PREGNANCY COMPROMISES: ROLE OF NEUROSTEROIDS IN NEURODEVELOPMENT AND BEHAVIOUR

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DECLARATION

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Signed:_____ Date:_____

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This thesis is dedicated to my mother,

Karin Kneis.

Without your love and support

I would not be where I am

or who I am today.

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CONTENTS

ABSTRACT

Pregnancy compromises impact a number of infants annually around the world. These include intrauterine growth restriction (IUGR), maternal psychological stress (prenatal stress; PS) and preterm birth (PTB). These compromises can result in alterations to the normal neurodevelopmental process in key brain regions, including the hippocampus, cerebellum and amygdala. These changes in fetal development are being increasingly attributed to the programming of fetal systems *in utero* towards negative *ex utero* events. In fetal life, IUGR, PS and PTB cause a delay and/or reduction in mature myelin, concurrent with an alteration in astrocyte activation. Infants born following IUGR, PS and PTB are more likely to develop cognitive deficits, anxiety, behaviours disorders and schizophrenia.

The progesterone metabolite, allopregnanolone is a key neurosteroid involved in fetal development. Allopregnanolone promotes myelination, inhibits astrocytosis, and has allosteric action at the γ-aminobutyric acid type A ($GABA_A$) receptor to modulate neural excitability. Allopregnanolone synthesis is upregulated following various acute neurological insults, including hypoxia and stress, protecting against excitotoxic cell death. Inhibition of allopregnanolone synthesis in late gestation, as may occur in chronic pregnancy compromises, reduces myelination and up-regulates astrocyte activation. In later life, dysregulation of allopregnanolone is implicated in several psychological disorders such as depression, and premenstrual dysphoric disorder. Little information exists on the effect of major pregnancy compromises, their contribution to neurosteroid dysfunction, and neurodevelopmental and behavioural outcomes of the offspring.

The effect of inhibiting allopregnanolone synthesis, using finasteride, in late gestation on postnatal cerebellar development was investigated in a guinea pig model. At 8 days postnatal age there was increased astrocyte activation and decreased expression of the allopregnanolone-sensitive $GABA_AR$ α6 subunit in the cerebella of neonates exposed to finasteride. This demonstrates the ongoing effects of a low neurosteroid environment in pregnancy extending into childhood. At 21 days postnatal age, females with *in utero* finasteride exposure displayed

increased neophobia-like responses to changes in their environment. This was without ongoing effects on myelin, astrocyte or GABAergic enzyme expression in the hippocampus or amygdala. These observations suggest that prenatal loss of neurosteroids programs an anxious phenotype in females, possibly by inducing deficits in the end-point targets of allopregnanolone action, and does not impact male anxiety development.

A model of combined IUGR+PS was used to investigate neurodevelopmental changes of offspring exposed to multiple pregnancy compromises at term. Circulating allopregnanolone and hippocampal myelination in males, whilst reduced in both IUGR and IUGR+PS, was not cumulatively affected by the combination of stressors. Interestingly, the addition of PS to IUGR had a potentially positive effect on subcortical myelination, suggesting the triggering of a protective mechanism, occurring in the fetal neurodevelopment, to preserve an already compromised brain.

The placenta contains all the essential enzymes and is the major contributor to fetal allopregnanolone for neurodevelopment Thus the health of the placenta is critical for the development of a healthy fetus. Many preterm infants do not survive the immediate neonatal period, with no overt indicators as to poor health. The placenta of surviving and non-surviving preterm guinea pigs were investigated to determine expression of the allopregnanolone synthesis enzymes. The expression of 5α-reductase type 2 was greatest in placentae from neonates that did not survive to 24 hours. This may indicate an upregulation of protective actions to increase allopregnanolone exposure, suggesting these neonates experienced an adverse *in utero* environment and were therefore more vulnerable to the insult of premature birth.

The current body of work indicates that fetal allopregnanolone plays a role in programming $GABA_A$ receptor subunit expression as well as juvenile female behaviour, and thus impaired supply of this steroid *in utero* may be a predisposing factor in the development of depression and anxiety. Allopregnanolone is implicated in the poor development of myelination following IUGR, yet PS may

have a neuroprotective action on myelin development in IUGR male brains. Based on this work, it is also postulated that placental expression of neurosteroid producing enzymes provide identification of neonates at risk of poor outcomes in the immediate neonatal period. Further studies investigating the protective effects of PS in IUGR, and their impacts on later behavioural development are warranted. Future work should investigate the potential of perinatal neurosteroid replacement for the improvement of mental health outcomes following pregnancy compromises.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

0.1M	0.1MOLAR
11β-HSD2	11β-HYDROXYSTEROID DEHYDROGENASE TYPE 2
17β-DIOL	17β-ESTRADIOL
3α-DIOL	3α-ANDROSTANEDIOL
5α-DHDOC	5α-DIHYDRODEOXYCORTICOSTERONE
5α, 3α-THDOC	5α, 3α- TETRAHYDRODEOXYCORTICOSTERONE
5α-DHP	5α-DIHYDROPROGESTERONE
5α-DHT	5α-DIHYDROTESTOSTERONE
5αR	5α-REDUCTASE
ADHD	ATTENTION DEFICIT HYPERACTIVITY DISORDER
AGA	APPROPRIATE FOR GESTATIONAL AGE
ANOVA	ANALYSIS OF VARIANCE
B_0	TRACER ANTISERA BINDING
BAT	BROWN ADIPOSE TISSUE
BLA	BASOLATERAL AMYGDALA
BLR	BRAIN-TO-LIVER RATIO
BSA	BOVINE SERUM ALBUMIN
CA	*CORNU AMMONIS*
CC	CORPUS CALLOSUM
CCAS	CEREBELLAR COGNITIVE AFFECTIVE DISORDER
CEA	CENTRAL AMYGDALOID NUCLEUS
CNS	CENTRAL NERVOUS SYSTEM
CPAP	CONTINUOUS POSITIVE AIRWAY PRESSURE
CREDITTS	CRITICAL RESEARCH DESIGN, INFORMATION TECHNOLOGY AND STATISTICAL SUPPORT
CRH	CORTICOTROPHIN RELEASING HORMONE

CSF	CEREBROSPINAL FLUID
CT	CYCLE THRESHOLD
DAB	3, 3'-DIAMINOBENZIDINE TETRAHYDROCHLORIDE
DG	DENTATE GYRUS
DHEA	DEHYDROEPIANDOSTERONE
DOHAD	DEVEVELOPMENTAL ORIGINS OF HEALTH AN DISEASE
DWM	DEEP WHITE MATTER
ECL	ENHANCED CHEMILUMINESCENCE
EDTA	ETHYLENEDIAMINETETRAACETIC ACID
EGL	EXTERNAL GRANULE LAYER
ELISA	ENZYME-LINKED IMMUNOSORBENT ASSAY
EPM	ELEVATED PLUS MAZE
FMRI	FUNCTIONAL MAGENTIC RESONANCE IMAGING
GA	GESTATIONAL AGE
GABA	γ-AMINOBUTYRIC ACID
$GABA_AR$	γ-AMINOBUTYRIC ACID TYPE A RECEPTOR
GAD67	GLUTAMIC ACID DECARBOXYLASE ISOFORM 67KDA
GAT1	GABA TRANSPORTER TYPE 1
GDNA	GENOMIC DEOXYNUCLEIC ACID
GEE	GENERALISED ESTIMATING EQUATIONS
GFAP	GLIAL FIBRILLARY ACIDIC PROTEIN
HPA	HYPOTHALAMIC-PITUITARY AXIS
HRP	HORSERADISH PEROXIDASE
HSD	HYDROXYSTEROID DEHYDROGENASE (3α, 17α ETC)
IGG	IMMUNOGLOBULIN G
IGL	INTERNAL GRANULE LAYER
IQ	INTELLIGENCE QUOTIENT

IUGR	INTRAUTERINE GROWTH RESTRICTION
IUGR+PS	INTRAUTERINE GROWTH RESTRICTION WITH PRENATAL STRESS
MAG	MYELIN ASSOCIATED GLYCOPROTEIN
MAP2	MICROTUBULE ASSOCIATED PROTEIN 2
MBP	MYELIN BASIC PROTEIN
ML	MOLECULAR LAYER
MRI	MAGNETIC RESONANCE IMAGING
mRNA	MESSENGER RIBONUCLEIC ACID
NCBI	NATIONAL CETNRE FOR BIOTECHNOLOGY INFORMATION
NeuN	NEURONAL NUCLEI
NMDA	N-METHYL-D-ASPARTATE
NSB	NON-SPECIFIC BINDING
NTC	NO-TEMPLATE CONTROL
OF	OPEN FIELD
Olig2	OLIGODENDROCYTE TRANSCRIPTION FACTOR
$P450_{17\alpha}$	17α-HYDROXYLASE
$P450_{AROM}$	P450 ENZYME COMPLEX, AROMATASE ENZYME
$P450_{SCC}$	P450 ENZYME COMPLEX, SIDE-CHAIN CLEAVAGE
PBS	PHOSPHATE BUFFERED SALINE
PCR	POLYMERASE CHAIN REACTION
PDGFRα	PLATELET-DERIVED GROWTH FACTOR RECEPTOR α
PLP	MYELIN PROTEOLIPID PROTEIN
PMDD	PREMENSTRUAL DYSPHORIC DISORDER
PMS	PREMENSTRUAL SYNDROME
PND	POSTNATAL DAY
PS	PRENATAL STRESS
PTB	PRETERM BIRTH

PTSD	POST-TRAUMATIC STRESS DISORDER
RF	RHINAL FISSURE
RIPA	RADIOIMMUNOPRECIPITATION ASSAY
RNA	RIBONUCLEIC ACID
RNASE	RIBONUCLEASE
RT	REVERSE TRANSCRIPTION
S100B	S100 CALCIUM-BINDING PROTEIN B
SCWM	SUBCORTICAL WHITE MATTER
SGA	SMALL FOR GESATIONAL AGE
TBS-T	TRIS-BUFFERED SALINE WITH TWEEN
TC	TOTAL COUNTS
TH	THALAMUS
TMB	TETRAMETHYLBENZIDINE
UV	ULTRAVIOLET
WWII	WORLD WAR II

PUBLICATIONS FOR INCLUSION

The work in this thesis describes the effects of intrauterine allopregnanolone loss via pharmacological inhibition on postnatal neurodevelopment, behavioural outcomes. It also investigates the impact of combined intrauterine compromises in fetal neurodevelopment and neurosteroid profiles at term, as well as placental expression of neurosteroid enzymes in relation to survival following premature delivery. As such, this thesis is divided into four publications, beginning with the loss of allopregnanolone in late gestation on postnatal cerebellar development, and then impact of low gestational allopregnanolone on behavioural outcomes at juvenility. The subsequent paper investigates the individual impacts of intrauterine growth restriction and the combined effects with prenatal stress on neurodevelopmental markers, and finally the mRNA expression of the 5α-reductases within the placenta of premature neonates who did not survive the immediate 24 hours period, comapred to preterm survivors and term neonates.

Cumberland AL, Palliser HK, Walker DW, Hirst JJ. Cerebellar Changes in Guinea Pig Offspring Following Suppression of Neurosteroid Synthesis During Late Gestation.
The Cerebellum. 2016:1-8. DOI: 10.1007/s12311-016-0802-0.
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Cumberland AL, Crombie GC, Palliser KH, Hirst JJ. Increased Anxiety-like Phenotype in Female Guinea Pigs Following suppression of Neurosteroid Synthesis In Utero. Submitted to *Developmental Neuroscience*.

Cumberland AL, Palliser KH, Rani P, Hirst JJ. Combination of IUGR and Prenatal Stress on Fetal Brain Development. Prepared for submission to *Journal of Developmental Origins of Health and Disease.*

Cumberland AL, Palliser HK, Hirst JJ. Increased placental neurosteroidogenic gene expression precedes poor outcome in the preterm guinea pig. *Journal of Developmental Origins of Health and Disease*. 2014;5(02):74-78. DOI: 10.1017/S2040174413000573.

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ADDITIONAL PUBLICATIONS

Palliser HK, Bennett GA, Kelleher MA, **Cumberland AL**, Walker DW, Hirst JJ. Models of Perinatal Compromises in the Guinea Pig: Their Use in Showing the Role of Neurosteroids in Pregnancy and the Newborn. Book Chapter, Chapter 10. Prenatal and Postnatal Determinants of Development. *Neuromethods*. New York, NY: Springer; 2016. p. 221-243. DOI: 10.1007/978-1-4939-3014-2_11.

Hirst JJ, **Cumberland AL**, Shaw JC, Bennett GA, Kelleher MA, Walker DW, Palliser HK. Loss of neurosteroid-mediated protection following stress during fetal life. Review. *The Journal of Steroid Biochemistry and Molecular Biology*. 2016;160:181-188. DOI: 10.1016/j.jsbmb.2015.09.012.

1 INTRODUCTION

Pregnancy compromises affect a significant number of births world-wide annually. These can include, but are not limited to, intrauterine growth restriction (IUGR), maternal psychological stress (prenatal stress; PS) and preterm birth (PTB). Individually, these complications impact on all developing systems within the fetus and neonate, and have long-term implications on the health and well-being of the infant in later life, with males being of greater vulnerability to a poor intrauterine environment than females[1]. Based on epidemiological records Barker and colleagues proposed, approximately 30 years ago, that adverse intrauterine events, such as those resulting in low birth weight and prematurity at birth, programmed the fetus for an increased risk of ischemic heart disease and non-insulin dependent diabetes in adulthood[2-4]. This gave rise to the developmental origins of health and disease (DOHaD) hypothesis, that the fetus exposed to adverse *in utero* events changes the developmental outcomes of many of its systems to prepare for an under nourishing *ex utero* environment (also known as fetal programming). However, these adaptations can prove detrimental if the *in utero* events do not match the predicted extra-uterine life. Since this hypothesis, Barker and numerous other researchers have linked pregnancy compromises, such as IUGR, PS and PTB to the development of many metabolic[5, 6], cardiovascular[7, 8] and neurological disorders[9-11].

1.1 Intrauterine Growth Restriction

Intrauterine growth restriction is characterised by the failure of the fetus to reach its genetic growth potential. There are many causes to pathological growth restriction including maternal factors such as pre-eclampsia and cardiovascular disease, as well as fetal factors including chromosomal abnormalities. However, the most common cause is placental insufficiency. This is where failure of the placenta to sufficiently invade into and through the decidua, or poor vascular development impairs transfer of nutrients, hormones and oxygen to the fetus. IUGR affects up to 10% of pregnancies annually[12], and is diagnosed by abnormal artery Doppler, reduced amniotic fluid and abnormal fetal ultrasound measures[13]. Currently, there are limited treatment options to correct poor *in utero* growth, with current practice to monitor both maternal and fetal health and induce delivery based on indicators of distress. Infants born following IUGR are at an increased risk of mortality and morbidities compared to appropriately grown infants[14], including predisposition to metabolic impairments, cardiovascular disorders and neuropathologies[15].

1.1.1 Symmetrical versus Asymmetrical Growth

Babies can be classified as small for gestational age (SGA) or intrauterine growth restricted (IUGR). Both are classified as having a birth weight less than the 10th percentile or 2 standard deviations below the population mean[13]. However, SGA babies are small, but are not necessarily growth restricted whereas IUGR fetuses that have not reached their genetically determined growth potential. The growth restricted fetus can be further catergorised as having developed symmetrical growth restriction, where it limits growth of all organ and physical measures to compensate for the limited nutrient supply from the placenta, and fall under the category of small for gestation age (SGA) IUGR. Alternatively, the fetus may develop asymmetrically. Asymmetrical growth restriction results from the redirection of blood flow away from the extremities and lower organs, such as the legs and liver, and increasing blood flow towards preserving key organs, the heart and brain. This is known as fetal circulatory redistribution or the “brain-sparing” effect[16]. Despite the changes in blood flow, these children remain at an increased

risk of developing neuropathologies, such as behavioural and cognitive disorders, throughout childhood and adolescence[17]. This indicates that this compensatory mechanism is still inadequate in many cases of growth restriction. Regardless, infants born IUGR, be it symmetrical or asymmetric growth restriction, have worse outcomes than infants born with a weight appropriate for gestational age (AGA). These can translate long term to poor cognitive development[18, 19], increased impulsivity[20], aggressive behaviours[21, 22] and internalising behaviours[23].

1.2 Prenatal Stress

Stress is a natural physiological response to the environment, and is hormonally characterised by the release of the stress hormones, the glucocorticoids, predominantly cortisol in the human. Throughout pregnancy, cortisol is steadily increasing from maternal adrenal output[24, 25], where the fetus utilises this in maturational processes within the brain and lungs[26]. Perturbations in this cortisol reaching the fetus can affect neurodevelopment in the immediate and long term.

Prenatal stress is predominantly of psychosocial origin, with stressors experienced by pregnant women including financial and socioeconomic concerns, extreme emotional distress such as the loss of a partner or family member and pregnancy-related stress such as the fear of becoming a mother or poor fetal health. Prenatal stress is highly subjective, with women perceiving the degree of stress to the same "threat" differently[27]. However, children born to mothers who report higher perceived stress during pregnancy were more likely to be delivered prematurely, and consequently of lower birth weight than children born to mothers reporting low stress levels[28]. Repeated glucocorticoid administration to pregnant ewes, similar to multiple stressful events during gestation, caused fetal growth restriction. Increasing number of doses resulted in greater reductions in fetal weight with concurrent delays in myelination[29]. Consistent with these observations, prospective human studies have found that preterm children had reduced birthweight ratios (a ratio value calculated from the individual's birthweight and the median population specific growth curve) at follow-up if their

mother received repeated glucocorticoid administration prior to delivery[30], and this was associated with increased behavioural deficits including increased externalisation of problems and distractability[31]. Low birth weight and prematurity have well characterised impacts on the infant, however, increasing epidemiological and animal studies are finding that abnormally high levels of circulating glucocorticoids associated with stress has substantial negative impacts on fetal neurodevelopment.

1.3 Preterm Birth

Premature delivery affects approximately 8% of births in Australia each year[32], and is the leading cause of neonatal morbidity and mortality. While some pregnancies are indicated for premature delivery, including IUGR, maternal/fetal infection and previous history of premature deliveries[33], a significant proportion of women who deliver have no overt indictors. Despite the increased risk of mortality associated with decreasing gestational age at delivery[34], there are relatively few predictors to assess which preterm infants may be more vulnerable to postnatal complications . Those preterm neonates that do survive the immediate neonatal period are at a larger risk of chronic health and psychological disorders. These children are more likely to develop behavioural and cognitive disorders, cardiopulmonary dysfunctions and physical morbidities than children born at term[35-37].

1.4 Neurodevelopmental Outcomes

Neurodevelopment commences approximately 2-3 weeks post conception and, in humans, continues well into the postnatal period[38]. The development of various brain regions occur at different time points throughout gestation[39] (figure 1-1). The neurodevelopment in the first half of pregnancy is dominated by neuronal proliferation and neural migration to form the key brain regions. From mid-gestation onwards, processes of apoptosis, synaptogenesis and myelination begin to occur[40]. The neural tissue requires tight control of growth factors and related hormones, to provide the correct signaling for these cells to develop. Disruption to

the optimal *in utero* environment such as those occurring with IUGR, PTB and PS can alter exposure to these substrates thus changing to developing brain towards less than optimal outcomes.

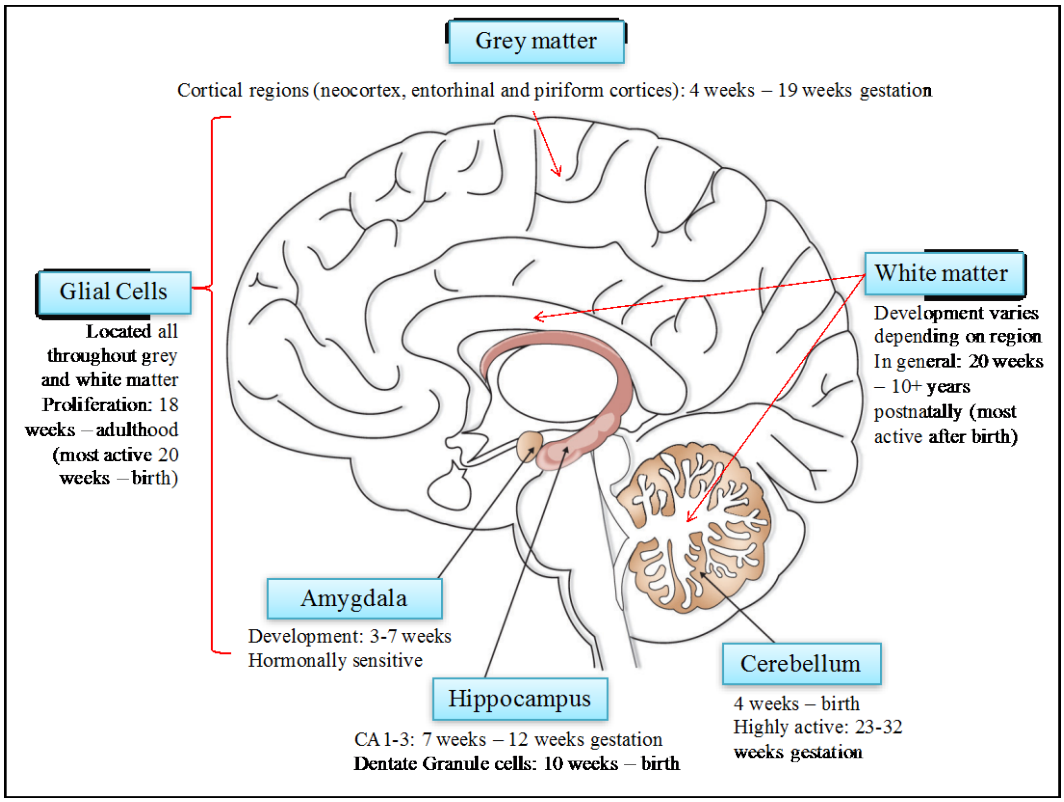


FIGURE 1-1 NEURODEVELOPMENTAL TIMELINE OF BRAIN CELLS AND REGIONS VULNERABLE TO DAMAGE. DIFFERENT BRAIN REGIONS DEVELOP DURING DIFFERENT PERIODS OF GESTATION. THE HIPPOCAMPUS BEGINS TO DEVELOP FROM APPROXIMATELY 7 WEEKS GESTATION UNTIL BIRTH. SIMILARLY, THE CEREBELLUM BEGINS EARLY IN GESTATION AND CONTINUES UNTIL TERM. CELLULAR DEVELOPMENT OF THE AMYGDALA COMMENCES AND FINISHES WITHIN THE FIRST TRIMESTER, THIS AREA REMAINS HIGHLY VULNERABLE TO HORMONAL CHANGES THROUGHOUT PREGNANCY. THIS LEAVES THE AMYGDALA VULNERABLE TO PROGRAMMING VIA FLUCTUATIONS IN CIRCULATING HORMONES. THE MACROGLIA, ASTROCYTES AND OLIGODENDROCYTES, BEGIN PROLIFERATION AND DIFFERENTIATION AT APPROXIMATELY 18-20 WEEKS AND CONTINUE TO DEVELOP WELL INTO ADOLESCENCE, LEAVING THIS POPULATION OF CELLS AT AN INCREASED RISK OF DAMAGE TO PREGNANCY COMPLICATIONS SUCH AS INTRAUTERINE GROWTH RESTRICTION AND PRENATAL STRESS. IMAGE ADAPTED FROM *BEGINNING PSYCHOLOGY*[41], UNDER Creative Commons by-nc-sa 3.0 LICENSE. FIGURE TEXT ADAPTED FROM TAU, 2009[38], BAYER, 1993[39] AND KNEUSEL, 2014[40].

Neurons and glia are the two main cell types in the brain. Glial cells are further categorised into macroglia and microglia. Macroglia are oligodendrocytes, the myelinating cells, and astrocytes, cells important in maintaining neural homeostasis. Microglia act as immune cells within the brain, interacting with astrocytes to release cytokines in the instance of damage or infection.

1.4.1 Oligodendrocytes and Myelination

Oligodendrocytes provide the myelin sheath to neurons within the central nervous system (CNS), and are essential for axonal propagation of action potentials. Progenitor cells of the oligodendrocyte lineage, arising from neuroepithelial cells in the ventral zone of the spinal cord[42, 43], begin proliferating early in gestation and undergo differentiation into pre-myelinating oligodendrocytes before further maturation into fully mature oligodendrocyte. Mature oligodendrocytes then begin to produce myelin, with this stage of neurodevelopment continuing into the neonatal period and adolescence. The health of the mature oligodendrocytes is essential in many neural processes, with damage to these cells leading to serious demyelinating conditions. The effects of demyelination depends on the region where it occurs, with demyelination within the hippocampus associated with memory loss and the inability to form new memories[44]. The loss of myelin in the cerebellum is associated with ataxia[45]. There is a natural decline of myelin as we age, however these demyelinating processes are accelerated in neurological disorders such as Alzheimer's disease and multiple sclerosis, disorders which affect memory and motor function, respectively. The major myelinating proteins are myelin basic protein (MBP; maintains structure between the multiple layers of the myelin sheath[46]), myelin proteolipid protein (PLP; involved in adhesion of the myelin layers[47]) and myelin associated glycoprotein (MAG; roles axon-glial interactions and myelination[48]) and thus are the most well studied proteins when investigating myelin deposition. These are usually assessed by immunohistochemical and western blot methods, to determine differences in protein expression. The structure of the myelin sheath can be assessed by electron microscopy to determine thickness (or loss) of myelin wrapped around axons.

1.4.2 Astrocytes and Reactive Astrocytosis

Astrocytes are the most abundant cells within the CNS. They have a large number of roles, including maintaining the blood brain barrier by supporting pericytes around endothelial cells, providing essential nutrients and ions to neurons and clearance and recycling of neurotransmitters from the synapse[49-52]. In early neural development, radial astrocytes are critical for providing scaffolding for the migration of neurons to form the layers of the cortex and substructures of the brain[53]. Activity and release of soluble factors from astrocytes in late gestation is also important in synaptic maturation[54]. Activation of astrocytes also occurs in instances of trauma and injury[55]. Part of the activation involves recruitment of microglia to the site of injury through chemokine signaling, and the formation of the glial scar. These processes are thought to be mechanisms that close the injured site and prevent further damage. The activation of astrocytes can be measured by assessing the expression of glial fibrillary acidic protein (GFAP), as this protein is upregulated in activated astrocytes, particularly in cases of neuronal damage and dysfunction.

1.4.3 Functional Outcomes of Pregnancy Compromises

1.4.3.1 The Impact of IUGR on Functional Outcomes

Magnetic resonance imaging (MRI) studies have demonstrated altered haemodynamic distribution in human preterm and term fetuses with IUGR[56, 57], and these changes in blood flow are associated with brain size[58]. Premature infants diagnosed with IUGR also display reduced brain volumes under MRI scanning, compared to age matched preterm controls[59]. Further studies show the brain volume does not “catch up” to appropriately grown preterm and term children at 12 months of age[60]. Being born late-preterm is associated with lower global and performance intelligence quotient (IQ), with increased risk of internalising and attention problems at 6 years of age[61]. At 5 years of age, children born prematurely with IUGR had lower global IQ and visual-motor integration than preterm children without IUGR[17], highlighting the deficits within an already vulnerable population. In a rabbit model of IUGR, researchers found that IUGR induces changes in regional patterning on functional MRI (fMRI) scans at one day

old, and these were associated with poorer neurobehaviour scores[62]. A follow-up study in this model showed that altered fractional anisotropy (a scalar value of the diffusive capability of water molecules along or among fibres in MRI scanning) in various grey matter regions of the brain, such as the amygdala and hippocampus, were associated with increased anxiety within open field testing of IUGR rabbits at postnatal day (PND) 70 as well as changes in white matter connectivity[63]. These studies show that following this pregnancy compromise there are the long-term decrements in cognition and behaviour that do not recover. Similar findings have been made clinically with the size of the infant at birth correlating with behavioural profiles found within school age children. Extensive studies of Leitner and Geva have found reductions in overall IQ scores and reduced academic achievement, coinciding with poor memory as well as reduced visuomotor and language skills in children born IUGR compared to age-matched AGA children[64, 65]. Being born SGA also correlates to having poorer intelligence scores at 17 years of age[66], and at 26 years of age being less likely to obtain higher level occupations[67]. Being born with IUGR does not only impact the cognitive development of an individual, but also places them at an increased risk of developing anxiety and depression. A study from Norway investigated the impact of growth restriction on socioeconomic and educational outcome as well the development of psychiatric disorders at 20-30 years of age[68]. The authors reported, paired with a lower achievement of education as measured by school completion, reduced socioeconomic function and a greater incidence of self-reported anxiety and depression among adults who were born growth restricted. An American study investigating the impact of being SGA on the prevalence of depression among teenaged males and females, reported the rates of depression among SGA females interviewed was 38.1%, compared to AGA females at 8.4%[69]. Being SGA did not affect the prevalence of depression in males. Lund *et al* have reported an increase in the development of mental health problems among individuals born SGA at term from 14 years of age until 20 years of age[70]. Individuals at 20 years age, with and without cognitive impairment, were more likely to self-report increased attention problems, depression/anxiety, internalising, externalising and aggressive behaviours compared to 20 year old individuals born appropriate weight at term[71]. Being born SGA was also

associated with fewer interactions with friends, and reduced feelings of social acceptance. These two studies[70, 71] also included a cohort of individuals born preterm, classified as very low birth weight, and found similar impacts on behaviour, attention and mental health, although not to the degree of being classified as SGA. Whilst all these studies used SGA as a proxy for growth restriction, and some authors acknowledge this classification includes those who may have had medically identified as IUGR[68, 70, 71], the evidence strongly shows sufficient *in utero* growth is essential for proper brain development and improved long-term outcome.

1.4.3.2 Prenatal Stress and Glucocorticoids

Glucocorticoids are a family of steroid hormones produced by the adrenal glands in response to stress. Administration of a single dose of a synthetic glucocorticoid, dexamethasone or betamethasone, reduces fetal sheep brain weight, length and volume[72], emphasising that a single stress event exposing the fetus to high cortisol concentrations can cause immediate impacts on brain development. Repeated doses of corticosteroids, particularly dexamethasone, is associated with the development of periventricular leukomalacia in preterm offspring regardless of gestational age and birthweight[73]. These studies emphasise that exposure of the fetus to synthetic glucocorticoids prematurely or excessively has detrimental effects on neurodevelopment, and this will later impact on their health and well-being.

Follow-up studies of women who report high prenatal stress during pregnancy show changes in child behaviour, even from infancy. Davis *et al* showed that at 4 months of age, infants from prenatally stressed mothers had strong negative responses to novel stimuli[74]. One study based prenatal stress on high or low maternal salivary cortisol at gestaional weeks 37 and 38, and assessed behaviour using mother-infant interactions during bathing up to twenty weeks of age. The researchers reported that, infants from mothers with high late-gestation cortisol displayed more negative behaviours of crying, fussiness, negative facial expressions (e.g. frowning) and fewer vocalisations towards their mothers during bathing[75]. Gutteling *et al* found at 27 months of age, mothers of prenatally

stressed children were more likely to report their child had restless behaviour and greater incidences of externalisations of problem behaviour[76]. Self-reported high state and/or trait anxiety during pregnancy, as well as the infant being male, was associated with reduced mental developmental scores at 2 years of age[77], and self-reported maternal stress during pregnancy negatively correlated to the child's school marks in reading, writing, mathematics and music at 6 years of age[78]. Several studies by Laplante and King followed women who were pregnant or became pregnant during the 1998 Quebec ice storms, and experienced greater stress during this period than unaffected women form surrounding areas. Ongoing follow-up studies show children from these pregnancies had lower IQ and vocabulary scores, and these deficits were seen in affected individuals from early childhood through to school ages, compared to their peers[79-83]. There were also greater presentations of autistic traits among male children[79-83]. Beversdorf *et al* surveyed mothers of children with and without autism at 9 years of age[84]. The survey included questions pertaining to the number of stressful events experienced during conception to 4 weeks post-birth, with different stress events rated on severity using the Social Readjustment Rating Scale[85]. Mothers of autistic children reported a greater number of stressful events throughout pregnancy compared to women of children without autism[84]. Gestational weeks 25-28 were also shown to have the highest number of stressful events, and greatest stressor severity scale from women with autistic children, highlighting a timing effect in prenatal programming. Two different studies investigated the impact of World War II (WWII) events on the psychological vulnerability of offspring. One study found that males of mothers pregnant during the German invasion of the Netherlands were at an increased risk of developing schizophrenia[86], whilst another study looking at the impact of paternal death in WWII during the pregnancy period found similar results[87]. These long term behaviour studies show how deregulation of stress related processes in pregnancy have long term impacts on cognition and mental health.

1.4.4 Neurodevelopmental Consequences: Key Brain regions

The brain is a complex structure, with many regions being affected by a poor intrauterine environment, with the majority of data coming from animal models

(see 1.7.1 for discussion on the pros and cons of these animal models). Human and animal studies have found regions, such as the white matter[88-90], cerebral cortex[60, 91], corpus callosum[92, 93], insula[60, 94], frontal lobe[95-97] and basal ganglia[98] being damaged following IUGR, PS and PTB. The hippocampus and cerebellum are two highly studied regions in perinatal research, due to these two regions being frequently damaged following pregnancy compromises. The amygdala, whist is a relatively underexplored region following IUGR and PS, is involved in regulating fear responses. These regions are also implicated in the development of many psychological, cognitive and behavioural disorders associate with IUGR, PS and PTB (see sections 1.4.4.1, 1.4.4.2 and 1.4.4.3, below), thus are a major focus of the studies in this thesis.

1.4.4.1 Hippocampus

The hippocampus, named after the seahorse due to its distinctive shape, is a limbic structure found within the temporal lobes of both hemispheres. It has a critical role in the consolidation of short term memories into long term storage, with links to other limbic structures such as the amygdala, and cortical regions to encode emotional[99] and declarative memory[100], respectively. Damage to this region following traumatic brain injury leads to significant reductions in the ability to form new memories in affected animals and individuals[101, 102]. Certain diseases, such as Alzheimer's Disease, cause a slow degeneration of neuronal tissue, with hippocampal cells some of the first to be affected leading to the onset of symptoms[103].

Formation of the hippocampus beings approximately 6-8 weeks gestation and continues to develop after birth. The hippocampal subfields of *Cornu Ammonis* 1-3 (CA1-3) develop first, followed by development of the dentate granule cells. The hippocampus is one of the most well characterised regions with regard to deficits following pregnancy compromises. IUGR causes reductions in hippocampal MBP (myelination) and astrocyte expression of GFAP in fetal guinea pigs at term[104, 105]. This reduction in MBP is attributed to a delay in myelination from IUGR brains, not a loss of cells[90]. This reduction in MBP expression, along with changes in receptor subunit composition of the excitatory

N-methyl-D-aspartate (NMDA) receptors, is also found in the hippocampus of juvenile male rats following uterine artery ligation during pregnancy[106]. Other rat studies of IUGR have found reductions in MBP of the hippocampus in both moderate and severe growth restriction, along with increased GFAP[107, 108]. Reductions in total volume, neuronal cell and oligodendrocyte precursor numbers have been reported in the hippocampus of PND0 IUGR rats, with this predominantly within males[109]. There are similar reductions in MBP within the hippocampus of a guinea pig model of preterm delivery, compared to term neonates without changes in GFAP or mature neuron marker microtubule-associated protein 2 (MAP2)[110]. At adolescent equivalence, reduced myelination as measured by MBP persists in male guinea pigs[111]. Reductions in myelination, with reduced oligodendrocyte cell counts, have also been found in human and rat studies[112, 113]. These data support the notion of the oligodendrocyte lineage being the most vulnerable population of cells within the late developing brain. Human data investigating specific cell populations within the brain of premature infant is unavailable; however, MRI has enabled researchers to investigate changes in brain volumes within all neural regions. Many studies show that premature delivery is associated with reduced grey matter volumes including the hippocampus, smaller white matter volumes and impaired brain folding that persist throughout all stages of life[89, 91, 114-116].

Administration of synthetic glucocorticoid dexamethasone to pregnant macaques with a single dose at 132 days gestation, or multiple dosing on 132 and 133 days displayed significant losses in many areas of the hippocampus[117]. There were fewer pyramidal and granule cells within the CA regions and the dentate gyrus, and greater degeneration of mossy fibre connections within the CA3 in fetuses exposed to a single dose compared to controls, with these deficits greater again in multiple dosing cohorts compared to single dose and control fetuses. This study, and others[118, 119] report the implications glucocorticoid treatment has on brain development in preterm infants exposed prior to delivery. However, these works also translate into the detrimental effects that abnormal glucocorticoid exposure, due to prenatal stress, may have on fetal brain development. Models of prenatal stress in the rhesus macaque have also shown impaired hippocampal development

and behavioural abnormalities in both early and late PS exposed offspring compared to control juvenile monkeys[120]. This protocol used acoustic startle at either gestational age (GA) 50-92 (early) or GA105-147 (late) to induce PS and markedly raise maternal cortisol levels. In these studies, offspring were assessed at 2.5 years of age for behaviour and hippocampal development. The prenatally stressed offspring showed less time exploring their environment compared to control juveniles, regardless of the timing of PS events. These animals also had increased circulating basal cortisol levels, reduced hippocampal size and neurogenesis in the dentate gyrus. In a guinea pig model of PS, male fetuses had significantly reduced hippocampal MBP, GFAP and MAP2, whilst females remained seemingly unaffected at term collection[121]. However, a follow-up study at PND18 in this model showed there was prolonged reduction in MBP and GFAP within the CA1 region of the hippocampus, and that this was found in both male and female juvenile guinea pigs[122]. These guinea pigs also displayed increased anxiety-like behaviour with reduced time spent in the inner zone of open field testing, and less time investigating a novel environment marker. These results highlight the impact of PS in the long lasting effects on macroglia, with reductions in these cells potentially the cause of behavioural disturbances.

1.4.5 Cerebellum

The cerebellum, Latin for “little brain”, is located at the base of the cerebrum, adjacent to the pons and medulla. One of its major functions is to refine motor control for timing and accuracy. However, more recent work has demonstrated that the cerebellum also has important roles in behaviour and cognition[123]. The cerebellum is made up of ten lobes characterised into the anterior (lobes I-V), posterior (lobes VI-IX) and flocculonodular (lobe X) lobes, with the hemispheres joined at the middle by the cerebellar vermis[124, 125]. The different lobes are responsible for different motor, cognitive and emotional outputs. MRI studies have found children with autism have smaller cerebellar lobes VI and VII, two lobes associated with cognition[126]. The size of the cerebellum also correlates to memory retention, cognitive scoring and development of attention deficit hyperactivity disorder (ADHD)[127, 128]. Cerebellar lesions occurring in the perinatal period through to adulthood are associated with the development of

cerebellar cognitive affective syndrome (CCAS)[129]. The manifestation of symptoms varies depending on cause and cerebellar location of the lesion, however can be characterised by changes in abstract reasoning, working memory and language skills, all of which are similar to deficits seen in autistic and ADHD children.

Cerebellar development, like the hippocampus, begins around 8 weeks gestation and continues to develop in the postnatal periods, again making this region highly vulnerable to perinatal compromises. The cerebellum is a highly organised structure consisting of four layers. The internal layer contains the white matter tracts, moving outwards through the granule layer, Purkinje cell layer to the outer most molecular layer. During gestation, the granule cells begin in the external granule layer (EGL) and migrate through the molecular layer to produce the internal granule layer (IGL). Granule cells release excitatory neurotransmitter glutamate, with synapses connecting to inhibitory γ-aminobutyric acid (GABA) releasing Purkinje cells. The Purkinje cells are the output cells of the cerebellum, and are most vulnerable cells to insults[130]. In a guinea pig model of IUGR, previous studies have shown reductions in Purkinje cell numbers within affected cerebella[131], consistent with these cells being vulnerable to damage[132-134]. In sheep models, the field of dendritic branching is significantly reduced with IUGR[135], which imposes the risk of increased excitability of the perinatal cerebellum without inhibitory control by these Purkinje cells. The cerebellum, much like the hippocampus, has also displayed a delay in the production of mature myelin within IUGR brains compared to control fetuses in a guinea pig model[90]. Preterm birth impedes the development of the cerebellum that normally occurs in late gestation such as volume expansion and white matter deposition[136]. The volume of the cerebellum does not appear to catch-up postnatally, with adolescent males born extremely preterm more likely to develop the associated reductions in cognitive performance related to cerebellar underdevelopment and defect[137]. The more moderate compromise of PS also lead to adverse changes in the cerebellum. Increased glucocorticoid exposure in rats and mice leads to a decrease in Purkinje cell numbers and early decline of migrating EGL cells within the cerebellum[138-140]. There is also decreased secondary folding in lobes VIII and

X of cerebellum[141], leading to immaturity, and underdevelopment of this region. Rat pups from mothers who were restrained during pregnancy have reduced neuron-to-synapse ratios in the granular cell layer of the cerebellum compared to control offspring, reducing the connectivity between the cells of this layer and other neuronal cells including Purkinje cells[142]. Reductions in this interneuronal connectivity may reflect impaired signaling between the layers, and therefore potentially between the cerebellum and cerebrum which may account for some of the cognitive changes seen in individuals with altered cerebellar structures.

1.4.6 Amygdala

The amygdala is one of the key regions responsible for emotional responses, and often implicated in anxiety disorders. This region controls fear responses to perceived threatening stimuli. The amygdala has direct and indirect links to the hippocampus, influencing memory formation for emotional events[143] and indirect links to the hypothalamus. Thus in response to an emotionally stressful event, the amygdala can signal the hypothalamic-pituitary-adrenal (HPA) axis to trigger the production and secretion of stress hormones e.g. glucocorticoids, which act to activate neurons in creating a memory with context between an emotion and a situation. However, excessive release of glucocorticoids can interfere with neural signaling, impairing memory. MRI studies have found that activation of the amygdala in anxious individuals is hyperactive compared to non-anxious individuals[144, 145]. This finding is consistent with studies showing that people with anxiety and depression have a hyperactive HPA system[146, 147].

The amygdala develops relatively early in gestation, however, remains highly sensitive to hormonal regulation. The amygdala consists of multiple nuclei, including the basolateral complex (BLA), medial nucleus and central amygdaloid nucleus (CeA; see figure 2-2 for location). The BLA is considered the input centre of the amygdala, where it processes signals from temporal structures as well as the CA1 region of the hippocampus and subiculum to send information to the CeA. This is to produce a rapid, unconscious response to remove oneself from danger. The CeA is therefore effectively the output area of the amygdala. Calbindin-positive GABAergic interneurons located within the BLA act to regulate

activation of cells within this region, potentially limiting the activation of the CeA. This in turn modulates the output behaviour and responsiveness of an individual to the external environment. As we age, the reactivity of the amygdala to emotional stimuli shifts from remembering negative stimuli to activation on processing positive stimuli[148], with reactivity also controlled by higher order centres in the medial prefrontal cortex[149]. However, in chronic stress states, such as post-traumatic stress disorder (PTSD), there is an increased activation of the amygdala in response to negative stimuli[150], which implies impaired regulation of GABAergic pathways within the amygdala. This in turn leads to greater amygdala output and presentation of fear/anxiety traits.

