluid protein to be reduced with aFGR (Jansson 1990; Jones 1983; Sparks 1985; Tisi 2004). It can be due to decreased rate of activity of amino acid transporter A because of reduced availability of rate-limiting sodium resulting in a fetal environment with decreased essential amino acids (Mehendran 1993). Many studies suggest hypoxia and oxidative stress as a mechanism for FGR. Various proteins can be indicative of mechanisms underlying aFGR and sFGR, high erythropoietin concentration is an indicator of hypoxic stress, high interleukin-10 concentrations suggest increased higher metabolic rates due to infection, and lower amniotic volume is a sign of poor placental perfusion (Buescher 1998, Heyborne 1994, Ostlund 2000, Tisi 2004). The expression of enzymes that utilize glucose suggest guinea pigs utilise glucose oxidatively at mid-gestation and increase in expression towards term (Booth 1980). Thus the hypoxia and oxidative stress may not only be from maternal or placental causes in oxygen availability but it could also be due to reduced oxygen extraction by the fetus. A compilation of these mechanisms could result in aFGR or sFGR contributing to morbidity and mortality by altering brain development.

6.3 CONCLUSIONS

In conclusion, this thesis was focused on determining the relationship between FGR and synaptic and myelination changes in term guinea pig. The major findings of this study were:

1) That synapse formation is reduced in FGR animals.

2) sFGR animals have reduced maturation of synapses in the hippocampus while aFGR animals maintain synapse formation
3) Myelination is reduced in FGR animals

These studies suggest a potential role for synapses and myelin in causing aberrant neuronal connectivity in FGR fetuses. This may lead to cognitive difficulties and neurological disorders.

6.4 REFERENCES

Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, and Kinney HC. (2001) Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci*. 21(4):1302-12

Banks R. (1989) The guinea pig: biology, care, identification, nomenclature, breeding, and genetics. *USAMRIND Sem Ser*.

Booth RFG, Patel TB, and Clark JB. (1980) The development of enzymes of energy metabolism in brain of precocial (guinea pig) and non-precocial (rat) species. *J Neurochem*. 34(1):17-25

Bourgeois JP. (1997) Synaptogenesis, heterochrony and epigenetics in the mammalian neocortex. *Acta Paediatr.* Suppl 422:27-33

Buescher U, Hertwig K, Wolf C, and Dudenhausen JW. (1998) Erythropoietin in amniotic fluid as a marker of chronic fetal hypoxia. *Int J Gynaecol Obstet.* 60:257-263

Cannon TD, van Erp TGM, Bearden CE, Loewy R, Thompson P, Toga AW, Huttunen MO, Keshavan MS, Seidman LJ, and Tsuang MT. (2003) Early and late neurodevelopmental influences in the prodrome to schizophrenia: Contributions of genes, environment, and their interactions. *Schizophrenia Bull.* 29(4):653-69

Chaichareon DP, Rankin JH, and Ginther OJ. (1976) Factors which affect the relative contributions of ovarian and uterine arteries to the blood supply of reproductive organs in guinea pigs. *Biol Repro.* 15:281-290

Clancy B, Kersh B, Hyde J, Darlington RB, An KJS and, Finlay BL (2007) Web-Based Method for translating Neurodevelopment from laboratory species to humans. *Neuroinformatics* 5-1:79-94. http://people.psych.cornell.edu/~blf2/pdfs/BCBKBLFNI07.pdf

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

De Rodriguez MCC, Mello RR, and Fonseca SC. (2006) Learning difficulties in school children born with very low birth weight. *J Pediatr* 82(1):6-14

Detmer A and Carter AM. (1992) Factors influencing the outcome of ligating the uterine artery and vein in a guinea pig model of intrauterine growth retardation. *Scand J Lab Anim Sci.* 19:9-15

Eckstein P, McKeown, T, and Record RG. (1955) Variations in placental weight according to litter size in the guinea pig. *J Endocrin.* 12:108-114

Fields RD. (2008) White matter in learning, cognition, and psychiatric disorders. *Trends Neurosci.* 31,361-370.

