Potential Neurosteroid Replacement Therapy Following Premature Birth and Fetal Growth Restriction

by

Meredith Anne Kelleher
Bachelor of Biomedical Science (Hons)

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

July, 2012

School of Biomedical Sciences & Pharmacy
Faculty of Health
University of Newcastle
Australia
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent for this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Signed: _____________________________  Date: _________
STATEMENT OF CONTRIBUTION TO JOINT PUBLICATIONS

I attest that I, Meredith Kelleher, have made a primary and original contribution to the publications, and manuscripts awaiting publication, included in this thesis, as detailed below and endorsed by my supervisors.

<table>
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<th>Chapter</th>
<th>Title</th>
<th>Status</th>
<th>Contribution</th>
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Signed (Candidate): _____________________________  Date: __________

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This thesis is dedicated to

Elizabeth Jane Mullier (1918-2010)

a great woman and the most steadfast, loving
and proud grandma, who is truly missed.
I hope that over the course of this PhD I have learnt much about “Science” and
at least a little about life. It is difficult not to descend into cliches and hyperbole
when trying to express the gratitude that I feel to all those people that have
supported me over the past four years. I have discovered that undertaking a PhD
is truly an all-encompassing, challenging, humbling and foolhardy endeavour. I
have also discovered the simple joy that can accompany a successful day in the
lab, the elation at finally producing that single graph of results and the pleasure
that comes with solving what seemed an impossible problem. In all these things,
the people around me have truly been the most important, inspirational and
encouraging part. Here, in this small way, I am trying to express my absolute and
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complete this thesis and PhD.

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advice and good humour are always appreciated. It has truly been an honour to
be able to work with you and I hope to have your advice and support in the
years to come as I continue to learn and grow as a scientist. Hannah, I consider
it a privilege to have been your first PhD student. I hope that (if my opinion
counts for anything) I can express how great a job you have done and how
significant you have been in my life and career so far. You encourage and inspire
me daily. I am so fortunate to have you as a friend, and as a colleague. I would
not have been able to reach this point without you. Thank you (and thanks to
Dave too).

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and supported me through it, you have been more important to me than you
could know and I will miss you greatly as I move on to new challenges. Bec
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Cheers.
# TABLE OF CONTENTS

Abstract .......................................................................................................................... xi
List of Figures & Tables ................................................................................................. xiii
List of Publications ........................................................................................................ xv
List of Conference Abstracts ....................................................................................... xvi
List of Abbreviations ...................................................................................................... xix

## Chapter One

### Introduction

1.1 Preterm Birth ............................................................................................................. 25
1.1.1 Rates of Preterm Birth ......................................................................................... 26
1.1.2 Causes of Preterm Birth ...................................................................................... 28
1.1.3 Risk Factors for Preterm Birth ........................................................................... 31
1.1.5 Postnatal Preterm Outcomes ............................................................................. 33
1.2 Intra-Uterine Growth Restriction ........................................................................... 35
1.2.1 IUGR & Low Birth-Weight .................................................................................. 36
1.2.2 Pathophysiology of IUGR .................................................................................. 38
1.2.3 Risk Factors and Causes of IUGR ...................................................................... 40
1.2.4 IUGR and Preterm Birth .................................................................................... 42
1.2.5 Postnatal Outcomes of IUGR Infants ................................................................. 43
1.3 Perinatal Brain Injury .............................................................................................. 45
1.3.1 Excitotoxicity ...................................................................................................... 46
1.3.2 Oxidative Stress ................................................................................................... 49
1.3.3 Inflammation ....................................................................................................... 50
1.3.4 Apoptosis ............................................................................................................ 51
1.3.5 Preterm Brain Injury and Periventricular Leukomalacia ..................................... 52
1.3.6 Term Brain Injury ............................................................................................... 56
1.4 Neurodevelopmental Sequelae .............................................................................. 57
1.4.1 Sensory-Motor Disorders .................................................................................... 58
1.4.2 Cognitive, Learning and Behavioural Disorders ................................................. 59
1.4.3 Neurological Disorders ....................................................................................... 60
1.5 Neuroprotective Strategies .................................................................................... 61
1.6 Progesterone ........................................................................................................... 63
1.6.1 Progesterone Synthesis ....................................................................................... 64
1.6.2 Progesterone Receptors & Signalling ................................................................. 67
1.6.3 Progesterone Functions & Concentrations During Gestation .............................. 67
1.6.4 Progesterone Effects on Neurodevelopment ...................................................... 70
1.6.5 Neuroprotection by Progesterone ...................................................................... 72
1.7 Neuroactive Steroids ............................................................................................. 75
1.7.1 GABA_A Receptor .............................................................................................. 78
1.7.2 Pregnanolone Isomers ....................................................................................... 82
1.8 Allopregnanolone .................................................................................................. 84
1.8.1 Allopregnanolone Synthesis ............................................................................. 86
Chapter Two