The volume of the amygdala is associated with neurocognitive disorders such as schizophrenia. Numerous studies have shown that patients with schizophrenia have reduced amygdala volume[151-153], and that these reductions are greater in older patients[154] leading authors to believe that this neurocognitive disorder is progressive. Keshavan *et al* examined unaffected children with a high risk of developing schizophrenia, meaning they were born to people with the disorder, and found these individuals had significantly smaller amygdala volumes compared to individuals born to people without a psychiatric history within self or first-degree family members, suggesting that this disorder is of developmental origin[155]. An MRI study investigated the differences in white matter and grey matter within preterm children born IUGR versus AGA preterm children and term born infants. The investigators found that growth restriction, not premature birth, is associated with reductions in the amygdala at 12 months corrected age[60]. The literature suggests that disorders such as generalised anxiety disorders in adolescents, and autism are associated with both reductions[156-158], and enlargements[159, 160] in amygdala volume. Animal models of PS also show increases in volumes of the lateral nucleus in adult male rats is in part due to increased numbers of neurons and glia[161]. Prenatal stress induces an increased fear responsiveness and prolonged corticosteroid release, and increased hyperactivity within the amygdala in adulthood compared to control animals with no previous PS exposure[162]. These data combined show amygdala volume and activity needs to be tightly controlled during fetal life to produce “normal” fear

responses in postnatal life into adulthood, and that perturbations that either enlarge or reduce the amygdala's volume and reactivity have long-term negative effects on behaviour.

Early life deprivation in rodents (PND1-14) is a form of perinatal stress occurring during postnatally in rats, which corresponds to a neurodevelopmental period that takes place *in utero* in humans and guinea pigs. This early life deprivation is associated with depressive-like behaviours in adult male rats[163] and is also associated reductions in GFAP-reactive astrocyte numbers in the BLA[164]. Reduced glial cell counts are also found in the amygdala of adults with major depression, without loss of total neuron numbers[165]. This reduction in glial cells may deprive the surrounding neural environment of essential nutrients and neurotransmitter clearance provided by astrocytes. Another group using a rat model of prenatal stress occurring from gestational age (GA) 14 through to delivery, found reductions in both glial and neuronal cell numbers within the BLA and CeA of the amygdala of offspring at PND25, however these numbers normalise to control counts at PND45[166]. This difference in neuronal count may be due to timing of the stress event in relation to what neurodevelopmental processes are occurring. However, these studies suggest that changes in the perinatal brain may predispose the adult brain to depressive disorders by loss of glial cells during key periods of life.

1.5 Steroids of Pregnancy

Pregnancy is a time of continually changing hormone profiles. Progesterone, the main hormone of pregnancy, continually increases in concentration as gestation advances due to placental growth and output[167]. Much of this progesterone is metabolised as it passes from placenta to the fetal circulation. These metabolites are precursors for the production of neurosteroids in the fetal brain[168]. In fetal development progesterone and its 5α-reduced metabolite, allopregnanolone, have major protective and trophic actions that are highly important for proper neurodevelopment[169]. Placental production of allopregnanolone, as well as the supply of precursors for synthesis within fetal systems, results in fetal brain

concentrations that are higher than any time in postnatal life[169]. There is a dramatic decline in allopregnanolone levels during the fetal to neonatal transition, due to loss of the placenta. Furthermore, our studies showed that there is a similar reduction in allopregnanolone concentrations within the neonatal brain following preterm delivery in guinea pigs[170].

Progesterone is a precursor to many other neurologically active steroids that act as neural activators and inhibitors, including cortisol (figure 1-2). Progesterone itself is a neuroactive steroid, and binds to progesterone receptors in the brain to induce neurodevelopmental processes during fetal life, such as myelination and Purkinje cell development. Studies of traumatic brain injury in rats found that female rats with high progesterone as well as males treated with progesterone had less edema than untreated male rats following injury[171, 172], as well as reducing damage caused by lipid peroxidation[173]. Progesterone also modulates neurotransmission by reducing glutamate activity, independent of GABA inhibition[174]. Maturation and dendritic branching of Purkinje cells in the cerebellum rely on progesterone, rather than its GABA-modulating derivative allopregnanolone[175]. However, it's conversion to allopregnanolone is important for Purkinje cell survival[176]. Both progesterone and allopregnanolone are important in the production of myelination. Incubation of rat cerebellar slices with progesterone produces significant increases in the intensity of MBP staining in the slices. This was reduced, but not completely suppressed, by the addition of an allopregnanolone synthesis inhibitor, yet restored with co-treatment with allopregnanolone[177]. Therefore an interplay exists between progesterone and its metabolite in promoting neurodevelopment and protection, thus interference in the metabolism of this steroid into allopregnanolone can impact these processes.

1.5.1 Allopregnanolone: Enzymes and Location for Synthesis

The term neurosteroid was first used by French scientist Etienne Baulieu, and was used to describe steroids produced within the brain[178], either by *de novo* synthesis from cholesterol or from steroid precursors supplied by the periphery. The term neuroactive steroid was coined by scientists Paul and Purdy[179], and refers to steroid hormones that exert their actions in the brain, but can be produced within

the brain, or from peripheral sources such as the gonads and placenta during pregnancy. Unlike conventional steroids that bind to nuclear receptors to modulate genomic expression of mRNA and subsequently proteins, allopregnanolone acts on membrane bound receptors to rapidly excite or inhibit neural circuits[179, 180]. The synthesis pathway of allopregnanolone involves the metabolism of progesterone into 5α-dihydroprogesterone (5α-DHP) by the 5α-reductase enzymes type 1 and 2 (5αR1 and 5αR2 respectively; figure 1-2). These enzymes form the rate-liming step in the production of allopregnanolone. These enzymes are also responsible for the metabolism of other steroids in to their 5α, 3α-reduced derivatives. These include converting stress steroid deoxycorticosterone into 5α, 3α-tetrahydrodeoxycorticosterone (THDOC) and androgen testosterone into 3α-androstanediol (3α-diol). These 5α, 3α-derivatives also have positive allosteric functions at the same target receptor as allopregnanolone, however, less is known of the role of these neuroactive steroids during pregnancy and in brain development.

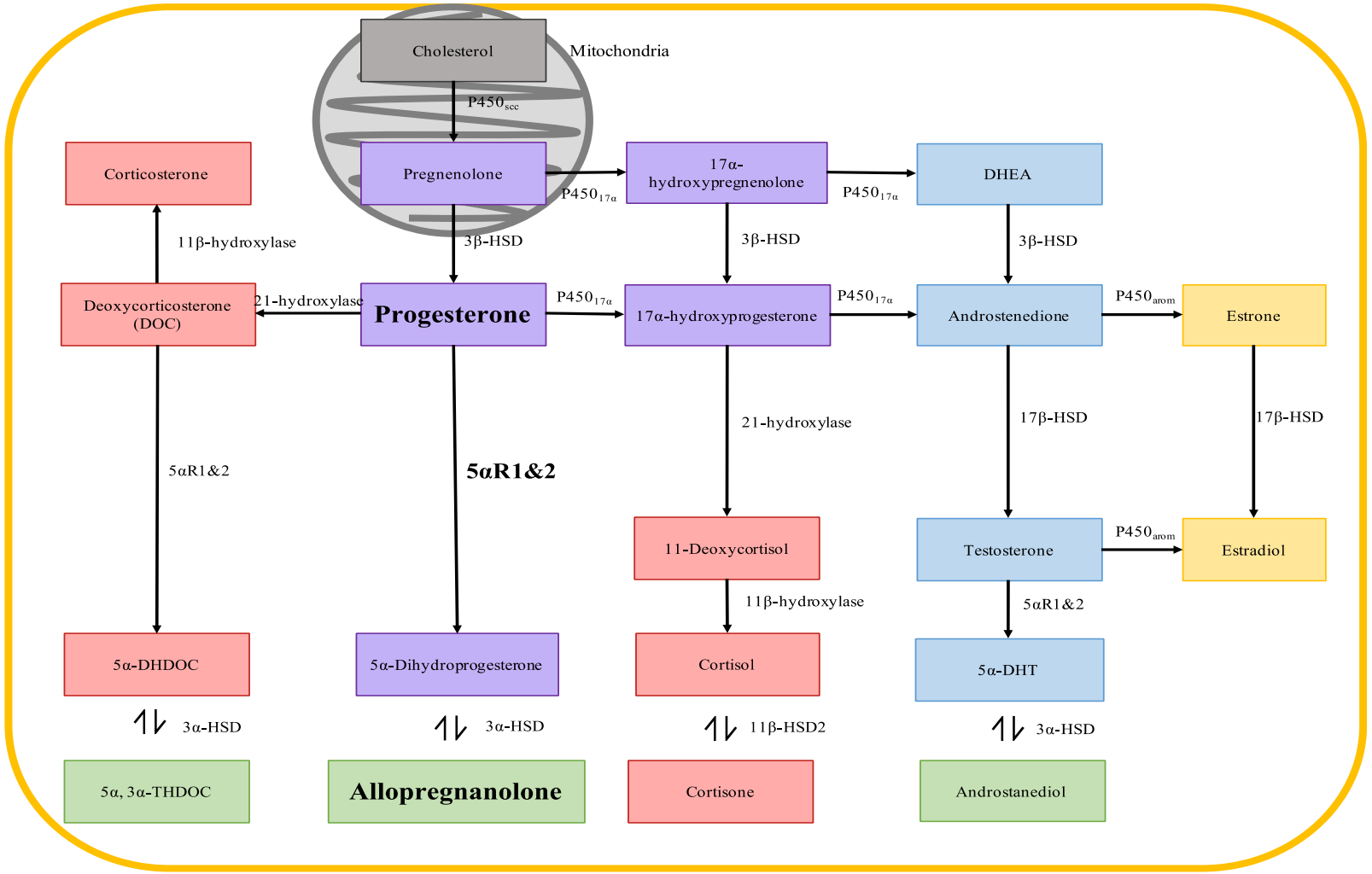

Cholesterol
Mitochondria
P450scc
Corticosterone
Pregnenolone
17α-hydroxypregnenolone
DHEA
P450 17α
P450 17α
11β-hydroxylase
3β-HSD
3β-HSD
3β-HSD
Deoxycorticosterone (DOC)
21-hydroxylase
Progesterone
P450 17α
17α-hydroxyprogesterone
P450 17α
Androstenedione
P450arom
Estrone
5αR1&2
5αR1&2
21-hydroxylase
17β-HSD
17β-HSD
11-Deoxycortisol
Testosterone
P450arom
Estradiol
11β-hydroxylase
5αR1&2
5α-DHDOC
5α-Dihydroprogesterone
Cortisol
5α-DHT
3α-HSD
3α-HSD
11β-HSD2
3α-HSD
5α, 3α-THDOC
Allopregnanolone
Cortisone
Androstanediol

FIGURE 1-2 NEUROSTEROIDOGENESIS. CELLULAR LOCALISATION OF NEUROSTEROID PRODUCTION FROM CHOLESTEROL. CONVERSION FROM CHOLESTEROL INTO PRECURSOR PREGNENOLONE OCCURS WITHIN THE MITOCHONDRIA OF CELLS, BEFORE TRANSLOCATION INTO THE CYTOSOL AND OTHER ORGANELLES WHERE METABOLISM INTO OTHER STEROIDS OCCURS. THE PREDOMINANT STEROID OF PREGNANCY IS ALLOPREGNANOLONE. PREGNENOLONE IS METABOLISED TO PROGESTERONE BY ENZYME 3β-HSD. SUBSEQUENTLY, ENZYMES 5αR1 AND 5αR2 IRREVERSIBLY PRODUCE 5α-DIHYDROPROGESTERONE FROM PROGESTERONE. ALLOPREGNANOLONE IS THEN, REVERSIBLY, METABOLISED BY THE 3α-HSD ENZYMES. OTHER 5α, 3α-REDUCED NEUROSTEROIDS ARE 5α, 3α-THDOC, PRODUCED FROM DEOXYCORTICOSTERONE, AND ANDROSTANEDIOL METABOLISED FROM TESTOSTERONE. THESE 5α, 3α-REDUCED METABOLITES THEN ACT ON $GABA_A$ RECEPTORS. GREEN BOXES = 5α, 3α-REDUCED NEUROSTEROIDS; PURPLE BOXES = PROGESTOGENS; BLUE BOXES = ANDROGENS; YELLOW BOXES = ESTROGENS; RED BOXES = GLUCOCORTICOIDS. DHEA = DEHYDROEPIANDOSTERONE; 5α-DHDOC = 5α-DIHYDRODEOXYCORTICOSTERONE; 5α, 3α-THDOC = 5α, 3α- TETRAHYDRODEOXYCORTICOSTERONE; 5α-DHT = 5α-DIHYDROTESTOSTERONE. $P450_{SCC}$ = P450 ENZYME COMPLEX, SIDE-CHAIN CLEAVAGE; $P450_{17\alpha}$ = 17α-HYDROXYLASE; HSD = HYDROXYSTEROID DEHYDROGENASE (3α-HSD, 3β-HSD, 17β-HSD); 5αR = 5α=REDUCTASE (TYPES 1 AND 2); $P450_{AROM}$ = AROMATASE ENZYME. ADAPTED FROM "FIGURE 1: BIOSYNTHESIS OF ALLOPREGNANOLONE AND PREGNENOLONE SULPHATE FROM CHOLESTEROL WITHIN NEURON AND GLIAL CELL" WANG, 2011[181] (OPEN ACCESS LICENSE).

Whilst the two 5αR enzyme isoforms metabolise the same substrates, these two enzymes share low homology (~50%) and have differing affinities for hormones. These two isoforms are also differentially expressed. The 5αR1 isozyme is located throughout the body, and has a more stable expression among tissue types[182]. The 5αR2 isozyme, however, is developmentally regulated and is implicated in being the main producer of neurosteroids in the placenta and brain during pregnancy[183]. Following the production of 5α-DHP, the 3α-hydroxysteroid dehydrogenase (3α-HSD) enzymes, reversibly, produce allopregnanolone. Neurons contain both 5αR1 and 5αR2, whilst macroglia contain 3α-HSD for the production of neurosteroids, allowing for alteration and regulation of local tissue specific concentrations[184]. Placental pathologies, particularly sheep models of IUGR, show increases in the expression of brain 5αR2 suggesting this is a compensatory mechanism adopted by the fetus in attempts to correct an imbalance within the intrauterine environment[185]. Work in adult rats has shown that PS interferes with the production of neurosteroids by changing the regional-specific expression of the synthesising enzymes within the brain[186]. These changes can also be corrected with exogenous neurosteroid administration.

1.5.1.1 $GABA_A$ Receptors: Structure, Expression and Function

Allopregnanolone is an allosteric modulator of the GABA type A receptor ($GABA_AR$)[187]. The $GABA_ARs$ are a pentameric structure with a central pore to allow for the influx of chloride ions, thus controlling the ability of a cell to reach its action potential threshold. At low nanomolar concentrations, allopregnanolone acts to enhance the inhibitory actions of the neurotransmitter GABA by increasing the frequency and duration of opening of the receptor channel[180, 188], allowing for a greater influx of chloride ions to hyperpolarise the cell. This reduces the membrane potential, such that a greater input is required to depolarise the cell, and so markedly decreases excitability. This in turn decreases the risk of over excitation and the potential for excitotoxic cell death. These actions are consistent

with the neuroprotective actions of $GABA_A$ agonists. The action of allopregnanolone at the $GABA_A$ receptor has also been shown to promote neurodevelopmental processes including myelination and dendritic formation. At micromolar concentrations allopregnanolone can exert these actions without the need for GABA[189]. There are approximately 19 $GABA_AR$ subunits and isoforms consisting of α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, and π. General formation consists of two α, two β and a single subunit of either γ_{1-3}, δ, ε, θ, or π (figure 1-3), with the most common receptor composition being $\alpha_1\beta_2\gamma_2$. $GABA_ARs$ have two functions in the CNS, phasic inhibition and tonic inhibition. Phasic inhibition is caused by receptors within the synaptic cleft of neurons, where GABA binds to rapidly produce hyperpolarization of neurons following binding, via influx of chloride ions into the cells. Tonic inhibition occurs at extrasynaptic sites (located outside of the cleft, usually on the axons). At these extrasynaptic sites ligands bind to slowly lower the basal resting potential of neurons and glia, modulating a cell's response to excitatory inputs. Previous studies have shown that allopregnanolone largely enhances tonic inhibition[190]. This is consistent with the lipophilic nature of this steroid which diffuses from its site of synthesis rather than being expelled in secretory granules.

The affinity of allopregnanolone binding to $GABA_ARs$ depends on the subunit composition of the receptors. Within the hippocampus receptors containing the α_4, α_5 and δ subunits, which are highly expressed within this region, bind allopregnanolone with a higher affinity than receptors containing γ subunits, whilst in the cerebellum $\alpha6$ and δ are more highly expressed. Regulation of the α_4 subunit is implicated in altered mood and anxiety states in response to acute allopregnanolone exposure and withdrawal following chronic exposure. The upregulation of mRNA expression of this subunit within the hippocampus coincides with increases in anxious behaviours[191, 192]. A study in female mice attributed increased anxiety in elevated plus maze testing to the decrease in δ subunit protein expression in the hippocampus during the estrus period (characterised by low progesterone, and allopregnanolone), concurrently with higher expression of neurosteroid insensitive subunit γ_2[193], although this study did not find any changes in α_4 subunit expression. Despite the differing findings in the

literature, the available evidence suggests both subunits, δ and α_4, are of key importance in the development of neuropsychological disorders in response to changes in neurosteroid concentrations.

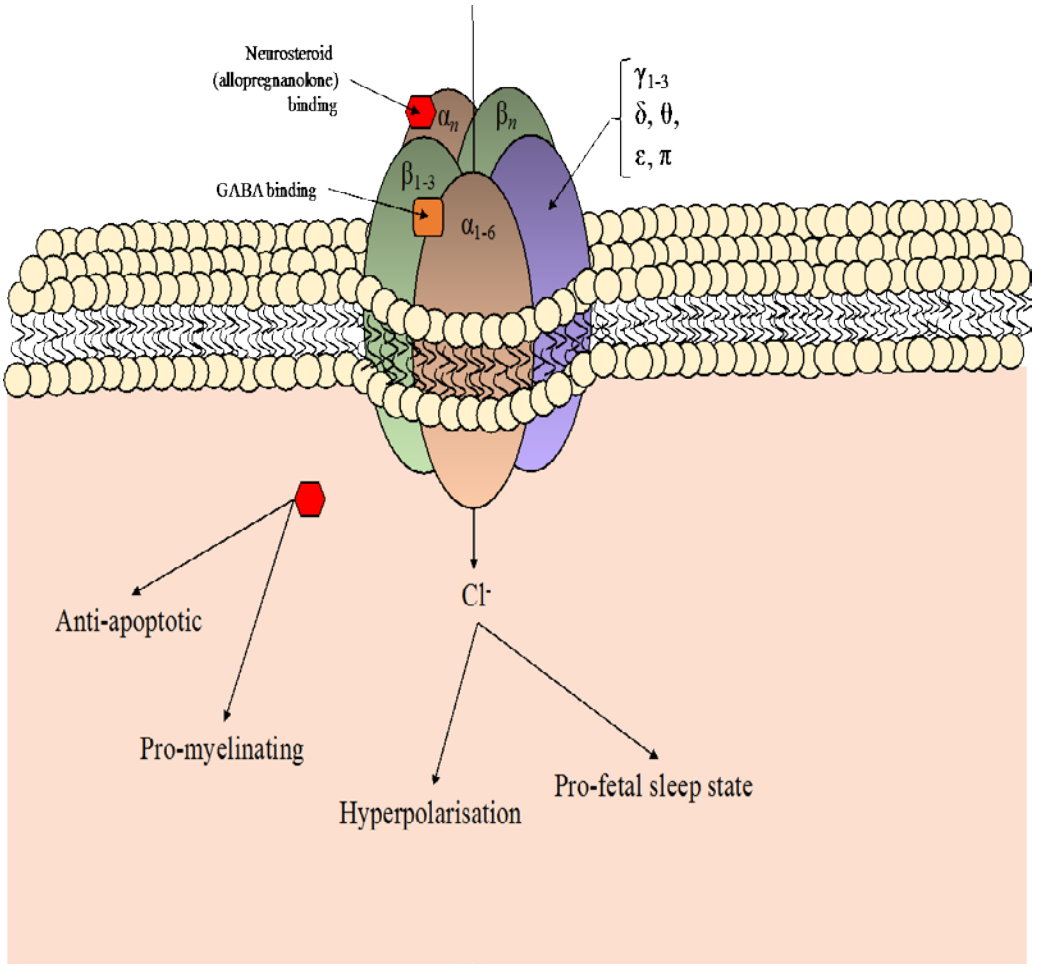


FIGURE 1-3 $GABA_A$ RECEPTOR SUBUNITS. THE $GABA_A$ RECEPTOR CONSISTS OF 5 SUBUNITS, 2 α_{1-6} SUBUNITS, 2 β_{1-3} SUBUNITS AND ONE OTHER SUBUNIT CONSISTING OF γ_{1-3}, δ, ε, θ, OR π. THE NEUROSTEROID BINDING SITE IS LOCATED ALLOSTERICALLY ON THE α SUBUNIT. THE COMBINATIONS OF THE α AND THE γ/δ SUBUNITS AND THEIR CELLULAR LOCATION DETERMINES THE SENSITIVITY OF THE $GABA_A$ RECEPTOR TO NEUROSTEROIDS. TRADITIONALLY, PAIRING $\alpha_{1,\ 2,\ 3\ OR\ 4}$ WITH THE γ_{1-3} PRODUCES A NEUROSTEROID-INSENSITIVE RECEPTOR AND IS LOCATED SYNAPTICALLY TO REGULATE SHORT-ACTING, PHASIC CONDUCTION OF ACTION POTENTIALS. PAIRING $\alpha_{4,\ 5\ OR\ 6}$ WITH THE δ SUBUNIT PRODUCES A NEUROSTEROID-SENSITIVE RECEPTOR, LOCATED EXTRASYNAPTICALLY TO REGULATE TONIC CONDUCTANCE OF RESTING MEMBRANE POTENTIAL USING AMBIENT GABA. THE ENHANCEMENT OF GABAERGIC ACTION VIA ALLOPREGNANOLONE PROMOTES THE FETAL SLEEP STATE IN LATE GESTATION, MAINTAINING MINIMAL NEURAL ACTIVITY TO PROMOTE NEURODEVELOPMENT AND REDUCE THE RISK OF EXCITOTOXIC CELL DEATH, VIA CELL HYPERPOLARISTATION, IN THE EVENT OF AN INSULT. INDIVIDUALLY, ALLOPREGNANOLONE CAN ACT

WITHIN THE CELL ITSELF TO PROMOTE MYELINATION FROM MATURE OLIGODENDROCYTES, AND LIMIT APOPTOSIS BY PREVENTING ACTIVATION OF THE CASPASE CASCADE. ADAPTED FROM JACOB *ET AL*, 2008[194] WITH PERMISSION.

1.5.1.2 Glutamic Acid Decarboxylase in Neurological Disorders

Little information is available on the impact pregnancy compromises have on the expression of glutamic acid decarboxylase kDa 67 (GAD67), one of the enzymes responsible for GABA production. However, many studies in adult animal models show that stress events modulate the expression depending on the duration of the stress event. Acute stress in adult rats increases the mRNA expression of GAD67 in a number of brain regions[195]. This is thought to be protective, by increasing the amount of GABA available to promote neuronal inhibition. However, chronic stress using immobilisation in mice down regulates the mRNA expression of this enzyme in the whole amygdala, whilst on the protein level reductions were found only in the medial amygdala[196]. Thus, differing exposure to glucocorticoids has programming effects on the GABAergic system in adulthood. Acute stress is known to increase allopregnanolone concentrations in fetal sheep brains[197], and in human adult circulation[198]. Allopregnanolone has also been shown to increase the expression of GAD67 in myelinating Schwann cells of the rat peripheral nervous system[199], and in the adult rat brain GAD67 co-localises with 5αR2 in the hippocampus and amygdala[200]. The co-localisation suggest these two enzymes act together to regulate local cellular excitability via substrate production[201], and alterations in these substrates may impact this ability.

Dysregulation of GAD67 has been found in rat models of mild traumatic brain injury[202]. Traumatic brain injury is associated with increased risks of developing anxiety and depression, and one study found this may be due to a decrease in GAD67-positive interneurons within the BLA following insult in both the side affected by injury (ipsilateral) and the unaffected (contralateral) side, without reductions in total numbers of neurons[202]. The expression of GAD67 is reduced in areas of the amygdala, including the BLA, in individuals with schizophrenia, bipolar disorder and major depression[203]. Post mortem studies in the cerebellum

of individuals that had autism showed a reduction in the expression of GAD67 mRNA within Purkinje cells compared to healthy matched controls[204], with reductions also found in protein expression albeit not statistically significant[205]. The authors suggest reductions in GABA synthesis within these cells disrupts the output to higher order cortices which in turn would affect behavioural output. Given that individuals born following IUGR, PS and PTB are more likely to develop disorders such as depression, autism and schizophrenia, it is plausible that programming *in utero* by these events impairs GABA synthesis, via reduction of enzyme expression in postnatal life following subsequent insults including death of a loved one, social pressures, career pressures and/or financial stress, and this may thus be a major factor in the development of mental health disorders.

1.5.1.3 Allopregnanolone in Neurobehaviour: Clinical Data

Allopregnanolone has a biphasic action within the brain in regards to anxiety and aggressive behaviours. Studies have shown an inverted-U shape action with nanomolar concentrations producing low levels of anxiety- and aggressive-like behaviours in women, but with these behavioural effects lessening at lower and higher concentrations[206]. This has been observed in rodents as well, with increasing allopregnanolone concentrations ≥10mg/kg producing aggression and place aversion. These negative behaviours were reversed with >30mg/kg doses[207, 208]. Allopregnanolone synthesis within the brain is transient, with concentrations increasing following acute stress periods[197], to limit potential damage by inflammatory components released. However, periods of chronic insults can reduce the production of allopregnanolone. Indeed, individuals with unipolar depression and premenstrual dysphoric disorder (PMDD) have lower circulating allopregnanolone concentrations and also a blunted allopregnanolone response upon stress testing[209, 210]. A significant proportion of women report suffering from mild to severe negative premenstrual symptoms, including irritability, depression and social anxiety around the time of menses. However, approximately 5% of women report having severe symptoms that significantly impair their day-to-day mental health and functioning and it's these women that meet the criteria for PMDD[211]. The corpus luteum is responsible for producing large quantities of progesterone and allopregnanolone (approximately 4nM) during the luteal phase

of the menstrual cycle. During the subsequent follicular phase (if fertilisation does not occur), concentrations of both hormones are much lower (approximately 2nM)[212]. It has been suggested that the drop in allopregnanolone to sub-luteal levels following high exposure alters $GABA_AR$ subunit composition[213] and produces ligand insensitivity of $GABA_ARs$[214], leading to the onset of negative mood. There is evidence to suggest that women with higher levels of allopregnanolone during this phase of the cycle have a lower risk of developing PMDD symptoms[212]. Allopregnanolone concentrations have been shown to be 60% lower in the cerebrospinal fluid (CSF) of individuals with unipolar depression[215]. A restoration in CSF and plasma concentrations of allopregnanolone, paired with improvements in symptoms, is reported following treatment with selective serotonin reuptake inhibitors fluoxetine or fluvoxamine[209, 215, 216]. The same level of reduction has been reported in the CSF of premenopausal women with PTSD[217]. This study further correlated the ratio of allopregnanolone and dehydroepiandosterone (DHEA, a negative $GABA_AR$ modulator) to degree of negative mood symptoms, leading the authors to suggest that CSF allopregnanolone is important in regulating inhibitory GABAergic vs. excitatory signaling and alterations in this ratio are important in the development of psychiatric disorders. Therefore, the levels of allopregnanolone supplied to, and produced by the brain during adulthood has major implications for mental health and related disorders. As outlined above, pregnancy, and hence the fetal brain is exposed to high levels of allopregnanolone produced and supplied by the placenta. Pregnancy compromises such as IUGR and PS that can interfere with this steroid pathway can impede the degree of myelination occurring within the brain, which may then in turn impair signal propagation. Alterations in neurosteroid utilisation may also program $GABA_ARs$ towards a more insensitive configuration. These changes away from a normal neurodevelopmental trajectory can potentially have negative connotations for later life fluctuations in neurosteroid concentrations, responses to stress events and onset of neurological disorders.

1.5.1.4 Allopregnanolone in Neurobehaviour: Animal Models

Neurosteroids are critical for fetal neurodevelopment, as well as adult neural signaling. Whilst MRI has allowed researchers to analyse activity and volumetric changes in gross neurological development following pregnancy compromises and adult disorders of living individuals, the examination of protein expression from specific cell types is reliant on post mortem studies. These, whilst these are highly informative for end stages of diseases, can be confounded by factors such as lifestyle and drug treatments that make it difficult to draw strong conclusions. Animal models aid research in investigating the mechanistic roles neurosteroids have on neurodevelopment, by allowing for various manipulations including pharmacological interventions, to elucidate where and how these steroids act. Animal models also aid in defining what changes occur when biological processes of interest are impeded while controlling for confounding factors. Pharmacological agent finasteride (*N*-(1,1-dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-ene-17-carboxamide) is an irreversible inhibitor of both 5αR enzymes. It inhibits the production of 5α-DHP, and subsequently allopregnanolone, and has been used in fetal studies to investigate the role allopregnanolone has in promoting neurodevelopment. Administration to fetal sheep causes increased fetal arousal during late gestation[218], and this finasteride-induced arousal was prolonged following a period of hypoxia/asphyxia (modelled by umbilical cord occlusion)[219]. The increase in arousal was reversed with co-infusion of synthetic allopregnanolone analogue alfaxalone, emphasising the role neurosteroids have in regulating fetal activity. Further studies have shown significant increases in apoptosis within the CA1 and CA3 regions of the hippocampus, as well as in the molecular and granular layers of the cerebellum following finasteride administration to fetal sheep[220]. Allopregnanolone controls apoptosis by binding to pro-apoptotic factor Bax, preventing its translocation to the cytosol of the cell[221]. This prevents the activation of the caspase/apoptosis cascade. However, without normal pregnancy levels of allopregnanolone, there is an uncontrolled increase in cell death, and this may have long term negative outcomes on the capability of the remaining cells to adapt and regulate changes. In guinea pig models of pregnancy, finasteride administration to the dams resulted in a 3 fold reduction in allopregnanolone concentrations within the fetal brain[105].

This was associated with decreases in the MBP within the CA1 region of the hippocampus, concurrently with increases in GFAP. Together this work supports the key role of allopregnanolone in maintaining neural health and neurodevelopmental processes.

The use of finasteride mimics changes in allopregnanolone supply to the fetus that occurs during pregnancy compromises, such as PTB and IUGR. Research from our group using the guinea pig model of PTB has shown reductions in plasma and brain allopregnanolone from high fetal levels to low term concentrations in prematurely delivered neonates[110, 222]. This was also associated with reductions in the area coverage of MBP within both the hippocampus[110] and the cerebellum[223] irrespective of sex. At term equivalence in this model, there is a tendency towards reduced myelination in males, as measured by MBP, PLP and oligodendrocyte progenitor cell marker platelet-derived growth factor receptor-α (PDGFRα), with a significant reduction in the expression of Olig2 (marker for oligodendrocytes in all stages of differentiation)[222] implying an impairment of oligodendrocyte proliferation in the male postnatal cerebellum. This is possibly due to males having less progesterone as a precursor, and thus allopregnanolone, to protect oligodendrocytes and promote cell survival in times of insult. This also shows that whilst there is some further development of myelination following premature delivery, without the high concentrations of allopregnanolone associated with intrauterine development myelination does not recover postnatally and males are the most vulnerable to oligodendrocyte loss. A fetal sheep model of placental insufficiency has demonstrated that there is an upregulation of the 5αR2 enzyme expression in numerous brain regions, including the hippocampus and cerebellum, following umbilical cord embolisation without differences in brain allopregnanolone concentrations[185], leading to a compensatory effect of increased enzyme expression to maintain neurosteroid levels in the face of chronic hypoxemia. However, there are still neurodevelopmental deficits following IUGR with hypoxemia during mid- and late gestation inducing white matter injury in fetal sheep. This occurs even after stabilisation of biological (physical and chemical) parameters[224]. IUGR induced hypoxemia also demonstrates an increase in glucocorticoids, indicating a premature activation of the fetal HPA axis in

IUGR[225]. Whilst the maintenance of neurosteroid production via upregulation of 5αR expression is a compensatory mechanism, it is not efficient to combat the potential negative actions glucocorticoids may be having, paired with hypoxemia, as evidenced by white matter injury.

1.5.1.4.1 Inhibition of Allopregnanolone Synthesis in Adult Animal Models

Examination of proestrous female rats, with high circulating levels of allopregnanolone, to diestrous females and males (low allopregnanolone) shows that proestous females have less anxious behaviour in open field testing, characterised by more entries into the inner zone, and less depressive behaviours in the forced swim test model, with fewer periods of immobility[226]. Two studies by Rhodes and Frye *et al* administered systemic or intrahippocampal finasteride to proestrous rats, and found in the open field test and forced swim test these animals became more anxious- and depressive-like in their behaviours. This was concurrent with the reductions in hippocampal allopregnanolone following both systemic and intrahippocampal administration, suggesting that these behaviours can be mediated by hippocampal levels of allopregnanolone[226, 227]. Treatment of male mice with finasteride for 7 days has been shown to significantly reduce the number of newborn cells in the hippocampus, and this is suggested to be a factoring role in the development of depression in men following finasteride administration for treatment of benign prostate hyperplasia or androgenic alopecia[228, 229]. Administration of finasteride directly into the amygdala of adult female rats increases anxiogenic behaviours under testing compared to control animals, whilst administration of allopregnanolone directly into the amygdala of adult rats decreases the anxiety-like behaviour under neophobic testing[230, 231]. This highlights an important interplay between the limbic structures in regulating anxious behaviours, and that aberrant allopregnanolone levels are important in the development of anxiety and depression in both males and females.

Extensive evidence indicates that in the postnatal period allopregnanolone has a role in programming behaviour. Neonatal administration of finasteride from PND5 to 9 in rats produced anxiogenic-like behavioural profiles in elevated plus maze (EPM) and passive avoidance tasks in adulthood (>PND90). Animals exposed to neonatal finasteride had shorter latency times to enter a dark box that

delivered an electric foot shock, compared to vehicle control animals indicating an impairment of memory in these animals[232]. The finasteride treated offspring also made fewer passes into the open arms of the EPM, and this corresponded with less time spent in the open arms, findings that suggest these animals had a greater anxiety-like phenoytype on testing compared to controls. Follow-up studies in male rats after neonatal finasteride administration showed that the treated animals displayed fewer exploratory measures in modified open-field testing compared to control males at mid- and post-adolescence (PND40 and PND60)[233]. These observations further indicate that lower brain allopregnanolone levels in the immediate postnatal period in rats programs for stronger negative emotional responses in later life.

These studies show that early life changes in allopregnanolone have negative impacts on perinatal neurodevelopment, and later onset of anxiety like behaviour. Whilst the behavioural aspects have been investigated, in male rats particularly, the follow-up of fetal neurodevelopmental deficits and how these are related to these behavioural changes have not been investigated into the postnatal period. It is important to understand how long these structural and/or molecular changes may be occurring in regions of the brain most vulnerable to loss of allopregnanolone and how far into postnatal life they are occurring in order to be able to implement effective treatments.

1.5.2 Effect of Changes in Cortisol Concentrations with Prenatal Compromises

Cortisol concentrations remain relatively stable throughout pregnancy, before rising exponentially within the last 10-15 weeks of gestation[234]. The concentration of cortisol reaching the fetus from the maternal circulation is tightly regulated by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme. This enzyme converts active cortisol to the less receptor-active cortisone within the placenta, maintaining lower circulating fetal concentrations compared with circulating maternal levels[235, 236]. Expression of this enzyme is correlated with maternal cortisol concentrations across gestation[237, 238]. Placentae from IUGR pregnancies show reduced 11β-HSD2 expression and activity compared to

placentae from appropriately grown preterm infants and healthy, term born infants[239]. Likewise, PS is associated with reductions in 11β-HSD2 enzyme expression within the placenta[240], and this is associated with increased cortisol concentrations in the fetal circulation. Therefore, the combination of IUGR and PS may overwhelm the normal placental enzymatic barrier to the passage of cortisol, allowing increasing levels to enter the fetal circulation where it can alter and inhibit the neurodevelopmental processes of astrocyte activation and myelination. The brain is capable of reducing cortisol to cortisone via 11β-HSD2, predominantly in the thalamus and the cerebellum[241, 242]. However, evidence shows there still exists detrimental effects on neurodevelopment[142, 243, 244] suggesting the expression of 11β-HSD2 in the fetal brain is not entirely effective in combatting stress-induced rises in cortisol.

As previously reported, acute periods of stress transiently increase allopregnanolone concentrations along with the rise in corticosteroids released from the adrenal glands[198, 245]. This rise is thought to be a buffer to protect against the negative effects of cortisol on neural development and survival. As indicated above, in chronic stress states such as depression and PTSD there is an associated reduction in levels of allopregnanolone. This is supported by animal models of socially isolated rats, shown to have reduced circulating allopregnanolone concentrations, along with development of anxiety/depressive like behaviours and loss of hippocampal progenitor cells[246]. In human brains from suicide victims there is increased corticotrophin releasing hormone (CRH, the initial substrate hormone in the stimulation of cortisol), coinciding with decreased $GABA_AR$ δ and α_4 subunit expression in the frontopolar cortex[247]. This implies long term exposure to stress hormones has a negative feedback role in programming neurosteroid production and GABAergic pathways. In the instance of PS, where the high circulating concentrations of cortisol pass from mother to fetus, where there is a programming effect for anxiety and depression in adulthood, this is potentially due to the loss of key structural proteins and neurons in vulnerable regions such as the hippocampus, cerebellum and amygdala in fetal life (see sections 1.4.4, 1.4.5 and 1.4.6 respectively). Therefore, in pregnancy compromises whereby the normal cortisol barrier is deficient, it may also interfere with the

production and supply of allopregnanolone to the fetus. Furthermore, this buffering system may be rendered inadequate leaving the fetal brain vulnerable to cell excitotoxicity and death, as well programming the remaining cells towards a more vulnerable phenotype later on in life. Thus it is necessary to fully elucidate what changes are occurring in these vulnerable brain areas during fetal neurodevelopment, whether the regions recover these lost cell populations and structural proteins and how these may program for the later development of psychiatric disorders. This information will allow the development of effective interventions and treatments to combat the ever-growing precedence of mental health disorders in society.

1.6 Placental Health Impacts Fetal Health

The placenta is the organ of pregnancy. Without implantation and development, the fetus does not survive. The placenta acts as a filter between the maternal and fetal circulation, supplies essential hormones and clears waste to maintain a healthy environment for the fetus to grow[248]. Poor placental implantation is associated with many prenatal complications including preeclampsia and IUGR[249]. Placental health can be used to identify fetuses at risk of poor outcomes e.g. sonography to identify placental abruption, although this method is not very sensitive[250], or poor placental growth (based on size). Post-delivery assessment of placental lesions can identify infants with greater risk of brain injury, with the greater number and severity of lesions correlated to the severity of neurological impairment and cerebral palsy[251]. However, in preterm birth (as defined by delivery before 37 completed weeks gestation), there are a large proportion of women who do not have the known risk factors, or give any indication deliver prematurely[252]. Preterm birth is the leading cause of neonatal mortality worldwide[253], with majority of premature deliveries being of moderate to late preterm (32-<37 weeks). Gestational age at the time of delivery is a strong indicator of survival. However these moderate to late preterm infants are at an increased risk of death compared to term infants[254], with some preterm neonates dying without overt indicators such as repeated periods of respiratory distress and need for mechanical ventilation.

The placenta, as previously stated, has the machinery to protect the fetus from adverse *in utero* events. It protects the fetus from the high maternal levels of cortisol via the presence of 11β-HSD2[235]. It also is responsible for the high levels of neurosteroids supplied to the fetus, with loss of the placenta following delivery resulting in dramatically reduced concentrations of allopregnanolone[110, 167, 255]. IUGR in the guinea pig is associated with increased mRNA expression of 5αR2, suggesting this organ upregulates allopregnanolone production following a stressor[104], much like the brain does[185, 197]. Thus, given the importance of this organ in fetal health, and in the supply of essential neurosteroids for fetal brain development, it is a relatively underappreciated source for potentially identifying infants that may have been exposed to previously undiagnosed intrauterine events that requires further investigation.

1.7 Rationale, Hypothesis and Aims

An appropriate neurosteroid environment is essential for the development of neuronal and glial connections, neurochemical signaling, myelination and apoptosis. Neurosteroids, in particular allopregnanolone, are essential for supporting and maintaining these critical neurodevelopmental processes. Intrauterine growth restriction, prenatal stress and preterm birth present a difficult start to life for affected offspring. All these pregnancy compromises cause detrimental neurodevelopmental outcomes in both the short and long term, highlighting the strong programming effects that *in utero* development has on the quality of life of the offspring.

These conditions interfere with allopregnanolone synthesis and the ability of this key neurosteroid to exert its actions, in part leading to the deficits seen in astrocyte regulation and myelination in fetal life. The importance of allopregnanolone's actions in modulating $GABA_A$Rs in the adult hippocampus and amygdala is well known. However, no studies that we are aware of investigate how suppressed allopregnanolone *in utero* impacts the postnatal programming of $GABA_A$Rs in the cerebellum, even though this region is highly

important in the development of emotional and cognitive disorders normally associated with hippocampal and amygdaloid dysfunction. Nor have studies followed up these structural and protein changes seen in fetal life (see section 1.4.4) within the juvenile brain and behaviour in a comparable animal model, and if there exists a persistent reduction in myelination and astrocyte activity that may (in part) explain the behavioural deficits associated.

A large amount of evidence exists on the combined negative impacts growth restriction imposes on the preterm infant in both neurodevelopment and psychological development. Similarly, individuals born following IUGR and maternal PS are at a greater disadvantage, neurodevelopmentally in fetal life and psychologically later in life. Yet, scant epidemiological data is available investigating the prevalence of these two issues of IUGR and prenatal stress occurring together. This warrants investigation given the high probability that a mother who received news her fetus is growth restricted may experience repeated stressful episodes worrying about her baby's health. To date, no study has combined intrauterine growth restriction and maternal prenatal stress to investigate if these complications induce a greater neurodevelopmental and neurosteroid deficit, nor how these changes may impact the different sexes.

The placental upregulation of the neurosteroid pathway in response to negative intrauterine events, for example hypoxia and IUGR, is well documented. However, it is often overlooked as a marker for identifying if a fetus has been compromised *in utero* beyond size, sonographic indicators of poor placentation or abruption and identification of infarcts.

The work in this thesis aims to improve and add to the current body of literature, of the role neurosteroids play in programming fetal neurological systems and gross neurodevelopment. This work uses our well-established guinea pig models, to investigate $GABA_A$Rs, behaviour and changes in structural proteins in postnatal life following suppression of allopregnanolone synthesis during late gestation. This work also intends to investigate if the combination of two common pregnancy compromises has an additive effect on neurodevelopment and

neurosteroid production. A smaller study in this thesis also aims to identify if the neurosteroidogenic capacity of the placenta will help identify preterm infants at a greater risk of negative outcomes.