Fletcher T, de Camilli P, and Banker G. (1994) Synaptogenesis in hippocampal cultures: Evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. *J Neurosci*. 14(11):6695-706

Geva R, Eshel R, Leitner Y, Fattal-Valevski A, and Harel S. (2008) Verbal short-term memory span in children: long-term modality dependent effects of intrauterine growth restriction. *J Pediatr*. 49(12):1321-30

Halliday HL. (2009) Neonatal management and long-term sequelae. *Best Prac Clin Obstet Gynaecol.* 23:871-880

Heyborne KD, McGregor JA, Henry G, Witkin SS, and Abrams JS. (1994) Interleukin-10 in amniotic fluid at midtrimester: immune activation and suppression in relation to fetal growth. *Am J Obstet Gynecol*. 171:55-59

Ibsen HL. (1928) Prenatal growth in guinea-pig with special reference to environmental factors affecting weight at birth. *J Exp Zool.* 51:51

Indredavik MS, Vik T, Evensen KAI, Skranes J, Taraldsen G, and Brubakk AM. (2010) Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr* 31(4):286-94

Isaacs EB, Lucas A, Chong WK, Wood SJ, Johnson CL, Marshall C, Vargha-Khadem F, and Gadian DG (2000) Hippocampal volume and everyday memory in children of very low birthweight. *Pediatr Res*. 47:713-20

Jansson T and Persson E. (1990) Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Ped Res.* 28:203-208

Jiang Changan and Schuman EM. (2002) Regulation and function of local protein synthesis in neuronal dendrites. *Biochem Sci.* 27(10):506-513

Jones CT and Parer JT. (1983) The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea pig. *J Physiol.* 343:525-537

Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, Kelsh RN, and Appel B. (2006) *In vivo* time-lapse imaging shows dynamic oligodendrocyte progenitor behaviour during zebrafish development. *Nature Neuroscience.* 9(12):1506-11

Mallard CE and Rees S. (1997) Effects of chronic placental insufficiency on brain development in fetal sheep. *Pediatr Res.* 43(2):262-70

Mehendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RDH, and Sibley CP. (1993) Amino acid (system A) transporter activity in microvillous membrane vesicles from placentas of appropriate and small for gestational age babies. *Pediatric Res*. 34(5):661

Mjaatvedt AE and Wong-Riley TT. (1988) Relationship between synaptogenesis and cytochrome oxidase activity in Purkinje cells of the developing rat cerebellum. *J comp neurol*. 277(2):155-18

Myatt L. (2006) Placental adaptive responses and fetal programming. *J Physiol*. 572(1):25-30

Nagerl UV, Kostinger G, Anderson JC, Martin KAC, and Bonhoeffer T. (2007) Protracted synaptogenesis after activity-dependent spinogenesis in hippocampal neurons. *J Neurosci.* 27(30):8149-56

Ostlund E, LindHolm H, Hemsen A, and Fried G. (2000) Fetal erythropoietin and endo-thelin-1:relation to hypoxia and intrauterine growth retardation. *Acta Obstet Gynecol Scand.* 79:276-282

Pallotto EK and Kilbride HW. (2006) Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynaecol*. 49(2):257-269

Peeters LLH, Sparks JW, Grutters G, Girard J, and Battaglia FC. (1982) Uteroplacental blood flow during pregnancy in chronically catheterized guinea pigs. *Pediatr Res.* 16:716-720

Rehn AE, Van Den Buuse M, Copolov D, Briscoe T, Lambert G, and Rees S. (2004) An animal model of chronic placental insufficiency: relevance to neurodevelopmental disorders including schizophrenia. *Neurosci.* 124: 381-391

Sparks JW, Girard JR, Callikan S, and Battaglia F. (1985) Growth of fetal guinea pig:physical and chemical characteristics. *Am J Physiol.* 248:E132-9

Synnes AR, Anson S, Arkesteijn A, Butt A, Grunau RE, Rogers M, and Whitfield MF. (2010) School entry age outcomes for infants with birth weight ≤800 grams. *J Pediatr.* 157(6):989-94

Tisi DK, Emard JJ, and Koski KG. (2004) Total protein concentration in human amniotic fluid is negatively associated with infant birth weight. *J Nutr.* 134(7):1754-8