Materials & Methods

2.1 Animal Ethics
2.2 Animal Housing
2.3 IUGR Surgery
2.4 Finasteride Treatment
2.5 Preterm Neonatal Model
2.6 Tissue Collection
2.7 Measurement of Allopregnanolone
2.8 Steroid immunoassays
2.9 Protein Western Blot Immunodetection
2.10 Brain Immunohistochemistry
2.11 Statistical Analyses
Chapter Three
Sex-Specific Effect of Inhibition of Neurosteroid Synthesis and Intra-Uterine Growth Restriction on Fetal Guinea Pig Brain Development ..... 132
3.1 Abstract .................................................................................................................................. 132
3.2 Introduction ................................................................................................................................ 133
3.3 Materials and Methods ............................................................................................................. 136
  3.3.1 Animals ........................................ 136
  3.3.2 Tissue Collection ........................................ 137
  3.3.3 Radio-Immunoassay ........................................ 138
  3.3.4 Western Blot Analysis ........................................ 138
  3.3.5 Immunohistochemistry ........................................ 140
  3.3.6 Statistical Analyses ........................................ 141
3.4 Results ..................................................................................................................................... 141
  3.4.1 Fetal Characteristics ........................................ 141
  3.4.2 Fetal Brain Allopregnanolone Concentrations ........................................ 142
  3.4.3 Brain 5α-Reductase Enzyme Expression ........................................ 144
  3.4.4 GFAP Expression ........................................ 144
  3.4.5 MBP Expression ........................................ 146
  3.4.6 Activated Caspase-3 Expression ........................................ 148
3.5 Discussion ................................................................................................................................. 150

Chapter Four
Changes in Neuroactive Steroid Concentrations after Preterm Delivery in the Guinea Pig ..................................................................................................................... 155
4.1 Abstract ..................................................................................................................................... 155
4.2 Introduction ................................................................................................................................ 156
4.3 Materials and Methods ............................................................................................................. 158
  4.3.1 Animals ........................................ 158
  4.3.2 Preterm and Term C-Section Delivery ........................................ 159
  4.3.3 Progesterone Treatment ........................................ 160
  4.3.4 Tissue and Plasma Collection ........................................ 160
  4.3.5 Western Blot Analysis ........................................ 160
  4.3.6 Immunohistochemistry ........................................ 161
  4.3.7 Steroid Radioimmunoassay and Enzyme Immunoassay ........................................ 162
  4.3.8 Statistical Analyses ........................................ 163
4.4 Results ..................................................................................................................................... 163
  4.4.1 Neonatal Animals ........................................ 163
  4.4.2 MBP Expression ........................................ 165
  4.4.3 GFAP Expression ........................................ 167
  4.4.4 MAP-2 Expression ........................................ 167
  4.4.5 Plasma Steroid Concentrations ........................................ 167
  4.4.6 Allopregnanolone Concentrations in the Brain ........................................ 170
  4.4.7 Brain 5α-reductase Expression ........................................ 171
4.5 Discussion ................................................................................................................................. 173
Chapter Five
Neuroactive Steroids in Preterm Guinea Pigs Following Postnatal Progesterone Therapy................................................................. 179
5.1 Abstract .................................................................................................................. 179
5.2 Introduction .............................................................................................................. 181
5.3 Materials and Methods ........................................................................................... 184
  5.3.1 Animals ................................................................................................................. 184
  5.3.2 Preterm C-section Delivery .................................................................................. 184
  5.3.3 Treatment Groups ............................................................................................... 185
  5.3.4 Neonatal Scoring & Behavioural Testing ............................................................... 186
  5.3.5 Plasma, Brain & Salivary Steroid Analysis ............................................................ 186
  5.3.6 Protein Analysis ................................................................................................... 188
  5.3.7 Immunohistochemistry ...................................................................................... 188
  5.3.8 Statistical Analysis .............................................................................................. 189
5.4 Results ..................................................................................................................... 190
  5.4.1 Neonatal Animals, Survival & Scoring ................................................................. 190
  5.4.2 Salivary Progesterone .......................................................................................... 193
  5.4.3 Plasma Steroids .................................................................................................. 195
  5.4.4 Brain Allopregnanolone ..................................................................................... 197
  5.4.5 Hippocampal 5α-Reductase Enzyme Expression .................................................. 197
  5.4.6 Immunohistochemistry ...................................................................................... 197
  5.4.7 Behavioural Analysis .......................................................................................... 200
5.5 Discussion ................................................................................................................. 203