1.7.1 The Guinea Pig as a Model for Pregnancy Compromises

There are a number of animal models currently used to investigate how prenatal compromises impact perinatal behaviour and neurodevelopment, and how these can then translate into human studies. These include studies in rats, sheep, guinea pigs and non-human primates. Each species has their pros and cons for the use in translating to human disease. The rat is the most commonly used model in IUGR and prenatal stress. The neurodevelopmental timeline for the rat has been well established, however the majority of neurodevelopment, equivalent to the late gestation human, in these rodents occurs postnatally [256, 257]. Therefore, the neurosteroid environment in these altricial species is reliant on brain and peripheral organ production of these steroid hormones, as opposed to humans which rely on placental supply. Long gestational species, such as sheep and guinea pigs, are precocial species whereby a significant proportion of neurodevelopment occurs *in utero*[258, 259]. The manipulations during gestation in these species can also be targeted towards specific developmental time points and correlated to human data. Mid gestation in the guinea pig (~GA35-40 days) equates to approximately 20-24 weeks of human gestation[38, 39]. This coincides with both myelination and hippocampal formation in both species [39, 131, 258]. Late gestation in the guinea pig (GA62-63) has been approximated to 34 weeks of human gestation[260], with myelination in the guinea pig brain peaking and slowing down in late gestation compared to human[259]. However, the brains of both species are relatively developed and mature at the time of delivery compared to rodents.

The guinea pig has a haemochorial placenta[261], like humans and non-human primates, with progesterone production under control of this organ. The guinea pig placenta and fetal brain both contain the necessary enzymes to produce allopregnanolone[104, 105], and neurodevelopment occurs in a high neurosteroid environment[110]. Sexual maturity occurs at approximately 4-6 weeks in females and 8-10 weeks in males[262], and behaviour can be assessed using established

behavioural tests during neonatal, adolescent and adult ages[111, 122, 263, 264]. Studies in guinea pigs can therefore occur in a shorter time frame compared to longer gestational and maturing species like sheep and non-human primates. It is, therefore, also more cost-effective to conduct guinea pig experiments compared to these species. Thus the guinea pig presents itself as an ideal animal model by use of surgeries, psychosocial stressors and pharmacological inhibition to mimic pregnancy compromises of IUGR, PS, PTB; of which these compromises have been well established within our lab[104, 105, 110, 111, 121, 122, 222, 223, 260, 265].

1.7.2 Hypothesis

Suppression of allopregnanolone synthesis, using pharmacological inhibition, in late gestation will have a programming influence on the early postnatal cerebellar development. The loss of allopregnanolone in late gestation will be associated with changes in behaviour of juvenile offspring, with the amygdala and hippocampus being key regions of deficit.

The individual effects of intrauterine growth restriction using a modified model of growth restriction induction will display negative neurodevelopmental sequelae in the fetal brain, paired with depreciated levels of allopregnanolone supply. This will be confounded with concurrent prenatal stress exposure.

Placental 5α-reductase mRNA expression will act as a marker of poor neonatal outcome within the first 24 hours of life in preterm guinea pig neonates, compared to term counterparts.

1.7.3 Specific Aims

1.a. To determine what ongoing effects pharmacological inhibition of allopregnanolone synthesis during late gestation has on cerebellar myelination, astrocyte activation and $GABA_AR$ subunit expression during the neonatal period (postnatal day 8) in guinea pig offspring.

1.b. To assess the behavioural deficits intrauterine inhibition of allopregnanolone synthesis causes at juvenile equivalence (postnatal day 21) in the guinea pig, and if these changes are associated with impaired neuronal proteins, astrocytosis and GABAergic pathways.

2. To determine the extent of how individually, intrauterine growth restriction reduces markers of neural maturation within vulnerable regions of the fetal brain, and how prenatal stress may further impair these neurodevelopmental markers in affected growth restricted fetuses, with a greater deficit to be seen in males.

3. To determine if the placental expression of the 5α-reductase enzymes correlates with preterm survival of guinea pig neonates within the first 24 hours of life, potentially reflecting exposure to suboptimal *in utero* conditions.

2 METHODS AND MATERIALS

2.1 Animal Ethics

All animal experiments were approved by the University of Newcastle Animal Care and Ethics Committee (approved ethics number A-2012-251), in accordance with National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals were monitored daily for signs of early or unexpected labour, distress and illness. Pregnant dams were weighed daily to monitor weight gain as an indicator of maternal health and fetal growth. All neonates were weighed and monitored daily for assessment of healthy growth and physical activity. Veterinary advice was sort in the event of abnormal symptoms according to ethical guidelines, and animals were humanely euthanised if necessary.

2.2 Animal Housing

Outbred, tricolour, pregnant guinea pigs were obtained from the University of Newcastle Research Support Unit. Time-mating of guinea pigs occurred during

the post-partum estrous period, and dams were housed with their recently delivered litter until 21 days postnatal age, when weaning occurred. Pregnancy was confirmed by gentle palpation of the abdomen to detect the conceptus. Subsequently, all animals were housed in individual cages within visual and vocal distance of each other, under 12 hour light/dark cycle. Guinea pigs were provided with commercial guinea pig feed pellets, loose hay and daily fresh water fortified with ascorbic acid, *ab libitum*. Following deliveries within protocol, neonates were housed with their mothers under the same conditions.

2.3 Finasteride Treatment

Finasteride was administered to pregnant guinea pigs to inhibit the increasing gestational production of allopregnanolone by the placenta, brain and other steroidogenic organs. Pregnant guinea pigs used for experiments in chapters 3 and 4 were allocated to receive either vehicle (45% cyclodextrin, 400µl/kg, Sigma Aldrich, Australia) or finasteride (25mg/400µL/kg, STERALOIDS) commencing at GA60 until delivery (term ~71 days gestation in this cohort). This range of gestation would equate to roughly 32 weeks through to term in the human. This period was chosen to administer finasteride as this period coincides with late term preterm birth. Infants born in the late preterm period (approximately 32 to less than 37 weeks gestation[260]), make up the majority of preterm birth and are a group at a greater risk of neurological and behavioural deficits compared to term born infants. The placenta provides the high concentrations of allopregnanolone provided to the fetus during gestation. Premature delivery of the infant, and hence placenta, results in the early loss of these high concentrations[110]. Finasteride in late gestation mimics this significant reduction, without introducing the confounding factors of organ immaturity (lung, heart, liver etc.) known to complicate preterm neurodevelopment. Administration occurred at approximately the same time each day (~8am), via the oral route. Briefly, guinea pigs were weighed and amount of drug calculated. Vehicle or finasteride was drawn up in a 1ml needleless syringe. The tip of the syringe was placed in the back of the mouth and the plunger slowly depressed until all liquid was administered. Following delivery of the neonates, administration of vehicle and finasteride ceased.

2.4 Spontaneous Delivery and Behavioural Testing

2.4.1 Spontaneous Delivery of Offspring

Dams allowed to spontaneously deliver were closely monitored from time of pubic symphysis opening (as assessed by gentle palpation). Following delivery all newborn pups were weighed, sexed and measured. Pups were allocated to either 8-day (neonatal) endpoint tissue collection or 21-day (juvenile) behavioural testing and tissue collection protocols. One male and female from each litter was given preference to each time point.

2.4.2 Juvenile Behavioural Testing

On postnatal day (PND) 20, all pups underwent behavioural testing, occurring in the morning between 0800 and 1200 hours. Juvenile animals were assessed in open field (OF) and environment exploration tasks. All tests were recorded using ANY-Maze software (Stoelting, Wood Dale, IL, USA) and Logitech webcams (logitech). The behavioural testing consisted of an arena (40cm x 40cm) set up in a brightly lit room. Grids were set up within the software program using the dimensions of the arena. Guinea pigs had pre-test saliva collection (see section 2.4.3 for collection protocol) before being placed in the arena along the bottom wall, facing away from the centre of the arena, and allowed to explore for 10 minutes. Total number of grid crossings and total number of entries into the inner zone as well as time spent in the inner zone were recorded using the tracking software. The total distance travelled and area grid crossings were used as a measure of locomotor activity, with total number of entries and time spent exploring the inner zone used as a measure of anxiety-like behaviour. Following the OF test, guinea pigs were placed in a holding cage and the arena set-up for the next test. Juvenile offspring were placed back in the arena with two objects of differing shape and colour. Guinea pigs were allowed to explore the objects for 10 minutes as a measure of willingness to investigate changes in their environment. The total amount of time spent investigating objects, as measured by time the head was within contact (biting, sniffing etc.) or within a 10% distance of the

object's boundary, was used as a measure of neophobic-like behaviour. The total amount of time spent investigating objects was used as a secondary measure of anxiety-like behaviour. Following the final test, post-test saliva was collected before offspring were placed back into their home cage with their mothers.

2.4.3 Saliva Collection from Juvenile Pups and Pregnant Dams

Saliva was collected immediately prior to behavioural testing for juveniles, and prior to induction of prenatal stress for pregnant dams by cotton tips. Samples were also collected immediately following behavioural testing and stress events for pups and dams, respectively. The cotton tips were inserted into the cheek pouches parallel to teeth and guinea pigs encourage to chew the tip. Cotton tips were left in the mouth for 2 minutes before being placed in sterile 1.5mL eppendorf tubes. The tubes were centrifuged for 5 minutes at >8000g at 4°C to collect the saliva out of the cotton tips. Once collected, the tips were removed from the tube and the saliva was stored at -40°C.

2.5 Intrauterine Growth Restriction Surgery

To examine the individual effects of IUGR on neurodevelopment and the combined effects of IUGR and PS, an experimental model of slow induction growth restriction was adapted from Dickinson *et al*[266] and Herrerra *et al*[267] for this study. Abdominal surgery was performed at mid-gestation to induce growth restriction in the fetuses. To allow for comparison between sham and IUGR animals, surgeries were performed on all animals as detailed below.

2.5.1 Surgical Preparation

Surgery was performed between 30-35 days gestation (term ~71 days) under strict aseptic technique. Food was removed 4 hours prior to surgery, with access to water remaining unrestricted. Buprenorphine (Temgesic; buprenorphine hydrochloride, 0.05mg/kg) and atropine sulphate (0.05mg/kg) were administered subcutaneously 30 minutes before anaesthesia induction. Anaesthesia was induced in a chamber with an isoflurane vapouriser with a flow rate of 6L/min medical

grade oxygen and 4% isoflurane. Once righting reflex was lost pregnant guinea pigs were placed on heat pads on the surgical table in the supine position with the head raised to maintain body temperature and aid respiration. Anaesthesia was maintained by mask inhalation of 2% isoflurane with 2L/min oxygen. Excess saliva was removed via cotton tip to the guinea pigs mouth to prevent inhalation and asphyxiation during the procedure. The abdomen was shaved and washed three times with chlorhexidine solution, starting at intended incision site and moving outwards with circular motions. The abdomen was wiped between each wash with clean pads ensuring no unclean area or hair was touched. Once cleaned, the shaved site was sprayed with betadine solution and rinsed with 70% ethanol prior to incision. The surgeon maintained aseptic technique by wearing hair net, facemask, autoclaved surgical gown and sterile gloves. All drapes and surgery instruments were autoclave sterilised or were from sterilised single-use packets.

2.5.2 Surgical Technique

An incision, approximately 4cm long, was made between the umbilicus and pubic symphysis to expose the subcutaneous muscle layer. Another incision was made along the *linear alba* to open the peritoneal cavity. The uterine arteries at both ovarian and cervical ends of the uterine horns were located, fat was carefully dissected away from the arteries. To induce growth restriction, sterilised medical-grade silicon tubing (Gecko Optical, Perth, WA, Aus; 4mm length, 1mm wall thickness, cut lengthways for opening) was placed around the artery at both the cervical (internal diameter 1.5mm) and ovarian ends (internal diameter 1.5-2mm; figure 2-1). Internal diameter size was decided on the basis that the tubing should not restrict blood flow at time of placement and could be moved along artery when shifted. This procedure was used on both horns of the uterus. Tubing was secured in place using sterile suture tied around the external wall to prevent slippage. Exposed tissue was kept moist using sterile saline solution (0.9% NaCl). The muscle layer was then closed using the simple interrupted suture pattern using Biosyn monofilament absorbable sutures. The subcutaneous layer and skin were then closed using a continuous horizontal mattress suture pattern with Daclon blue nylon suture. After surgery the incision site was sprayed with Chloromide antiseptic spray and terramycin antibiotic spray.

2.5.3 Sham Surgery

Pregnant guinea pigs allocated to the sham protocol had their fat pads and uterine arteries manipulated in the same manner as in 2.5.2, however did not have tubing placed around the arteries.

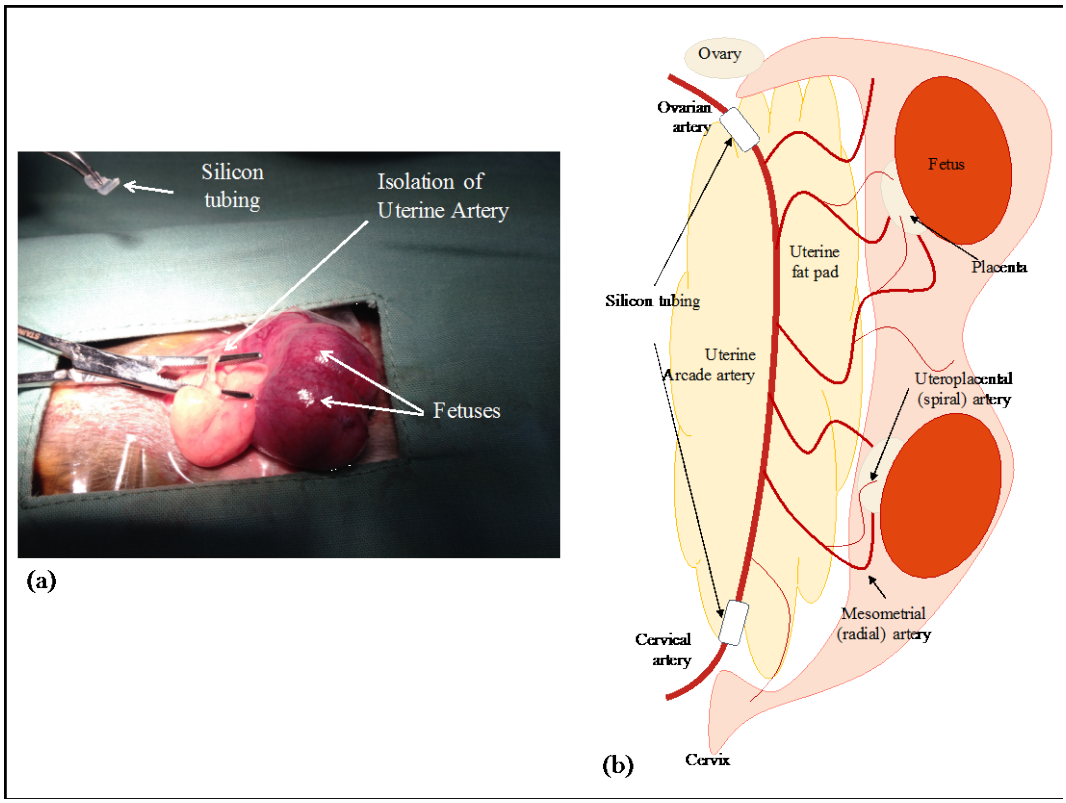


FIGURE 2-1 UTERINE ARTERY RESTRICTION. PHOTO OF UTERINE HORN (A) AT ~GA30 DEPICTING TWO FETUSES. STERILISED FORCEPS WERE USED TO ISOLATE THE UTERINE ARTERY FROM THE SURROUNDING FAT PAD BEFORE THE SILICON TUBE IS WRAPPED AROUND THE ARTERY AND FASTENED IN PLACE WITH STERILE SUTURE. SCHEMATIC OF LEFT HORN OF GUINEA PIG UTERUS (B). THE GUINEA PIG HAS A BICORNUATE UTERUS SUPPLIED TWO UTERINE ARTERIES WITH OVARIAN AND CERVICAL ENDS, THAT CONNECT INTO THE ARCADE ARTERY. THIS ARCADE ARTERY BRANCHES INTO MESOMETRIAL AND UTEROPLACENTAL ARTERIES TO SUPPLY THE PLACENTA, AND SUBSEQUENTLY THE FETUS DURING GESTATION. IUGR WAS INDUCED BY WRAPPING STERILE SILICON TUBING AROUND THE ARTERY AT THE CERVICAL AND OVARIAN ENDS, BELOW AND ABOVE THE BRANCHING MESOMETRIAL ARTERIES RESPECTIVELY TO PREVENT BLOOD REDISTRIBUTION TO MINOR ARTERIES AS COMPENSATION. UTERINE ARTERY RESTRICTION PROCEDURE ADAPTED FROM DICKINSON ET AL[266] AND HERRERA ET AL[267]. SCHEMATIC ADAPTED FROM KAUFMANN AND DAVIDOFF[261] AND KELLEHER[268].

2.5.4 Post-surgery Recovery

On completion of surgery, isoflurane was turned off and animals placed in the prone position with 100% oxygen. Once reflexes were returned, the oxygen was turned off and the animals were allowed to recover from the anaesthetic, after which they were returned to their home cage with the heat pad. Guinea pigs were closely monitored until normal activity was restored. Animals received a second dose of buprenorphine (0.05mg/kg) 8 hours post-surgery. Guinea pigs were monitored daily after surgery and sutures removed 10 days post-operation. Suture sites were monitored closely for signs of dampness, infection or nibbling, with terramycin spray reapplied as required.

2.6 Stress Induction

Prenatal stress was induced by strobe light in a subset of IUGR surgery dams to investigate the combined effects of growth restriction and prenatal stress on fetal brain development.

2.6.1 Strobe Light Induction of Prenatal Stress

Prenatal stress was induced between 0900 and 1100 hours using a strobe light stressor, previously validated by other research groups and ourselves[121, 122, 265, 269, 270]. Briefly, pregnant guinea pigs were placed into a darkened environment for 2 hours, commencing on gestational day 40, as this is equivalent to human gestation of ~20-24 weeks. This period in the human encompasses processes such as synaptogenesis, myelination, astrogliosis and the development of regions such as the hippocampus [38, 39]. as it does in the guinea pig[131, 258]. The stress events were repeated on days 45, 50, 55, 60 and 65. Psychosomatic stress was induced by a strobe light (75 joules) within a custom built box. Guinea pigs remained in their homes cages inside the box, with unrestricted access to food and water. Immediately before and after the stressor event a stress salivary sample was collected as detailed in section 2.4.3.

2.6.2 Control Conditions

Dams allocated to the control (no stress) group also had saliva samples collected at the same time points. However, these guinea pigs remained in their home cages within the housing room during the allocated 2 hour period, without exposure to the strobe light and acted as non-stressed controls.

2.7 Tissue Collection

All fetal, PND8 and PND21 animals and their dams were euthanised via CO_2 inhalation (Coregas) for approximately 7 minutes. Pedal and palpebral reflexes were tested before commencement of tissue and fluid collection. The secondary death measure of excision of the diaphragm and cessation of heartbeat were also used for confirmation. All bloods from dams, fetuses and neonates/juveniles were collected via cardiac puncture with 23G with 1mL syringe (fetal and neonatal animals) or 21G needle attached to 3mL syringe (dams), and collected into ethyldiaminetetracetic acid (EDTA) blood collection Vacuette tubes (Griener Bio-One, Monroe, NC, USA). Blood samples were centrifuged in a refrigerated centrifuge for 10 minutes and plasma pipetted into sterile tubes and snap frozen.

2.7.1 Collection of Neonatal and Juvenile Finasteride Tissues

At experimental endpoint of PND8 or PND21 offspring were weighed before euthanasia. After blood collection the brain was dissected by making a mid-sagittal incision along the skull and scalp moved away from the skull. The skull bone was peeled back using rongeurs to expose the brain. The whole brain was removed, weighed before a sagittal cut was made down the middle of the two hemispheres. The left hemisphere was then coronally cut into three planes – region A (containing the pre-frontal cortex), region B (containing the hippocampus, amygdala and subcortical structures) and region C (consisting of one half of the cerebellum, cut along the vermis). Tissue blocks were fixed in 10% neutral buffered formalin overnight at 4°C before storage in 0.1M PBS with 0.05% sodium azide. The other hemisphere was dissected to remove and weigh the hippocampus, which was then snap frozen in liquid nitrogen. The right

hemisphere was dissected into prefrontal cortex, the cortex overlying the hippocampus, the hippocampus, half the cerebellum and leftover subcortical structures (including the thalamus). The right hippocampus and half cerebellum were weighed, before all brain regions were placed in individual sterile Eppendorf tubes and snap frozen in liquid nitrogen. The heart, liver, adrenals and kidneys were collected and weighed before adrenals were snap frozen. A portion of the right lobe of the liver was taken for formalin processing and whilst another part of the same lobe was snap frozen in liquid nitrogen.

2.7.2 Collection of fetal IUGR/Stress Tissues

Euthanasia of dams, and subsequently fetuses, was performed as stated in 2.7. An incision was made in the abdomen and the uterus exteriorised. The uterine wall was carefully opened using a scalpel blade without damaging the amniotic sac. Amniotic fluid of all pups was collected via needle and syringe into sterile Eppendorf tubes and immediately frozen in liquid nitrogen. Fetuses were then removed from the sac and immediately weighed and sexed before blood collection as previously described. Brains were removed and collection of the left hemisphere for immunohistochemistry was as described in 2.7.1. The right hemisphere was dissected, weighed and frozen as described in 2.7.1. Placenta from each fetus was cleaned, weighed and dissected into two halves. One half was fixed in 10% neutral buffered formalin overnight at 4°C before storage in 0.1M PBS with 0.05% sodium azide. The other half was placed in sterile Eppendorf tubes and snap frozen in liquid nitrogen.

2.7.2.1 Classification of IUGR fetuses

Fetuses were classified as symmetrically growth restricted if their body weight was less than the 25th percentile for weight[271-273] (below 75g within our colony, unpublished data), but displayed a normal brain to liver ratio (BLR; <0.9[105]) at term, and included in this cohort. Fetuses were classified as asymmetrically growth restricted if their BLR>0.9. No fetuses >75g had a BLR>0.9.

2.7.3 Collection of Placenta from Surviving and Non-surviving Preterm Neonates and Term Controls

All animal work was for this experiment was carried out by Dr. Meredith Kelleher and Dr. Hannah Palliser as a part of Dr. Kelleher's PhD thesis and detailed methodology has been published[105, 110, 222, 268]. Placental samples and survival data from these animals were provided for the current study. Briefly, pregnant guinea pigs were randomly allocated to term and preterm delivery groups. Preterm neonates (n=36) were obtained by caesarean section at approximately 62-63 days gestation (equivalent to late preterm delivery, ~34 weeks gestation in humans). Term neonates (n=22) were obtained by caesarean section at approximately 69 days gestation or when the pubic symphysis was determined to be >2cm by palpation (as an indicator for imminent labour). Normal gestational length for guinea pig colony used for this study was ~ 71 days. At the time of caesarean section, placental samples were collected and stored as described in section 2.7.2.

Preterm guinea pig neonates were further grouped into preterm survivors (n=21), those that survived to 24 hours when they were euthanized, and preterm non-survivors (n=15) which included those that did not survive to 24 hours. Guinea pig pups that died within the first 2 hours were excluded from this study to remove bias for those neonates who never obtained normal respiration activity after delivery due to lung immaturity.

2.7.3.1 Caesarean Section Delivery Procedure and Respiratory Support for Preterm Neonates

Dams allocated to both preterm and term delivery received antenatal betamethasone (s.c.; 1mg/kg; betamethasone sodium phosphate/betamethasone acetate; Celestone Chronodose, Schering-Plough, North Ryde, NSW, Australia), given 24 hours, and 12 hours pre-delivery. All neonates were delivered via caesarean section, under anaesthesia, with a midline incision to expose the uterus as described in 2.5.1. Once the uterus was exposed, pup locations within the myometrium were identified. An incision was made into the myometrium, and the amniotic was sac removed from pups. The umbilical cord was tied with surgical silk and then cut. Newborn neonates were dried, warmed and stimulated to breath,

given a dose of surfactant (50μL of Curosurf, 80mg/mL Poractant alfa, Douglas Pharmaceuticals, Baulkam Hills, NSW, Australia) and administered continuous positive airway pressure (CPAP; animal anaestheisa mask (Harvard apparatus, Holliston, MA, USA) attached to a Neopuff infant T-piece resuscitator (Fisher & Paykal Healthcare, Melbourne, Australia) to aid breathing. Following delivery all term and preterm neonates received continuous thermal, respiratory and feeding support, while being housed in a humidified incubator (small animal intensive care incubator, Thermocare, Incline Village, NV, USA). Preterm neonates who did not achieve stable respiration within 2 hours were euthanised humanely. Placenta from each newborn guinea pig was weighed and collected as per 2.7.2 until use.

2.8 Allopregnanolone Radioimmunoassay

Radioimmunoassay was performed by Britt Saxby to detect circulating and placental concentrations of steroid allopregnanolone as per previously published protocols[274-276].

2.8.1 Steroid Extraction

2.8.1.1 Placental Preparation

100mg of crushed frozen placental tissue was weighed into a plastic tube. Ice-cold 50% methanol with 1% acetic acid was added to the tubes at a volume of 1mL. The mixture was homogenised for 3x30 second bursts using the T25 Ultra-Turrax Homogeniser (IKA), before centrifugation in a J-6 M/E centrifuge (Beckman Coulter, Gladesville, NSW, Australia) at 4°C, for 25 minutes at 1800 x g. The supernatant was decanted; with an additional 1mL of 50% acidified methanol added to the pellet, rehomogenised and centrifuged. Supernatant for each sample was collected into glass tubes (12 x 75mm, Kimble Chase, Vineland, NJ, USA) and stored at -20°C until further processing.

2.8.1.2 Plasma and Placental Homogenate Preparation

Plasma was spun down at 4°C, for 15 minutes at 1800 x g in a J-6 M/E centrifuge to remove particulates. 60µL of plasma, or 1mL of placental supernatant was added to glass tubes. 600µL of radioactive allopregnanolone tracer (approximately 2000cpm/600µL, 5α-pregnane-3α-ol-20-one, 5α-[9, 11, 12, ^{3}H(N)], PerkinElmer Life and Analytical Sciences, Boston, MA, USA; see section 2.8.2.2) and 2mL 50% acidified methanol was added to each tube for plasma and placental supernatant. Solid-phase extraction was carried out in Sep-Pak Classic C_{18} cartridges (360mg, 55-105mm, Waters Corporation, Milford, MA, USA) primed with 2.5mL of 100% methanol, 2.5mL of 50% methanol and 2.5mL of 50% acidified methanol. Homogenate samples were added to the cartridges and washed with 2.5mL 50% acetic acid and 50% acidified methanol. Steroids were eluted from the mixture by adding 3mL of 100% methanol to the cartridges. Eluent was then dried at 50° Celsius under a nitrogen gas stream (Coregas) using a dry block heater (Ratek, Boronia, Vic, Australia).

2.8.1.3 Progesterone Oxidation

Dried extracts were re-formed in 450µL of distilled water, with 50µL of 5% potassium permanganate solution in order to remove non-saturated steroids including progesterone [274, 277]. Mixtures were then incubated at room temperature for 30 minutes. Steroids were extracted by addition of 2mL of 50% n-hexane and 50% diethyl-ether mixture. All samples were vigorously mixed before allowing the separation of the aqueous and organic layers. The aqueous layer containing the steroids was frozen on dry ice for 1 minute and the organic (n-hexane/diethyl-ether) layer was separated into a fresh tube. The addition of the n-hexane/diethyl-ether mixture was repeated a total of 3 times to improve steroid recovery. Samples were again dried at 50° Celsius under a nitrogen stream on a dry block heater.

2.8.1.4 Extraction Recoveries

600µL of assay buffer (0.05M PBS, 0.025M EDTA, 0.1% bovine serum albumin (BSA), and 0.1% sodium azide) was added to the dried samples. 50µL of each sample, along with 5mL of BCS liquid scintillation cocktail (GE Healthcare, Rydalmere, NSW, Australia) was added in duplicate to mini Poly-Q polyethylene

scintillation vials (Beckman Coulter, Gladesville, NSW, Australia) for determination of allopregnanolone recovery. β-radiation from samples was counted using a LS3801 liquid scintillation counter (Beckham Coulter). Recovery was determined and extraction loss accounted for in final determination of allopregnanolone concentration for each sample. The average recovery of allopregnanolone from plasma was 74.31±1.52%, and 55.43±0.70% from placental tissue.

2.8.2 Counting and Determination of Allopregnanolone Concentrations in Samples

2.8.2.1 Allopregnanolone Standards

Standards for allopregnanolone were made from a primary stock (Steraloids, New Port, RI, USA) in 100% ethanol to a concentration of 200μg/mL. Secondary stocks were also diluted by using 100% ethanol, with all standards stored at -20°C until use. Standard curves were prepared by serial dilution in assay buffer, with concentrations ranging from 25 – 8000pg/mL. Medium (400pg/mL) and high (2000pg/mL) allopregnanolone concentrations were also prepared as quality control samples and run in each assay. The limit of detection was 25pg/mL, with inter- and intra-assay coefficients of variation 9.39 and 2.75% respectively.

2.8.2.2 Allopregnanolone Tracer

Radioimmunoassay to determine allopregnanolone concentrations used a tritium-labelled allopregnanolone tracer (PerkinElmer Life and Analytical Sciences). Tracer stocks were diluted out using 100% ethanol and further diluted using assay buffer to a working concentration of approximately 7800cpm of allopregnanolone in each tube (concentration determined by 250μL of tracer in 5mL of scintillation fluid, counted by β-counter).

2.8.2.3 Allopregnanolone Antisera

Allopregnanolone polyclonal antibody (hen anti-allopregnanolone, Total IgY in PBS pH 8.0, 0.02% sodium azide) was obtained from Agrisera (Sapphire Bioscience, Vannas, Sweden), and stored at 4°C until use. On the day of the assay, the antisera was diluted to a working concentration of 1:2000.

2.8.2.4 Assay Procedure

250µl of allopregnanolone standards, unknown samples and quality control samples were added to glass tubes, all kept at 4°C for the duration of the assay. 250µL of antisera at working concentration was added to each tube and incubated for 15 minutes before adding 250µL of tracer and then incubated at 4°C overnight. 200µL of charcoal-dextran mixture (0.5%w/v Norit-A activated charcoal, 0.1%w/v Dextran T70 (Pharmacosmos, Holbaek, Denmark), 0.05% γ-globulin) was added to the samples the following day. Tubes were then centrifuged for 10 minutes at 4°C at 2500rpm (J-6 M/E centrifuge, Beckman Coulter, Gladesville, NSW, Australia). This separates out the bound antisera from the unbound tracer, with the bound fraction collected, and used in determining the concentration of the bound tracer. 500ul of the supernatant was added to scintillation vials with 5mL of scintillation cocktail, and then counted on a β-counter. Standard curves were generated from the counts produced from the known allopregnanolone standards, from which the unknown sample concentrations were then calculated. Each assay also had vials for determining total counts (TC), non-specific binding (NSB) and tracer-antisera binding (B_0). Recoveries as determined in section 2.8.1.4 were also incorporated into calculation of final allopregnanolone concentration.

2.9 Salivary Cortisol Enzyme-Linking Immunoassay

Salivary cortisol concentrations were determined using a salivary cortisol enzyme-linked immunoassay (ELISA; Salimetrics Inc., State College, PA, USA), as per manufacturer's instructions. The salivary cortisol ELISA, with reagents and plate, were conducted at room temperature. The kit provided a 96 well plate, cortisol standards (concentrations 3.0, 1.0, 0.333, 0.111, 0.037 and 0.012µg/dL), and high and low cortisol controls (0.98µg/dL and 0.1µg/dL respectively). Unknown samples were diluted to a working concentration of 1:10 with assay buffer provided. Standards, controls, non-specific binding wells, and unknown samples were plated in duplicate. Enzyme conjugate buffer (with cortisol linked horseradish peroxidase; 1:1600 dilution) was added to each well, plate sealed and

mixed for 5 minutes at 500rpm on an ELISA plate mixer. The plate was incubated for a further 55 minutes, allowing for competitive binding of cortisol in standards and samples with the horseradish peroxidase to the antibody binding sites. The plate was then washed 4 times with 1x manufacturer's wash buffer, before addition of TMB (tetramethylbenzidine) solution to each well. The plate was covered in aluminium foil to prevent light from reaching with the substrate, and was mixed again for 5 minutes, and incubated for a further 25 minutes. The reaction was ceased by addition of 3M sulphuric acid stop solution, and mixed for 3 minutes at 500rpm. The plate was read on a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany) at 450nm, with corrections at 490nm. Resulting optical densities corresponding to the amount of bound horseradish peroxidase, which is inversely proportional to the amount of cortisol in standards and unknown samples. A 4-parameter sigmoid minus curve fit was used to calculate the concentrations of cortisol for each sample. Sensitivity of the ELISA was 0.012μg/dL to 3.0μg/dL, with an intra-assay coefficient of 5.52%.

2.10 Measurement of Placental 5a-Reductase types 1 and 2 and Brain $GABA_AR$ mRNA Expression

2.10.1 Tissue Crushing and RNA Extraction

2.10.1.1 Tissue Preparation

Frozen tissues (cerebellum, chapter 3 and placenta, chapter 6) were dry crushed using a pestle and mortar, into a fine powder before transfer into a precooled, sterile Eppendorf tube. Samples and equipment were kept cold using dry ice and liquid nitrogen.

All ribonucleic acid (RNA) was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen Pty Ltd, Chadstone Centre, VIC, Australia). All samples were extracted according to the recommended protocol. The kit provided all necessary reagents and buffers (including RLT Plus, RW1 and RPE). All reagents were used at room

temperature and necessary centrifugation was at room temperature at ≥8000g for 15 seconds according the manufacturer's instructions.

For cerebellum and placental tissue homogenisation, 20mg of crushed tissue was weighed into an extraction tube. Following weighing, 600µL of RLT Plus buffer containing 10µL/mL β-mercaptoethanol (Sigma-Aldrich Co. LLC, Castle Hill, NSW, Australia) was added to the tube.

Placental tissue samples were homogenised using a T25 Ultra-Turrax Homogeniser for 30 seconds and cooled for 20 seconds on ice. The process was repeated a total of three times before homogenates were left to settle on ice until all samples were homogenised. Placental lysate was carefully transferred to clean eppendorf tubes. Cerebellum tissue samples were homogenised in the Precellys 24 dual tissue homogeniser (Bertin Corp, France), for 3x30 second bursts with 20 seconds rest periods. All brain and placenta lysates were centrifuged at ≥8000g; 4°C for 3 minutes.

2.10.1.2 Column RNA extraction

Supernatant was pipetted into a gDNA elimination column in a 1.5mL collection tube provided in the kit. Samples were centrifuged for 30 seconds and the gDNA column removed. 600µL of 70% ethanol was added to the flow-through and mixed by pipetting up and down. Following this, 700µL of the mixture was added to a spin column in a 1.5mL collection tube and centrifuged. The remaining ethanol mixture was added to the spin column and the process repeated. After, 700µL of RW1 buffer was added to the spin column and centrifuged. Flow-through was disposed of before 500µL of RPE buffer was added to the column. After centrifuging, flow-through was disposed of and another amount of RPE buffer added. Samples were centrifuged for 2 minutes before the spin column was transferred to a fresh collection tube and centrifuged for 1 minute at full speed to further dry the membrane. The spin column was transferred to a fresh 1.5 mL tube, and 30µL of distilled RNase free MilliQ water (EMD Millipore Corporation, Billerica, MA, USA) was pipetted directly onto the spin column membrane and centrifuged for 1 minute. The process was repeated with a second volume of water

to give a total volume of 60μL. 1μL of RNase inhibitor was added to each sample before quantification. All samples were stored at -80° Celsius.

2.10.1.3 Assessment of RNA Quality, Purity, Integrity and Quality

RNA concentrations were quantified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DEM, USA). The NanoDrop was calibrated by pipetting 2μL of distilled MilliQ water (Millipore) onto the fibre optic point. Another 2μL of MilliQ water was used as a blank. Following the blank, 2 μL of each sample was placed on the fibre optic point. Absorbance at 260nm, 280/260 and 230/260 ratios as well as concentrations in ng/μL for each sample were recorded. The fibre optic point was wiped clean using a clean, lint-free cloth between samples. RNA purity was assessed by ratio of optical densities at the 260nm and 280nm ranges. The 260/280 and 260/230 ratios of pure RNA are expected to be approximately 2 and 2.2 respectively. Deviations from these expected values indicate contamination by proteins, organic compounds or low RNA concentrations. Samples with readings of 1.6 suggested contamination, and were discarded and the RNA extraction was repeated.

The quality of RNA samples was confirmed by 1% agarose gel. The RNA gel apparatus was soaked in 0.1M sodium hydroxide (NaOH, sodium hydroxide, distilled MilliQ water) for 30 minutes to remove RNases. Samples were made up with a 6x Blue-Orange loading dye (Promega, Alexandria, NSW, Australia), with the loading dye at a 1/6 concentration of sample. Agarose powder was slowly dissolved in 1xTBE electrophoresis buffer (tris-base, boric acid, 0.5M EDTA; pH 8.0) at a concentration of 1%, by heating in a microwave. 1μL of SYBR safe (Invitrogen, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) was added to the liquefied gel and poured into a gel cast to set for a minimum of 30 minutes. The set gel was placed in RNase free gel tank, and filled with 1xTBE buffer. The gel comb was then removed and 5μL of 1kb TrackIt DNA ladder (Invitrogen) added to the first well. A total of 5-10μL of sample was loaded to each well. The gel was left to run at approximately 90V for 60 minutes. Gels were imaged using a UVP benchtop UV transilluminator chamber (BioDoc-It Imaging System, Upland, CA, USA) to observe for 18S and 28S bands at a 1:2 ratio and any smearing indicative of RNA degradation. Samples showing signs of degradation

were discarded and further extractions performed as described in previous sections.

2.10.2 Reverse Transcription PCR

Placental and cerebellar cDNA was synthesised using the protocol recommended by the Superscript III RT (Invitrogen) kit, as follows. All reagents and samples were kept on ice. Reverse transcription was performed in two tubes for each sample. One tube contained the sample with reverse transcriptase to make cDNA (labelled RT+) and one tube contained all the same reagents, however received MQ water instead of the reverse transcriptase enzyme. This tube (labelled RT-) acted as a control for DNA contamination of the sample. Each RT sample was also supplemented with $5x10^6$ copies/µL of Alien qPCR Inhibitor RNA alert (Invitrogen), to assess for reverse transcription inhibition. Presence of reverse transcription inhibitors in samples were assessed by comparing cycle threshold values of alien in the RT+ sample cDNA and in the alien alone control cDNA (see Real Time section 2.10.3 below). Samples demonstrating similar RT efficiency were expected to reveal similar Ct values following real time measurement for Alien by the following protocol, 1µg of sample RNA, $5x10^6$ copies of Alien RNA, 50ng random hexomers, 10mM dNTP mix was added to each of the tubes and incubated for 5 minutes at 65°C on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia). Samples were incubated on ice while the RT reagent mixtures were made. RT+ samples received 10x RT buffer, 25mM magnesium chloride, 0.1M DTT and 200U of Superscript III reverse transcriptase (Invitrogen). RT- samples received the same reagents; however MQ water replaced the Superscript III reverse transcriptase.

Samples were gentle mixed then spun down before reverse transcription thermocycling. Briefly, RNA strands were denatured for 10 minutes at 25°C before cDNA synthesis of RNA strands. This occurred for 50 minutes at 50°C, before the process was terminated at 85°C for 5 minutes. Samples were then chilled on ice before the addition of 2 units of RNase H to each tube. The samples were incubated at 37°C for 20 minutes to catalyse RNA and complete the cDNA synthesis.

The final concentration of total cDNA obtained for placental samples was 50ng/μL, and 33.33ng/mL for cerebellar samples. All samples were diluted to a working concentration of 10ng/μL and stored at -20° until use.

2.10.3 Real-Time PCR

Forward and reverse primers were obtained for 5α-Reductase type 1 and 5α-Reductase type 2, and $GABA_AR$ subunits α6 and δ as well as for the housekeeping gene β-actin. The β-actin housekeeping gene is routinely checked to make sure no differences occur in cycle thresholding between samples and experimental groups. Alien RNA is used as the internal housekeeping control, should β-actin expression change. The guinea pig specific primers for each transcript, listed in table 2-1, were previously designed and used in our laboratory[104, 223]. Primers for β-actin were designed using a known mRNA sequence for the guinea pig (GeneBank accession number AF508792). 5α-reductase type 1 and 2 primers were designed from sequences obtained from the UCSD Genome Browser and the February 2008 CavPor3 draft assembly of the guinea pig from the Broad Institute. Primer sequences for the $GABA_AR$ α5 and δ subunits were designed from the predicted guinea pig sequences obtained from the National Centre for Biotechnology Information (NCBI). The primers were designed to cover exon boundaries, the amplicon size was assessed to be between 50-150 base pairs for optimal efficiency and the qPCR product was sequenced to confirm the correct gene sequence was targeted and amplified. Primer efficiency for all mRNAs of interest were previously determined to be similar. The alien primer master mix (Invitrogen) contained the forward and reverse primers at a concentration of 2.5μM.

TABLE 2-1 PRIMER SEQUENCES FOR REAL-TIME POLYMERASE CHAIN REACTION

Transcript Target	Forward Primer Sequence	Reverse Primer Sequence	Primer Concentrations (nM)	Amplicon Length (bp)
5α-Reductase type 1	CGA GGA GGG AAG CCA ACA	TAA CCA CAA GGC ACA ACC AGC	400	158
5α-Reductase type 2	TCA GAA AGC CTA GAG AAG TCA TC	CCG AGG AAA CAA AGC GTG AA	800	165
$GABA_AR$ α6	ACG AAA GCA AAG CAT ACA GC	ATA AGG AGT CAG TCC CAG CA	600	147
$GABA_AR$ δ	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC	400	153
β-actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT	400	72

PRIMER SEQUENCES OBTAINED FOR GUINEA PIG 5A-REDUCTASE TYPE 1, 5A-REDUCTASE TYPE 2, $GABA_AR$ α6, $GABA_AR$ δ SUNUBITS AND B-ACTIN GENE EXPRESSION. PRIMER SEQUENCE FROM 5'-3' FOR FORWARD AND REVERSE PRIMERS.