Tolcos M, Harding R, Loeliger M, Breen S, Cock M, Duncan J, and Rees S. (2003) The fetal brainstem is relatively spared from injury following intrauterine hypoxemia. *Dev Brain Res*. 143:73-81

Tolcos M and Rees S. (1997) Chronic placental insufficiency in the fetal guinea pig affects neurochemical and neuroglial development but not neuronal numbers in the brainstem: A new method for combined stereology and immunohistochemistry. *J comp neurol.* 379:99-112

Turner AJ and Trudinger BJ. (2009) A modification of the uterine artery restriction technique in the guinea pig fetus produces asymmetrical ultrasound growth. *Placenta.* 30:236-240.

Walker DM and Marlow N. (2010) Neurocognitive outcome following fetal growth restriction. *Arch Dis Child Fetal Neonatal Ed.* 93:F322-25

Karolina Piorkowska

Curriculum Vitae

EDUCATION

Master of Science in Physiology-Developmental Biology from the University of Western Ontario in Dr. Bryan Richardson's laboratory. Completed July 2012

Bachelor of Medical Sciences with a Specialization in Physiology from the University of Western Ontario awarded June 2008

PUBLICATIONS

Thompson JA, Gros R, Richardson BS, **Piorkowska K**, and Regnault TR (2011). Central stiffening in adulthood linked to aberrant aortic remodelling under suboptimal intrauterine conditions. *Am J Physiol Regul Integr Comp Physiol*. Dec;301(6):R1731-7

RESEARCH PRESENTATIONS:

May 2012 Poster presentation- 3rd annual Developmental Biology Research day, UWO

May 2012 Oral presentation- 8th annual Paul Harding Research day (Obstetrics/Gynecology), UWO

March 2012 Poster presentation- Lawson Health Research Institute Research Day, UWO

March 2012 Poster presentation- Society for Gynecologic Investigation 59th annual meeting, San Diego, California (Awarded CHRI travel award, CIHR travel award and won Best New Investigator award-Poster)

March 2012 Oral presentation- Talks on Fridays (TOFS), UWO

November 2011 Oral presentation- 35th annual Perinatal Investigator Meeting, Kingston, Ontario

November 2011 Poster presentation- Physiology and Pharmacology Research Day, UWO

June 2011 Poster presentation- Neurodevnet Brain Development Conference, Vancouver, British Columbia 2011 (Awarded Neurodevnet travel award 2010 and 2011)

May 2011 Poster presentation-7th annual Paul Harding Research Day (Obstetrics & Gynecology), UWO

May 2011 Poster presentation- 2nd annual Developmental Biology Day, UWO

March 2011 Poster presentation- Lawson Health Research Institute Research Day, UWO

March 2011 Poster presentation- Society for Gynecologic Investigation 58th annual meeting, Miami Beach, Florida (awarded CHRI travel award)

December 2010 Oral presentation- Talks on Fridays (TOFS), UWO

November 2010 Poster presentation- 34th annual Perinatal Investigator Meeting, Kingston, Ontario

August 2010 Poster presentation- Aspen Perinatal Biology Conference in Aspen, Colorado (Awarded USDA funded Travel award)

June 2010 Poster presentation- Neurodevnet Brain Development Conference, Montreal, Quebec (Awarded Neurodevnet travel award 2010)

May 2010 Poster presentation- 1st annual Developmental Biology Day, UWO

May 2010 Poster presentation- 6th annual Paul Harding Research Day (Obstetrics & Gynecology), UWO

February 2010 Oral presentation- Talks on Fridays (TOFS), UWO

RELATIVE WORK EXPERIENCE

November 2011-Present, The Biotron: institute for environmental climate change research, UWO

Research Associate part-time, Imaging and data analysis module

October 2009- April 2011, University of Western Ontario

Teaching Assistant, Physiology 3130Y

RELATIVE VOLUNTEER EXPERIENCE

2009-2012, UWO

PPGSC council member, Phys/Pharm Communication and Outreach commissioner, Phys/Pharm off-campus representative, Social Committee member, SOGS council representative, Let's Talk Science member