Chapter Six
Cerebellar Development in the Neonatal Guinea Pig following Preterm Birth & Progesterone Replacement Therapy ................................ 212
6.1 Abstract ................................................................................................................... 212
6.2 Introduction .............................................................................................................. 213
6.3 Methods .................................................................................................................... 216
  6.3.1 Tissue Collection .............................................................................................. 216
  6.3.2 Determination of Cerebellar Lobule Thickness ................................................... 218
  6.3.3 MBP Staining of Cerebellar White Matter Tracts ............................................... 218
  6.3.4 Calbindin Staining for Purkinje Cell Measurements ......................................... 218
6.4 Results ..................................................................................................................... 219
  6.4.1 Cerebellar Growth and External Granular Layer Thickness ............................... 219
  6.4.2 MBP in Cerebellar White Matter Tracts ............................................................... 219
  6.4.3 Purkinje Cells .................................................................................................... 222
6.5 Discussion ................................................................................................................. 222
Chapter Seven

Discussion & Conclusions ........................................................................................................228

7.1 Discussion ............................................................................................................................228

7.1.1 Allopregnanolone in the Late Gestation Fetal and Preterm Neonatal Guinea Pig 229

7.1.2 Progesterone and Allopregnanolone Replacement in Preterm Guinea Pigs 234

7.1.3 Neurosteroids and Perinatal Myelination in the Guinea Pig 241

7.1.4 Neurosteroids, Neuropathology and Behaviour in the Perinatal Guinea Pig 243

7.2 Conclusions ..........................................................................................................................248

References ...................................................................................................................................250

Appendix A

Plasma Allopregnanolone Concentrations in Fetal Guinea Pigs with IUGR and Finasteride Treatment ....................................................................................................................302

Appendix B

Preterm and Term Guinea Pig Lungs ..........................................................................................303

Appendix C

Rights and Permissions ..............................................................................................................304
ABSTRACT

Events during gestation and changes in the intrauterine environment contribute to abnormal development and injury in the immature brain, influencing health and disease throughout life. Progesterone and its neuroactive steroid metabolite, allopregnanolone, are present in high concentrations during pregnancy. Allopregnanolone signalling at the GABA_A receptor has important trophic and neuroprotective effects. The disruption of neuroactive steroid concentrations due to complications such as intrauterine growth restriction (IUGR) or preterm birth may therefore adversely affect brain development and increase perinatal brain injury.

Inhibition of allopregnanolone synthesis was assessed in fetal guinea pigs after surgery to induce IUGR. Both fetal brain and plasma allopregnanolone concentrations were reduced by finasteride treatment. Finasteride treatment and IUGR were associated with reduced myelination and IUGR with increased astrocyte activation in the brain.

A model of premature birth (0.87 gestation) was developed in the guinea pig to assess the effect of preterm postnatal changes in neuroactive steroid concentrations on the developing brain. Preterm guinea pigs exhibited less activity, higher mortality rates, reduced allopregnanolone concentrations and lower expression of steroid synthetic enzymes. Myelination in the hippocampus and cerebellum was also suppressed.

The potential of postnatal replacement of neuroactive steroids by progesterone treatment was examined in preterm neonates. Following progesterone therapy, cortisol levels were elevated, with implications for development. Sex differences were noted in plasma neuroactive steroid concentrations. Brain allopregnanolone concentrations in preterm neonates were increased at postnatal days 1 and 8 by progesterone administration. Exploratory behaviours were altered in progesterone treated preterm animals, demonstrating changes in brain function associated with treatment.
This thesis identifies changes in the perinatal guinea pig brain associated with altered neuroactive steroid concentrations and establishes the efficacy of progesterone replacement therapy in augmenting the endogenous synthesis of allopregnanolone in the preterm brain. Long-term studies to establish the developmental outcomes of postnatal progesterone/neuroactive steroid replacement after preterm birth and in combination with complications such as IUGR, hypoxic insults and infection are needed to identify new, safe and effective treatment options.
LIST OF FIGURES & TABLES