SYBR green (Applied Biosystems, containing 2X SYBR green, dNTPs with dUTPs and passive reference 1 (ROX)) was used to analyse PCR products for all genes examined. Master mixes (containing SYBR green, forward and reverse primers and MQ water) for 5αR1, GABAAR δ, and β-actin were made up to final primer concentrations of 400nM, $GABA_AR$ α6 made up to 600nM and 5αR2 made to a final concentration of 800nM. These primer concentrations had previously been assessed and determined to provide similar real time PCR efficiencies of all genes of interest. All RT+ samples were run in duplicate, with its corresponding RT- sample also run to confirm the removal of DNA contamination. A calibrator sample was also run in triplicate on every real time plate to allow for normalisation of samples across plates. No-template controls (NTC) were also run on the plate to assess for contamination of the PCR plate or

mixes and also to assess for primer-dimer formation. The protocol was as follows: 20ng of sample cDNA, calibrator and no-template controls (MilliQ water) were pipetted into a DNA/RNA/RNase free PCR plate (Applied Biosystems). Following this step 8μL of SYBR green primer mix was added to each well and covered with a PCR cover-shield. The plates were gently mixed then spun for 1 minute in a Heraeus refrigerated benchtop multifuge (Heraeus 3XR multifuge, Thermo Fisher Scientific) to combine all reagents in the bottom of the wells and to remove air bubbles that could affect the reading.

Real time PCR was performed on the 7500 ABI real-time machine (Applied Biosystems) and analysed using the Sequence Detection Software (SDS) v1.4 (Applied Biosystems). Plates were placed in the real-time machine and selected for detection of gene of interest. Cycle threshold (Ct) for detection was automatically set for log scale of 0.2 fluorescence of cDNA expression above the threshold. A dissociation curve was obtained for each plate using the SYBR green to assess for non-specific amplification (including contamination and primer-dimers). Samples containing differing melt curve plots from the target sequences, and were not the result of primer-dimer formation, were assumed to be contaminated or of poor quality and were rerun or resynthesised.

Inhibition of the RT reaction was assessed by comparison of Ct values of alien cDNA in the RT+ spiked samples and the alien only control. Spiked sample Ct values were deducted from alien only Ct values to assess reaction efficiency. A high value was indicative of reaction inhibition in the RNA samples. If this occurred, the real time was repeated and if similar results were obtained the reverse transcription was repeated. Continued poor results lead to re-extraction of the RNA sample.

2.10.4 Analysis of 5a-Reductase and $GABA_A$Rs Relative Expression

The Ct values for the duplicates of each sample and calibrator were averaged. To obtain the ΔCt for each sample, the averaged Ct value of β-actin was subtracted from the averaged sample values for the 5αRs in the placenta, and $GABA_A$R

subunits in the cerebellum. Following this, the ΔΔCt value was obtained by subtracting the ΔCt calibrator value from the ΔCt sample. Finally, the relative fold change difference of samples were collected by taking the –ΔΔCt value to the power of 2 ($2^{-\Delta\Delta Ct}$).

2.11 Brain Immunohistochemistry

2.11.1 Tissue Processing

Fixed brain tissue was dehydrated in graded ethanol series, embedded in paraffin wax and mounted onto tissue cassettes. Embedded tissue was cooled before sectioning using a Leica RM2145 microtome (Leica Biosystems Pty Ltd, North Ryde, Sydney, NSW). Sections were cut into slices of 8μm thickness in serials of 3, mounted onto SuperFrost plus glass slides (Menzel-Glaser, Braunschweig, Germany) and left to air-dry overnight. Slides were dewaxed in a series of 3 xylene washes for 5 minutes each before dehydrating in 100% ethanol, twice for 3 min and once for 2 min.

2.11.2 Cerebellar Immunohistochemistry

2.11.2.1 Myelin Basic Protein and Glial Fibrillary Acidic Protein Immunohistochemistry

Slides to be assessed for MBP and GFAP expression were then incubated for 20 min in 100% methanol with 3% hydrogen peroxide to block endogenous peroxidase activity. The slides were then slowly rehydrated in 70% ethanol for 2 minutes before incubating in distilled MilliQ water (Millipore) for another 5 minutes. Antigen retrieval was performed in Reveal-It solution (ImmunoSolutions Pty Ltd, Australia) exactly as indicated in the manufacturer's instructions. In short, slides were incubated in a beaker of Reveal-It solution (pH 6) at 80° C for 10 minutes, before being cooled and washed for 5 minutes in distilled MilliQ water. Slides were washed twice for 15 minutes in PBS, before being blocked at room temperature for 30 minutes in 1x Block solution (0.5% BSA, 0.05% Saponin, 0.1M PBS, 0.05% sodium azide). Slides were incubated with either

MBP (rat anti-MBP, 1:3000 (M9434, Sigma-Aldrich)) or GFAP (mouse anti-GFAP; 1:3000 (G3893, Sigma-Aldrich)) overnight at room temperature. Sections were washed with PBS before 1hour incubation with biotinylated secondary antibodies: anti-rat IgG (1:300, B7139, Sigma-Aldrich) and polyclonal anti-mouse (1:300, E0354, Dako). Tertiary reagent, VECTASTAIN ABC kit (Abacus ALS, Brisbane, Australia), was added to PBS and incubated for 30 min at room temperature, as per manufacturers instructions. All antibodies had a no-primary (secondary-only) control slide run simultaneously to ensure specific staining for primary antibodies of interest. Control slides had diluent on for the primary incubation period, without primary antibody. During secondary antibody incubation, appropriate secondary antibodies were used as per protocol.

2.11.2.2 Neuronal Nuclei (NeuN) and S100B Immunohistochemistry

Slides analysed for NeuN (mature neuron cell body stain) and S100B (astrocyte cell body stain) were rehydrated in 70% ethanol for 2 minutes, following 100% ethanol, before incubating in distilled MilliQ water (Millipore) for another minute. All subsequent PBS washes occurred for 3x5 minutes. Slides were washed in PBS, before antigen retrieval was performed using 10mM citrate buffer, pH 6.0, for 12 minutes in the microwave followed by cooling in distilled water and washes in PBS. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS. Following PBS washes slides stained for NeuN were blocked overnight (2% goat serum, 0.4% BSA, 0.3% Triton X-100), at room temperature and followed by incubation with primary antibody for NeuN (mouse anti-NeuN, 1:500 (MAB377, Millipore Chemicon, Kilsyth, Victoria, Australia)), for 1 hour at room temperature. Slides for S100b were blocked for 1 hour (2% goat serum, 0.4% BSA, 0.3% Triton X-100) at room temperature and then incubated with primary antibody S100B (rabbit anti-S100B, 1:500, Millipore) overnight at 4°C. Slides were again washed in PBS, before secondary antibody incubation with polyclonal anti-mouse (1:300, E0354, Dako) and anti-rabbit (1:300, RP1004, Amersham) respectively, for 1 hour at room temperature. Following another series of PBS washes, slides were incubated with VECTASTAIN ABC for 30 min at room temperature. See section 2.11.2.1 for no-primary (secondary-only) controls.

2.11.3 Amygdala and Hippocampal Immunohistochemistry

The immunohistochemically procedure for slides containing the amygdala and hippocampus, contained slight changes in protocol. As such, antigen retrieval was performed using 10mM citrate buffer, pH 6.0 for 25 minutes at ~95°C on a hotplate, before cooling for 15 minutes and washed with MilliQ water as per section 2.11.2.1. Endogenous peroxidases were blocked in 3% hydrogen peroxide in PBS following antigen retrieval and washes. The blocking reagent used for hippocampal and amygdaloid tissue was a goat serum block (2% goat serum, 0.4% BSA, 0.3% Triton X-100 in 0.1M PBS) for 1 hour at room temperature. All subsequent antibody incubation and PBS washes procedures occurred as outlined above in section 2.11.2.2.

2.11.3.1 MBP, GFAP, Microtubule-Associated Protein 2 (MAP2), Glutamate Decarboxylase 67 (GAD67)

Following blocking, hippocampus and amygdala slides were incubated with MBP (rat anti-MBP, 1:1000, Sigma) and GFAP (mouse anti-GFAP, 1:1000, Sigma), MAP2 (mouse anti-MAP2; 1:20000, (M9942; Sigma Aldrich)) and GAD67 (mouse anti-GAD67; 1:1000, (ab26116, Abcam, Melbourne, Victoria, Australia)) at room temperature. Primary incubation for GAT1 (rabbit anti-GAT1; 1:500, (ab426, Abcam)) occurred overnight at 4°C. Following PBS washes, MBP was incubated with anti-rat IgG (1:300, B7139, Sigma-Aldrich) as above in section 2.11.2.1. MAP2, GFAP and GAD67 were incubated with anti-mouse IgG (goat anti-mouse; 1:300, (B6649, Sigma Aldrich)); and GAT1 was incubated with anti-rabbit (donkey anti-rabbit IgG, 1:300, Amersham) for 1 hour at room temperature. After PBS washes slides, were incubated with tertiary antibody biotin conjugated Streptavidin-horseradish peroxidase (HRP; 1:400, (ab7403, Abcam)) for 1 hour at room temperature.

2.11.4 Immunodetection

Slides were stained in chromagen 3,3’-diaminobenzidine tetrahydrochloride solution (metal enhanced DAB substrate kit #34065; Pierce). One section was stained with cresyl violet for morphological analysis. Slides were then mounted using DEPX (Merck, Kilsyth, Vic, Australia).

2.11.5 Imaging and Analysis

Blinded analysis of immunohistochemical sections occurred for all antibodies imaged, until the acquisition of all raw data. Group allocations were released for statistical analysis.

2.11.5.1 Cerebellar Imaging and Analysis

Cerebellar sections were examined using a Nikon Ni-U microscope (Nikon). Images were acquired using a Nikon camera. MBP and GFAP immunostaining were examined in lobes VIII and X of the cerebellum, and in the deep white matter (figure 2-2). NeuN immunostaining was examined in lobes VIII and X only, and S100B was imaged in lobes VIII and X as well as the deep cerebellar nuclei. The MBP, GFAP and NeuN immunoreactivities were analysed by densitometry using ImageJ (version 1.49d, National Institutes of Health, Bethesda, MD, USA) and made binary by adjusting the threshold manually. GFAP, MBP and NeuN were expressed as the percent area of coverage recorded for 3 fields (MBP, GFAP, figure 2-2c) or 4 fields (NeuN, figure 2-2c) of view per region on two sections per animal. S100B cell counts were collected using ImageJ, using 3 fields of view for each region (figure 2-2b). Cells counts were obtained by using the known magnification scale for each image. From this the known pixel distance per μm measurement was used to produce the area per image. Cell numbers within these images were counted and averaged per region These average cell numbers gave the number of cells per image region. Subsequently, $1mm^2$ was divided by the image area value to obtain the factor difference for each region. The average cell count was multiplied by this factor to produce cells/mm^2.

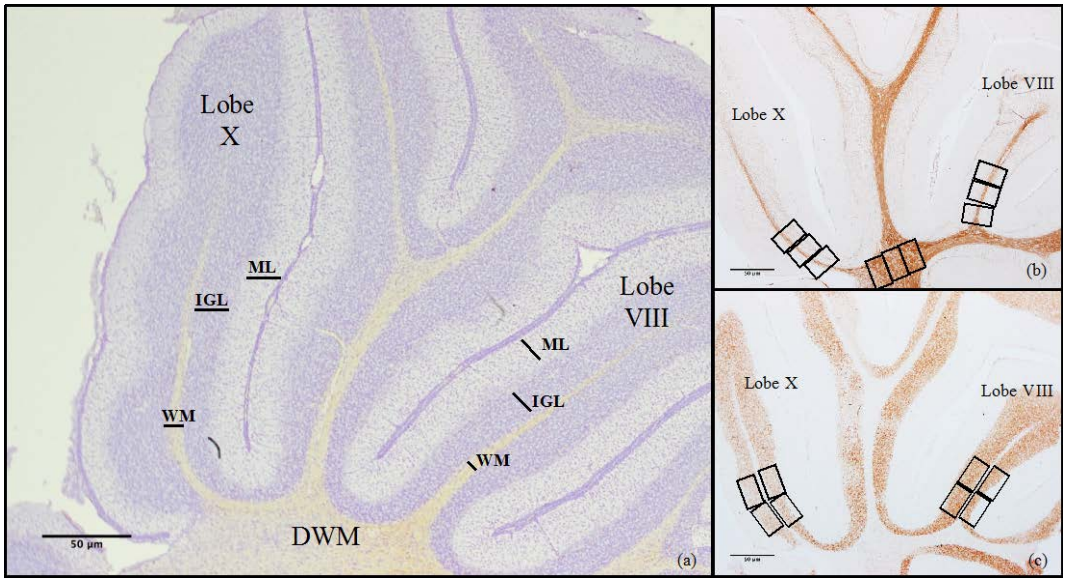


FIGURE 2-2 REPRESENTATIVE CEREBELLUM IMMUNOHISTOCHEMISTRY IMAGES. CRESYL STAINED CEREBELLUM (A) DISPLAYING DEEP WHITE MATTER (DWM), LOBES X AND VIII WITH CEREBELLAR LAYERS. LOBE X AND LOBE VIII WERE ANALYSED AS LOBE X IS AN EARLY DEVELOPING LOBE, WHILST LOBE VIII DEVELOPS RELATIVELY LATE IN CEREBELLAR DEVELOPMENT. IGL = INTERNAL GRANULE LAYER; ML = MOLECULAR LAYER; WM = WHITE MATTER TRACTS. REPRESENTATIVE IMAGING SCHEMATIC FOR MBP, GFAP AND S100B (B); WITH THREE IMAGES PER REGION. REPRESENTATIVE IMAGING SCHEMATIC FOR NEUN (C), WITH FOUR REGIONS OF INTEREST PER LOBE OF INTEREST. SCALE BARS = 50µM.

2.11.5.2 Hippocampal and Amygdala Imaging and Analysis

Hippocampal and amygdala brain sections were imaged using Aperio AT2 scanner (Leica Biosystems). Images for regions of interest were captured using Aperio ImageScope software (version 12.1.0.5029) at 20x magnification.

Images for MBP, MAP2, GFAP and GAD67 were obtained from 4 fields of view, for 2 sections per animal, for the CA1 region of the hippocampus (MBP, MAP2, GFAP and GAD67) and subcortical white matter (MBP and GFAP, figure 2.-3b). Immunoreactivites for MBP, GFAP and MAP2 were assessed using ImageJ as described in 2.11.5.1, and expressed as percent area coverage. GAD67 cell counts were collected as described above, and expressed as GAD67+ cells/mm^2.

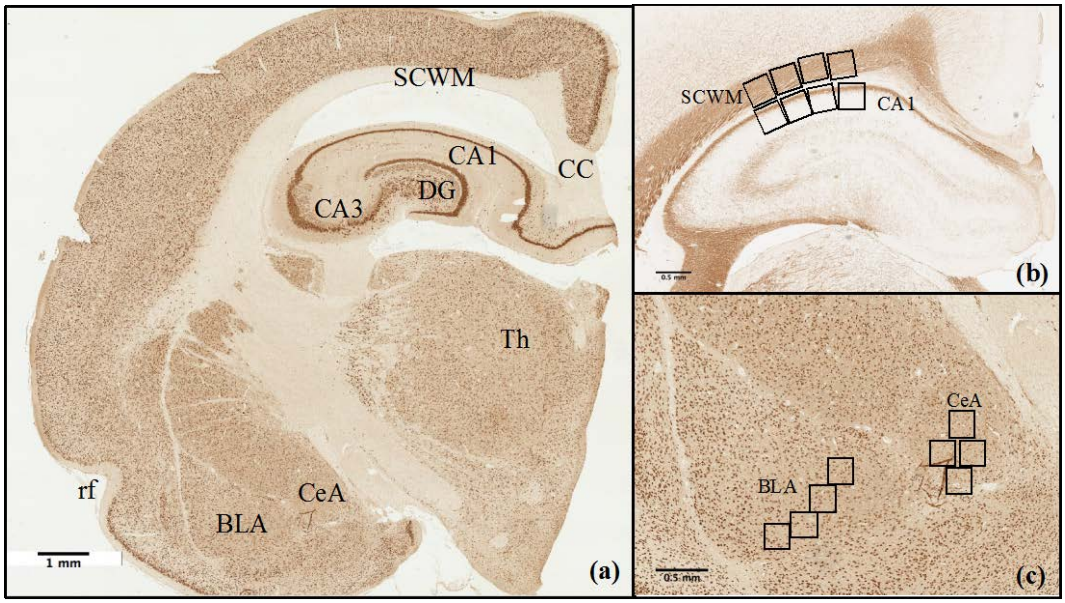


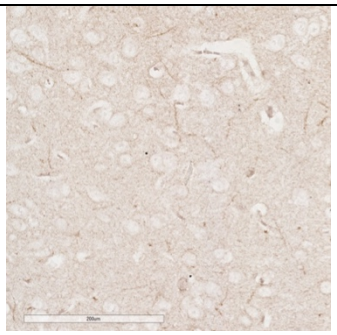
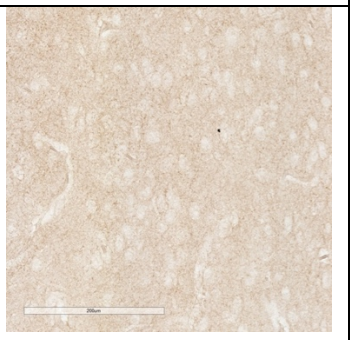
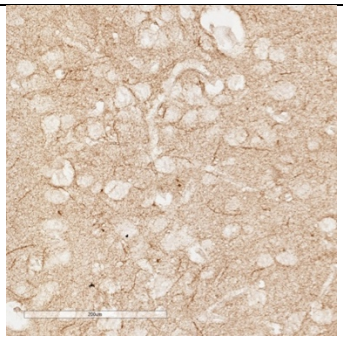
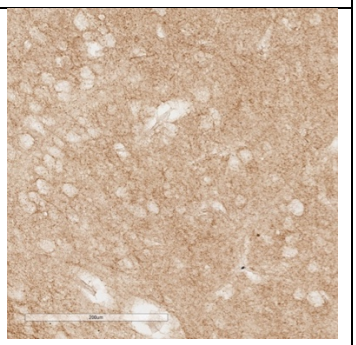
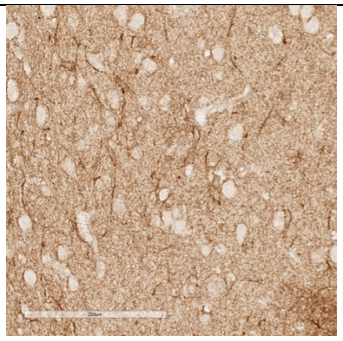
FIGURE 2-3 REPRESENTATIVE HIPPOCAMPUS AND AMYGDALA IMMUNOHISTOCHEMISTRY (A) DEPICTING BLA = BASOLATERAL NUCLEUS OF THE AMYGDALA; CA1 = *CORNU AMMONIS* REGION 1; CA3 = *CORNU AMMONIS* REGION 3; CC = CORPUS CALLOSUM; CEA = CENTRAL NUCLEUS OF THE AMYGDALA; DG = DENTATE GYRUS; RF = RHINAL FISSURE; SCWM = SUBCORTICAL WHITE MATTER; TH = THALAMUS. REPRESENTATIVE IMAGES OF REGIONS OF INTEREST WITHIN THE (B) HIPPOCAMPUS AND (C) AMYGDALA. BOXES REPRESENT THE FOUR IMAGES ANALYSED PER REGION IN TWO SECTIONS PER ANIMAL. SCALE BARS: (A) = 1MM; (B, C) = 0.5MM.

Images for GFAP, MAP2, GAD67 and GAT1 were obtained within the basolateral amygdala (BLA) and central nucleus (CeA), using the rhinal fissure as the landmark locating feature (figure 2-3). Four fields of view were collected within each region, using two sections per animal (figure 2-3c). Immunoreactivities of GFAP and MAP2 were assessed using ImageJ as in section 2.11.5.1.

Cells counts for GAD67 were collected using ImageJ, with area per image calculated and expressed as GAD67+ cells/mm^2. See section 2.11.5.2

GAT1 was qualitatively assessed using a grading system[278], with weak positivity (+) being described as few puncta staining within BLA and CeA regions. Moderate positive staining (++) described as stronger puncta staining and some axonal staining. Intense positive staining (+++) was described as sections containing dark stained puncta and multiple stained axons (table 2-2).

TABLE 2-2 QUALITATIVE ASSESSMENT OF GABA TRANSPORTER 1 (GAT1) STAINING

Qualitative Assessment	Description	Example of staining in BLA	Example of staining in CeA
+	Weak staining; few puncta in surrounds or cell bodies		
++	Moderate staining; many positive puncta, some axonal staining		
+++	Intense staining/ numerous puncta surrounding cytoplasm/outlining axons		No CeA image classified as intense

2.12 Western Blot Immunodetection

2.12.1 Frozen Tissue Protein Extraction

Frozen cerebella were dry crushed and 60mg tissue weighed into precellys tubes with ceramic beads. RIPA buffer (400µl; 50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, Sigma, St Louis, USA), with inhibitors, was added to each tube before being homogenised 5 times in 30 second bursts in Procellys 24 dual homogeniser (Bertin Corp, France). Samples were centrifuged at 10000 x g, at 4°C for 10 minutes, and the supernatant collected into sterile tubes. Protein was quantified using a commercially available BCA protein assay kit (BCA Protein Assay Kit, Pierce, Rockford, IL, USA), and protein concentrations were determined as per manufacturers instructions. Briefly, 6µl of each sample was made up to a 1 in 10 dilution using MilliQ water. Bovine serum albumin standards were made up to a 1 in 10 dilution using RIPA buffer/MilliQ water. 25µl of each sample and standard was aliquoted on a 96 well plate in duplicate. Working reagent was made up to 50 parts reagent A and 1 part reagent B, and 200µl added to each well. The plate was incubated for 30 minutes at 37°C for a colour change. After incubation the plate was left to settle to room temperature before analysis. Concentrations were analysed using Fluorstar Optima Plate Reader (BMG Labtech) and software at absorbance at 570nm. Sample and standard duplicate values were averaged and corrected for background. Standard values were used to generate a standard curve from which concentrations were determined.

2.12.2 Western Blot for 5a-Reductase

Protein extracts (100µg for 5αR1, 70µg for 5αR2) were separated by electrophoresis using 12% Bis-Tris polyacrylamide gels and transferred to PVDF membranes. Guinea pig total brain protein was used as an internal control, and was run simultaneously on gels for normalisation of expression. Membranes were blocked in 5% bovine serum albumin/5% skim milk in 0.1% TBS-Tween for 1 hour. Membranes were then incubated with either goat anti-5αR1 (goat anti-

SRD5a1; (NB100-1491, Novus)) or 5αR2 (goat anti-SRD5a2; (GTX47547, Genetex)) antibodies in blocking solution, both at dilution 1:1000 overnight at 4°C. Blocking peptides were run with primary antibody during the optimisation process to ensure specificity of the antibodies to the 5αR1 and 2 proteins. Protein band products were also sequenced to confirm antibody specificity. Membranes were incubated with secondary antibody rabbit anti-goat IgG/horseradish peroxidase (HRP) conjugate (Dako, Glostrup, Denmark Dako) at dilution 1:5000 in 3% skim milk in 0.1% TBS-Tween for 1 hour. Chemiluminescence of 5αR1 and 5αR2 was detected using Amersham ECL kit (Amersham, GE Healthcare, Buckinghamshire, England) and visualised in a Fuji LAS-3000 imager (Fuji Photo Film, Japan). Densitometry was performed using Multigauge software (version 3.0, Fuji Film).

2.12.3 Immunodection for Loading Control

Membranes were stripped with 0.2M sodium hydroxide for 10 minutes before reactivation in 100% methanol for 1 minute. Membranes were washed with TBS-T before blocking for 1 hour with 3% skim milk in TBS-T at room temperature. Blots were then probed using rabbit anti-β-actin (rabbit anti-β-actin; (ab8227, Abcam)) at a dilution of 1:8000 for 2 hours at room temperature, followed by TBS-T washes and probing with secondary antibody anti-rabbit (Upstate, 12-384). Membranes were imaged and analysed as above in 2.11.2. Densitometry values for β-actin are compared for all samples across all experimental groups to ensure no differences in "expression".

2.12.4 Normalisation and Relative Expression

5αR expression was normalised to internal control samples and expressed as an arbitrary expression relative to housekeeping gene β-actin.

2.13 Statistical Analysis

2.13.1 Statistical Analysis of Cerebellar Data (Chapter 3)

All statistical analysis was performed using GraphPad PRISM v.6 Software (GraphPad Software Inc., La Jolla, CA, United States of America). Differences between means was analysed by two-way ANOVA with subsequent Tukey multiple comparisons test was used to determine significant differences between control and finasteride-exposed males and females. If data is not normally distributed as identified by a significant Bartletts p-value, the Mann-Whitney U test was performed.

2.13.2 Statistical Analyses for Juvenile Brain and Behaviour (Chapter 4)

This study used the service of Hunter Medical Research Institute's Critical Research Design, Information Technology and Statistical Support (CReDITSS) team, with assistance from Kerrin Palazzi. For juvenile data collected in Aim 1b (Chapter 4), only one male and one female from each litter was used from each pregnancy for analysis. An independent samples t-test was used to assess differences between treatment groups in physical parameters, behaviour and neurodevelopmental markers within each sex, with a Levene's test for equality of variances. Statistical significance was set as $p<0.05$. All statistical data in text presented as means; 95% CI; and p-values. All data in tables and graphs are represented as mean±SEM.

Statistical analysis was performed using IBM SPSS Statistics software (version 24; SPSS Inc.; IBM Corporation, Armonk, NY, USA). Graphs were created using GraphPad Prism software (version 7.0a, GraphPad Software Inc., La Jolla, CA, USA).

2.13.3 Statistical Analyses of Fetal IUGR and Prenatal Stress (Chapter 5)

This study used the service of Hunter Medical Research Institute's Critical Research Design, Information Technology and Statistical Support (CReDITSS) team, with assistance from Kerrin Palazzi. For fetal data collected in Aim 2 (Chapter 5), due to ethical constraints on animal numbers allowed for this study multiple fetuses per pregnancy were used based on IUGR classification. Naturally occurring growth-restricted fetuses were included in this study, with appropriately grown siblings as controls to allow for greater use of animals. For this study, maternal pregnancy characteristics were analysed by one-way ANOVA. For fetal data a generalised estimating equation (GEE) was used to compare prenatal group (control, IUGR or IUGR+PS) and sex of the fetus (male or female). This model was applied to allow for analysis of correlated data of multiple fetuses per pregnancy. Where a significant effect of both group and sex were identified, an interaction term (Prenatal Group*Sex) was included in the analysis. Statistical significance was determined using the Wald-Chi squared type III test, and post-hoc pairwise comparisons to identify between group differences with an alpha of $p<0.05$ to identify an effect. Unless specified, all post-hoc data is presented as mean difference; 95% CIs; and p-values, all data in tables is presented as estimated marginal means with 95% CI, and graphs are represented as mean±SEM.

Statistical analysis was performed using IBM SPSS Statistics software (version 24; SPSS Inc.; IBM Corporation, Armonk, NY, USA). Graphs were created using GraphPad Prism software (version 7.0a, GraphPad Software Inc., La Jolla, CA, USA).

2.13.4 Statistical Analysis of Placental 5a-Reductase Expression (Chapter 6)

Data were analysed using GraphPad PRISM Software (version 5.01, Graphpad Software Inc, La Jolla, CA, USA). Data were analysed using a one-way ANOVA

with Tukey multiple comparisons test to determine significance between term, preterm survivor and preterm non-survivor groups, with sexes combined.

3 CEREBELLAR CHANGES IN GUINEA PIG OFFSPRING FOLLOWING SUPPRESSION OF NEUROSTEROID SYNTHESIS DURING LATE GESTATION

This chapter examines the ongoing effects of intrauterine suppression of allopregnanolone synthesis on the neurodevelopment of the cerebellum. This

article was published in journal *Cerebellum,* first published online 03 June 2016, DOI 10.1007/s12311-016-0808-0. For consistency, it is presented with minor edits to the original manuscript.

STATEMENT OF AUTHOR CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
Angela L. Cumberland	Experimental design Animal protocols and tissue collection Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K. Palliser	Experimental design Animal protocols and tissue collection Laboratory procedures Data analysis Manuscript corrections	
David D. Walker	Experimental design Manuscript corrections	
Jonathan J. Hirst	Experimental design Data analysis Manuscript corrections	

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Assistant Dean Research Training 31/08/16

3.1 Abstract

Elevated gestational concentrations of allopregnanolone are essential for development and neuroprotection of the fetal brain. Preterm birth deprives the fetus of these high levels of allopregnanolone, which may contribute to the associated adverse effects on cerebella development. Preterm birth alters expression of $GABA_A$ receptor subunit composition, which may further limit neurosteroid action. The objective of this study was to determine the effects of suppression of allopregnanolone levels of the markers of development and functional outcome. Pregnant guinea pigs were treated with finasteride at a dose (25mg/kg maternal weight) shown to suppress allopregnanolone between 60 days of gestation until delivery (term ~71 days). Cerebella from neonates, whose mothers were treated with finasteride or vehicle during pregnancy, were collected at postnatal age 8. Pups that received finasteride displayed significantly greater GFAP area coverage and reduced $GABA_A$ receptor α6-subunit mRNA within the cerebellum than pups that were exposed to vehicle. These findings indicate that loss of neurosteroid action on the fetal brain in late gestation produces prolonged astrocyte activation and reductions in $GABA_A$ receptor α6-subunit expression. These changes may contribute to the long term changes in function associated with preterm birth.

3.2 Introduction

Preterm birth is associated with poor neurodevelopmental outcomes. The cerebellum is vulnerable to suboptimal growth and damage following preterm birth; and the resultant deficits contribute to the ongoing impairments observed after birth. The neurosteroid, allopregnanolone is present in relatively high concentration in late gestation throughout the fetal brain, including the cerebellum, and declines after birth with the removal of the placenta [167]. Allopregnanolone acts by binding to an allosteric modulation site on the γ-aminobutyric acid type A ($GABA_A$) receptor to enhance the effects of neurotransmitter GABA [279], leading to cell hyperpolarisation and reduced

susceptibility to excitotoxicity, thus producing its neuroprotective action in pregnancy. In addition to protective roles, allopregnanolone has major neurotrophic roles [280]. During pregnancy the placenta is the key organ for the production and supply of allopregnanolone precursors [167], for synthesis that supports fetal brain concentrations [281].

Allopregnanolone is synthesised from progesterone by 5α-reductase types 1 and 2 (5αR1 and 5αR2) that catalyse the rate-limiting conversion to 5α-dihydroprogesterone. This precursor is reduced to allopregnanolone by enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD). 5αR enzymes are localised in neurons, with high levels of expression in Purkinje cells, whilst 3α-HSD is largely expressed in astrocytes and oligodendrocytes [184, 282]. The expression of these enzymes leads to high levels of allopregnanolone in the cerebellum in the area of GABAergic neurones.

The $GABA_A$ receptor ($GABA_AR$) is a pentameric arrangement of subunits, the composition of which differs based on brain region. There are 19 subunit types (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, and π) [283], which can be expressed at the synapse or extrasynaptically. Synaptically located receptors mostly comprise the configuration $\alpha_{1-6}\beta_{1-3}\gamma_{1-3}$, and the commonly expressed extrasynaptic $GABA_AR$ comprising $\alpha_6\beta\delta$ [284]. The α6 subunit is expressed on mature granule cells of the cerebellum [285] with expression throughout fetal life. However expression of this subunit has been found to be reduced following preterm labour [223]. This change may cause a reduction in sensitivity to neurosteroids and thus GABAergic inhibitory activity in the immature cerebellum leading vulnerability to excitatory injury. The loss of cerebella α6 subunits in knock-out stargazer mice, has been shown to result in ataxia in these animals, suggesting the importance of this subunit in correct granule cell signaling [286].

Preterm birth results in the delivery of neonates with reduced myelination in the cerebellum and immaturity of the oligodendrocyte linage [59, 131, 287-289]. These deficits may result from a reduced exposure to allopregnanolone during the preterm neonatal period. Previous studies have shown maternal treatment with the

5αR inhibitor, finasteride, during pregnancy reduces concentrations of allopregnanolone in the fetal brain [220], as well as similar deficits in brain development. Finasteride treatment has been shown to significantly increase apoptosis within fetal sheep brains [290], with these apoptotic cells co-localised with astrocytes and neurons. Finasteride-induced allopregnanolone suppression has also been associated with an increase in astrocyte activation, indicated by glial fibrillary acid protein (GFAP) levels, and a decrease in mature myelin in the hippocampus of newborn guinea pigs [105].

The cerebellum is vulnerable to damage following insults in pregnancy and preterm birth; and the associated deficits in cerebellar function contribute to adverse outcomes following compromises in pregnancy. These include effects on precision, accuracy and timing of fine motor control as well as in behaviour, memory and learning [123]. The long period of development starting at 8 weeks gestation, and continued development after birth [291], leaves this region of the brain highly vulnerable to gestational insults and damage over this period. Indeed, many prenatal insults such as hypoxia, intrauterine growth restriction, and prematurity have all been shown to adversely influence cerebellar development [136, 243, 292, 293]. Magnetic resonance imaging studies of premature infants have found significant reductions in overall size and volume of the cerebellum [91, 294], and are at a greater risk of developing cerebellar cognitive affective syndrome [295].

Allopregnanolone levels are high in the cerebellum during late gestation and the expression of neurosteroidogenic enzymes in Purkinje cells and astrocytes suggests local production has a key role in maintaining these levels. Given the effects of pregnancy compromises on the cerebellum may be mediated by the loss of normal allopregnanolone levels, this study aimed to investigate the potential programming effect of suppressing neurosteroid concentrations during gestation on cerebellar neurodevelopment during early childhood in a guinea pig model.

3.3 Materials and Methods

3.3.1 Animals and Finasteride Administration

Time-mated, outbred guinea pigs were acquired from the University of Newcastle Research Support Unit. Guinea pigs were randomly allocated to either vehicle (45% β-cyclodextrin, 400µl/kg, Sigma Aldrich, Castle Hill, NSW, Australia) or finasteride (25mg/400µL/kg orally, Steraloids, Newport, RI, United States) administration groups commencing at gestational age 60 until delivery (term ~71 days). This dose has been shown to reduce brain allopregnanolone concentrations approximately three-fold in the guinea pig (from a reported average of 11.63 ± 2.33ng/g in fetal vehicle brains)[105]. Dams were allowed to spontaneously deliver, and were closely monitored from time of pubic symphysis opening. Within 2 hours of delivery all newborn pups were weighed, sexed and measured. On postnatal day (PND) 8 all neonates (vehicle n=14; males=8, females=6; finasteride treated in gestation, n=17, males=8, females=9) were weighed and measured before plasma and brain collection for analysis as previously described [223].

3.3.2 Immunohistochemistry

Fixed, paraffin-embedded cerebella tissue were sectioned at 8µm, dewaxed, incubated in Reveal-It solution for antigen retrieval and immunostained for myelin basic protein (MBP) as a marker of mature myelination and glial fibrillary protein (GFAP) as a marker of astrocyte activation as previously described [105]. Briefly, immunodetection of cerebellar tissue for NeuN (mature neuron marker) and S100B (astrocyte cell counter stain) occurred following dewaxing and incubation in citrate buffer for 12 minutes in the microwave. Sections for NeuN were blocked overnight (2% goat serum, 4% BSA, 0.3% Triton X-100), at room temperature, then primary incubation (mouse anti-NeuN, 1:500 (MAB377, Millipore Chemicon, Kilsyth, Victoria, Australia)) occurred for 1 hour. Tissues for S100B immunoreactivity were incubated in blocking solution for 1 hour at room temperature and primary incubation (rabbit anti-S100B, 1:500, Millipore) overnight at 4°C. Secondary antibody incubation for NeuN and S100B used

polyclonal anti-mouse (1:300, B6649, Sigma) and anti-rabbit (1:300, RPN1004, Amersham, GE Healthcare, Silverwater, NSW, Australia) respectively, for 1 hour at room temperature. Sections were incubated for 30 min with tertiary reagent, VECTASTAIN ABC kit (Abacus ALS, Brisbane, Australia), as per manufacturers instructions.

Slides were stained in chromagen 3,3'-diaminobenzidine tetrahydrochloride solution (metal enhanced DAB substrate kit #34065; Pierce, ThermoFisher Scientific, Scoresby, VIC, Australia). Slides were then mounted using DEPX (Merck, Kilsyth, Vic, Australia) and examined using a Nikon Ni-U microscope (Nikon Instruments Inc., New York, USA) with a Nikon DS-Ri1 camera attached (Nikon) and NIS-Elements Advanced Research software (Nikon). MBP and GFAP immunostaining were examined in lobes VIII and X of the cerebellum, and in the deep white matter. NeuN and S100B immunostaining was examined in lobes VIII and X only. The MBP, GFAP and NeuN immunoreactivities were analysed by densitometry using ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA) and made binary by adjusting the threshold manually. GFAP, MBP and NeuN were expressed as percent area of coverage recorded for 3 fields (MBP, GFAP) or 4 fields (NeuN) of view per region on two sections per animal. S100B cell counts were collected using ImageJ, using 3 fields of view for each region.

3.3.3 Real-Time PCR

Frozen cerebella (20mg) were dry crushed and RNA extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen Pty Ltd, Chadstone Centre, VIC, Australia) following the manufacturer's recommendations. Concentrations of RNA were determined using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA quality was assessed using the A260/A280 ratios and integrity verified by running RNA on a 1% agarose gel. Reverse transcription was performed using the Superscript III Reverse Transcription kit (Invitrogen, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) and the GeneAmp 9700PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) and final product was diluted to

a working concentration of 10ng/μl. Primers designed to detect guinea pig sequences of $GABA_AR$ α6 and δ, as well as housekeeping gene β-actin, were run as previously published by members of our group [223]. The SYBR Green (Applied Biosystems) method of detection was utilised using 7500 ABI real-time PCR machine (Applied Biosystems) to assess levels of expression. Relative fold change is was determined using the comparative $2^{-\Delta\Delta Ct}$ method for expression of α6 and δ, with β-actin and a calibrator sample used as controls.

3.3.4 Radioimmunoassay

Plasma allopregnanolone concentrations were determined using radioimmunassay (RIA) as previously published[105, 110]. Plasma was treated with 1% acetic acid and 50% methanol and homogenised before being added to $Sep\text{-}Pak_{18}$ cartridges. The extracts were treated 5% in potassium permanganate in water to reduce cross-reactivity of progesterone in samples, before re-extraction with 50%v/v diethyl-ether/n-hexane[296]. Concentrations of allopregnanolone were quantified using polyclonal antibody to allopregnanolone (Agrisera, Sapphire Bioscience, Vannas, Sweden), and tritium-labelled allopregnanolone tracer (5α-[9, 11, 12, ^{3}H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) as previously described[122]. Average recovery of allopregnanolone after extraction was 74.31±1.52%, and individual sample recoveries were used in the final calculation of allopregnanolone concentrations. The intra-assay coefficient of variation was 2.75%.

3.3.5 Western Blot Analysis

Frozen cerebella were dry crushed and extracted into protein extraction buffer (50 mmol/L Tris- HCl, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deox-ycholate, and 0.1% sodium dodecyl sulfate) with protease and phosphotase inhibitors. Protein was quantified using commercial BCA protein assay kit (Pierce) with bovine serum albumin as standard, as per manufacturers instructions. Relative expression of 5αR1 (~26kDa; NB100-1491, Novus Biologicals, Littleton, Colorado) and 5αR2 (~22kDa; GTX47547, Genetex,

Irvine, CA, USA) were determined using methods previously described by members of our group [110].

3.3.6 Statistical Analysis

Data are presented as mean ± SEM, and analysed using PRISM software (version 6.01, GraphPad Software Inc, La Jolla, CA, USA). Significance was set at $p<0.05$. For normally distributed data, two-way ANOVA was performed, using multiple comparisons. For non-normally distributed data a Mann-Whitney test was performed.

3.4 Results

3.4.1 Neonatal Physical Measures

There were no differences in body weight, nose-rump length, head circumference or brain weight between neonates that were exposed to finasteride during pregnancy compared with vehicle exposed neonates at time of tissue collection, at 8 days of age (table 3-1).

TABLE 3-1 NEONATAL PHYSICAL CHARACTERISTICS AT POSTNATAL DAY 8

Sex	Treatment	n	Body Weight (g)	Brain Weight (g)	Nose-Rump Length (cm)	Head Circumference (cm)
Female	Vehicle	*6*	125.833±6.08	2.442±0.068	16.917±0.507	10.08±0.417
	Finasteride	*9*	135.733±6.744	2.592±0.06	18.056±0.386	9.611±0.2
Male	Vehicle	*8*	138.25±6.638	2.59±0.051	17.5±0.354	10.125±0.245
	Finasteride	*8*	134.11±3.344	2.572±0.054	17.778±0.118	9.667±0.167

ALL MEASUREMENTS PRESENTED IN METRIC UNITS. NO DIFFERENCES WERE FOUND IN BODY OR BRAIN WEIGHT, BODY LENGTH OR HEAD CIRCUMFERENCE AT TIME OF TISSUE COLLECTION BETWEEN NEONATES FROM FINASTERIDE AND VEHICLE EXPOSED PREGNANCIES. DATA ANALYSED BY TWO-WAY ANOVA. DATA PRESENTED AS MEAN±SEM.

3.4.2 Myelin Basic Protein, Glial Fibrillary Acidic Protein, S100B, and NeuN

A significant effect of finasteride treatment was found for GFAP expression in lobes VIII and X, as well as the DWM (p=0.0052, p=0.0103 and p=0.0079 respectively, figure 3-1) independent of sex, with markedly increased GFAP area coverage observed in the cerebella of females from finasteride exposed pregnancies within lobe VIII (p=0.0312; figure 3-1a; d) compared to vehicle exposed females. No differences were seen between sexes for GFAP coverage in lobe X (figure 3-1b; e). However, in the DWM finasteride-exposed male neonates had significantly greater area coverage compared to control males (p=0.05; figure 3-1c; f).

No differences were identified in % area coverage for MBP (figure 3-2) or NeuN (data not shown) within lobes VIII and X and DWM of the cerebellum, between any of the experimental groups. There was also no differences in the number of S100B positive cells within lobe VIII or lobe X between any exposure groups (data not shown).

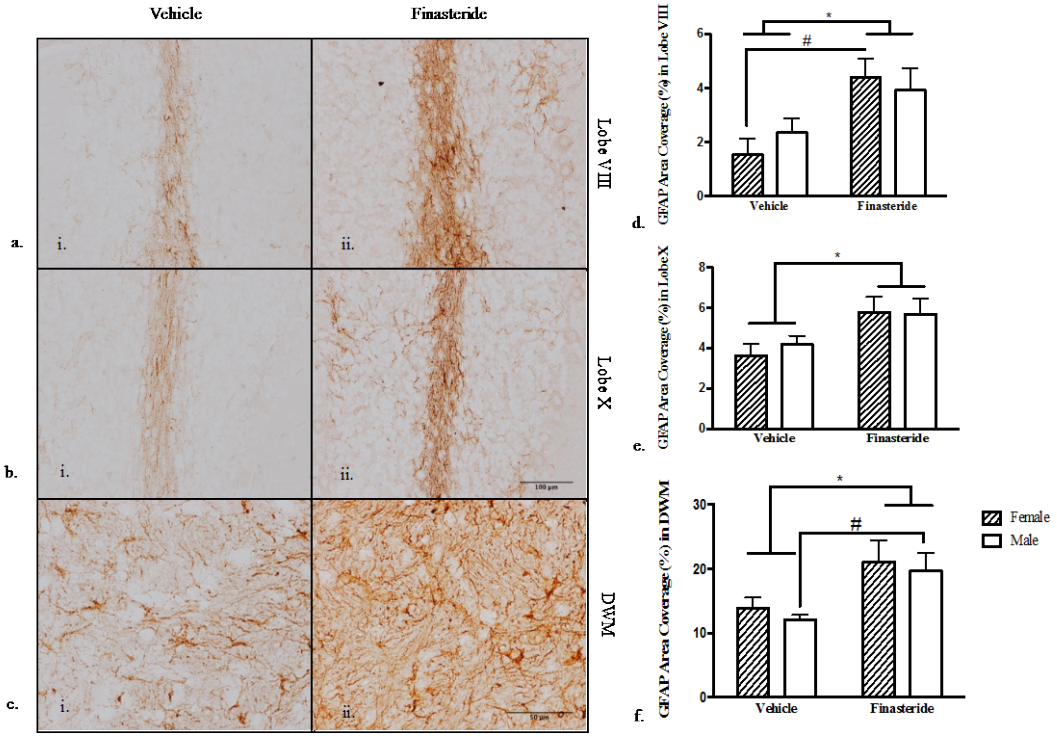


FIGURE 3-1 GLIAL FIBRILLARY ACIDIC PROTEIN IN THE CEREBELLUM (GFAP). REPRESENTATIVE MICROGRAPHS SHOWING GFAP STAINING IN THE CEREBELLUM IN LOBE VIII (A); LOBE X (B) AND DEEP WHITE MATTER (DWM; C) FROM VEHICLE EXPOSED (I) AND FINASTERIDE EXPOSED (II) CEREBELLA. AREA COVERAGE IN LOBE VIII (D); LOBE X (E) AND DWM (F) OF THE CEREBELLUM OF VEHICLE OR FINASTERIDE EXPOSED MALE AND FEMALE NEONATES FROM FINASTERIDE PREGNANCIES (HASHED BARS FEMALE N=4-5, WHITE BARS MALE N=6-7). LOBE VIII P=0.005; LOBE X P=0.010; AND DWM P=0.008. FINASTERIDE EXPOSED FEMALES HAD MARKEDLY INCREASED GFAP IN LOBE VIII (#P=0.031), WHILST FINASTERIDE EXPOSED MALES HAS SIGNIFICANTLY HIGHER STAINING IN THE DWM (#P=0.05). DATA ANALYSED BY TWO-WAY ANOVA. MEAN ± SEM. SCALE BAR (A, B) = 100µM, (C) = 50µM.

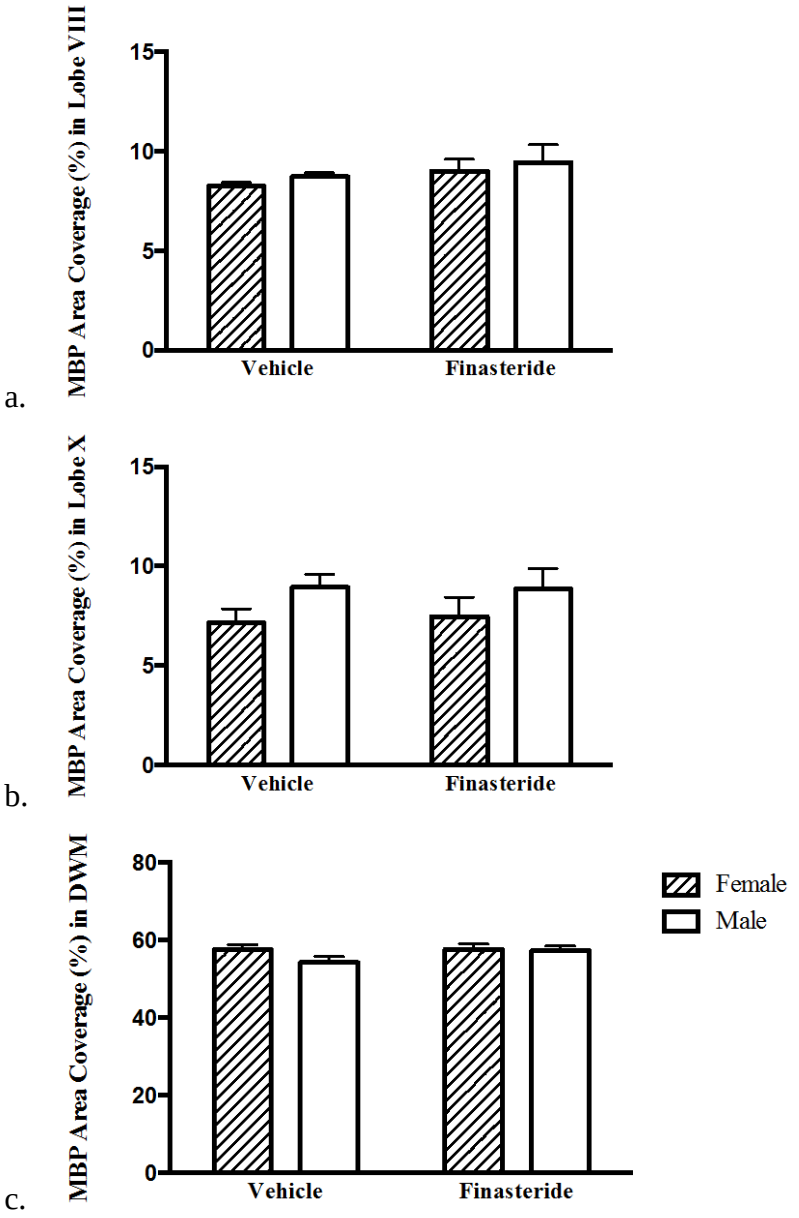
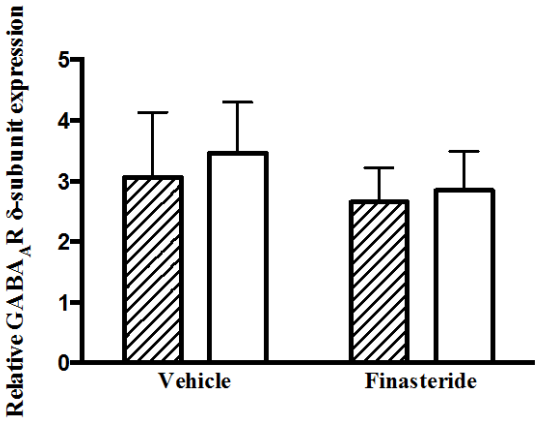


FIGURE 3-2 MYELIN BASIC PROTEIN (MBP) AREA COVERAGE (%) IN THE CEREBELLUM OF LOBE VIII (A); LOBE X (B) AND DWM (C) OF THE CEREBELLUM OF VEHICLE OR FINASTERIDE-EXPOSED NEONATES (HASHED BARS FEMALE N=4-5, WHITE BARS MALE N=6-7). DATA ANALYSED BY TWO-WAY ANOVA. MEAN ± SEM.

3.4.3 $GABA_AR$ Expression in the Cerebellum

Neonates exposed to finasteride in late gestation displayed significant reductions in α6 mRNA levels (p=0.0058), with females showing greater reductions compared to their control counterparts (p=0.0476; figure 3-3b). In contrast no differences were found between vehicle and finasteride exposed neonates in the expression of the δ-subunit (figure 3-3a).

a.



b.

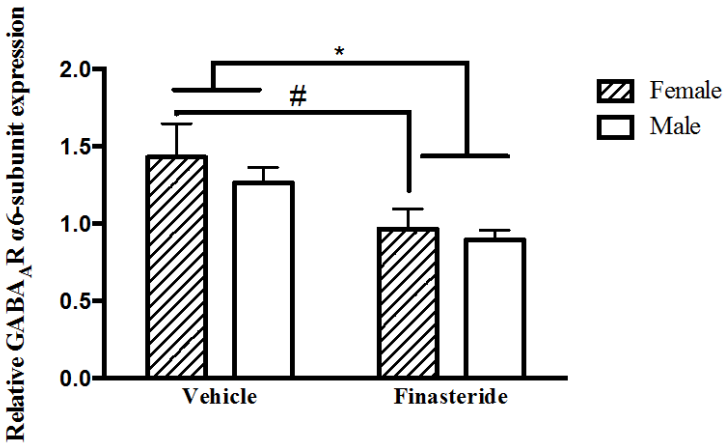


FIGURE 3-3 RELATIVE $GABA_A$ RECEPTOR MRNA EXPRESSION IN THE CEREBELLUM OF VEHICLE VS. FINASTERIDE-EXPOSED NEONATES (HASHED BARS FEMALE N=6-9; WHITE BARS MALE N=6-8). EXPRESSION LEVELS OF THE δ-SUBUNIT DID NOT DIFFER BETWEEN GROUPS (A). SIGNIFICANT REDUCTIONS OF THE α6-SUBUNIT WERE FOUND IN CEREBELLA TAKEN FROM NEONATES EXPOSED TO FINASTERIDE DURING LATE GESTATION (B; *P=0.006); WITH FEMALES SHOWING A GREATER DIFFERENCE (#P=0.048). DATA EXPRESSED AS ARBITRARY UNITS. DATA ANALYSED BY TWO-WAY ANOVA. MEAN ± SEM.

3.4.4 Plasma Allopregnanolone Concentrations and Cerebellar 5α-Reductase Expression

There was no difference in plasma allopregnanolone concentrations between finasteride or vehicle males; or vehicle and finasteride-exposed females (n=5 for each group) indicating that the suppressive action of finasteride on synthesis has been lost by 8 days of age. When the males and female were combined there was a trend toward lower levels in the finasteride treated animals. Although this trend was not significant (p=0.078; figure 3-4), it could indicate a residual effect of finasteride on plasma steroid levels. There was also no difference in expression of 5αR1 or 5αR2 in the cerebellum of neonates between treatments or with sex (figure 3-5).

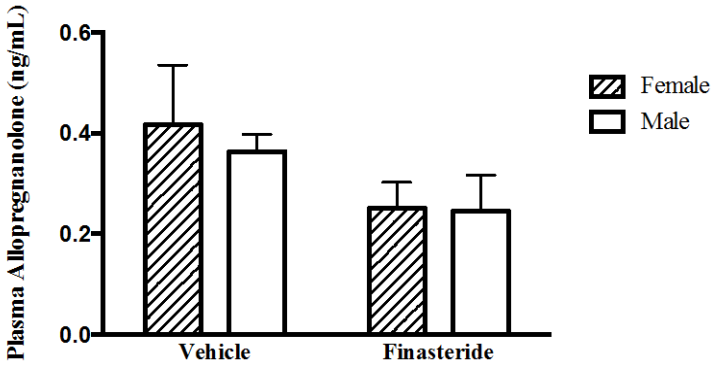
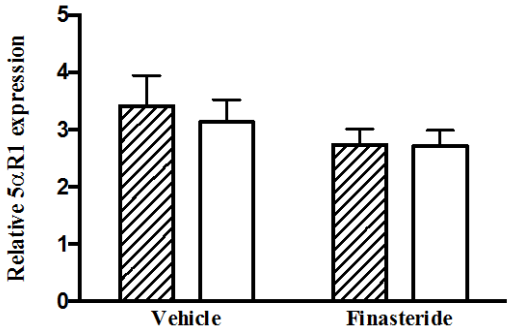


FIGURE 3-4 PLASMA ALLOPREGNANOLONE LEVELS (NG/ML) OF 8 DAY OLD NEONATES FROM VEHICLE AND FINASTERIDE PREGNANCIES (HASHED BARS FEMALE N=5 EACH; WHITE BARS MALE N=5 EACH). DATA ANALYSED BY TWO-WAY ANOVA. MEAN ± SEM.

a.



b.

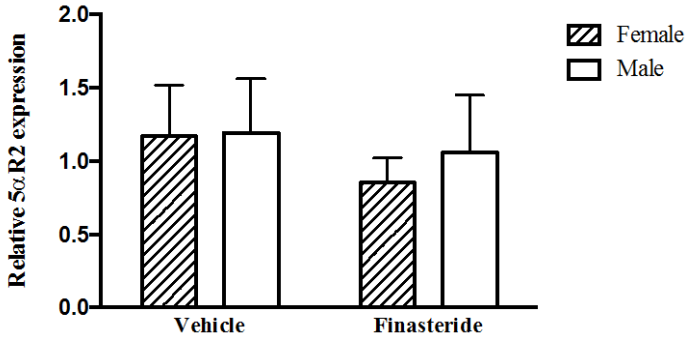


FIGURE 3-5 RELATIVE 5α-REDUCTASE PROTEIN EXPRESSION IN THE NEONATAL CEREBELLUM FOLLOWING *IN UTERO* VEHICLE OR FINASTERIDE EXPOSURE (HASHED BARS FEMALES N=6-9, WHITE BARS MALES N=6-8). THERE WERE NO SIGNIFICANT DIFFERENCES IN RELATIVE 5αR1 (A) OR 5αR2 (B) PROTEIN EXPRESSIONS BETWEEN FINASTERIDE OR VEHICLE-EXPOSED NEONATES. DATA EXPRESSED AS ARBITRARY UNITS. DATA ANALYSED BY TWO-WAY ANOVA. MEAN ± SEM.

3.5 Discussion

This study shows that reduced prenatal exposure to allopregnanolone results is an increase in astrocyte activation and a reduction in α6-subunit mRNA expression in the postnatal cerebellum of guinea pig neonates. Astrocytes have pivotal roles in synaptogenesis and synaptic pruning, as well as a role in the promotion of myelination via electrical signaling. Ishibashi *et. al* showed increased myelin staining from rat dorsal root ganglion cocultured with astrocytes [297]. This increase in myelination coincided with increases in GFAP expression from the astrocytes. We found significant up-regulation of GFAP expression within all regions of the cerebellum analysed, indicating an increase in reactive astrocytes.

Allopregnanolone can influence the activation of astrocytes, with studies showing that administration of allopregnanolone into lesions following traumatic brain injury in rodent model suppressed astrocyte activation [298]. Niemann-Pick C is a congenital neurodegenerative disorder with impaired cholesterol trafficking that consequently impairs neurosteroidogenesis. Individuals with the disorder have progressive Purkinje cell loss, and display increases in GFAP staining in the cerebellum, signifying upregulation of astrocyte activity in a compromised neurosteroid environment [299, 300]. Restoration of homeostasis can be induced by exogenous allopregnanolone administration in rat and mouse models [176, 301], emphasising positive action of this steroid on neurological recovery. The fall in α6-subunit mRNA expression may reduce the action of allopregnanolone, particularly at extrasynaptic sites and contribute to potentially excessive astrocyte activation.

As myelination continues after birth, the increase in astrocyte activation seen here may be promoting myelination postnatally, compensating for the lack of allopregnanolone and its pro-myelinating affects in late gestation. Inhibition of 5α-reductases limits the conversion of not only progesterone to allopregnanolone, but also inhibits the production of 5α-dihydrotestosterone and 5α-deoxycorticosterone, both important precursors to other $GABA_AR$-modulating neurosteroids. However, it does not impede the production of other neuroactive

steroids with positive neurodevelopmental effects. Other steroid hormones may also be compensating for the loss of allopregnanolone in terms of myelination. We cannot exclude the possibility that in the presence of finasteride progesterone, or its immediate precursor, pregnenolone, may be increasingly metabolised down other pathways. More pregnenenolone may also be metabolised into dehydroepiandosterone (DHEA) in the mitochondria of cells. The high concentrations of both DHEA and progesterone can, separately, be metabolised to 17β-estradiol (17β-diol). DHEA and 17β-diol exert pro-myelinating effects via binding to estrogen receptors[302], which activate the required synthesis cascades within oligodendrocytes[303, 304]. As DHEA is the most abundant circulating hormone [305], and combined with the high concentrations of progesterone found in pregnancy, this may consequently be resulting in the apparent "catch-up" of MBP coverage within the brain of these neonates following *in utero* finasteride exposure. Additionally, we cannot exclude effects of the greater astrocyte activation at 8 days age observed in this study. Therefore further studies of the longer-term outcomes are warranted. The finding that there was no differences in number of astrocyte cells identified by S100B positive staining suggests that the increase in GFAP area coverage is due to increased ramification of the astrocytes. Previous studies in adult brains from depressed suicides found that GFAP positive astrocytes increased their branching in the anterior cingulate white matter compared to normal subjects [306]. Whether this is a cause or response to the compromised neural environment is yet to be fully elucidated.

Progesterone and 17β-diol have been also been shown to be important in promoting spinogenesis and dendritic branching of Purkinje cells [307]. Blocking the actions of progesterone by RU486, or 17β-diol by tamoxifen at their receptors individually inhibit Purkinje cell branching in the cerebellum [175, 307]. While allopregnanolone, and other 5α-reduced $GABA_AR$ modulators do not promote branching and spinogenesis directly, they are critical for cell survival of neurons including Purkinje and granules cells [221, 300]. Whilst the production of progesterone and 17β-estradiol are not affected by finasteride, inhibition of allopregnanolone may leave these cerebellar cells vulnerable to insults, such as

hypoxia, and thereby impeding neuronal development by increasing the risk of cell death.

The α6-subunit of the $GABA_A$ receptor is largely an extrasynaptically located subunit, indicating it has a role in regulating tonic activity. This subunit type is expressed predominantly on the granule cells of the cerebellum, cells that release excitatory neurotransmitters and have links to GABAergic Purkinje cells. Previous studies have shown, in a guinea pig model of preterm birth, prematurely delivered pups have significantly lower levels of allopregnanolone and 5αR2 [110] as well as reduced expression of the α6 subunit in the cerebellum [223]. This study with finasteride pharmacologically mimics the premature withdrawal of allopregnanolone seen in preterm delivery, and whilst it did not find reductions in allopregnanolone and 5αR2, it did have the similar finding of reduced α6 expression. Together these studies suggest that alterations in this subunit may be driven by changes in allopregnanolone. This is also supported by previous work in the rat brain investigating $GABA_AR$ subunit changes in pregnancy. These previous findings showed a pregnancy-induced reduction in mRNA expression of the γ_{2L} subunit; and that this decrease was prevented by finasteride [308]. A further study examined the differences in $GABA_AR$ subunit expression of cultured rat neonatal granule cells in response to progesterone exposure. This study showed decreases in $GABA_AR$ $\alpha_{1, 3, 5}$ and γ_2 following progesterone treatment, and these changes were blocked by the administration of finasteride to the cultures. This indicates that allopregnanolone suppressed expression of these subunits[309]. Combined, these results indicate finasteride-induced suppression of allopregnanolone concentrations will alter α6-subunit expression. However, cerebellar culture studies with finasteride and allopregnanolone replacement are required to confirm this relationship. Allopregnanolone is a potent allosteric modulator of $GABA_A$ receptors, particularly at extrasynaptic sites, and reductions in allopregnanolone action along with reduced α6 expression may lead to long term changes in cerebellar signaling as well as potential loss of Purkinje cells, of which astrocyte upregulation may not be able to compensate for in the long term.

3.5.1 Conclusion

The findings of this study indicate that a reduction in allopregnanolone exposure has ongoing postnatal effects on the cerebellum, with lasting increases in astrocyte activation and modifications in $GABA_A$ receptor α6-subunit expression. There is varying information on the effect of prolonged astrocyte activation, therefore these results must be further explored to determine if these mature markers of brain development are maintained in adulthood or if there is long term programming and potential later cell loss due to astrocyte over-excitation. The reductions in the α6-subunit, and potential reduction in tonic neurosteroid-mediated inhibition may lead to ongoing vulnerability of the cerebellum to excititoxic activity. Future studies will be needed to determine the long-term effects on the cerebellum of changes in neurosteroid sensitivity and function.

4 INCREASED ANXIETY-LIKE PHENOTYPE IN FEMALE GUINEA PIGS FOLLOWING REDUCED NEUROSTEROID SUPPLY IN UTERO

A truncated version of this chapter has been accepted for publication in the *International Journal of Developmental Neuroscience* (February 2017).

STATEMENT OF AUTHOR CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
Angela L. Cumberland	Experimental design Animal protocols and tissue collection Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K. Palliser	Experimental design Animal protocols and tissue collection Laboratory procedures Data analysis Manuscript corrections	
Gabrielle K. Crombie	Laboratory procedures Data analysis	
Jonathan J. Hirst	Experimental design Data analysis Manuscript corrections	

Professor Robert Callister
Assistant Dean Research Training 31/08/16

4.1 Abstract

Neurosteroids are essential for aiding proper fetal neurodevelopment. Pregnancy compromises such as preterm birth, prenatal stress and intrauterine growth restriction are associated with an increased risk of developing behavioural and mood disorders, particularly during adolescence. These pathologies involve the premature loss or alteration of oxygen, nutrients and hormones to the fetus during pregnancy that results in impaired neurodevelopment. However, the specific programming mechanisms are yet to be fully elucidated. In later life, dysfunctions of allopregnanolone action are prevalent in individuals with depression, post-traumatic stress disorder and anxiety disorders without prior known fetal complications. The objective of this study was to assess if changes in concentrations of the neurosteroid, allopregnanolone, may be a fetal programming factor in priming the brain towards a negative behavioural phenotype during juvenility in a guinea pig model. Pregnant guinea pigs received either vehicle (45% β-cyclodextrin) or allopregnanolone synthesis inhibitor, finasteride (25mg/kg maternal weight) from gestational age 60 until spontaneous delivery (~71 days gestation). Male and female offspring from vehicle and finasteride treated dams were tested at postnatal day 20 (juvenile-equivalence) in an open field arena, and hippocampus and amygdala subsequently assessed for neurological changes in gross markers of development and inhibitory GABAergic production 24 hours later. Females with low allopregnanolone *in utero* displayed increased neophobic-like responses to a change in their environment compared to female controls ($p=0.009$). However, there were no differences in neuronal markers, MBP, GFAP or GAD67 within the hippocampus or amygdala between intrauterine finasteride or vehicle exposure at juvenility. This study shows an intrauterine reduction in the supply of allopregnanolone programs vulnerability of female offspring to anxiety-like disorders in juvenility. As long term levels of allopregnanolone did not change, these data suggest the end target receptors of neurosteroids may be more important in this programming effect.

4.2 Introduction

Behavioural and mood disorders remain a major clinical problem in today's society. Mood disorders can incorporate anxiety disorders, bipolar disorder and depression, whilst behavioural disorders include attention deficit hyperactivity disorder (ADHD) and conduct disorder among others[310]. The prevalence of these disorders differs between the sexes, with women more often developing mood disorders whilst males tend to develop behavioural disorders[311]. Childhood and the juvenile period are vulnerable periods for onset of many of these problems.

Physiological responses to stress involve important hormonal changes, particularly in steroid hormones, that aid in adaptation to the stressful conditions. Neurosteroids, hormones that can rapidly alter neural signaling also respond to stressful conditions. One of the most important neurosteroids in fetal life is allopregnanolone, a 5α-, 3α-reduced metabolite of progesterone[277]. Allopregnanolone acts as an agonist at the γ-aminobutyric acid type-A receptor ($GABA_AR$), where it binds to an allosteric modulatory site, enhancing the actions of GABA[187, 312]. This interaction leads to the hyperpolarisation of cells and a reduction in excitability. This in turn decreases the risk of excitotoxicity in times of neural trauma and is responsible for the neuroprotective action of allopregnanolone.

The placenta provides both allopregnanolone and precursors for synthesis during gestation. Concentrations are highest in the third trimester[167], which coincides with growth of the placenta and with rapid development of the fetal brain. While allopregnanolone can be synthesised from precursor hormones, such as progesterone, derived from peripheral endocrine organs, it can be synthesised *de novo* from cholesterol within the brain itself[313]. Neurons and glial cells contain the required enzymes to produce neurosteroids in the local tissue[184, 314]. Glial cells, astrocytes and oligodendrocytes, are key sites of allopregnanolone synthesis in neural tissue[184]. Inhibition of allopregnanolone synthesis during late gestation using 5α-reductase inhibitor finasteride causes significant changes within the fetal brain. In fetal sheep studies, administration of finasteride causes significant cell death within the hippocampus[290], and has been shown to reduce expression of

myelin basic protein (MBP, mature myelin marker) as well as increase astrocyte activation, as assessed by increased glial fibrillary acidic protein (GFAP) expression, within the fetal guinea pig brain[105]. Cases of placental insufficiency, where the placenta cannot adequately supply the demand for nutrients, is associated with poor fetal development and may result in impaired neurosteroid synthesis, with the deficit in neurosteroids further confounding fetal neurodevelopment[219].

Allopregnanolone is an endogenous anxiolytic hormone in adult life because it enhances GABAergic inhibition. In addition, synthesis is increased during acute stress events, in a response that has been suggested to counteract the potentially harmful effects of the stress-induced cortisol release[313]. In addition, administration of allopregnanolone to male rats prior to a stressful event has been shown to reduce the release of corticosterone following the stressor[315]. These observations highlight the role of allopregnanolone in remodeling the stress response. Furthermore, the deregulation of allopregnanolone levels has been implicated in mood disorders. Women suffering premenstrual dysphoric disorder have lower circulating allopregnanolone than women with no history of the disorder (PMDD)[210, 316], as well as displaying a blunted allopregnanolone response to stress[317]. Individuals with post-traumatic stress disorder (PTSD) also display lower concentrations of allopregnanolone in their cerebrospinal fluid than individuals without the condition[217]. Allopregnanolone concentrations are stabilised after treatment of selective-serotonin re-uptake inhibitors in major depressive patients and in animal models of depression[209, 246, 318]. This suggests that improvement of symptoms is related to the up-regulation of this neurosteroid synthesis within the brain.

The amygdala is the key region of the brain for memory, emotional responses to stimuli and anxiety states. It is comprised of a group of cellular regions located medially within the temporal lobes. The basolateral amygdala (BLA) is a major input area of the amygdala and is associated with the development and storage of conditioned fear[319]. Neuronal connections from this region to the hippocampus are involved in modulating long-term depression, and its influence on memory

acquisition of hippocampal-dependent tasks[320]. The central nucleus of the amygdala (CeA) is the major output area of this region. Hyperactivity of the CeA may be involved in the development of anxiety states[321]. The CeA links the hypothalamus via the stria terminalis, whereby it can modulate the production of corticotrophin releasing hormone (CRH) from the paraventricular nucleus, and this in turn stimulates the hypothalamic-pituitary-adrenal axis to increase production of cortisol[322]. Inhibition of allopregnanolone production within the adult amygdala has been shown to increase anxiety-like behaviours in test animals[323], whilst administration of allopregnanolone into the CeA produces anxiolytic affects[230]. The involvement of the hippocampus has been well established in the production of anxiety and memory behaviours[324-327]. Similar to behavioural changes found following manipulations within the amygdala, administration of finasteride directly into the hippocampus of adult rats resulted in fewer inner zone entries in open field testing and greater periods of immobility in Porsolt swim test[226]. Conversely, infusion of allopregnanolone into the rat hippocampus produces anxiolytic effects on behaviours in Bossier and elevated plus maze testing[328]. Therefore, it is important to consider the neurosteroidogenic influence on both regions in relation to behavioural output.

The evidence indicating that allopregnanolone has long term programming effects on GABAergic inhibitory pathways suggest that alterations in levels may affect the production and transport of GABA by the glutamic acid decarboxylases (GADs) and GABA transporter (GAT) proteins, respectively. GABA is synthesised by enzymes GAD65 and GAD67, with GAD67 producing the majority of GABA from inhibitory interneurons within the brain. One study showed that inhibition of GAD67 in the BLA diminished fear extinction in a mouse model[329]. Therefore, inhibiting the production of GABA, and hence reductions in GABAergic modulation of excitatory pathways, may lead to hyperactivity in the amygdala causing the increase in anxiety/fear-like behaviour. GAT1 knockout mice display reduced depression- and anxiety-like behaviour than wild-type mice[330] and this is due to a decreased clearance of GABA from synapses that in turn modulate the tonic inhibition of cells[331]. However, very little information is available on the effects of allopregnanolone inhibition on these

regulatory pathways, particularly in gestational programming of susceptibility to psychiatric disorders.

Allopregnanolone levels in the adult brain have been shown to be important in hyperactivity disorders, however it remains unclear if alterations in allopregnanolone exposure during pregnancy can affect vulnerability to these disorders. Furthermore, if reduced allopregnanolone levels lead to an increased risk of behavioural changes these will develop during juvenility, a time when there is a higher incidence in the onset of mood and behavioural disorders. The objective of this study was to determine if a reduction in allopregnanolone levels during late gestation alters long term brain development and behaviour in juvenile guinea pigs.

4.3 Methods and Materials

4.3.1 Animals and Finasteride Administration

Time-mated, outbred guinea pigs were acquired from the University of Newcastle Research Support Unit. Guinea pigs were randomly allocated to either vehicle (45% β-cyclodextrin, 400µl/kg, Sigma Aldrich, Castle Hill, NSW, Australia) or finasteride (25mg/400µL/kg[105] orally, Steraloids, Newport, RI, United States) administration groups commencing at gestational age 60 until delivery (term ~71 days). Dams were allowed to spontaneously deliver, with sex, birth weight and nose-rump length recorded. Pups were maintained with dams in normal housing conditions until testing. On postnatal day (PND) 21 all juveniles (prenatal vehicle treatment n=18, males=9, females=9; prenatal finasteride treatment, n=15, males=8, females=7) were weighed and measured before plasma and brain collection for analysis as previously described[223].

4.3.2 Behavioural Testing

On postnatal day (PND) 20 all offspring underwent behavioural testing. The tests were performed in the morning between 0800hrs and 1200hrs. Guinea pig pups

were assessed for anxiety-like behaviours in open field (OF) and environmental exploration testing. All behaviour tests were videoed using Stoelting ANY-maze 4.99m software[122] (Wood Dale, IL, USA).

The open field test consisted of an arena (40cm x 40cm) set up in a brightly lit room. Grids were set up within the software program using the dimensions of the arena. Guinea pigs had pre-test saliva collected by placing a cotton tip in the mouth for 30 seconds. Animals were placed in the arena along the bottom wall and allowed to explore the arena for 10 minutes. Total distance travelled, number of grid crossings, and entries into the inner zone as well as time spent in the inner zone were recorded by the tracking software using whole body positioning of the animal[122]. These parameters were used as measures of exploratory and motor behaviour.

Neophobia-like responses to changes in the environment were assessed by willingness of offspring to interact with unknown objects. Briefly, immediately after the open field test offspring were placed in the arena with two fixed objects placed on the inner zone boundary of the open field testing. Guinea pigs were allowed to explore the objects for 10 minutes. The total amount of time spent investigating objects, as measured by time the head was within contact or within a 10% distance of the object's boundary, was used as a measure of neophobic-like behaviour. Following the test, post-test saliva was collected as above. The arena and objects were thoroughly cleaned between each test to remove olfactory cues within the arena.

4.3.3 Brain Immunohistochemistry

Fixed, paraffin-embedded sections containing hippocampus and amygdala tissue were sectioned at an 8μm thickness in serials of 3, and immunostained for glial fibrillary protein (GFAP, astrocyte activation), NeuN (mature neuron cell count), microtubule-associated protein 2 (MAP2, mature neuron processes), myelin basic protein (MBP)[121], glutamic acid decarboxylase isoform 67 (GAD67, GABA synthesis enzyme) and GABA transporter 1 (GAT1, GABA transporter). Briefly, tissues were dewaxed and incubated in 10mM Citrate buffer, pH6.0 solution for

antigen retrieval. Sections were blocked for 1 hour (2% normal goat serum, 0.4% BSA, 0.3% Triton X-100), at room temperature, before overnight incubation in primary antibodies GFAP (mouse anti-GFAP, 1:1000, (G3893, Sigma)), NeuN (mouse anti-NeuN, 1:500 (MAB377, Millipore Chemicon, Kilsyth, Victoria, Australia)), MAP2 (mouse anti-MAP2, 1:20000 (M9942, Sigma)), MBP (rat anti-MBP, 1:100 (M9439, Sigma)) or GAD67 (mouse anti-GAD67, 1:1000, (ab26116, Abcam), at room temperature. Primary antibody for GAT1 (rabbit anti-GAT1, 1:500, (ab426, Abcam)) was incubated overnight at 4°C. Secondary antibody incubation for GFAP, GAD67, NeuN and MAP2 used polyclonal goat anti-mouse (1:300, B6649, Sigma), MBP used goat anti-rat (1:300, B7139, Sigma) whilst GAT1 used donkey anti-rabbit (1:300, Sigma). Secondary incubation occurred for 1 hour at room temperature. Tertiary reagent, biotinylated streptavidin-horseradish peroxidase (1:400, ab7403, Abcam, Melbourne, Victoria, Australia) was incubated for 1 hour at room temperature in PBS before colourmetric staining.

Slides were stained in chromagen 3,3'-diaminobenzidine tetrahydrochloride solution (metal enhanced DAB substrate kit #34065; Pierce, ThermoFisher Scientific, Scoresby, VIC, Australia). Slides were then mounted using DPX (Merck, Kilsyth, Vic, Australia) and slide images compiled using the Aperio AT2 slide scanner (Leica Biosystems). Image acquisition occurred using Imagescope (v12.1.0.5029, Leica Biosystems) at 20x magnification. GFAP, MAP2 and MBP immunoreactivities were analysed by densitometry using ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA) and made binary by adjusting the threshold manually. GFAP, MAP2 and MBP staining was expressed as percent area of coverage recorded for 4 fields of view per region on two sections per animal. Regions of interest were the BLA and CeA (GFAP and MAP2); and CA1 (GFAP, MAP2 and MBP) and subcortical white matter (SCWM; GFAP and MBP). GAD67 and NeuN cell counts were collected using ImageJ, again using 4 fields of view in two sections in both regions of interest. GAT1 qualitative staining was assessed by a staining scale of + (weak staining, with few puncta); ++ (moderate staining, some puncta and axonal staining) and +++ (intense staining, numerous puncta and axons recognisable)[278], in both amygdaloid and hippocampal regions.

4.3.4 Determination of Steroid Concentrations

Plasma allopregnanolone concentrations were determined using radioimmunassay (RIA) as previously published[105, 110]. Plasma was treated with 1% acetic acid and 50% methanol and homogenised before being added to Sep-Pak$_{18}$ cartridges. The extracts were treated 5% in potassium permanganate in water to reduce cross-reactivity of progesterone in samples, before re-extraction with 50%v/v diethyl-ether/n-hexane[296]. Concentrations of allopregnanolone were quantified using polyclonal antibody to allopregnanolone (Agrisera, Sapphire Bioscience, Vannas, Sweden), and tritium-labelled allopregnanolone tracer (5α-[9, 11, 12, ^{3}H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) as previously described[122]. Average recovery of allopregnanolone after extraction was 74.31±1.52%, and individual sample recoveries were used in the final calculation of allopregnanolone concentrations. The intra-assay coefficient of variation was 2.75%.

Salivary cortisol concentrations from samples collected pre and post behavioural testing of guinea pig offspring were determined using Salimetrics enzyme-linked immunoassay kit as per manufacterers instructions (Salimetrics Inc., State College, PA, USA) and as previously described[121, 122, 270]. Sensitivity of the assay was 0.012µg/dL to 3µg/dL and intra-assay coefficient of variation was 5.52%.

4.3.5 Statistical Analyses

One male and one female from each pregnancy was used for analysis. An independent samples t-test was used to assess differences in physical parameters, behaviour and neurodevelopmental markers between prenatal exposure groups in each sex, with a Levene's test for equality of variances. Statistical significance was set as $p<0.05$. Data in-text for t-test results are presented as mean difference; 95% CIs; and p-values. All data in tables and graphs are represented as mean±SEM. Statistical analysis was performed using IBM SPSS Statistics software (version 24; SPSS Inc.; IBM Corporation, Armonk, NY, USA). Graphs

were created using GraphPad Prism software (version 7.0a, GraphPad Software Inc., La Jolla, CA, USA).

4.4 Results

4.4.1 Juvenile Physical Measures

At birth, pups from vehicle and finasteride pregnancies did not differ in body weight or nose-rump length (data no presented). At juvenility, no differences were found between prenatal exposure groups for overall body weight in females or males. However, finasteride-exposed females were significantly longer in nose-rump length (1.38; 2.59, 0.18; p=0.028), and had heavier brain weight (0.17; 0.35, 0.001; p=0.049) and hippocampus weight (0.03; 0.05, 0.01; p=0.003) compared to vehicle exposed females (table 4-1). Vehicle and finasteride-exposed males did not differ in any of these parameters. All other organs weights, including heart, liver, kidneys and adrenals did not change between prenatal exposure within sex.

TABLE 4-1 PHYSICAL MEASUREMENTS AT POSTNATAL DAY 21

	Female		**Male**	
	Vehicle	*Finasteride*	*Vehicle*	*Finasteride*
n	*9*	*7*	*9*	*8*
Body weight (g)	226.27±11.42	248.57±13.15	250.27±12.9	258.38±21.07
Nose-rump length (cm)	21.33±0.42	22.71±0.34*	22.94±0.42	22.81±0.95
Brain weight (g)	2.749±0.049	2.922±0.066*	2.869±0.083	2.887±0.12
Hippocampus weight (g)	0.098±0.005	0.127±0.006*	0.122±0.009	0.122±0.005

PHYSICAL MEASUREMENTS IN METRIC UNITS. DATA ANALYSED BY INDEPENDENT SAMPLES T-TESTS FOR EACH SEX. DATA PRESENTED AS MEAN±SEM. *P<0.05 FINASTERIDE-EXPOSED VS. VEHICLE-EXPOSED FEMALES

4.4.2 Juvenile Behaviour is Affected by *In Utero* Allopregnanolone

In open field testing, offspring from vehicle and finasteride treated mothers did not significantly differ in locomotor activity measured by total distance travelled

and total number of line crossings (table 4-2). Nor did juvenile offspring differ statistically in the number of passes into the inner zone or time spent in this area of the arena.

TABLE 4-2 OPEN FIELD MEASURES AT JUVENILITY

	Female		**Male**	
	Vehicle	*Finasteride*	*Vehicle*	*Finasteride*
n	*9*	*7*	*9*	*8*
Total distance (m)	5.63±1.26	3.26 ±1.42	8.00±2.13	6.88±2.37
Total Line Crossings	81.33±21.81	47.43 ±15.39	105.33± 28.03	77.88± 20.61
Inner Zone Time (s)	45.03±40.61	6.7±4.8	23.58±9.99	76.3±34.83
Inner Zone Crossings	2.44±0.88	2.86±2.38	8.11±2.34	6.63±2.96

MEASUREMENTS IN METRIC UNITS. DATA ANALYSED BY INDEPENDENT SAMPLES T-TESTS FOR EACH SEX. DATA PRESENTED AS MEAN±SEM.

In environment exploration testing, finasteride-exposed females spent less time investigating changes in the arena compared to vehicle-exposed females (18.71; 6.14, 31.27; p=0.009; figure 4-1a). Females from finasteride and vehicle pregnancies travelled similar distances during this test (Figure 4-1b). Males did not differ in the total time spent investigating the changes, nor in the distance travelled during the 10 minute test (Figure 4-1c; d).

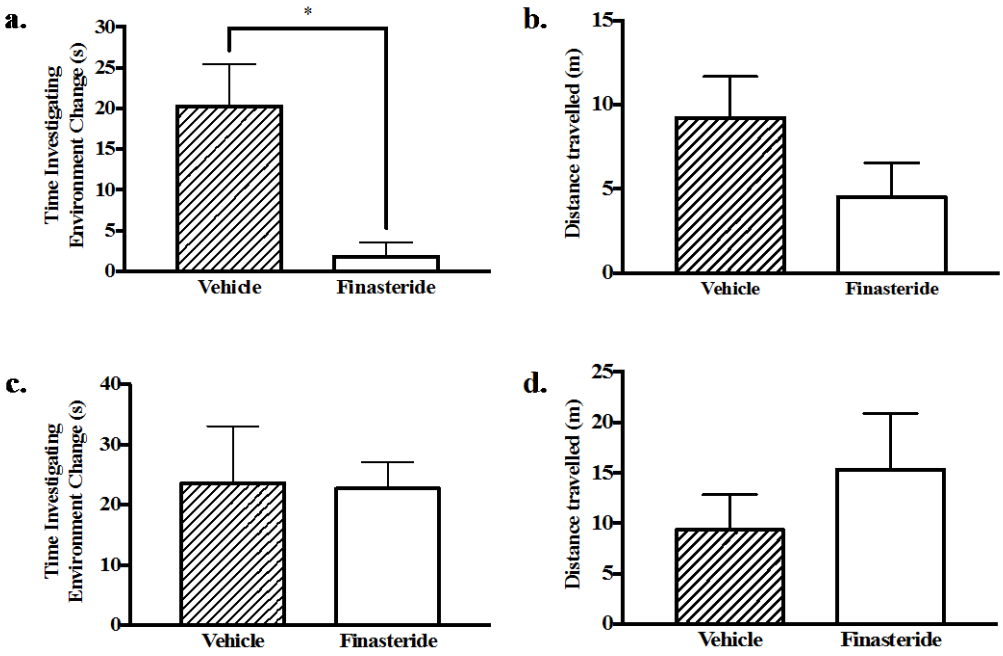


FIGURE 4-1 ENVIRONMENT EXPLORATION OF JUVENILE OFFSPRING. PRENATAL VEHICLE EXPOSURE INDICATED BY THE HASHED BARS (FEMALES N=9, MALES N=9); PRENATAL FINASTERIDE EXPOSURE INDICATED BY THE WHITE BARS (FEMALES N=7, MALES N=8). FEMALES EXPOSED TO FINASTERIDE IN UTERO SPENT SIGNIFICANTLY LESS TIME INVESTIGATING ENVIRONMENTAL CHANGES (A) COMPARED TO VEHICLE FEMALES (P=0.009). NO DIFFERENCES WERE FOUND IN LOCOMOTOR ACTIVITY OF FEMALES DURING THIS TEST (B). MALES DID NOT DIFFER IN TOTAL TIME SPENT EXPLORING CHANGE (C) OR IN TOTAL DISTANCE TRAVELLED (D). DATA ANALYSED BY INDEPENDENT SAMPLES T-TESTS FOR EACH SEX. DATA PRESENTED AS MEAN±SEM. *P<0.05.

4.4.3 Amygdala Immunohistochemistry

4.4.3.1 Mature Neurons in the Amygdala

Staining of mature neuron processes, as measured by % area coverage of microtubule-associated protein 2 (MAP2), and mature cells numbers, as measured by NeuN cell staining, did not differ in the BLA or CeA between vehicle and finasteride exposure, or between the sexes (data not shown).

4.4.3.2 Glial Fibrillary Acidic Protein in the Amygdala

Within the BLA and CeA of the amygdala, there were no effects of prenatal finasteride exposure found on GFAP % area coverage (figure 4-2) in females or males.