Figure 1.1 Mechanisms of Cell Death in Perinatal Brain Injury ......................... 47
Figure 1.2 Pathways of Steroid and Neuroactive Steroid Synthesis from Cholesterol ......................................................... 66
Figure 1.3 Mechanisms of Progesterone and Allopregnanolone Signalling 68
Figure 1.4 Pregnan Isomer Synthesis and Structure .................................. 85
Figure 1.5 Schematic Diagram of Specific Aims ........................................ 102

Figure 2.1 Uterine Blood Supply in the Guinea Pig and Site of Radial Artery Ablation for IUGR Surgery ........................................ 106
Table 2.1 Neonatal Scoring ........................................................................... 111
Table 2.2 Cross Reactivity of Sheep Allopregnanolone Antisera with Related Steroid Compounds ............................... 120

Figure 3.1 Fetal Brain Allopregnanolone Concentrations ............................. 143
Table 3.1 Fetal Animal Body and Organ Weights ......................................... 143
Figure 3.2 Fetal Brain 5α- Reductase Enzyme Expression ............................. 145
Figure 3.3 GFAP Immunostaining in the Fetal Guinea Pig Brain ................. 147
Figure 3.4 MBP Immunostaining in the Fetal Guinea Pig Brain .................. 149

Table 4.1 Animal Characteristics and Organ Weights of Preterm and Term Neonatal Guinea Pigs ................................................................. 164
Figure 4.1 Myelin Basic Protein Expression in Neonatal Guinea Pigs ............. 166
Figure 4.2 Glial Fibrillary Acidic Protein in Neonatal Guinea Pig Brains 168
Figure 4.3 Plasma Steroid Concentrations in Fetal and Neonatal Guinea Pigs .................................................................................. 170
Figure 4.4 Fetal and Neonatal Brain Allopregnanolone Concentrations 171
Figure 4.5 Expression of 5α-reductase in Fetal and Neonatal Guinea Pig Brains .................................................................................. 172
Table 5.1  Animal Characteristics and Organ Weights of Preterm and Progesterone Treated Neonatal Guinea Pigs.......................... 192

Figure 5.1  Daily Condition Scores in Preterm Guinea Pigs with Postnatal Progesterone Treatment ........................................... 193

Figure 5.2  Neonatal Salivary Progesterone Concentrations in Preterm Guinea Pigs with Postnatal Progesterone Treatment .......... 194

Figure 5.3  Plasma Steroid Concentrations in Preterm Guinea Pigs following Postnatal Progesterone Treatment ........................ 196

Figure 5.4  Brain Allopregnanolone Concentrations in Preterm Guinea Pigs following Postnatal Progesterone Treatment .............. 198

Figure 5.5  Hippocampal 5α-Reductase Enzyme Expression in Preterm Guinea Pigs following Postnatal Progesterone Treatment ..... 198

Figure 5.6  Myelin Basic Protein Immunoreactivity in Preterm Guinea Pig Brains following Postnatal Progesterone Treatment ......... 199

Figure 5.7  Glial Fibrillary Acidic Protein Expression in Preterm Neonatal Guinea Pig Brains following Postnatal Progesterone Treatment .............................................................. 201

Figure 5.8  Microtubule Associated Protein 2 Expression in Preterm Neonatal Guinea Pig Brains following Postnatal Progesterone Treatment ............................................................... 201

Table 5.2  Open Field Activity and Novel Object Recognition in Preterm Neonates with Progesterone Treatment ......................... 202

Figure 5.9  Novel Object Recognition by Preterm Progesterone Treated Neonates ........................................................................ 202

Figure 6.1  - Lobules and Layers in the Guinea Pig Cerebellum .......... 217

Figure 6.2  - Cerebellar Lobule Development and Extracellular Granular Layer Thickness in Preterm and Progesterone Treated Preterm Guinea Pigs ................................................................. 220

Figure 6.3  - Myelin Basic Protein (MBP) Immunoreactivity in Cerebella of Preterm and Progesterone Treated Preterm Guinea Pigs .... 221

Figure 6.4  - Calbindin Staining in Purkinje Cells in Cerebella of Preterm and Progesterone Treated Preterm Guinea Pigs .......... 223

Figure A.1  - Fetal Plasma Allopregnanolone Concentrations........... 302

Figure B.1  - Term and Preterm Neonatal Guinea Pig Lung Sections .... 303
LIST OF PUBLICATIONS

Publications Arising from this Thesis:

Kelleher MA, Palliser HK, Hirst JJ
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Changes in neuroactive steroid concentrations after preterm delivery in the guinea pig.