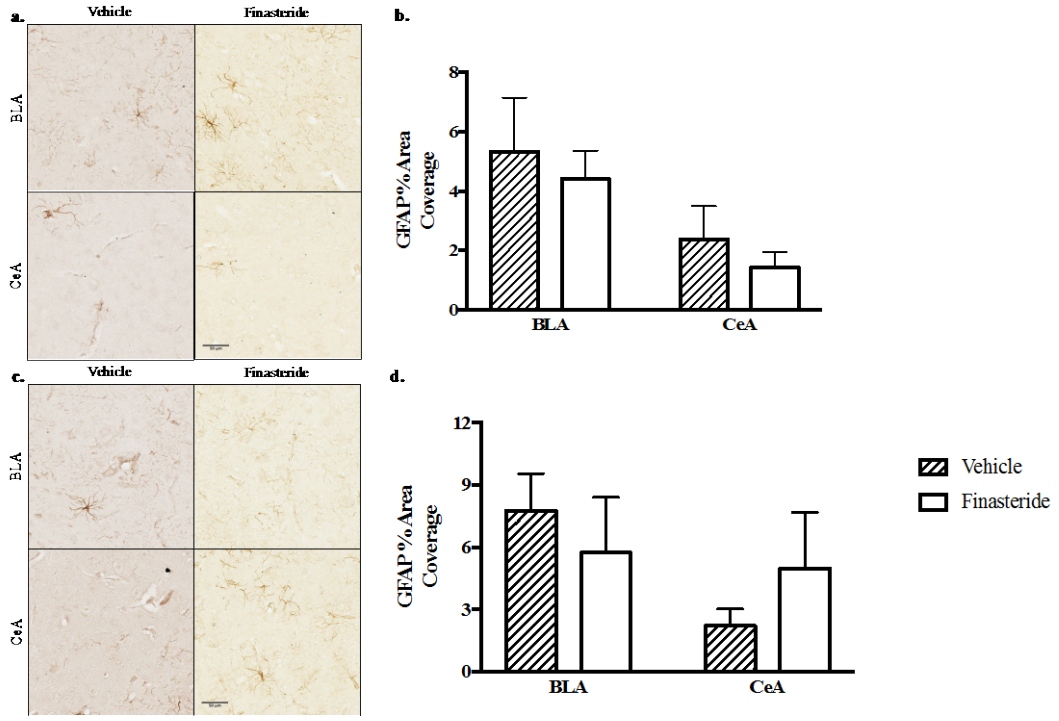


FIGURE 4-2EFFECT OF FINASTERIDE TREATMENT ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) % AREA COVERAGE WITHIN THE AMYGDALA. REPRESENTATIVE MICROGRAPHS OF GFAP OF FEMALES (A) AND MALES (C); IN THE BLA AND CEA FOR VEHICLE AND FINASTERIDE-EXPOSED JUVENILES. NO CHANGES IN GFAP AREA COVERAGE WERE FOUND FEMALE (B) OR MALE (D) OFFSPRING IN THE BLA OR CEA. PRENATAL VEHICLE EXPOSURE INDICATED BY THE HASHED BARS (FEMALE N=5, MALE N=5); PRENATAL FINASTERIDE EXPOSURE INDICATED BY THE WHITE BARS (FEMALE N=6, MALE N=5). DATA ANALYSED BY INDEPENDENT SAMPLE T-TESTS. DATA PRESENTED AS MEAN ± SEM. *P<0.05.

4.4.3.3 Glutamic Acid Decarboxylase 67 in the Amygdala

There was no effect of prenatal finasteride exposure on GAD67 positive cells within the BLA or CeA of female or male guinea pig offspring (figure 4-3).

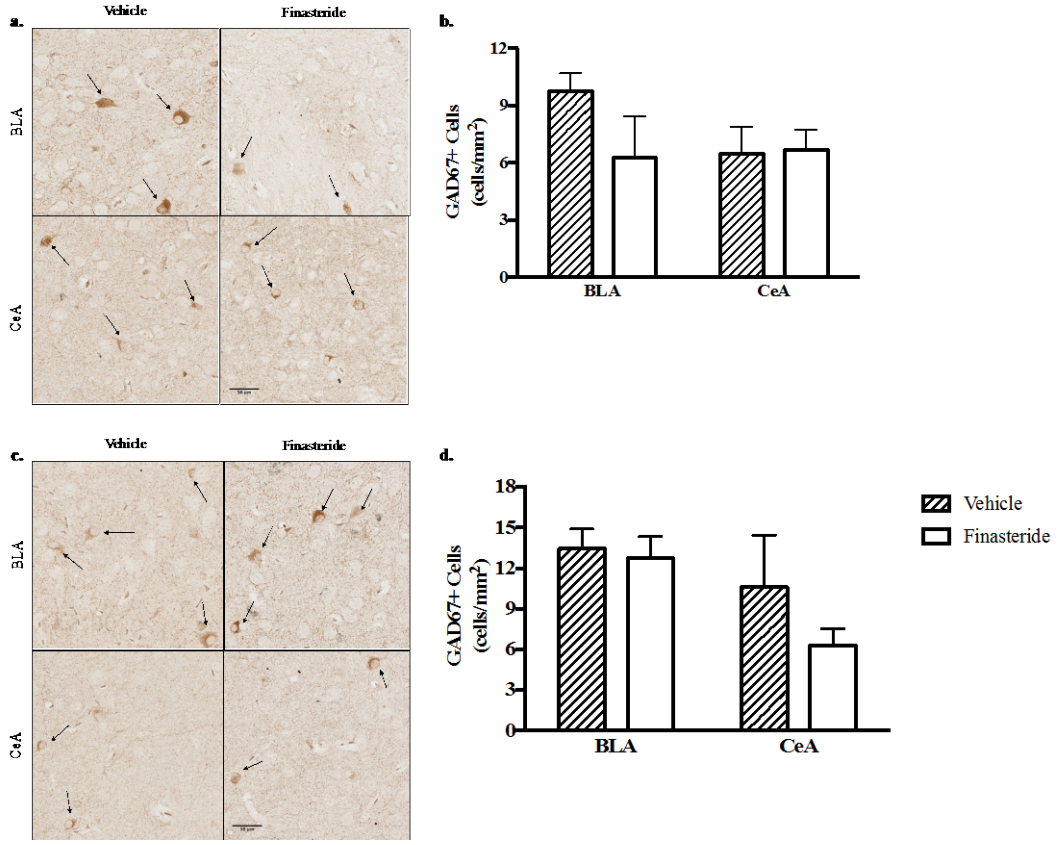


FIGURE 4-3 GAD67+ CELL COUNTS WITHIN THE AMYGDALA FOR FEMALE (A, B) AND MALE (C, D) JUVENILE GUINEA PIGS. REPRESENTATIVE MICROGRAPHS FOR GAD67 IN THE BLA AND CEA OF FEMALE (A) AND MALE (C) OFFSPRING. ARROWS INDICATE GAD67+ CELLS. SIMILAR GAD67+ CELL NUMBERS (CELLS/MM^2) WERE FOUND IN THE BLA AND CEA OF THE AMYGDALA IRRESPECTIVE OF PRENATAL EXPOSURE (FEMALES, B; MALES, D). PRENATAL VEHICLE EXPOSURE INDICATED BY THE HASHED BARS (FEMALES N=4, MALES N=4); PRENATAL FINASTERIDE EXPOSURE INDICATED BY THE WHITE BARS (FEMALES N=4, MALES N=4). DATA ANALYSED BY INDEPENDENT SAMPLES T-TEST. DATA PRESENTED AS MEAN±SEM. SCALE BARS = 50µM. *P<0.05.

4.4.3.4 GABA Transporter 1 (GAT1) in the Amygdala

Qualitative assessment of GAT1 in the BLA and CeA of the amygdala revealed stronger GAT1 staining in both the BLA and CeA of vehicle exposed females compared to finasteride exposed females (table 4-3, figure 4-4). Males did not differ in their qualitative staining.

TABLE 4-3 GAT1 QUALITATIVE ASSESSMENT IN THE AMYGDALA

Sex	n	Prenatal exposure	BLA	CeA
Female	4	Vehicle	++	++
	4	Finasteride	+	+
Male	4	Vehicle	+++	++
	4	Finasteride	+++	++

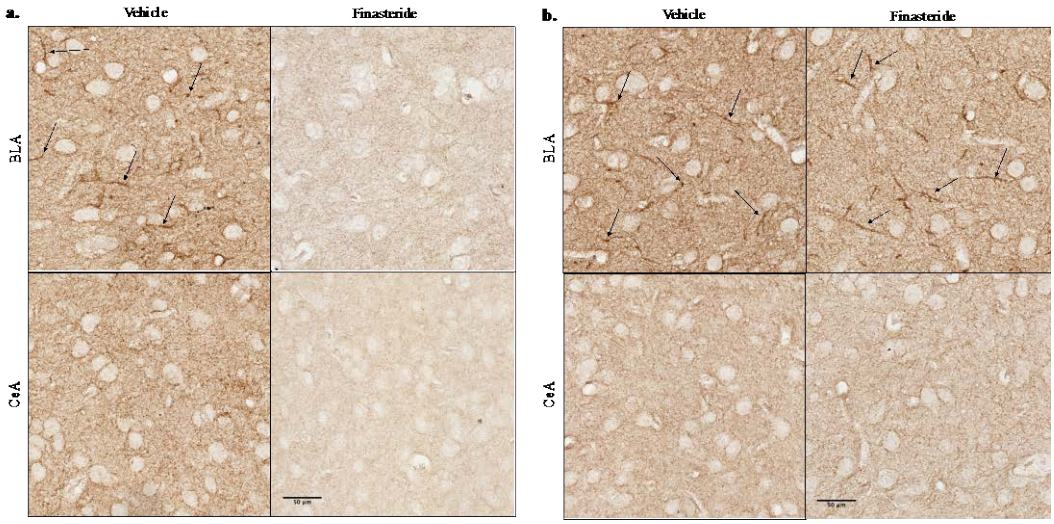


FIGURE 4-4 REPRESENTATIVE MICROGRAPHS OF GAT1 STAINING WITHIN THE BLA AND CEA IN JUVENILE FEMALES (A) AND MALES (B). ARROWS INDICATE INTENSE AXONAL AND PUNCTA STAINING WITHIN THE BLA. STRONGER STAINING OF PUNCTA SEEN IN THE BLA CEA OF VEHICLE FEMALES COMPARED TO FINASTERIDE FEMALES. MALES DID NOT DIFFER IN THEIR GAT1 STAINING. SCALE BAR = 50μM.

4.4.4 Hippocampal Immunohistochemistry

No differences were found in hippocampal markers MBP, GFAP (see appendix 1), MAP2 and NeuN; or within GABAergic modulation via GAD67 and GAT1 staining (data not presented).

4.4.5 Plasma Allopregnanolone and Salivary Cortisol Concentrations

There was no effect of prenatal treatment on plasma allopregnanolone concentrations at juvenility in female or male guinea pigs (figure 4-5). This indicates finasteride during gestation did not affect levels in the offspring 21 days after birth.

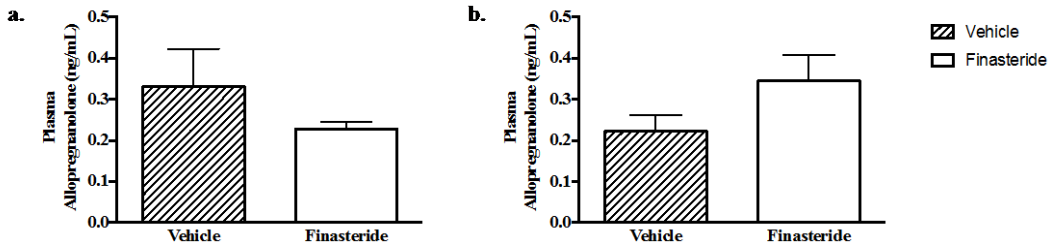


FIGURE 4-5 CIRCULATING PLASMA ALLOPREGNANOLONE CONCENTRATIONS AT JUVENILITY. NO STATISTICAL DIFFERENCES WERE FOUND WITHIN FEMALES (A) OR MALES (B) IN CIRCULATING ALLOPREGNANOLONE CONCENTRATIONS. PRENATAL VEHICLE EXPOSURE INDICATED BY THE HASHED BARS (FEMALES N=5, MALES N=5); PRENATAL FINASTERIDE EXPOSURE INDICATED BY THE WHITE BARS (FEMALES N=5, MALES N=5). DATA ANALYSED BY INDEPENDENT SAMPLES T-TEST. DATA PRESENTED AS MEAN ± SEM.

No effect of prenatal finasteride treatment was found in pre-testing salivary cortisol concentrations. However, there was a non-significant effect of finasteride treatment in females (p=0.058, figure 4-6). Males did not differ at either time point (data not presented).

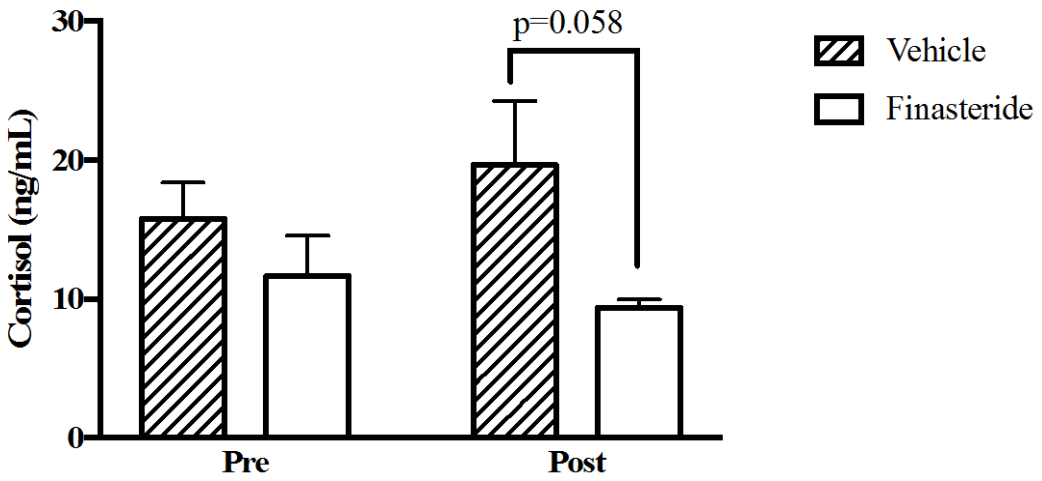


FIGURE 4-6 PRE AND POST-TESTING SALIVARY CORTISOL CONCENTRATIONS IN FEMALES. NO DIFFERENCE IN PRE-TESTING SALIVARY CORTISOL CONCENTRATIONS WERE FOUND BETWEEN FEMALES FROM FINASTERIDE (WHITE BARS, N=5) OR VEHICLE (HASHED BARS, N=5) PREGNANCIES. FEMALE JUVENILE GUINEA PIGS EXPOSED TO FINASTERIDE DURING PREGNANCY TENDED TOWARDS REDUCED SALIVARY CORTISOL CONCENTRATIONS POST-TESTING, COMPARED TO VEHICLE EXPOSED FEMALES (P=0.058). DATA ANALYSED BY INDEPENDENT SAMPLES T-TEST. DATA PRESENTED AS MEAN ± SEM. *P<0.05.

4.5 Discussion

The hypothesis examined in this study that reduced allopregnanolone exposure during late gestation can alter long term developmental outcome was supported by the overall findings. Female guinea pig offspring that were exposed to suppressed neurosteroid levels during late gestation displayed increased fear/anxiety-like responses in behavioural testing at juvenility, with a blunted cortisol response to stress. These findings are consistent with previous studies in the postnatal rat, showing alterations in neurosteroids early in life produce behavioural deficits in rats at adolescent and adult equivalence[332, 333]. However, the previous rat study excluded female offspring, a group in which decreased allopregnanolone has strongly been implicated in the development of mood disorders. The difference in findings between sexes in our study and the previous work involving male rats may also be due to the timing of finasteride administration and dosage. Guinea pigs have a long gestational period, with much of the fetal brain development occurring in utero and with the supply of allopregnanolone coming from the placenta[110, 258]. The drug regime was performed postnatally in the rat model, where much of the brain development occurs in a neurosteroid environment reliant on neonatal brain production. Therefore, in the rats, whilst brain development may be at a reasonably similar stage to fetal humans and guinea pigs[257, 259], it does not have the abundance of placental supply of allopregnanolone. The rat study also used a dose of finasteride (50mg/kg) known to induce behavioural changes in adults[332], that was twice the dose of that used in the present work. Therefore, whilst the dose of 25 mg/kg reduced fetal brain allopregnanolone concentrations in guinea pigs by approximately three-fold[105], it is plausible that the remaining supply of allopregnanolone to the male guinea pig fetuses was sufficient to suppress programming of the neophobic/fear responses at juvenile ages in these animals. Also, females are more likely to suffer negative mood states, including social anxiety and depression, during their schooling career than males[334]. Social anxiety can encompass a general anxiety to socialising as well as fear of new social situations, affecting relationships with peers[335], whilst depression has many negative impacts on social and emotional interactions and mental wellbeing.

We have previously shown that allopregnanolone levels are markedly reduced by the finasteride dosing used in this study[105]. The finding that allopregnanolone concentrations were not reduced in the offspring at 21 days old suggests that synthetic capacity and expression of the 5αR enzymes in the offspring were not permanently suppressed by in utero finasteride treatment. However, we cannot exclude the possibility that there were effects on the transient induction of allopregnanolone, its utilisation or sensitivity. Reduced levels of allopregnanolone are associated with increases in anxiety and depressive symptoms during puberty. A number of women suffer premenstrual syndrome (PMS) and the more severe form PMDD during their reproductive cycle, disorders that commonly display these negative mood behaviours. Paired with reductions in circulating allopregnanolone levels[336], women with PMS/PMDD have poor allopregnanolone responses following a stress test than women who do not have symptoms[317]. However, in contrast to effects at the time of suppression, the current work indicated that the deficiency during pregnancy alters gestational development with long term negative effects 21 days after birth. Research into the guinea pig prenatal stress model showed prenatally stressed male offspring had an exaggerated cortisol response on HPA stress testing[269] whilst prenatally stressed female guinea pig offspring exhibited a blunted cortisol release on exposure to a stressor[270]. This is consistent with human studies of chronic stress exposure that show males with PTSD have higher salivary cortisol levels during and following cognitive challenge than healthy males and females with PTSD[337]. Human data in young adult females with PTSD or major depression having an attenuated cortisol response to social stress testing, suggesting this is a female adaptation[338]. As the female offspring with low fetal allopregnanolone exposure in this study also displayed a blunted cortisol response following testing, this supports the notion of reduced in utero neurosteroid supply programming the hypothalamic-pituitary-adrenal (HPA) axis in response to stressors.

The normal hormonal cycle produces highs (luteal phase) and lows (follicular phase) in circulating allopregnanolone[212], and these fluctuations are thought to induce $GABA_A$ receptor subunit changes that impact on how allopregnanolone

acts. A comprehensive study by Shen et al[339] investigated the anxiolytic and anxiogenic properties of allopregnanolone during puberty in female mice, and compared this to outcomes from pre-pubertal and adult behaviour in elevated plus maze testing. These researchers found that during the equivalent period of puberty in these mice the elevated allopregnanolone concentrations caused an anxiogenic-like phenotype, whilst in pre-pubertal and adult mice this steroid was anxiolytic. This difference in the effect of allopregnanolone on behavioural properties was attributed to changes in $GABA_AR$ subunit composition following acute exposure and withdrawal of allopregnanolone. Acute exposure of neurons to increased allopregnanolone levels has been found to increase expression of the α_4 $GABA_AR$ subunit, which is implicated in producing anxious behaviours. These anxiety behaviours were also associated with the allopregnanolone-sensitive δ subunit[339]. Knockout mice lacking this δ subunit did not display anxious-like behaviours compared to pubertal mice with normal expression levels. Additionally, finasteride administration to neonatal male rats increases both α_4 and δ subunit expression in response to low allopregnanolone, and these profiles were maintained in adulthood upon behavioural testing in the elevated plus maze[340]. Therefore, these two subunits interact and change expression to produce biphasic mechanisms in response to allopregnanolone concentrations. Whilst we cannot comment on what is occurring at the time of behavioural testing in the animals in the current study, we cannot exclude that there is a stress-induced changes in brain allopregnanolone sensitivity leading to anxiogenic-like behaviours in female guinea pigs.

There was an increase in brain weight of females exposed to finasteride compared to vehicle females. However, we did not find changes in gross neurodevelopmental markers MAP2, NeuN, MBP or GFAP that could explain this alteration in the hippocampus or the amygdala. Finasteride, whilst known to impair neurodevelopment in fetal life[105, 218, 290], does not have a long term effect on these markers, yet it does impact on later life vulnerability. The hippocampus is a well-studied area for behavioural changes and anxiety, however the hippocampus interacts with and is regulated by the amygdala and vice versa. These two areas have reciprocal connections, creating a feedback circuit to

modulate memory and fear formation[143]. Lesion studies have shown that the amygdala is responsible for acquisition of fear responses regardless of stimuli (unconscious response), and the hippocampus is responsible for contextual fear acquisition[341]. The hippocampus produces reductions in freezing time following unsignaled foot shock in rats, and less anxious behaviour in the light/dark box testing[342]. Bilateral lesions of the CeA in rhesus monkeys produced similar behavioural changes compared to control animals, with reductions in time taken to retrieve a treat in the presence of a threatening stimuli , and reduced freezing time on exposure to an unknown human intruder[343]. Taken together, both regions are important in the modulation of fear and anxiety responses of the individual, and activity of both must be regulated together to maintain homoeostasis. So while the female offspring of this study did not show gross structural protein deficits, it cannot be excluded that the hippocampus and/or amygdala is under aberrant excitability following behavioural testing in this study.

There were no changes in the number of GAD67+ cells within both regions of the amygdala and hippocampus investigated. However, the authors cannot rule out whether prenatal reductions in allopregnanolone had a programming effect on the ability of GABAergic cells to upregulate GAD67 expression. Acute stress events upregulate the expression of GAD67 in a number of adult brain regions in the rat, peaking 1 hour post stress events[195]. Allopregnanolone has been shown to upregulate the expression of GAD67 in Schwann cells of the peripheral nervous system[199], and GAD67 is co-expressed with the 5αR2 isozyme, important in allopregnanolone production[200]. Studies inhibiting the expression of GAD67 within the amygdala of male mice have shown that GAD67 has a major role in regulating anxiety and fear extinction[329]. Bilateral injection of siRNA for GAD67 into the amygdala of these male mice led to prolonged decay of freezing response to conditioned foot shocks compared to scrambled control mice. Furthermore, lesion studies of the BLA have shown this region to be important in social behaviours and development of anxiety in rodents[202, 344]. Similar behavioural deficits are seen when allopregnanolone production is inhibited in the amygdala by finasteride in adult female rats[230]. As allopregnanolone and GABA act in co-ordination to regulate cell excitability and, based on behavioural studies, also

regulate behavioural output, changes in the levels of allopregnanolone may program an altered expression of GAD67 under stress events. Interestingly, females exposed to finasteride during pregnancy had reduced GAT1 immunostaining in the amygdala compared to vehicle female counterparts. GAT1 is responsible for the clearance of GABA from the synaptic cleft, and lower expression is associated with reduced anxiety-like behaviours in knockout animal models[330]. Little information is available on how allopregnanolone interacts or regulates this enzyme; thus whether this reduction found in the finasteride-exposed females is a compensatory mechanism following reduced allopregnanolone in utero requires further investigation.

Loss of allopregnanolone in females does not alter hippocampal or amygdalar mature neurons, GAD67 enzyme expression or astrocytosis at juvenility in guinea pigs. Fetal and early postnatal studies show that finasteride up-regulates GFAP area coverage in the hippocampus[105], however the present finding show this is not sustained into juvenility in the guinea pig. The finding of changes in female behaviour supports the notion that fetal programming has occurred even when neurodevelopmental proteins recover, which perhaps involves very subtle changes. The present findings have implications for the long term mental welfare of children, particularly girls, born following compromised pregnancies or preterm birth where neurosteroid exposure is reduced. Pregnancy compromises are associated with premature loss of allopregnanolone from the placenta (preterm birth) or impaired function of the placenta (IUGR, prenatal stress). The offspring of these pregnancies are more likely to develop disorders such as ADHD, anxiety, depression and schizophrenia in adolescence, periods of well-known fluctuation in neurosteroid utilisation. Given females are at a greater predisposition to mood disorders simply due to hormonal changes in the menstrual cycle, children born from compromised pregnancies who are already at a disadvantage neurodevelopmentally may be susceptible towards psychological deficits due to *in utero* programming by neurosteroids. Whilst this study did not assess changes in GABAergic signaling at the time of testing, previous evidence supports the contention that allopregnanolone does change $GABA_AR$ phenotype in response to fluctuating levels, and paired with suboptimal levels *in utero*, fetal exposure of

allopregnanolone does have a programming effect on the predisposition towards poor mental states at juvenility, particularly in females.

5 COMBINATION OF IUGR AND PRENATAL STRESS ON FETAL BRAIN DEVELOPMENT

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STATEMENT OF AUTHOR CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
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5.1 Abstract

Intrauterine growth restriction and maternal stress during pregnancy (PS) are two compromises that negatively impact neurodevelopment, and increase the risk of developing later life neuropsychiatric disorders such as schizophrenia, depression and behavioural disorders. Neurosteroids, particularly allopregnanolone, are important in protecting the developing brain and promoting many essential neurodevelopmental processes. Individually, IUGR and PS reduce myelination and neurogenesis within affected fetal brains, however less information is available on the combined effects of these two disorders on the term fetal brain. This study aimed to investigate how IUGR and PS impairs the neurosteroid pathway when combined using a guinea pig model, and how these then disrupt the neurodevelopment of the fetus. Uterine artery blood flow restriction was used to induce growth restriction, whilst PS was induced by exposure of the dam to a strobe light during gestation. Exposure in this model caused reductions in hippocampal MBP immunostaining of male fetuses in both IUGR alone and IUGR+PS paradigms in the CA1 but only by IUGR in the subcortical white mater, compared to control males. Plasma allopregnanolone was reduced by both stressors irrespective of sex, whereas GFAP or MAP2 expression were not affected by either stressor. Female neurodevelopment, as assessed by these markers, was unimpeded by these compromises. These observations indicate reduced placental perfusion produces mild deficits in neurodevelopment, and interestingly does so in a sex dependent manner.

5.2 Introduction

A healthy pregnancy is essential for proper fetal development. Complications, such as intrauterine growth restriction (IUGR) and maternal stress during pregnancy (prenatal stress, PS), can have acute effects on the fetus as well as on-going adverse effects after birth. IUGR is seen in up to 10% of pregnancies worldwide, and children that fail to reach their *in utero* growth potential are at an increased risk of mortality and morbidities ranging from mild behavioural deficits to physical and neurological impairments[19, 345, 346]. Infants born IUGR are

commonly small for gestational age (SGA), and can either have symmetrical growth restriction, whereby all physical and visceral growth is reduced equally, or fetuses may grow asymmetrically. Asymmetrical growth restriction is due to a redirection of blood flow resulting in at least a partial maintenance of normal brain growth and size, but reduced lower limb size, abdominal circumference and visceral organ growth[347]. The most common cause of IUGR is placental insufficiency, whereby inadequate placental supply of nutrients to the fetus impedes growth.

Neurodevelopmental delays are common in children born growth restricted. Two longitudinal studies by Leitner et al[65] and Geva et al[64] saw that at 9-10 years of age, children born growth restricted presented with significantly greater memory and learning difficulties, visuomotor and language disturbances as well as reduced IQ and school performance in the overall areas of verbal knowledge, reading and maths. Being born growth restricted is associated with increased withdrawn behaviour at 12 months of age[348] and a greater risk of presenting with attention deficit hyperactivity disorder (ADHD) at 4 ½ years age[349]. Changes in behaviour are evident from infancy with evidence showing, that even being born at term age, SGA infants have poorer attention, habituation and social-interaction scores at 24 months old than infants born appropriate for gestational age[350]. In a British cohort of infants born SGA, researchers found that individuals at follow-up ages of 5, 10 and 16 years of age had significant reductions in academic achievement, and were more likely to be within the lower ranks of school performance and to be recommended for special education. At 26 years of age, while those born SGA showed no differences in school completion or employment than people born at an appropriate size, they were less likely to be in professional or managerial jobs and earning less income[67]. Analysis of conscripted males in Sweden found that term delivered SGA males were at an increased risk of poor school achievement and cognitive performance at 17 years of age, and that males had a lower IQ score than females [66, 351].

Analysis of hippocampal sections from growth restricted guinea pig showed a reduction in CA1 neurons compared to control neonates[131]. Reductions were also

seen in the overall volume of the hippocampus, particularly in the stratum oriens, with increased ventricular volume. In a rabbit model of IUGR, Eixarch and co-workers found poor behavioural outcomes of 1 day old neonates correlated with changes in regional fractional anisotropy of various brain areas compared to control animals, including the hippocampus, using MRI imaging[62]. This suggests that growth restriction induced reorganisation of regional microstructures within the fetal brain, which, along with the reductions in cell numbers and brain volumes, may contribute to the associated behavioural deficits of affected children. MRI imaging of neonatal rat brains at postnatal age (P) 7, P14, P21 and P28 also supported the sustained reduction in total brain volume of IUGR neonates, yet also showed either a delay in myelination (as measured by myelin basic protein (MBP) immunostaining) or reduction in available axons at both P7 and P21[288]. This delay and/or reduction in myelination is a common neurodevelopmental deficit associated with IUGR. In a guinea pig model of IUGR, Nitsos and Rees[287] showed that growth restricted fetuses had a delay in the initiation of myelin production, with evidence of myelination occurring in the corticospinal tract of control fetuses at gestational age (GA) 52 days, but none in growth restricted fetuses. At GA62 myelination had commenced in IUGR fetuses, however the number of myelinated fibres were fewer than controls. A rat study using uterine artery ligation showed that moderate (body weight 1-2 standard deviations (SDs) below control average) and severely (>2 SDs below control average) growth restricted pups had less MBP optical density in white matter regions, including hippocampal areas, at P7[107, 108]. This resolved by P14 in moderately growth restricted pups indicating a delay in maturation. However, severely growth restricted pups maintained this reduction in MBP, due to fewer mature oligodendrocytes[107], with this persisting to P60 in rats[108], suggesting the degree of growth restriction differentially impacts on the maturation or loss of oligodendrocytes. Recent studies have shown reductions in MBP within the subcortical white matter in a guinea pig model of growth restriction[105], informing of the detrimental environment a fetus suffers when presented with inadequate placental perfusion. Reductions in myelin and decreased brain volume are suggested to translate long term into behavioural deficits such as ADHD and learning disabilities[352, 353]. Many studies report astrogliosis (as measured by

GFAP staining) as another injurious process occurring in growth restricted brains[107, 108], indicating neural inflammation and damage. However, the upregulation of GFAP is inconsistent in the literature[105, 287], although, this may be due to variability in the severity of placental insufficiency[107].

Stress is a natural physiological response to internal and external events, predominantly characterised by the release of glucocorticoids such as cortisol. During pregnancy, cortisol gradually increases until a large cortisol surge in late gestation, predominantly of maternal origin. The fetal brain uses this steroid in the maturation of neuronal axons and dendritic processes. However, a growing body of evidence is showing that premature exposure to high levels of glucocorticoids, either natural or synthetic, negatively impacts these neurodevelopmental processes. In a rat model of PS[289], pups exposed to high corticosterone levels displayed reduced neurogenesis of hippocampal cells, whilst research from our lab into PS has demonstrated that male fetuses have reduced hippocampal MBP, GFAP and microtubule associated protein 2 (MAP2, a marker of mature neurons), whilst females only showed reduction in MAP2[121]. This suggests that females have mechanisms that tend to protect their macroglia from the degenerative effects of corticosteroids *in utero*, with males having a higher vulnerability to cortisol. This exposure to PS also raises basal levels of cortisol within affected offspring, supporting the contention these increased resting levels may lead to further loss of neural cells. A follow-up study using this guinea pig model showed that PS animals had reductions in MBP as well as GFAP within the hippocampus at adolescent equivalence, irrespective of sex. Thus, male neurodevelopment does not catch up postnatally, and female offspring cannot maintain this protection of glial cells, highlighting that these brains are still vulnerable post *in utero* insult. These PS exposed offspring also displayed increased anxiety-like responses with less inner zone entries in open field testing and less time investigating unknown objects in exploration testing[122]. The amygdala, a limbic structure involved in anxiety and fear behaviours, is hormonally regulated and cortisol release in times of stress can activate this region, which can then signal the hypothalamic-pituitary-adrenal axis to release more cortisol in a feed-forward mechanism[322, 354]. Rat offspring from PS paradigms display altered postnatal developmental

trajectories of the amygdala, with reductions in total volume, length and cell number within amygdalar nuclei[166]. Psychological disorders such as generalised anxiety disorder, post-traumatic stress disorder and ADHD, commonly poor outcomes following pregnancy compromises, have shown abnormal activation of the amygdala[144, 321, 355]. The amygdala is a relatively underexplored brain region in investigating the impact of PS, with little information available on cellular changes during fetal neurodevelopment. Given its involvement in the development of behavioural and emotional disorders throughout life, alterations in the neural environment that may affect cellular composition and predisposition to deficits warrants investigation.

The duration of the stressor, and the time when it occurs during gestation can have differential effects on fetal outcome. Stress events occurring in the first trimester do not show any sex differences in alterations to fetal neurodevelopment. Stressors occurring within the second to third trimester have greater effects on male fetuses with an increased susceptibility to adverse outcomes[86]. Laplante and colleagues investigated the long-term effects of prenatal stress caused by the 1998 Quebec ice storm. They followed women who were pregnant during the storm; or became pregnant within the 3 months following the disaster and investigated the effects of the storm on perinatal and long-term outcomes of the children[82, 80]. Their results showed that neonates born to mothers who reported high levels of stress were of lower birth weight, had a smaller head circumference as well as smaller head circumference to body length ratios, and that males were more greatly affected by negative birth outcomes than females[356]. At 2 years of age, the same cohort of children had impairments in cognition, if prenatal stress occurred in the first or second trimester. There was also a significant reduction in productive and receptive language ability, regardless of when PS occurred during pregnancy, along with less mature functional play[79, 81]. These deficits in cognition and intelligence persisted at 5 ½ years of age. Numerous other studies have found consistently similar results[75, 77, 78, 84], whilst investigating the effects of maternal state and/or trait anxiety, depression, or life events such as loss of work or loved one, on child temperament and development. These data highlight that prenatal

stress has chronic detrimental effects on the offspring, which can impact their quality of life well into adulthood.

The brain possesses innate protective mechanisms to prevent neural loss following injury, with neurosteroids forming a major group of protective hormones. These neuroactive steroids are produced in both peripheral steroidogenic organs, and *de novo* in the brain from cholesterol. During pregnancy, the markedly increased amounts of progesterone act as a precursor that is metabolised to the key neurosteroid, allopregnanolone, by the enzymes 5α-reductases (5αRs) types 1 and 2, and then by the 3α-hydroxysteroid dehydrogenases (3α-HSDs). Allopregnanolone is critical in neurodevelopment as it promotes processes such as myelination by oligodendrocytes[177], prevents apoptosis[220, 221], and protects against excitotoxic cell death via its actions at the γ-aminobutyric acid type A receptor ($GABA_ARs$)[357]. At these receptors, it binds to an allosteric modulatory site to enhance the actions of GABA, leading to cell hyperpolarisation, thus greater action potentials are required to activate neural cells. The placenta is responsible for the majority of allopregnanolone and precursor supply to the fetus for neurodevelopment[167], resulting in much higher concentrations within the fetal brain than any other time in postnatal life.

Acute periods of stress increase local levels of allopregnanolone, concurrently with cortisol concentrations, in fetal sheep brains[197], as well as in adult rat brains[245]. This is proposed as a protective mechanism to protect cells from the deleterious effects of cortisol, as well as restoring GABAergic tone following stress[245]. In a sheep model of placental insufficiency, fetal brain allopregnanolone concentrations are maintained by the upregulation of 5αR2 protein in numerous brain regions including the hippocampus[185], supporting its role as a neuroprotective agent. As well as regulating $GABA_AR$ activity to promote cellular quiescence, it has also been shown to upregulate the expression of GABA synthesising enzymes glutamic acid decarboxylases 65 and 67 (GAD 65 and GAD67, respectively) in Schwann cells, the myelinating glial cells of the peripheral nervous system[199] and this may act to both increase myelination and regulate neuronal excitation. A study in adult rat brain has shown that GAD67 and

5αR2 are co-localised in the hippocampus and amygdala[200]. This suggests that these two enzymes may act together in an autocrine/paracrine manner to regulate neural activity[201], and changes in allopregnanolone may alter the production of GABA.

There is little information on the combined neurodevelopmental effects of IUGR and PS. A substantial number of women with an IUGR fetus may suffer psychological distress, and PS is associated with an increased risk of IUGR. Individually, both conditions can be significantly detrimental to fetal neurodevelopment and the increased risk of cognitive and psychological disorders, therefore it seems important and relevant to investigate the combined neurodevelopmental effects of these two complications. This study aimed to determine if IUGR in combination with PS in a guinea pig model has a cumulative effect on the development of vulnerable brain regions of the hippocampus, subcortical white matter and amygdala of fetal brains. Furthermore, this study intended to examine if the heightened vulnerability of males may lead to a potentiation of the two stresses with greater deficits in neurodevelopment compared to females.

5.3 Methods and Materials

5.3.1 Animals and Intrauterine Growth Restriction Surgery

Time-mated, outbred guinea pigs were acquired from the University of Newcastle Research Support Unit. Guinea pigs were randomly allocated to either control or growth restriction (IUGR) surgery, performed between gestational age (GA) 30 and 35. Surgeries, performed under aseptic conditions, were adapted from previously published methods[267]. Briefly, anaesthesia was induced in dams before a midline incision was made. The uterine arteries at both ovarian and cervical ends of the uterine horns were located and fat was carefully dissected away. To induce growth restriction, sterilised medical-grade silicon tubing (Gecko Optical, Perth, WA, Aus; 4mm length, tube wall thickness 1mm, one side cut lengthways for opening) was placed around the artery at both the cervical (internal diameter

1.5mm) and ovarian ends (internal diameter 1.5-2mm). Internal diameter size was selected such that the tubing should not restrict blood flow at time of placement and could be moved freely along the artery initially. This procedure was performed on both horns of the uterus. Tubing was secured in place using sterile suture tied around the external wall to prevent slippage. Pregnant guinea pigs allocated to the control protocol had their uterine arteries manipulated in the same manner, however did not have tubing placed around the arteries. The uterus was returned to its original position before the incision was sutured closed and dam allowed to recover.

5.3.2 Prenatal Stress Induction

IUGR dams were further allocated to either control or stress protocols, commencing GA40 and repeated on GA45, 50, 55, 60 and 65. Stress was induced using previously published protocols[121, 122, 269, 270]. Briefly, guinea pigs allocated to the stress procedure were placed inside a dark box with a strobe light for two hours, in their home cages with food and water provided *ad libitum*. Control and non-stressed IUGR animals remained in their home cages in normal holding facilities and were not exposed to the strobe light. All guinea pigs had saliva collected via cotton tips immediately prior and post stress/control timings[121, 122, 265].

5.3.3 Fetal Physical Measurements

Fetal tissue collection occurred at GA69 (term ~71 days), or on determination that the pubic symphysis had begun separation and had reached >2cm for 2 consecutive days (indication of imminent labour)[110, 260]. All fetuses were sexed, with physical measurements collected along with body, organ, peripheral (brown adipose tissue (BAT) of neck and back) and visceral (kidney BAT) fat weights. Plasma and brain were collected as previously described[104]. Due to ethical constraints on animal numbers allowed for this study, multiple fetuses per pregnancy were used. Some naturally occurring growth-restricted fetuses were included in this study (n=2 males, and n-2 females; fetal placement within the uterus did not differ between naturally occurring and surgically induced growth restricted fetuses), with appropriately grown siblings used as controls to allow for

greater use of animals. Fetuses less than the 25th percentile for weight[271-273] (below 75g within our colony, unpublished data) and/or a brain-to-liver ratio (BLR) of greater than 0.9[105] (indicating asymmetrical growth) were classified as growth restricted.

5.3.4 Radioimmunoassay for Allopregnanolone

Plasma and placental allopregnanolone concentrations were determined using radioimmunassay (RIA) as previously published[105, 110]. Plasma and placental tissue were treated with 1% acetic acid and 50% methanol and homogenised before being added to Sep-Pak$_{18}$ cartridges. Plasma was washed with graded methanol before oxidation using 5% potassium permanganate to reduce cross-reactivity of progesterone in samples, before re-extraction with 50%v/v diethyl-ether/n-hexane. Concentrations of allopregnanolone were quantified using polyclonal antibody to allopregnanolone (Agrisera, Sapphire Bioscience, Vannas, Sweden). Tritium-labelled allopregnanolone tracer (5α-[9, 11, 12, ^{3}H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) was used to determine sample recovery concentrations. Average recovery of allopregnanolone was 74.3±1.5% for plasma and 55.4±0.70% for placenta, with individual recovery values used to account for extraction loss and determine final allopregnanolone concentrations. Radioactivity was measured using a β-counter (LS65000, Beckman Coulter Australia Pty Ltd, Sydney, NSW, Australia). Limit of detection was 25pg/mL, with inter-assay and intra-assay coefficients were 9.39 and 2.75% respectively.

5.3.5 Brain Immunohistochemistry

Fixed, paraffin-embedded brain sections (8μm) were dewaxed, incubated in Citrate buffer (10mm, pH 6.0) for antigen retrieval and immunostained for myelin basic protein (MBP) as a marker of mature myelination, microtubule-associated protein 2 (MAP2) as a marker of mature neuronal processes, glial fibrillary protein (GFAP) as a marker of astrocyte activation and glutamate decarboxylase 67 (GAD67), a stain for GABA-producing cells. All samples were blocked for one hour (2% normal goat serum, 0.4% BSA, 0.3% Triton X-100), at room

temperature, followed by primary incubation in mouse anti-MAP2 (1:20 000, M9942, Sigma,); mouse anti-GFAP (1:1000, G3893, Sigma); mouse anti-GAD67 (Abcam, 1:1000) and rat anti-MBP (1:100, M9434, Sigma) overnight at room temperature. Secondary antibody incubation for MAP2, GFAP and GAD67 was anti-mouse (1:300, B6649, Sigma) and for MBP, anti-rat (1:300, B7139, Sigma) for 1 hour at room temperature. Tertiary antibody, Streptavidin-horseradish peroxidase (1:4001, ab7403, Abcam) was incubated on sections for 1 hr at room temperature. Slides were stained in chromagen 3,3'-diaminobenzidine tetrahydrochloride solution (metal enhanced DAB substrate kit #34065; Pierce, ThermoFisher Scientific, Scoresby, Australia). Slides were then mounted using DEPX (Merck, Kilsyth, Vic, Australia) and imaged using Leica Aperio AT2 imager (Leica Biosystems). Regions of interest were captured using Imagescope software (Leica Biosystems) at 20x magnification. MBP and GFAP immunostaining were examined in the CA1 of the hippocampus and adjacent subcortical white matter (SCWM), MAP-2 and GAD67 were imaged in the CA1 alone. Within the amygdala, GFAP, MAP2 and GAD67 were imaged in the basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA). The immunoreactivities of MBP, GFAP and MAP2 were analysed by densitometry using ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA) and made binary by adjusting the threshold manually. GFAP, MBP and MAP2 were expressed as percent (%) area of coverage recorded for 4 fields of view per region on two sections per animal. GAD67 cell counts were collected using ImageJ, using 4 fields of view for each region and two sections per animal.

5.3.6 Statistical Analysis

Maternal pregnancy characteristics were analysed by one-way ANOVA. A generalised estimating equation (GEE) was used to compare prenatal group (control, IUGR or IUGR+PS) and sex of the fetus (male or female). Where a significant effect of both group and sex were identified, an interaction term (Prenatal Group*Sex) was included in the analysis. Statistical significance was determined using the Wald-Chi squared type III test, and post-hoc pairwise comparisons to identify between group differences with an alpha of $p<0.05$ to identify an effect. In-text data for pairwise comparisons are reported as the

estimated difference between the group means; 95% Wald confidence intervals of the difference (CIs); and p-values. Unless specified, all data in tables are presented as estimated marginal means (95% CI), and graphs are represented as mean±SEM. Statistical analysis was performed using IBM SPSS Statistics software (version 24; SPSS Inc.; IBM Corporation, Armonk, NY, USA). Graphs were created using GraphPad Prism software (version 7.0a, GraphPad Software Inc., La Jolla, CA, USA).

5.4 Results

5.4.1 Presentation of Labour

Dams allocated to IUGR+PS protocols presented with signs of labour prior to GA69 which necessitated tissue collection significantly earlier than control dams (1.15; 0.35, 2.26; p=0.042, table 5-1). There were no differences between the groups for the number of fetuses at the time of surgery. Nor did IUGR or the addition of PS affect the percent of surviving fetuses at the time of collection.

TABLE 5-1 MATERNAL PREGNANCY CHARACTERISTICS

	Prenatal Group		
	Control	*IUGR*	*IUGR+PS*
Gestational Age on Signs of Labour	68.25 (67.8, 68.84)	67.57 (66.84, 68.3)	67.1 (66.31, 67.89) *
Number of Fetuses at Surgery	3.33 (1.62, 5.05)	3.5 (2.93, 4.07)	3.8 (3.14, 4.46)
Survival of Fetuses at Collection	87.78 (67.8, 107.77)	80.57 (64.29, 96.84)	94.0 (84.34, 103.57)

GESTATIONAL AGE (GA) OF GROUPS AT END OF EXPERIMENT: MAXIMUM OF GA69 OR BASED ON THE ONSET OF LABOUR (PUBIC SYMPHYSIS DILATATION >2CM FOR 48HOURS). PREVIOUS STUDIES HAVE SHOWN THAT DELIVERY IS IMMINENT AFTER THIS TIME[110, 260]. GESTATIONAL AGE ON SIGNS OF LABOUR IS IN DAYS; NUMBER OF FETUSES AT SURGERY IS IN ARBITRARY UNITS; SURVIVAL OF FETUSES AT SURGERY IS PERCENT. DATA ANALYSED BY ONE-WAY ANOVA. *P<0.05 IUGR+PS VS. CONTROL.

5.4.2 Fetal Physical Measures

There was a significant effect of prenatal group on body weight ($p<0.001$), peripheral ($p=0.011$) and visceral fat ($p<0.001$), brain ($p=0.003$), liver ($p<0.001$) and placenta ($p<0.001$) weights, nose-rump length ($p<0.001$) and brain-to-liver ratio (BLR, $p<0.001$) between control, IUGR and IUGR+PS fetuses. IUGR and IUGR+PS did not affect hippocampal weight (Table 5-2).

IUGR male and female fetuses were found to have reduced body weight, nose-rump length, visceral and peripheral fat weight, as well as brain, adrenal, kidney and liver weight. Male and female IUGR fetuses also had significantly greater BLR compared to control fetuses. IUGR and IUGR+PS fetuses did not differ in their physical measures. Physical parameters did not differ between naturally occurring and surgically restricted fetuses. IUGR+PS fetuses also displayed similarly reduced parameters, however, they did not differ in brain or adrenal weights in relation to control fetuses (summarised in table 2; see supplementary table S1 for mean comparative difference, 95% Wald CI and p-values).

TABLE 5-2 PHYSICAL CHARACTERISTICS OF CONTROL, IUGR AND IUGR+PS FETUSES AT TERM

	Females			**Males**			**Effect of group**	
	Control (n=5)	*IUGR (n=6)*	*IUGR+PS (n=6)*	*Control (n=6)*	*IUGR (n=6)*	*IUGR+PS (n=6)*	*Control vs. IUGR*	*Control vs. IUGR+PS*
Body Weight (g)	94.0 (84.49, 103.51)	66.23 (58.91, 73.54)	63.95 (56.60, 71.30)	102.08 (93.51, 110.65)	66.51 (57.47, 75.53)	59.76 (51.44, 68.08)	***	***
Nose-Rump (cm)	15.47 (14.42, 16.52)	13.15 (11.96, 14.34)	14.02 (13.19, 14.86)	16.56 (15.66, 17.45)	13.63 (13.14, 14.11)	13.55 (12.8, 14.3)	***	***
Visceral BAT (g)	0.494 (0.37, 0.62)	0.699 (0.56, 0.84)	0.392 (0.24, 0.54)	0.885 (0.71, 1.06)	0.529 (0.4, 0.66)	0.466 (0.35, 0.58)	**	***
Peripheral BAT (g)	1.467 (1.01, 1.92)	1.089 (0.88, 1.29)	0.882 (0.73, 1.04)	1.509 (1.13, 1.89)	0.802 (0.29, 1.32)	0.903 (0.74, 1.06)	*	**
Brain Weight (g)	2.27 (2.21, 2.32)	2.13 (2.03, 2.23)	2.255 (2.18, 2.33)	2.3 (2.22, 2.84)	2.235 (2.17, 2.3)	2.206 (2.08, 2.33)	**	ns
Hippocampus Weight (g)	0.088 (0.07, 0.10)	0.074 (0.05, 0.10)	0.091 (0.08, 0.10)	0.090 (0.08, 0.10)	0.089 (0.08, 0.09)	0.089 (0.08, 0.10)	ns	ns
Placental Weight (g)	5.69 (4.64, 6.74)	3.63 (3.2, 4.06)	3.87 (3.38, 4.36)	5.6 (5.92, 7.28)	4.16 (3.56, 4.76)	3.93 (3.47, 4.36)	***	***
Adrenal Weight (g)	0.036 (0.03, 0.04)	0.022 (0.02, 0.03)	0.026 (0.02, 0.03)	0.032 (0.03, 0.04)	0.022 (0.02, 0.03)	0.027 (0.01, 0.04)	**	ns
Kidney Weight (g)	0.74 (0.68, 0.80)	0.52 (0.42, 0.61)	0.56 (0.49, 0.62)	0.78 (0.69, 0.86)	0.55 (0.53, 0.58)	0.53 (0.47, 0.59)	***	***

Liver Weight (g)	4.42 (3.56, 5.35)	2.49 (2.11, 2.88)	2.661 (2.18, 3.14)	5.11 (4.77, 5.45)	2.62 (2.12, 3.11)	2.28 (1.81, 2.75)	***	***
Brain-to-Liver Ratio	0.53 (0.39, 0.67)	1.18 (0.68, 1.67)	0.91 (0.73, 1.08)	0.45 (0.37, 0.52)	0.98 (0.80, 1.16)	1.15 (0.84, 1.45)	***	***

ALL MEASUREMENTS ARE PRESENTED IN METRIC UNITS, EXCEPT BRAIN-TO-LIVER RATIO WHICH IS PRESENTED AS A RATIO VALUE OF BRAIN AND LIVER WEIGHTS. THIS VALUE INDICATIVE OF ASYMMETRICAL GROWTH RESTRICTION (BRAIN-SPARING) WHEN THE VALUE IS >0.9. PERIPHERAL BAT REFERS TO BROWN ADIPOSE TISSUE (BAT) ON THE FETUS' NCEK+BACK. VISCERAL BAT REFERS TO BAT ENCASING KIDNEYS. DATA PRESENTED AS MEANS (95%CI). *P<0.05; **P<0.01; ***P<0.001 FOR POST-HOC PAIRWISE COMPARISON BETWEEN CONTROL VS. IUGR, AND CONTROL VS. IUGR+PS GROUPS (MALE AND FEMALE FETUSES COMBINED). NO DIFFERENCES WERE FOUND BETWEEN IUGR AND IUGR+PS FETUSES.

5.4.3 Plasma and Placental Allopregnanolone Concentrations

There was no interaction between group and sex, however there was a significant effect of group alone (p=0.003, figure 5-1a), thus sexes were combined in each group. Pairwise comparison found less circulating allopregnanolone in IUGR (p=0.001) and IUGR+PS fetuses (p=0.029) than control fetuses. No differences in placental allopregnanolone concentrations were identified between control, IUGR or IUGR+PS fetuses (figure 5-1b).

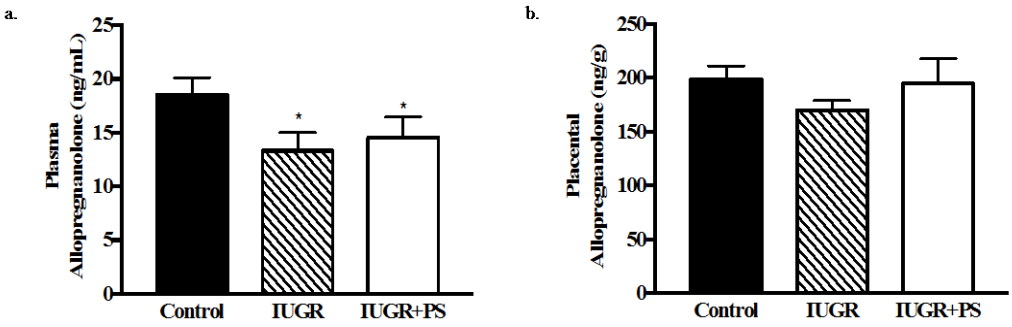

FIGURE 5-1 CIRCULATING PLASMA AND PLACENTAL TISSUE ALLOPREGNANOLONE CONCENTRATIONS IN CONTROL (BLACK BARS, N=10); IUGR (HASHED BARS, N=10); AND IUGR+PS (WHITE BARS, N=12) FETUSES. IUGR AND IUGR+PS FETUSES HAD SIGNIFICANTLY LESS CIRCULATING ALLOPREGNANOLONE THAN CONTROL (P=0.001 AND P=0.029, RESPECTIVELY; A). PLACENTAL ALLOPREGNANOLONE CONCENTRATIONS DID NOT DIFFER BETWEEN GROUPS (B). DATA ANALYSED BY GEE WITH GROUP COMPARISON. GRAPHS PRESENTED AS MEAN ± SEM. *P<0.05.

5.4.4 Hippocampal Immunohistochemistry

Significant interaction of prenatal group and sex was observed for MBP area coverage in the CA1 region of the hippocampus ($p<0.001$). IUGR and IUGR+PS male fetuses had significantly less MBP area coverage in the hippocampal CA1 region compared to control males ($p=0.005$; and $p<0.001$ respectively, figure 5-2). No difference in CA1 MBP coverage was identified between IUGR and IUGR+PS fetuses. An interaction of prenatal group and sex was seen in the subcortical white matter (SCWM; $p=0.033$). IUGR males had less MBP area coverage in the SCWM compared to controls ($p=0.003$), IUGR+PS males did not differ to control or IUGR alone males. No differences were found between females from any group in MBP area coverage in either region.

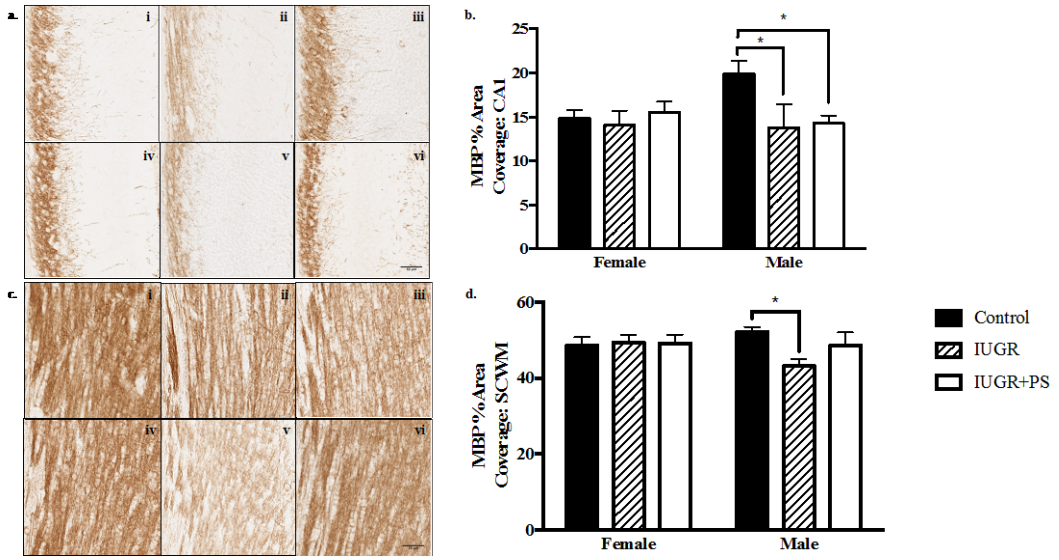

FIGURE 5-2 % AREA COVERAGE OF MYELIN BASIC PROTEIN (MBP) IN THE CA1 REGION OF THE HIPPOCAMPUS AND SUBCORTICAL WHITE MATTER (SCWM). REPRESENTATIVE MICROGRAPHS OF HIPPOCAMPAL CA1 (A) AND SCWM (C) MBP AREA COVERAGE FOR CONTROL (FEMALE (I); MALE (IV)); IUGR (FEMALE (II); MALE (V)) AND IUGR+PS (FEMALE (III); MALE (VI)) BRAINS. % AREA COVERAGE OF CA1 (B) AND SCWM (D) OF THE HIPPOCAMPUS IN CONTROL (BLACK BARS), IUGR (HASHED BARS), AND IUGR+PS (WHITE BARS) FETUSES. N=5 FOR MALES AND FEMALES IN EACH GROUP. DATA ANALYSED BY GEE WITH PAIRWISE COMPARISONS. GRAPHS PRESENTED AS MEAN ± SEM. SCALE BARS = 50μM. *P<0.05.

No differences were seen in GFAP area coverage in the CA1 region or SCWM of control, IUGR or IUGR+PS fetuses from either sex (figure 5-3a and b). Similarly, no changes in area coverage of MAP-2 was found in the hippocampal CA1 (figure 5-3c) region between any prenatal group, nor in GAD67+ cell numbers (figure 5-3d). Naturally occurring growth restricted fetuses were not at the extremes of neurodevelopmental proteins assessed.

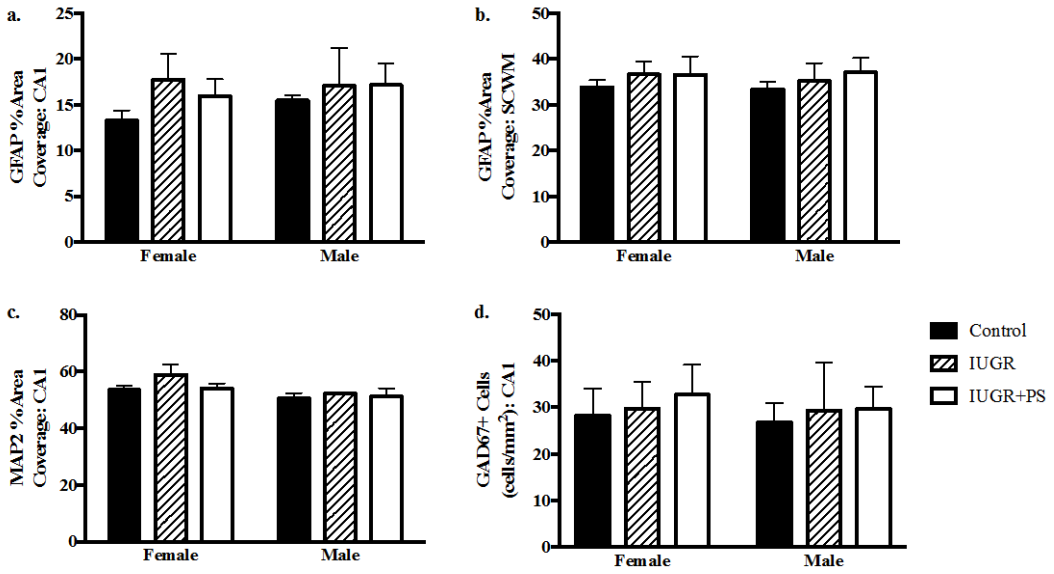

FIGURE 5-3 ASTROCYTE, NEURON AND GABAERGIC MARKERS IN THE HIPPOCAMPUS. % AREA COVERAGE OF GLIAL FIBRILLARY ACIDIC PROTEIN IN THE CA1 REGION (A) AND SUBCORTICAL WHITE MATTER (B) OF THE HIPPOCAMPUS; MICROTUBULE-ASSOCIATED PROTEIN 2 IN THE CA1 REGION (MAP2; C); AND POSITIVE STAINING OF GAD67 CELLS WITHIN THE CA1 (CELLS/MM^2; D). NO DIFFERENCES WERE FOUND IN GFAP AND MAP2 % AREA COVERAGE; NOR IN THE NUMBER OF GAD67+ CELLS WITHIN THE HIPPOCAMPAL REGION FOR CONTROL ANIMALS (BLACK BARS); IUGR (HASHED BARS); OR IUGR+PS FETUSES (WHITE BARS). FEMALES N=5; AND MALES N=5 FOR EACH GROUP. DATA ANALYSED BY GEE WITH PAIRWISE COMPARISONS. GRAPHS PRESENTED AS MEAN ± SEM. *P<0.05.

5.4.5 Amygdala Immunohistochemistry

No differences in GFAP or MAP2 % area coverage were found in the BLA or CeA of the amygdala. Additionally, no changes were identified in GAD67+ cell numbers in these regions between control, IUGR and IUGR+PS fetuses (data not presented).

5.5 Discussion

This study is the first to investigate the combined effects of intrauterine growth restriction and prenatal stress on allopregnanolone concentrations and fetal brain development. The overall findings of this study is that compromised male fetuses demonstrated decreased hippocampal MBP expression. Interestingly, the addition of prenatal stress seemed to induce a protective effect on subcortical white matter in male fetuses; and on brain and adrenal weights of IUGR fetuses. However, growth restriction, alone or in combination with prenatal stress, did not inhibit the gross neurodevelopmental markers of GFAP, MAP2 and GAD67. This is supported by previous work from our lab showing astrocyte reactivity was not impacted by growth restriction in a previous model used by our group[105].

This study showed a male vulnerability to pregnancy compromises within white matter. Reductions in mature myelination area coverage in the CA1 of male IUGR and IUGR+PS hippocampi, as well as the overlying SCWM were found in IUGR males only. It is well established that any compromise in pregnancy, including IUGR and PS, leads to reductions in myelination[90, 265, 272, 358]. Not only is there a reduction and/or delay in myelination following IUGR *in utero*, there is also loss of oligodendrocyte precursors[108]. Myelin is critical for the signal transduction and connectivity of neurons. Deficits in myelination in adolescence and adulthood are associated with poor school performance in childhood, as well in disorders such as schizophrenia[359] (a disorder in which males have a higher risk of developing[360, 361]) and major depression[362]. Therefore, the loss of myelination in infants that develop IUGR, paired with *in utero* severity and the degree of underdevelopment postnatally, is a major determining factor in the development of later life behavioural and cognitive disorders.

The fetuses in this study had reduced circulating allopregnanolone levels, however placental synthesis was not impacted by IUGR or IUGR+PS. This suggests an impairment of allopregnanolone and/or precursor transfer from placenta to the fetus. Despite these reductions in circulating allopregnanolone,

females were able to preserve myelination within the CA1 of the hippocampus and SCWM. Interestingly, there was no additive effect of PS to damage caused by IUGR within the SCWM of males, however, it seemingly conferred a protective effect on the maturation of myelination in these fetuses. Acute stressors, including hypoxia and psychological stress are known to increase allopregnanolone concentrations[198, 363]. The combination of IUGR and PS may have induced a stress event that could have increased levels of allopregnanolone periodically enough to preserve myelination in this region. As allopregnanolone is neuroprotective against excitotoxic cell death, potential changes in local production in the brain paired with changes in myelin structure and receptor subunit composition in response to IUGR may be programming the brain for a greater predisposition in the development of later life disorders commonly associated with pregnancy compromises. Whilst allopregnanolone is a key neurosteroid of pregnancy, there are other 5α, 3α-derived neurosteroids, including adrenal derived 5α, 3α-tetrahydrodeoxycorticosterone (THDOC), and androgen derived 3α-androstanediol (3α-diol). These are also positive allosteric modulators of the $GABA_AR$[364, 365]. Stress is known to increase circulating concentrations of deoxycorticosterone (DOC) as well as cortisol released from the adrenal glands[245]. The same enzymes that convert progesterone to allopregnanolone, convert DOC to THDOC, where it can act as an anxiolytic and anticonvulsant at the $GABA_AR$ by similar mechanisms as allopregnanolone[366]. We did not measure THDOC in these animals, however, given that prenatal stress causes a chronic increase in cortisol within the fetus[367], we cannot exclude that there is a potential upregulation of THDOC in these IUGR+PS animals that may be providing a protective effect not seen in the males exposed to IUGR alone. Nor can we exclude any effects prenatal stress may have on the levels of 3a-diol within the male brain.

Exposure to elevated glucocorticoid concentrations in fetal life can program the hypothalamic-pituitary-adrenal (HPA) axis. Previous studies in guinea pigs have shown that prenatal stress resulted in decreased MAP2, GFAP and MBP in the fetal hippocampus, particularly among male fetuses[121]. At the equivalence of late childhood, offspring of stressed guinea pigs showed increases in anxiety-like

behaviour and reductions in myelination and astrocyte activation in the hippocampus, regardless of sex[122]. Other researchers using the same model demonstrated that prenatally stressed male offspring had higher resting cortisol concentrations than control males, and also an exaggerated cortisol response on HPA stress testing[269]. Conversely, prenatally stressed female guinea pig offspring exhibited a blunted cortisol release on exposure to a stressor along with increased glucocorticoid receptor (cortisol target receptor) mRNA expression[270]. This is consistent with human studies of chronic stress exposure that show males with PTSD have higher salivary cortisol levels during and following cognitive challenge than healthy males and females with PTSD[337]. A blunted cortisol response appears to be a female HPA adaptation to chronic stressors with human data in young adult females with PTSD or major depression having an attenuated cortisol response to social stress testing[338]. However, the model in this study identified that the combination of IUGR and prenatal stress conferred a protective effect on neurodevelopment compared to the previous stress alone studies, with preservation of GFAP, MAP2 and GAD67 in both the hippocampus of male and female fetuses. There was also restoration of MBP in the SCWM of males. This suggests that IUGR+PS is less damaging than IUGR alone, and based on previous studies, better than PS alone[121, 122, 265]. Further work will be required to investigate if the combination of PS and IUGR improves behaviour and cognition compared to the known deficits in IUGR offspring and PS offspring[84, 368-371].

The neurodevelopment as measured by MAP2 and GFAP, and the expression of GABA synthesising enzyme GAD67 was not altered in the amygdala of IUGR or IUGR+PS fetuses. The amygdala commences and ceases development early in gestation, compared to the hippocampus which develops throughout the entirety of pregnancy[39]. This suggests that during the period of IUGR and prenatal stress induction, the window of vulnerability may have already passed for this region, resulting in no structural changes in GFAP and MAP2 in the CeA and BLA. Despite the development of the amygdala being completed prior to the onset of insults, IUGR fetuses remain more likely to develop neuropsychological disorders, such as ADHD, schizophrenia and anxiety in later life[349, 372]. We didn't find differences in mature neuron dendrite area coverage in this study, however

these markers do not account for cell functionality. MRI data in brains of children born following IUGR, compared to appropriately grown controls[373], show that IUGR alters global and regional connectivity within the brain, similar to changes in signaling seen in ADHD and schizophrenia[374, 375]. The finding of the present work does not exclude potential changes in the receptors of neurons and glia in these areas, which would not be reflected in the structural proteins investigated.

The method of induced growth restriction in the current studies is a less severe insult at the time of surgery, compared to previous artery ligation and ablation models. The restriction of blood flow from the uterine artery is gradual[266, 267], as opposed to instantaneous in previous models[131, 376, 377], and we contend is more representative of human onset of IUGR than the previous methods. The changes seen in MBP and allopregnanolone in this model may reflect a late onset IUGR, more than previous models that display changes in neuronal markers reflecting an early onset[131, 378]. However, many studies, particularly involving children from premature deliveries, suggest that even offspring with seemingly normal neurodevelopment following an impaired *in utero* environment are still at a greater risk of developing disorders such as schizophrenia, ADHD, depression and anxiety. The model used here may provide greater insight with longitudinal studies, in investigating the changes of the neural microenvironment and changes in behaviour at neonatal and juvenile equivalence, and whether the seemingly neuroprotective effects of PS in IUGR is beneficial to behavioural development at these time points. This in turn may aid the development of interventions or treatments to prevent the onset of these problems within this vulnerable population.

6 INCREASED PLACENTAL NEUROSTEROIDOGENIC GENE EXPRESSION PRECEDES POOR OUTCOME IN THE PRETERM GUINEA PIG

This chapter investigated the difference in neurosteroidogenic enzyme gene expression in the placenta from preterm guinea pig neonates who did and did not survive the first 24 hours of life, to determine if placental expression may be an indicator neonatal outcome. This article was published in *Journal of Developmental Origins of Health and Disease* (2014;5(02):74-8), as a brief report.

For consistency, this article is presented with minor edits to the original manuscript.

STATEMENT OF AUTHOR CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
Angela L. Cumberland	Experimental design Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K. Palliser	Experimental design Animal protocols and tissue collection Laboratory procedures Data analysis Manuscript corrections	
Jonathan J. Hirst	Experimental design Data analysis Manuscript corrections	

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6.1 Abstract

Placental 5α-reductase (5αR) is influenced by in utero compromises and has a role in regulating neuroactive steroid concentrations in the fetus. The objective of this study was to determine if changes in placental 5α-reductase (5αR) were associated with neonatal outcome after birth. Guinea pigs were delivered by caesarean section at term (GA69, n=22) or preterm (GA62, n=36, human equivalent ~34 weeks gestation) and the placenta collected. Preterm neonates were maintained for 24 hours unless their condition deteriorated before this time. Enzyme mRNA expression of 5αR type-1 and 5αR type-2 were determined using real-time PCR. All preterm neonates had significantly higher 5αR2 expression in their placenta compared to placentae from term neonates (p<0.0001). Expression was also markedly higher in the placentae from neonates that did not survive until 24hrs, compared to surviving preterm neonates (p=0.04). These findings suggest differences of in utero neurosteroidogenic capacity between surviving and non-surviving preterm guinea pig neonates. The increased 5αR2 mRNA expression in the placenta of non-survivors suggests an induction of the neurosteroid pathway due to prior exposure to an in utero compromise, with such exposure possibly a predisposing factor that contributed to their poor ex utero outcome.

6.2 Introduction

Placental steroidogenesis has a key role in regulating levels of neuroactive steroid concentrations in the fetus during late pregnancy [379]. These steroids include the progesterone derivative 3α-hydroxy-5α-pregnan-20-one (allopregnanolone). Allopregnanolone is a potent modulator of gamma aminobutyric acid type A ($GABA_A$) receptors and markedly influences their inhibitory activity. This action suppresses CNS activity and maintains the generally low levels of excitability that typifies fetal life in late gestation. Neuroactive steroids also inhibit apoptotic pathways, promote synaptogenesis, and stimulate proper glial cell activity and myelin formation by oligodendrocytes [177, 187, 380], all of which are critical for optimal fetal brain development.

Progesterone, supplied by the placenta, is essential for the maintenance of pregnancy with levels increasing steadily in the maternal circulation as gestation progresses [167]. The placenta also makes a major contribution to circulating concentrations of allopregnanolone, with levels in maternal and fetal plasma also increasing towards term[381]. 5α-reductases type 1 and 2 (5αR1 and 5αR2) catalyse the rate-limiting step in the metabolism of progesterone into intermediate hormone 5α-dihydroprogesterone. The enzyme 3α-hydroxysteroid dehydrogenase then reversibly converts this precursor to allopregnanolone. These isoforms have low homology and their activity differs with development. The type 1 isoform is found in many tissues at relatively stable levels, whereas type 2 is found mostly within the sex organs, the brain and the placenta during pregnancy and shows greater developmental regulation [382, 383]. Both 5αR isoforms are expressed in the human placenta with expression increasing with advancing gestation [255]. After birth, and subsequent removal of the placental supply of progesterone and its metabolites, concentrations of neuroactive steroids fall dramatically in maternal as well as neonatal plasma of humans and sheep [167, 384].

Pharmacological inhibition of neuroactive steroid production by finasteride, a 5αR inhibitor, during pregnancy reduces allopregnanolone concentrations resulting in marked reductions in myelination, and an increase in cell death within fetal sheep and guinea pig brains [105, 220]. Acute and chronic in utero stressors, including hypoxic episodes and intrauterine growth restriction, are associated with poor neonatal outcome and adverse effects on long term health [385, 386]. Previous studies in sheep and rats have shown that these insults raise fetal brain 5αR enzyme protein expression, as well as central and circulating neuroactive steroid concentrations [197, 387]. These findings suggest an upregulation of 5αR enzyme expression following compromise, leading to higher levels of protective neuroactive steroids acting to minimise adverse effects in the fetus.

Preterm birth is a leading cause of infant morbidity and mortality [388] and may lead to neurological and behavioural deficits [37]. However, these complications may not arise until later in neonatal life or childhood. In addition, chronic in utero

stressors that often precede preterm birth may potentiate adverse outcomes after premature delivery [389].

These stressors may raise neuroactive steroid production in the placenta; with a consequent reduction in the risk of developing neurological deficits, as occurs in the brain [185]. Therefore, elevated placental 5αR expression may indicate prior in utero fetal stress and thus provide a marker of in utero compromise and subsequently potential problems in neonatal or juvenile life. The objective of this study was to determine 5αR mRNA expression in placenta from term and preterm guinea pig neonates and compare expression with their outcome during the immediate 24 hours after delivery, and to elucidate whether elevated expression was seen in neonates with poor outcome.

6.3 Methods and Materials

6.3.1 Animals and Preterm Delivery

Time-mated, tri-color, outbred pregnant dams were obtained from the University of Newcastle Research Support Unit. Dams were randomly allocated to either a term delivery (GA69) or preterm delivery (GA62) group. Neonates were delivered no more than 24h apart within the term and preterm groups (68-69 and 62-63 days respectively). Term deliveries were performed as close to spontaneous term labour (approximately 71 days for our colony) as possible. Animals that displayed signs of cervical change predictive of delivery (assessed by pubic symphysis separation) were delivered at GA68 to avoid using animals in active labour.

Previous pilot studies from our group have seen a maximum survival of 5% of preterm guinea pig neonates delivered at GA62 without maternal betamethasone administration. Thus, all dams received 1mg/kg of betamethasone (Celestone Chronodose, Schering-Plough, North Ryde, NSW, Australia) subcutaneously 24 hours and 12 hours prior to delivery, a dosing regime that improves survivability as is seen in humans. Anaesthesia was induced in dams in a chamber with 4% isoflurane in 6L medical grade oxygen. Anaesthesia was maintained via mask

inhalation of 2% isoflurane in 2L oxygen, whilst pups were delivered via caesarean section. Following delivery dams were euthanized, and the placenta from each pup was weighed and snap frozen in liquid nitrogen.

All neonates had their airways cleared of fluid and received 50μL of surfactant (Curosurf 80mg/mL Poractant alfa, Douglas Pharmaceuticals, Baulkam Hills, NSW, Australia). Neonates received thermal support by heat lamps and pads and received a short period of continuous positive airway pressure (CPAP) to aid in establishing functional residual capacity. Once stable breathing was achieved (<2 hours) all guinea pig neonates were weighed and sexed before being housed in a humidified incubator and monitored continuously. Neonates received 24 hour care, and were scored every 2 hours for their ability to maintain respiration, posture and activity. The scoring system was based on a scale of 1 to 4 for each criterion, with 1 being poor and 4 being good. A total score out of 12 was given to each neonate at the time of scoring. Preterm neonatal guinea pigs that did not survive to 24 hours, due to cessation of respiration, formed a preterm non-survivor experimental group. Neonates who died within the first 2 hours after delivery were excluded from this study (term n=0, preterm n=8). All remaining neonates were euthanized at the 24-hour end point, and one male and one female neonate per litter used in this study, resulting in 3 study groups – term (n=22; male n=12, female n=10), preterm surviving (n=21 male n=10, female n=11) and preterm non-surviving (n=15 male = 8, female n=7) neonates.

6.3.2 Real-Time PCR

Frozen placental tissue was crushed on dry ice and RNA was extracted using a commercial kit (RNeasy Plus Mini Kit, Qiagen Pty Ltd, Chadstone, VIC, Australia) and quality tested before undergoing reverse transcription. Placental RNA (1μg) was reversed transcribed to cDNA using Superscript III First Strand Synthesis Reverse Transcription kit (Invitrogen, Life Technologies Pty Ltd, Mulgrave, VIC, Australia). Real-time PCR, using SYBR green detection, was performed using primer sequences previously designed and validated by our group to detect guinea pig 5αR1 and 5αR2 sequences [104]. Relative fold changes in

expression were determined using the $-2^{\Delta\Delta Ct}$ method, with expression data normalised to β-actin for each sample.

6.3.3 Statistical Analysis

Statistical significance was set at p=0.05. A Fishers exact test was used to assess survival data from the entire cohort of animals. Differences between means were analysed by one-way ANOVA with subsequent Tukey multiple comparisons test to determine statistically significant differences between term, preterm survivor and preterm non-survivor placenta samples. All data are presented as mean ± SEM.

6.4 Results

No statistically significant sex differences were found between neonates within the term and preterm groups and hence male and female data has been merged. Term pregnancies were delivered at 68.3 ± 0.2 days whilst preterm neonates were delivered at 62.2 ± 0.1 days. The gestational age at delivery of those neonates who did not survive the immediate neonatal period was not significantly different to those preterm neonates who did survive (62.4 ± 0.2 vs. 62.0 ± 0.2 days respectively, p=0.30). The range of survival for non-surviving neonates was 3 to 22 hours, with a mean survival time of 8.16 ± 1.24 hours. Whilst the survival rates showed no significance between males and females, sex ratios of surviving versus non-surviving neonates were 1:1.5 for males and 1:1 for females for the overall cohort. Survival rates of the neonates were independent of the litter, with no patterns of reduced survival within litters.

No differences were found among placental weights collected from term and preterm guinea pigs. As expected, body weight (post mortem) of term guinea pig neonates (83.1±2.9g) was significantly more than preterm survivors and non-survivors (64.0±1.9g and 62.9±1.2g respectively, p <0.0001). Body weight of the neonates did not differ significantly from time of delivery to the time of post mortem. Non-surviving preterm neonates had a reduced ability to maintain respiration, posture and activity than surviving preterm neonates (average scores 6.2±0.5 vs. 8.1±0.3 respectively, p<0.0002). Both preterm groups expectedly performed worse in the 24-hour time period than neonates born at term (average score 11.5±0.1, p<0.0001*)*.

No significant differences were seen in placental 5αR1 expression at time of delivery between the term, preterm surviving and non-surviving groups (Fig 6-1*a)*. In contrast 5αR2 mRNA expression was markedly lower in placenta collected at term compared to levels in the placenta from surviving and non-surviving prematurely delivered neonates (p =0.0004 and p <0.0001 respectively, Fig 6-1*b*). Interestingly, expression in placenta from preterm non-surviving neonates was significantly greater than placenta from preterm neonates that did survive to 24

hours (p =0.04). As mentioned above, no sex differences were found and when split by sex placental 5αR mRNA expressions followed the same patterns.

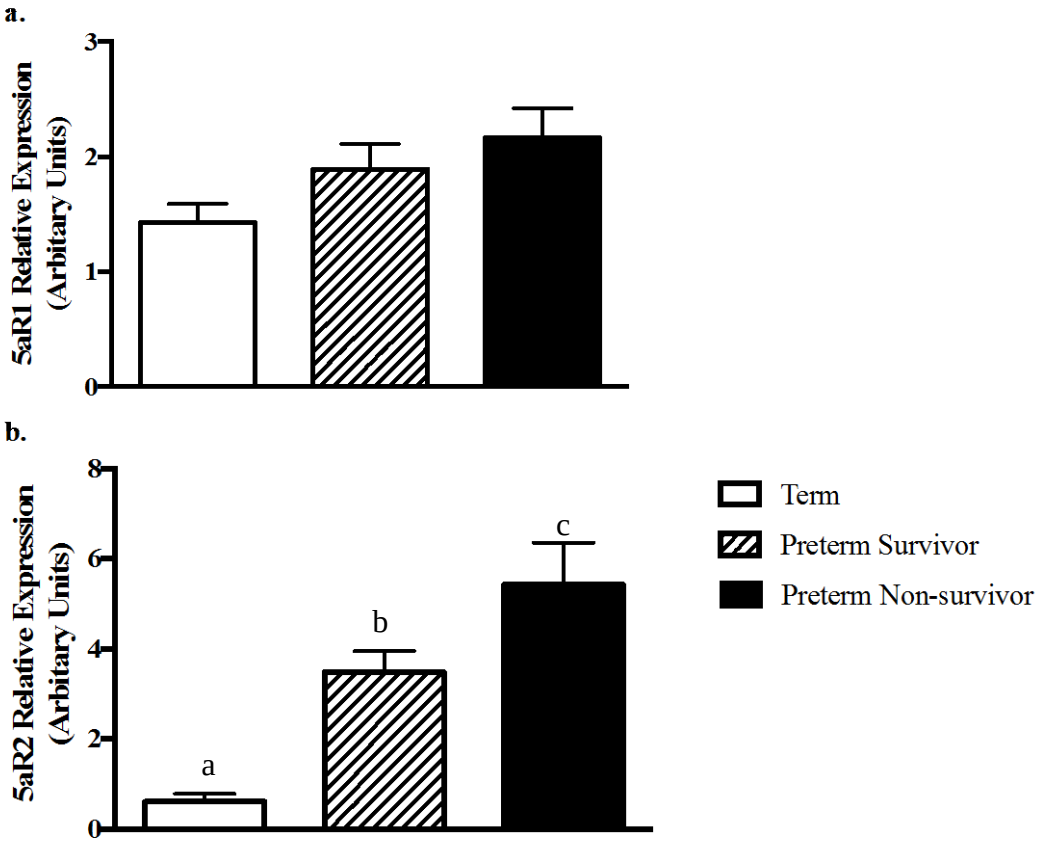

FIGURE 6-1 PLACENTAL 5α-REDUCTASE TYPE 1 (A) AND TYPE 2 (B) MRNA EXPRESSION FROM TERM (OPEN BARS), PRETERM SURVIVING (STRIPED BARS) AND PRETERM NON-SURVIVING (CLOSED BARS) GUINEA PIG NEONATES. RELATIVE EXPRESSION PRESENTED AS ARBITRARY UNITS. DATA PRESENTED AS MEAN ± SEM. BARS WITH DIFFERENT LOWER CASE LETTERS ARE SIGNIFICANTLY DIFFERENT FROM EACH OTHER (P <0.05).

6.5 Discussion

Our findings are the first to show that placental neurosteroidogenic capacity is elevated in the placenta of non-surviving preterm guinea pig neonates. In utero compromises are often major contributors to adverse perinatal outcomes and underlying non-detected insufficiencies have been suggested to potentiate hypoxic/ischemic injury at birth [389]. Guinea pigs occasionally have fetuses that suffer in utero death, which may be due to placental insufficiencies similar to that seen in human pregnancies. This suggests that some fetuses that do not die in utero may be exposed to periods of less severe hypoxia, which may be related to placental implantation. The present findings suggest elevated 5αR2 expression is indicative of insufficiency, potentially due to a hypoxic episode or multiple episodes, and organ underdevelopment that may have contributed to the reduced survival of the immature pups. The relative contribution of 5αR2 in the placenta and brain to central allopregnanolone concentrations is unclear, however the placenta is likely to not only supply the allopregnanolone precursor progesterone but also contribute to the supply of allopregnanolone itself. During fetal life 5αR2 has the key role in producing allopregnanolone, with expression developmentally regulated. After birth expression of 5αR2 in the brain declines with advancing postnatal age and neurosteroid production becomes more dependent on 5αR1 [183].

Previous studies by us investigating the effects of betamethasone on 5α-reductase expression showed a marked reduction in 5αR2 mRNA in the placenta of betamethasone treated fetuses [104]. However, the McKendry study employed multiple administrations of betamethasone over a prolonged period. Hence, the current study administered betamethasone to dams prior to both term and preterm delivery to reduce variability among experimental groups. This chronic exposure could have potentially caused the significant reductions in placental 5αR2. Our study used fewer administrations over a shorter time period, mimicking protocols undertaken in human pregnancies at high risk of preterm delivery, to aid in lung maturation and increase the survival of the preterm guinea pig neonates. Studies using human placenta found no differences in preterm placental 5αR1 and 5αR2 expression with and without prior betamethasone exposure [255]. Additionally

whilst it is known that betamethasone can be metabolised by the enzyme 11β-hydroxysteroid dehydrogenase type 2, this is slow compared to endogenous glucocorticoids [390]. Therefore levels of exposure between the term and preterm groups are likely to have been similar.

Allopregnanolone concentrations decline rapidly in sheep neonatal plasma and brain after birth, demonstrating the critical role of the placenta in producing allopregnanolone and its precursors during late gestation [384]. Concentrations in the brain, including the brainstem, also decline after birth; which increases excitability and may increase respiratory activity [384]. In this study the neonates that did not survive died of respiratory failure, often following repeated apnoeic episodes. Allopregnanolone levels in the neonatal rat brain are increased by chronic exposure to stress [391], albeit from a lower base, but these changes required repeated exposure and are unlikely to influence neonatal survival over the first 24 hours of life. This supports the contention that in utero stress and consequent effects on development may have a major influence on outcome. The increased levels of 5αR2 gene expression observed in the placenta of non-surviving preterm neonates is consistent with the induction of the protective neurosteroid pathway, which may potentially be due to an in utero stressor which was not sufficient to influence fetal weight.

Previous observations in sheep models suggest that moderate intrauterine growth restriction causes an increase in 5αR2 protein expression in the brain [185]. Although there were no differences in birth weight between this study's preterm groups, less severe compromise toward the time of delivery may have raised 5αR2 transcription and lead to sub-optimal development. As brain to body weight ratio was not available at delivery, evaluation of asymmetric growth was not possible. Furthermore, the upregulation of neuroactive steroid pathways and an increase in neurosteroid-mediated trophic actions may have improved brain development but not other organ systems. This type of in utero stressor may have predisposed these non-surviving neonates to their poor outcome in the first 24 hours after delivery, particularly when faced with premature delivery.

Despite the known contribution of the placenta to fetal neurosteroidogenesis, recent studies have focussed on 5αR expression within the fetal brain in response to gestational challenges such as intrauterine growth restriction (IUGR) and hypoxia, with the recent human placental studies characterising 5αR1 and 2 protein expression within uncomplicated term and preterm placentae [255]. As such, this is the first study to suggest that alterations in placental neuroactive steroid synthesis may be associated with an intrauterine compromise and a consequent poor outcome. Previous studies have shown that male preterm neonates are at a greater risk of morbidity and mortality than females [392]. This may potentially be reflected in their neuroactive enzyme protein levels and activity; and potentially steroid concentration profiles in times of compromise. Whilst allopregnanolone is the most well characterised neurosteroid of pregnancy, we cannot rule out the influence of other neurosteroids such as cortisol-derived tetrahydro-deoxycorticosterone and testosterone-derived 3α-androstanediol, and how these may influence the sexes differentially. Future studies could also examine if sex differences are present in brain 5α-reductase protein levels and neuroactive steroid concentrations at birth or in the neonatal period. In conclusion, the finding of markedly higher 5αR2 mRNA expression in the placenta of neonates with poorer outcomes support the measurement of expression of this enzyme as a potential marker of in utero compromise that may have ongoing influences on postnatal development.

7 DISCUSSION

Early life events, especially those occurring *in utero*, have life-long impacts on the health and wellbeing of an individual. The brain is a particularly vulnerable region to developmental programming by compromises that impact on the supply and/or utilisation of neurosteroids, such as intrauterine growth restriction, prenatal stress and preterm birth. The studies in this thesis show that pharmacologically induced reductions in allopregnanolone concentrations during pregnancy alter $GABA_A$ receptor $\alpha 6$ subunit expression in the immediate postnatal period (8 days) irrespective of sex, as well as programs an increased anxiety-like phenotype in juvenile female offspring (21 days), even in a healthy postnatal environment and normal levels of postnatal circulating allopregnanolone. This work also showed that late gestation allopregnanolone levels are not as critical in programming behaviour male behaviour.

The investigation of intrauterine growth restriction alone, and combined with prenatal stress results in reduced circulating allopregnanolone in male and female fetuses, but not in their placenta. These data also showed IUGR and IUGR+PS caused a marked deficit in mature hippocampal myelin in males but not within the female cohort. Interestingly myelin deficit in the subcortical white matter was seen in the IUGR only affected males but not in those also exposed to prenatal stress. Together these observations highlight the sex differences even prior to birth

in the offspring, in response to changes or impairment in the neurosteroid environment.

7.1 Effects of prenatal suppression of allopregnanolone concentrations on the postnatal brain and subsequent behaviour

The studies in chapter 3 and 4 indicate that whilst gross neurodevelopment and circulating allopregnanolone is able to recover following reductions in neurosteroid concentrations *in utero*, the $GABA_A$ receptors are more sensitive to these changes. This reduction also produces an anxiogenic-like phenotype upon stressful situations later in life.

The importance of the cerebellum is becoming increasingly recognised as an area controlling cognition, behaviour and emotional responses[129, 393]. The current studies found that inhibiting neurosteroid production in late gestation reduces the expression of $GABA_AR$ α6 mRNA within the cerebellum in females. The α6 subunit is largely located extrasynaptically and has a high affinity for allopregnanolone. This subunit is exclusively expressed on the excitatory granule cells within the cerebellum[394]. Granule cells make up the majority of total neuron numbers of the brain; and are the most abundant cell within the cerebellum with as many as 3000 granules cells synapsing with 1 Purkinje cell in the human brain[395, 396]. The tonic conductance of these cells in the cerebellum has been found to inhibit, to differing degrees, cell activation; increasing the capacity for information storage and finer coordination in terms of motor control[397]. As reduced tonic regulation of these cells has been known to produce impaired motor function[398], it is possible altered tonic inhibition from granule cells may impact cognitive and behavioural output from the cerebellum.

The cerebellum has interconnections with the amygdala and hippocampus[393, 399], whilst the amygdala and hippocampus contain reciprocal connections[143]. Lesion studies of the cerebellum[400, 401], amygdala[402, 403] and hippocampus[342, 403]

individually produce deficits in anxiety and fear responses. Therefore, reductions in the α6 subunit, such as those found in the current thesis may lead to distortions in the excitability and connectivity of these regions. This in turn can impact on the development of anxiety. The cerebellum and amygdala produce the highest levels of allopregnanolone in the female brain, and are both activated during emotional tasks[404]. This supports the role of the cerebellum in emotional disorders and suggests feedback between the cerebellum and limbic structures in emotional states. Functional MRI of the cerebellum and amygdala display impaired connectivity in adolescents with generalised anxiety disorder compared to adolescents with no diagnosis[405]. Paired with reported changes in subunit expression, it feasible that premature loss of allopregnanolone *in utero* in female fetuses begins to shift receptor expression from neurosteroid sensitive to insensitive subtypes. Subsequently, a secondary challenge such as fearful situations, new environments and puberty, which are known factors in altering neurosteroid concentrations, may induce a receptor subunit switch, in turn producing anxiogenic and neophobic responses to challenge.

There are discrepancies in the literature as to whether or not reductions in allopregnanolone concentration during late pregnancy result in changes in behaviour in females alone, or if it impacts male behaviour as well. Paris *et al* administered prenatal finasteride (50mg/kg) to pregnant rats from GA17-21[406]. This concentration of finasteride has been described to reduce allopregnanolone production by 50-75% in adult female rat brains. These investigators reported that prenatal allopregnanolone suppression decreased object recognition regardless of sex in the offspring at PND21, but did not affect anxiety-like behaviour as measured by total gridline crossings in open field testing. This overall effect, but the absence of sex differences may be due to the timing of finasteride administration. In terms of neurodevelopment, the end of gestation in the rat (GA22) equates to approximately 12-15 weeks of human pregnancy, thus this prenatal treatment regimen occurred before the critical window of brain development, and pre-sexual differentiation[256, 407, 408]. Therefore, the observation that no sex differences were found may be due to a global impact on neurogenesis in this rat study. In other postnatal rat studies, a dose of 50mg/kg of finasteride

was also used as the authors state this concentration has proven to modify behaviour in rats[332]. Finasteride was administered from PND5-9 in male rats, a period of neurodevelopment equivalent to late gestation in humans and guinea pigs[259]. They found early postnatal suppression of allopregnanolone levels produced deficits in locomotor activity, head dipping and central zone entries in the Bossier test of anxiety and novelty at PND40 (mid-adolescence) and PND60 (post adolescence)[233]. We did not see an effect in male adolescent behaviour in our studies, however this may potentially be due to the higher doses of finasteride administrated (50mg/kg) compared to the doses used in this thesis (25mg/kg). Previous work from our lab had shown finasteride administration (25mg/kg) reduced fetal brain allopregnanolone concentrations from 11.63±2.33ng/g to 4.2±0.51ng/g (up to 65%)[105]. Nevertheless, a follow-up study in these rats using the same dosing and timing regime did not produce deficits in adult anxiety measures in the same tests at PND100-105[332], or in elevated plus maze and passive avoidance[409], implying that neonatal loss of allopregnanolone may be not a strong contributor to behavioural outcomes in adulthood in the male rat. Despite these behavioural changes in these rat studies, females were not investigated and thus comments cannot be made on what potential changes may have occurred in their behaviour. However, the current studies showed that female behaviour was affected by intrauterine reductions of allopregnanolone in late gestation, with an increase in neophobia-like behaviour at juvenility. This shows that, in this study, female behaviour is prenatally programmed by allopregnanolone, and warrants further investigation to determine if these behaviours persist into adulthood.

Discrepancies between these rat studies and the behaviour of guinea pigs seen in this study may also be due to concentrations of allopregnanolone present at the time of finasteride administration. In long gestation species such as humans and guinea pigs, allopregnanolone is produced in large quantities by the placenta. In rats and mice elevated gestational levels rely on progesterone production by the corpus luteum of pregnancy. In rats, the equivalent periods of rapid brain growth, synaptogenesis and maturation of oligodendrocytes are occurring postnatally[257, 259], thus the neonatal brain and peripheral organs are responsible for supplying the precursors for neurosteroid production. Therefore, allopregnanolone is no longer

at high gestational concentrations, nor is it necessarily the predominant neurosteroid. In males, testosterone can be metabolised into 5α, 3α-reduced neurosteroid 3α-androstandione, which is also an allosteric modulator of the $GABA_AR$ acting in the same manner as allopregnanolone. The changes in males following finasteride exposure may be more due to suppression of testosterone derivatives and allopregnanolone, than by the loss of allopregnanolone alone. These previous studies also did not investigate the effects of finasteride on females, as they were excluded from the studies. Extensive previous studies have found that progesterone withdrawal, and subsequently allopregnanolone, are implicated in numerous female emotional disorders such as PMS[210] and PMDD[317], highlighting that females are highly sensitive to changes in progesterone derived neurosteroids, more so than males. Thus, how prenatal reductions in allopregnanolone affect female offspring may provide evidence for those at a greater risk of developing emotional disorders at the onset of puberty.

Modol and co-workers have investigated the expression of the α_4 and δ $GABA_AR$ subunits in the hippocampus during finasteride administration (PND6&9) and immediately after cessation in rats at PND10, 12 and 15[340]. These authors found increases in α_4 on PND6 (1 day after commencement) and on PND10 (1 day after cessation) following finasteride treatment in neonates. The expression of the δ subunit was increased on PND9 (last day of administration) compared to vehicle neonates. Increases in the δ subunits were also seen in finasteride exposed neonates at PND10 compared to finasteride neonates at PND9, and vehicle PND10 neonates. The differential expression of these two subunits during finasteride administration suggests differing regulation in response to acute versus chronic neurosteroid withdrawal, however both are upregulated with restoration of neurosteroids, as evident by the increase on PND10. Continuing this study, investigators also pre-treated the finasteride-exposed rats with 25mg/kg of progesterone (a dose that results in physiological concentrations of allopregnanolone in females[410]) for 3 days at adulthood with rats undergoing behavioural testing 20 minutes post final progesterone administration[340]. Males exposed to finasteride neonatally and subsequent progesterone exposure in adulthood were found to have stronger anxiogenic behaviours in EPM than

control rats. This anxiogenic behaviour was also found in pubertal female mice administered allopregnanolone prior to testing[339], and seen in our juvenile female guinea pigs following prenatal finasteride exposure. These results support the notion that prenatal loss of allopregnanolone and subsequent changes in neurosteroids during the onset of puberty in females program for an anxiogenic phenotype.

The authors of the above two studies attribute changes in behaviour to altered expression of $GABA_AR$ subunits[340]. The expression of the α_4 and δ subunit in the hippocampus of the male rats with neonatal finasteride exposure had increased α_4 expression immediately following testing compared to control male rats, irrespective of progesterone or vehicle treatment at adulthood. However, prenatal finasteride exposed rats with vehicle administration at adulthood had greater expression of the δ subunit compared to their progesterone treated counterparts. In the study of female mice with acute treatment or withdrawal of allopregnanolone[339], the authors also showed that alterations in neurosteroid concentrations causes receptor subunit changes with increased $\alpha_4\beta_n\delta$ GAB_AARs. Whilst there is discrepancy between whether or not it is the α_4 or δ subunit upregulation that promotes anxiety-like behaviours, the overall observations are consistent with changes in allopregnanolone levels early in life, and subsequent progesterone withdrawal in later life programming behavioural deficits. The studies presented here did not find differences in the capacity of females to produce GABA in the amygdala or hippocampus, nor in their end-point concentrations of circulating allopregnanolone. Although based on the recent finding it is plausible the finasteride exposed female guinea pigs potentially had a blunted allopregnanolone response and altered $GABA_AR$ subunit composition at the time of behvioural testing that may explain the neophobia-like responses produced.

Events including preterm birth, IUGR and prenatal stress impact neurosteroid supply to the fetus, either by reducing synthesis or reducing sensitivity at the receptor. These effects may alter the neural networks leading to prenatal programming. Whilst the studies in this thesis did not investigate hippocampal or

amygdaloid levels of $GABA_AR$ subunits, based on previous studies it is possible allopregnanolone may alter sensitivity of the $GABA_ARs$ towards a less sensitive phenotype, and this in turn may dysregulate the cerebellar-limbic pathways predisposing to the development of anxiety-like phenotype in females. Taken together, whilst allopregnanolone is of key importance for the prenatal neurodevelopment of the fetus regardless of sex, the present studies suggest a sexually dimorphic effect on behaviour that needs to be further investigated.

7.2 Effects of intrauterine growth restriction and prenatal stress

This work used a new modification of previous artery ligation methods to induce growth restriction. This modification involved restricting the capacity of the uterine arteries to expand as gestation progresses, producing growth restriction. This modification gradually limits the expansion of the uterine arteries as pregnancy advances[266, 267]. This produces a less severe insult at the time of surgery, compared to previous artery ligation and ablation models which produced an instantaneous restriction of blood flow[131, 376, 377]. We contend this modification is more representative of human onset of IUGR than the previous methods. In this study of IUGR and prenatal stress, both IUGR alone and IUGR+PS offspring showed alteration of myelination, without the changes in astrocytosis that have been recently reported by members of our group using prenatal stress alone[265]. Interestingly, the combination of IUGR and prenatal stress appeared to have restorative aspects on mature myelination in the subcortical white matter of males. These findings suggest that maternal prenatal stress stimulates the production of neurosteroids and neurodevelopment in an already compromised fetus, thereby inducing neuroprotection.

Acute asphyxia/hypoxia exposure *in utero* leads to temporarily increased brain allopregnanolone levels in what has been described as a protective process from excitatory neurotransmitter-induced cell death[197]. However, animal models of more chronic insults such as IUGR report conflicting data on brain allopregnanolone levels. In fetal sheep, umbilicoplacental embolization, which

mimics placental insufficiency, does not change brain concentrations of neurosteroids, however does elevate plasma concentrations[185]. This highlights the observation that plasma concentrations do not necessarily reflect the brain's capability to maintain neurosteroid production. This may explain the observation of reduced circulating allopregnanolone concentrations from IUGR and IUGR+PS fetuses, yet no changes in gross neuronal and astrocyte structural proteins found in this model. Similar to the sheep, in guinea pig models of IUGR using ablation, where 50% of the spiral arteries are cauterised, brain concentrations of allopregnanolone remain unaffected in IUGR fetuses[104, 105]. However, in a rat model using uterine artery ligation, whereby one end of the uterine artery and vein is tied off, rat pups classified as growth restricted exhibited increased brain allopregnanolone concentrations[387]. Whether this is an effect of degree of severity in the restriction method or in timing is uncertain. Uterine artery ligation produces an instantaneous reduction in blood, oxygen and nutrient supply to the uterus, whereas the embolization, ablation and silicon tubing methods are less severe in this reduction. This may produce a milder neurosteroid response to the insult that readjusts over time. Rat models induced growth restriction at GA18, and then collect samples on GA20. This likely represents the response to a more acute *in utero* insult, which as indicated above, results in increased brain neurosteroid concentrations which may not have settled by the end point 2 days later. The embolisation studies in fetal sheep spanned from GA114 induction to GA131-137 (term 148 days) tissue collection, whilst in the guinea pig models, including the new modification used in the current studies, are performed between GA30-35 with end point collection occurring approximately GA65-69[104, 105]. This potentially allowed for neurosteroid levels to stabilise in these chronic models, thereby ameliorating any local differences in brain neurosteroid concentrations. While there is limited data on brain neurosteroid concentrations in these two guinea pig models[104, 105], increases in both mRNA and protein expression of neurosteroidogenic enzymes have been demonstrated in other studies[104, 185, 387]. The brain has the capacity to produce neurosteroids independent of peripheral sources. The upregulation of key enzymes, primarily 5αR2, in the brain but no differences in circulating neurosteroid concentrations found in other studies suggests that the brain may have compensated for the detrimental environment.

As observed with IUGR, prenatal stress has not been shown to change allopregnanolone profiles within the plasma of offspring[121, 411]. However, like the previous studies, allopregnanolone was only assessed at the end of gestation and not at the time of the stress events. Nevertheless, prenatal stress does induce similar decrements in neurodevelopmental markers MAP2 and MBP, as found following the induction of IUGR in multiple models. These changes are due to the negative impact of glucocorticoids on neurodevelopment. Markedly increased glucocorticoid exposure above baseline levels at any point in life is neurotoxic, causing increased cell death[412, 413] and demyelination[414]. The neuroprotective role of allopregnanolone also involves regulation of the hypothalamic-adrenal-pituitary (HPA) axis. Allopregnanolone inhibits the activation of cells within the parvocellular paraventricular nucleus (pPVN), which in turn limits the release of CRH and downstream production of glucocorticoids[415]. Prenatal stress does acutely increase cortisol concentrations and persistent stress raises baseline concentrations above levels involved in fetal maturational processes. Glucocorticoids are known to program the HPA axis, with excess exposure prenatally inducing a hyper-responsiveness of the HPA axis to stressors in offspring from PS pregnancies[416-418]. However, exogenous administration of allopregnanolone to female rats, or the testosterone derived neurosteroid, 3β-androstanediol to male rats who were exposed to prenatal stress *in utero* has been reported to restore normal HPA signaling following immune-challenge[186]. These data suggest that glucocorticoid concentrations induced by PS overwhelm the protective levels of allopregnanolone, without necessarily reducing concentrations in the fetal brain. This in turn may program the developing HPA axis towards contributing to negative outcomes. Regardless of the degree of neurodevelopment, offspring from pregnancies complicated by IUGR and PS are more likely to develop behavioural and emotional disorders than appropriately grown and "unstressed" offspring. Bennett *et al*. profiled behaviour at adolescence in prenatally stressed guinea pigs[265]. Despite the absence of overt differences in neurodevelopmental markers in fetal life, these investigators showed that offspring stressed from mid-gestation had increased anxiety-like behaviours during open field and environmental exploration testing[265].

The present studies found a male vulnerability in myelination of the hippocampus and subcortical white matter. Previous studies using a guinea pig model of IUGR displayed a delay in the development of mature myelin within the brains of IUGR fetuses[90]. Similar findings were seen in an animal model of preterm birth, a compromise that impairs the supply of neurosteroids to the fetal brain[222]. The oligodendrocytes are the most vulnerable cells within the brain, and are highly sensitive to oxidative, excitotoxic and inflammatory damage[419]. Reductions in white matter are implicated in a number of disorders including bipolar disorder, schizophrenia, attention deficit hyperactivity disorder, post-traumatic stress disorder and major depression[420-424]. Whilst no changes in myelination were found in the female fetuses in this study others have reported a loss of myelination in the female offspring brain following prenatal stress. Female guinea pig offspring exposed to prenatal stress, whilst showing no changes in myelination within the CA1 of the hippocampus during fetal life, did show marked reductions at adolescence[122, 265]. Therefore, IUGR and PS may be priming factors for the early loss of myelination during postnatal development. Secondary insults, such as changes in hormones associated with puberty, environmental or inflammatory stressors may provide additional insult later in life triggering the onset of these disorders.

Interestingly, prenatal stress appeared to have less of a negative effect in IUGR offspring, with recovery of MBP in the SCWM of males. This is in contrast to the hypothesis that the combination of stressors would have an additive detrimental effect. There have been previous reports of negative pregnancy factors having paradoxical effects on outcomes. For example maternal smoking during very early pregnancy brings a reduced risk of preeclampsia[425, 426] but with this there is an associated increased risk of neonatal morbidity and mortality at later ages[426]. It is possible that the addition of PS in this model may have caused an upregulation of (as yet unknown) compensatory mechanisms. Given the previous evidence indicating poor health outcomes of PS and IUGR individually, it is most likely the positive effects seen in IUGR+PS fetuses may be due to the ages that

measurements were made, and that the life-long health of these offspring may be negatively affected compared to control counterparts.

In the human population, fetuses with late onset IUGR grow asymmetrically, as opposed to early onset IUGR where the fetus is constitutionally small[427], although this classification is not universal. The current diagnostic criteria for IUGR is abnormal umbilical artery Doppler, however this excludes a number of infants who are growth restricted but have no Doppler recording abnormalities[428, 429]. Regardless, infants who were born growth restricted have been reported to show changes in brain metabolic profiles without overt abnormal brain structures on MRI scanning[430], suggesting that these babies have changes in the molecular microenvironment compared to appropriately grown offspring. Thus, in our study, while there were deficits in myelination, without changes in astrogliosis and GABA producing enzymes, we cannot exclude changes in developmental profiles of neurotransmitters and associated receptors that may be altering the excitability of these cells, increasing their susceptibility to postnatal behavioural disorders. Future investigation of the brain and paired placentae of these offspring would provide greater insight into what changes are occurring within the neurosteroid pathways, and their downstream targets, and how these will have impacted on the recovery of neurodevelopment and steroid profiles seen after birth and into adolescence.

The present study was limited by the number of neurodevelopmental markers that have previously been found to be sensitive to changes in the neurosteroid environment. Future investigations could move towards the expression of neurosteroidogeneic enzyme and target receptor expressions within specific brain regions. This would involve quantifying brain allopregnanolone and other neurosteroid concentrations to determine key steps and changes that may be compensating within the combined IUGR+PS model. Postnatal studies will further investigate behaviour changes occurring in offspring with IUGR and PS exposure, and investigate what long term neurodevelopmental losses or preservations may be occurring.

7.3 Conclusions and Future Directions

Barker and colleagues put forward the idea of the fetus to adapt a "thrifty phenotype" in response to *in utero* complications 30 years ago[2, 3], which has evolved into the developmental origins of adult disease hypothesis. As the incidences of preterm birth, intrauterine growth restriction and prenatal stress remain major determinants of perinatal outcome, the long term outcomes of these compromises on the programming of fetal systems is becoming of greater importance in the prediction and development of mental health, cognitive and behavioural disorders. The studies in this thesis show that neurosteroids play an integral role in fetal programming for later life deficits among females, and are involved in the vulnerability of males to neurological damage in fetal development. This sexually dimorphic effect emphasises that, even during intrauterine life, the sexes need to be treated differently in their immediate and long term treatment. These neurodevelopmental outcomes were not attributed to changes in the structural proteins of neurons and astrocytes nor in the number of cells present to synthesise GABA, suggesting that the machinery for responding to neurosteroids *in utero* is responsible for these changes. The findings presented in chapter 6 highlight a way to determine which infants may be at a greater risk of poor outcomes in the immediate postnatal period following premature delivery. Placental health is of great importance in determining overall fetal health and development. The supply of neurosteroids for appropriate development, and their upregulation following acute insults, is essential for maintaining key neurodevelopmental processes. Thus future investigations into the neurosteroidogenic capacity of the placenta may also be a useful tool in identifying which infants born following IUGR and IUGR+PS experienced repeated, or a greater severity of *in utero* insults and therefore target infants in need of replacement and supportive therapies.

Further investigation is needed to fully elucidate the changes in neurosteroid pathways occurring in response to intrauterine stresses. This will aid in the future development and use of neurosteroid based therapies as protective and preventative treatments. Replacement studies using allopregnanolone, or synthetic analogues such as ganaxolone, should focus on perinatal administration to

determine if this reduces the risk of developing anxiety-like phenotypes in later life. This may then provide a strategy for improving the long term mental health of females that suffered an adverse neurosteroid disrupting event *in utero*. Future work extending from these studies should also aim to characterise behavioural outcomes of offspring following IUGR+PS to investigate whether or not the seemingly protective effects of the combined compromises prevent negative behavioural changes. Altogether, this works presents useful and relevant models to help improve the life-long health and well-being of infants born following development in an adverse intrauterine environment.

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9 APPENDICES

APPENDIX 1 ADDITIONAL FIGURES: HIPPOCAMPAL MBP AND GFAP

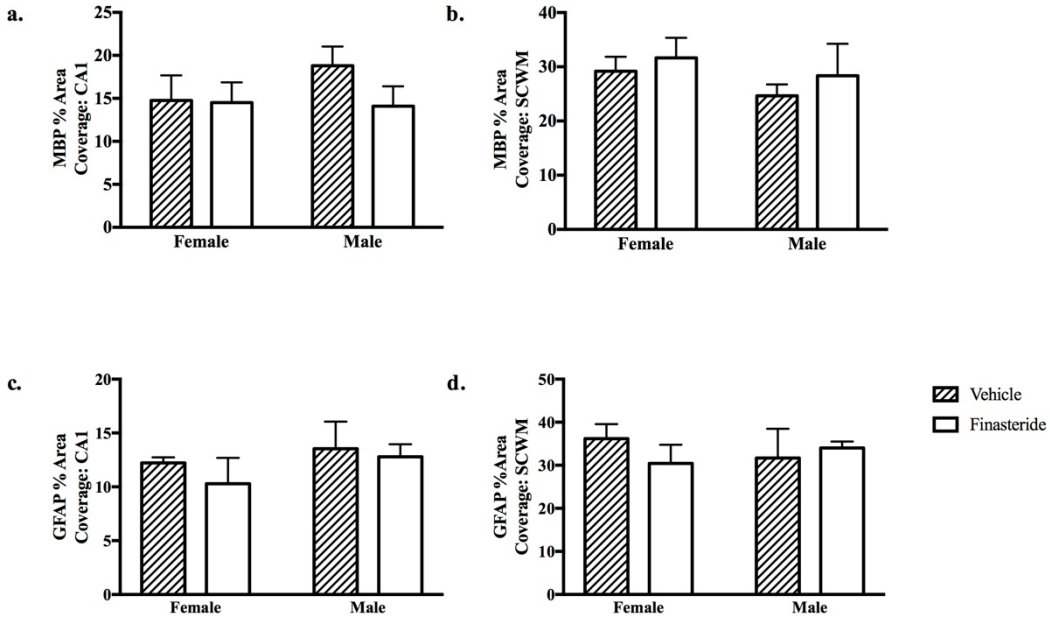

FIGURE A1 MYELIN AND ASTROCYTE MARKERS IN THE HIPPOCAMPUS. % AREA COVERAGE OF MYELIN BASIC PROTEIN IN THE CA1 REGION (MBP; A) AND SUBCORTICAL WHITE MATTER (B) OF THE HIPPOCAMPUS; GLIAL FIBRILLARY ACIDIC PROTEIN IN THE CA1 REGION (GFAP; C); AND SUBCORTICAL WHITE MATTER (D). NO DIFFERENCES WERE FOUND IN MBP AND % AREA COVERAGE WITHIN THE HIPPOCAMPAL REGION FOR VEHICLE ANIMALS (HASHED BARS) AND FINASTERIDE-EXPOSED ANIMALS (WHITE BARS) FEMALES N=5; AND MALES N=5 FOR EACH GROUP. DATA ANALYSED BY INDEPENDENT SAMPLES T-TEST. GRAPHS PRESENTED AS MEAN ± SEM.

APPENDIX 2 SUPPLEMENTARY DATA FOR CHAPTER 5

Birth weight

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	980.237	.000
Group	78.220	.000
Sex	1.299	.254

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	35.98119[a]	.000	27.58457	44.37780
	IUGR/Stress	38.01948[a]	.000	27.08429	48.95466
IUGR	IUGR/Stress	2.03829	.678	-7.57383	11.65041

a= mean difference significant at $p<0.05$

Nose-rump length

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	6989.081	.000
Group	49.742	.000
Sex	1.334	.248

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference Lower	Upper
Control	IUGR	2.6117[a]	.000	1.8285	3.3948
	IUGR/Stress	1.9756[a]	.000	1.2156	2.7357
IUGR	IUGR/Stress	-.6360	.137	-1.4746	.2026

a= mean difference significant at p<0.05

Visceral fat

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	284.581	.000
Group	20.706	.000
Sex	7.007	.008

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference Lower	Upper
Control	IUGR	.21048[a]	.003	.07309	.34786
	IUGR/Stress	.35092[a]	.000	.19709	.50474
IUGR	IUGR/Stress	.14044	.058	-.00460	.28548

a= mean difference significant at p<0.05

Peripheral fat

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	212.302	.000
Group	9.023	.011
Sex	.068	.794

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	.58925[a]	.034	.04498	1.13351
	IUGR/Stress	.63591[a]	.003	.21679	1.05503
IUGR	IUGR/Stress	.04666	.780	-.28082	.37415

a= mean difference significant at p<0.05

Brain

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	12868.324	.000
Group	11.332	.003
Sex	6.837	.009

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	.10499[a]	.001	.04342	.16656
	IUGR/Stress	.03976	.404	-.05362	.13314
IUGR	IUGR/Stress	-.06523	.162	-.15659	.02612

a= mean difference significant at p<0.05

Hippocampus Weight

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	618.394	.000
Group	.998	.607
Sex	.831	.362

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	.00646	.388	-.00822	.02114
	IUGR/Stress	-.00214	.740	-.01475	.01047
IUGR	IUGR/Stress	-.00860	.334	-.02606	.00886

a= mean difference significant at $p<0.05$

Placenta Weight

Test of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	880.601	.000
Group	94.555	.000
Sex	2.560	.110

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	2.46515[a]	.000	1.95574	2.97457
	IUGR/Stress	2.33479[a]	.000	1.63267	3.03691
IUGR	IUGR/Stress	-.13036	.687	-.76430	.50357

a= mean difference significant at p<0.05

Adrenal Weight

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	273.462	.000
Group	10.785	.005
Sex	.065	.799

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	.01147[a]	.001	.00462	.01832
	IUGR/Stress	.00686	.180	-.00318	.01690
IUGR	IUGR/Stress	-.00461	.333	-.01394	.00472

a= mean difference significant at p<0.05

Kidney Weight

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	2271.388	.000
Group	42.534	.000
Sex	.244	.621

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	.22623[a]	.000	.14889	.30356
	IUGR/Stress	.21423[a]	.000	.14535	.28311
IUGR	IUGR/Stress	-.01200	.702	-.07346	.04946

a= mean difference significant at p<0.05

Liver weight

Tests of model effect

Source	Wald Chi-Square	Sig.
(Intercept)	575.847	.000
Group	76.631	.000
Sex	.805	.370

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	2.37701[a]	.000	1.81081	2.94321
	IUGR/Stress	2.40346[a]	.000	1.74440	3.06252
IUGR	IUGR/Stress	.02644	.928	-.54734	.60022

a= mean difference significant at p<0.05

Brain-to-liver ratio

Tests of model effects

Source	Wald Chi-Square	Sig.
(Intercept)	165.138	.000
Group	44.595	.000
Sex	1.073	.300

Pairwise comparison

(I) Group	(J) Group	Mean Difference (I-J)	Sig.	95% Wald Confidence Interval for Difference	
				Lower	Upper
Control	IUGR	-.56966[a]	.000	-.83760	-.30171
	IUGR/Stress	-.47455[a]	.000	-.67039	-.27872
IUGR	IUGR/Stress	.09510	.592	-.25307	.44327

a= mean difference significant at $p<0.05$

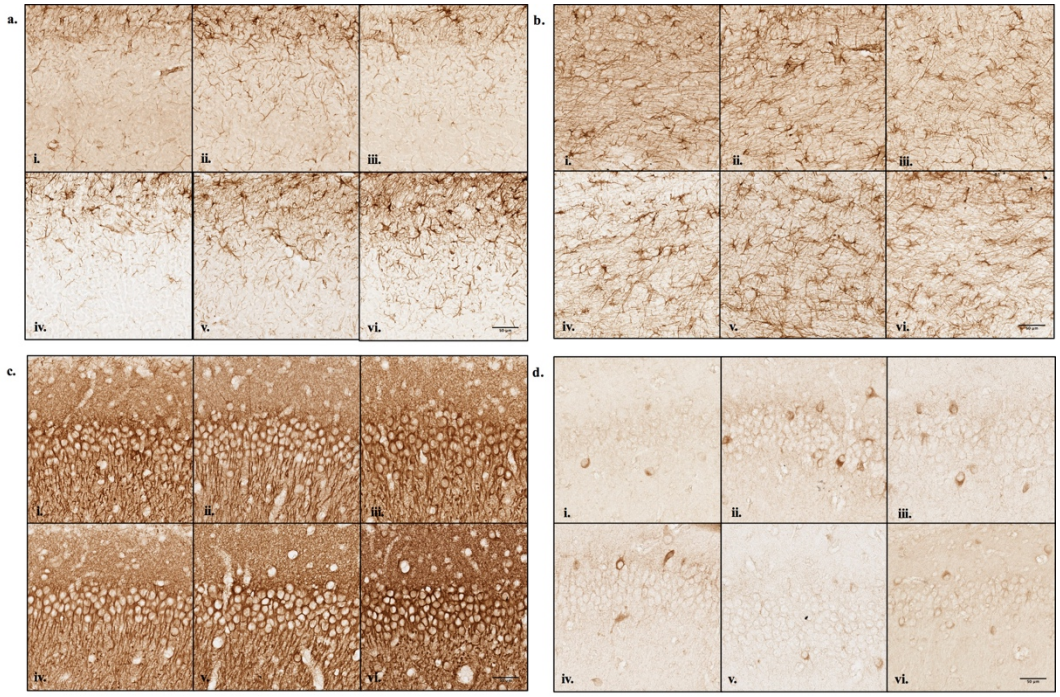

FIGURE A2 ASTROCYTE, NEURON AND GABAERGIC MARKERS IN THE HIPPOCAMPUS. REPRESENTATIVE IMAGES OF GLIAL FIBRILLARY ACIDIC PROTEIN IN THE CA1 REGION (A) AND SUBCORTICAL WHITE MATTER (B) OF THE HIPPOCAMPUS; MICROTUBULE-ASSOCIATED PROTEIN 2 IN THE CA1 REGION (MAP2; C); AND POSITIVE STAINING OF GAD67 CELLS WITHIN THE CA1 (CELLS/MM^2). CONTROL ANIMALS (FEMALES (I.); MALES (IV.)); IUGR (FEMALES (II); MALES (V.)); OR IUGR+PS FETUSES (FEMALES (III.); MALES (VI.)). SUPPLEMENTARY IMAGES TO FIGURE 5-3 (CHAPTER 5; SECTION 5.4.4). SCALE BAR = 50μM.

APPENDIX 3 SUPPLEMENTARY DATA FOR CHAPTER 6

Table A3-1 Scoring of Neonatal Guinea Pigs in first 24 hours of Life

Scoring	*0*	*1*	*2*	*3*	*4*
Respiration	No breathing	Gasping only	Gasping with some regular breaths	Irregular and/or shallow breaths	Regular breathing achieved
Posture	No muscle tone, inability to support themselves	Poor muscle tone, could not sit up, neck and limb spasticity	Difficulty supporting head, some spasticity, could sit up	Sit upright, walk but poor coordination	Walk and support body without difficulty
Movement and Alertness	No movement	Movement on stimulation	Some spontaneous movement	Some activity and alertness	Strong movement and alertness
Total	Animal scores added together to give total out of 12 Very poor: 0-3; Poor: 4-6; Good: 7-9; Very good: 10-12				

SCORING OF BEHAVIOUR WAS OBTAINED EVERY 2 HOURS FOR THE 24 HOURS (OR LESS) OF LIFE OF THE NEONATE. BEHAVIOUR WAS BASED ON THE ANIMALS' RESPIRATION, POSTURE AND MOVEMENT. EACH CATEGORY WAS GIVEN A SCORE FROM ZERO TO FOUR BASED ON SPECIFIC CRITERIA, WITH ZERO BEING THE POOREST SCORE AND FOUR BEING THE OPTIMAL ACHIEVED. EACH SCORE WAS ADDED TOGETHER TO GIVE A TOTAL OUT OF 12 FOR EACH ANIMAL.

Table A3-2 Pregnancy Characteristics and Neonatal Well-being Scores

Group	*Sex*	*Birth Weight (g)*	*Post Mortem Weight (g)*	*Placenta Weight (g)*	*Brain Weight (g)*	*Liver Weight (g)*	*Brain-to-liver Ratio*	*Last Score (/12)*	*Average Score (/12)*
Term	*Female (n=10)*	87.23 ± 4.78	81.88 ± 4.56	4.01 ± 0.25	2.29 ± 0.04	3.47 ± 0.24	0.70 ± 0.04	11.85 ± 0.11	11.40 ± 0.15
	Male (n=12)	89.65 ± 3.49	84.19 ± 3.85	4.01 ± 0.26	2.33 ± 0.04	3.32 ± 0.33	0.68 ± 0.04	11.92 ± 0.08	11.62 ± 0.36
Preterm Survivors	*Female (n=12)*	66.70 ± 3.20[a]	62.89 ± 2.96[a]	3.82 ± 0.23	2.04 ± 0.03[a]	2.89 ± 0.19	0.32 ± 0.04	8.04 ± 0.68[a]	7.59 ± 0.44[a]
	Male (n=11)	69.53 ± 2.61[a]	65.20 ± 2.25[a]	3.85 ± 0.22	2.11 ± 0.03[a]	2.90 ± 0.17	0.75 ± 0.04	9.36 ± 0.41[a]	8.80 ± 0.30[a]
Preterm Non-survivors	*Female (n=10)*	62.95 ± 1.46[a]	62.23 ± 1.37[a]	3.55 ± 0.15	2.04 ± 0.04[a]	3.04 ± 0.23	0.70 ± 0.05	5.55 ± 0.57[a, b]	5.69 ± 0.58[a, b]
	Male (n=12)	65.51 ± 1.93 [a]	63.37 ± 1.86[a]	4.26 ± 0.18	2.09 ± 0.04[a]	3.26 ± 0.20	0.66 ± 0.03	6.29 ± 0.49[a, b]	6.71 ± 0.36[a, b]

SUPERSCRIPT 'A' INDICATES A SIGNIFICANT DIFFERENCE FROM TERM WITHIN SEX ($P<0.05$). SUPERSCRIPT 'B' INDICATES A SIGNIFICANT DIFFERENCE BETWEEN THE PRETERM SURVIVORS AND PRETERM NON-SURVIVORS GROUPS WITHIN EACH SEX ($P<0.05$). DATA PRESENTED AS MEAN ± SEM.

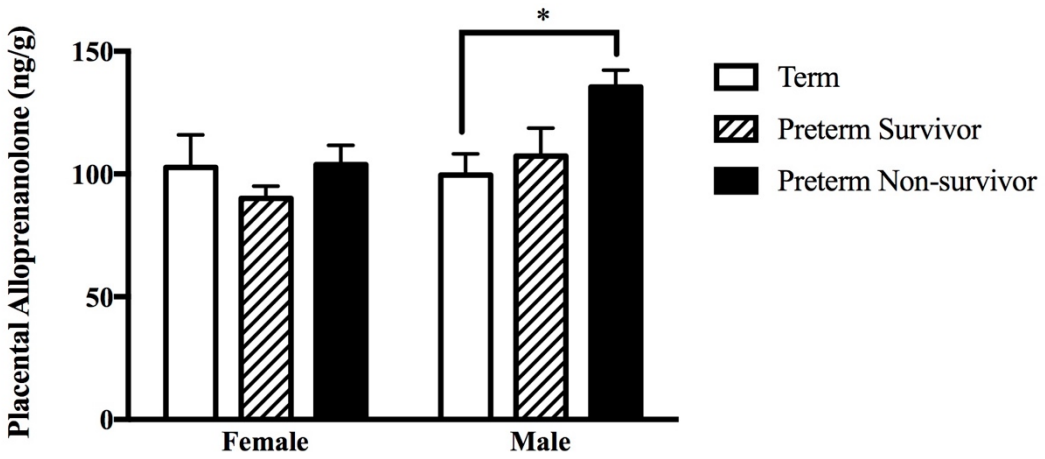

FIGURE A3-1 PLACENTAL ALLOPREGNANOLONE CONCENTRATIONS. NO SIGNIFICANT DIFFERENCES WERE FOUND IN THE PLACENTAL ALLOPREGNANOLONE CONCENTRATIONS AMONG THE FEMALE NEONATES (TERM N= 5, PRETERM SURVIVOR N= 8, PRETERM NON-SURVIVOR N= 7). NO SIGNIFICANT DIFFERENCE IN PLACENTAL ALLOPREGNANOLONE CONCENTRATIONS WAS FOUND BETWEEN MALE TERM (N= 10) AND PRETERM SURVIVING (N= 8) NEONATES. A SIGNIFICANT INCREASE WAS FOUND IN THE PLACENTA OF NON-SURVIVING MALES (N= 6) COMPARED TO PLACENTA OF TERM MALES (P=0.0203). DATA PRESENTED AS MEAN ± SEM. STATISTICAL SIGNIFICANT WAS DETERMINED USING TWO-WAY ANOVA. DIFFERENCES BETWEEN GROUPS DETERMINED USING TUKEY MULTIPLE COMPARISONS TEST. CONCENTRATION PRESENTED AS NG/G OF WET WEIGHT TISSUE. * P<0.05.

A negative correlation between placental 5αR2 expression and the average wellbeing score of neonates (r=-0.6021, p<0.0001), which supports the notion that worse off animals were exposed to an in utero event, leading to the upregulation of placental 5αR2 prior to delivery.

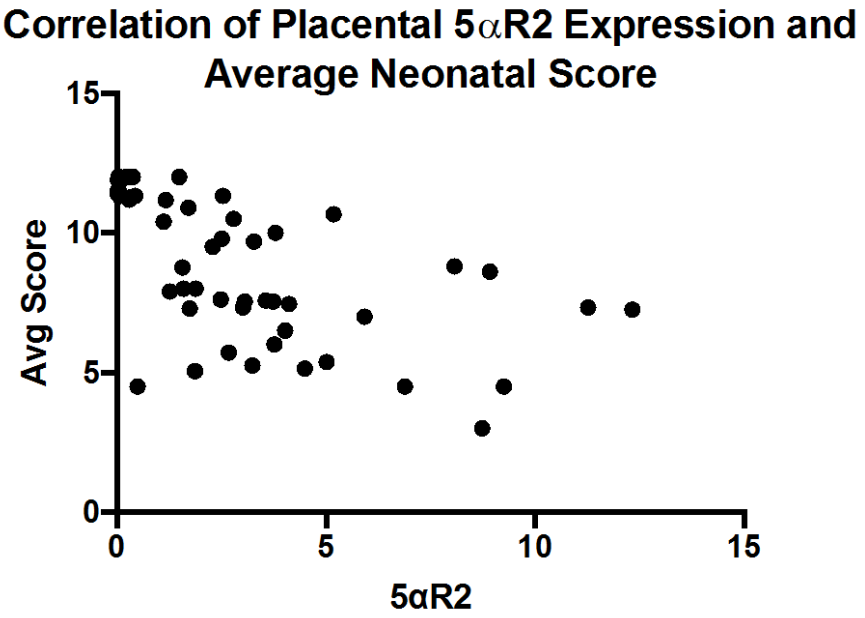

FIGURE A3-2 CORRELATION OF PLACENTAL 5α-REDUCTASE TYPE 2 EXPRESSION AND THE AVERAGE NEONATAL SCORE WITHIN THE ≤24HRS. A SIGNIFICANT NEGATIVE CORRELATION WAS FOUND BETWEEN PLACENTAL 5αR2 EXPRESSION AND THE AVERAGE SCORE OF NEONATES (R=-0.6021, P<0.0001). DATA ANALYSED USING PEARSON'S CORRELATION.

The driver of increased placental 5αR2 could possibly be an interference with blood supply, that impairs fetal oxygenation without inducing growth restriction (possibly occurring in the last days of gestation). The pups were assessed for IUGR post-mortem and were not found to have indicators of growth restriction based on BLR and physical measurements (Appendix table A3-2). The increase in 5αR2 expression would lead to a prediction of increased allopregnanolone concentrations. The fetal embolisation study displayed increases in plasma allopregnanolone of fetuses exposed to chronic embolisation [185].

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