Kelleher MA, Palliser HK, Walker DW, Hirst JJ (2011)

Publication Arising from this Thesis, results not presented:

Dyson RM, Palliser HK, Kelleher MA, Hirst JJ, Wright IMR (2012)
LIST OF CONFERENCE ABSTRACTS


Kelleher MA, Palliser HK, Hirst JJ (2011). Neurosteroid replacement therapy in the preterm neonate. 38th Annual Meeting of The Fetal and Neonatal Physiological Society, Palm Cove, Queensland, Australia. **Abstract 133**


Dyson RM, Palliser HK, Kelleher MA, Hirst JJ, Wright IMR (2010) Preterm birth and intrauterine growth restriction: effect on microvascular function in the neonatal guinea pig. *Annual Scientific Meeting of The Endocrine Society of Australia, Sydney, Australia. Abstract 475*


<table>
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<tr>
<td>3α,5α-THP</td>
<td>3α,5α-tetrahydroprogesterone; allopregnanolone</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>5α-DHP</td>
<td>5α-dihydroprogesterone</td>
</tr>
<tr>
<td>5αR</td>
<td>5α-reductase enzyme</td>
</tr>
<tr>
<td>5αR1</td>
<td>5α-reductase enzyme type 1</td>
</tr>
<tr>
<td>5αR2</td>
<td>5α-reductase enzyme type 2</td>
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<tr>
<td>AC</td>
<td>adenylate cyclase</td>
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<tr>
<td>ADHD</td>
<td>attention deficit and hyperactivity disorder</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B&lt;sub&gt;o&lt;/sub&gt;</td>
<td>tracer-antisera binding</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2 protein</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BLR</td>
<td>brain to liver weight ratio</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CA1</td>
<td>cornu ammonis area 1 of the hippocampus</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td>cyclic adenosine monophosphate</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>CPAP</td>
<td>continuous positive airway pressure</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
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Cu^{2+}  copper ion
DAB  3,3'-diaminobenzidine
DHEA  dehydroepiandrosterone
DHEAS  dehydroepiandrosterone sulfate
DHT  dihydrotestosterone
DNA  deoxyribonucleic acid
ECL  enhanced chemiluminescence
EDTA  ethylenediaminetetraacetic acid
EGL  external granular cell layer
EIA  enzyme immunoassay
ERK  extracellular signal-regulated kinase
FGR  fetal growth restriction
Fin  finasteride
GA  gestational age
GABA  γ-amino-butyric acid
GABA_{A}  γ-amino-butyric acid type A receptor
GFAP  glial fibrillary acidic protein
H_{2}O  water
hCG  human chorionic gonadotropin
HCl  hydrogen chloride
HRP  horseradish peroxidase
IgG  immunoglobulin G
IGL  internal granular cell layer
IL  interleukin
i.p.  intraperitoneal
IQ  intelligence quotient
IUGR  intrauterine growth restriction
IVH  intraventricular haemorrhage
K^{+}  potassium ion
KCC2  potassium chloride co-transporter 2
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<td>KMnO₄</td>
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<td>LPS</td>
<td>lipopolysaccharide; endotoxin</td>
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<td>MAP-2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
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<td>myelin basic protein</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mPR</td>
<td>membrane progesterone receptor</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>Na²⁺</td>
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<td>NaN₃</td>
<td>sodium azide</td>
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<td>phosphate buffer</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PEEP</td>
<td>positive end expiratory pressure</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PGRMC1</td>
<td>progesterone receptor membrane component 1</td>
</tr>
<tr>
<td>PI3K/Akt</td>
<td>phosphoinositide 3-kinase/protein kinase B</td>
</tr>
<tr>
<td>PIP</td>
<td>peak inspiratory pressure</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
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<tr>
<td>PPROM</td>
<td>preterm premature rupture of membranes</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<tr>
<td>PRE</td>
<td>progesterone response element</td>
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<tr>
<td>Pre-T</td>
<td>preterm postnatal day 1</td>
</tr>
<tr>
<td>Pre-T8</td>
<td>preterm postnatal day 8</td>
</tr>
<tr>
<td>+Prog</td>
<td>preterm postnatal day 1 with progesterone treatment</td>
</tr>
<tr>
<td>+Prog8</td>
<td>preterm postnatal day 8 with progesterone treatment</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>PVL</td>
<td>periventricular leukomalacia</td>
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<tr>
<td>RDS</td>
<td>respiratory distress syndrome</td>
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<tr>
<td>RIA</td>
<td>radio-immunoassay</td>
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<tr>
<td>ROP</td>
<td>retinopathy of prematurity</td>
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<tr>
<td>RU486</td>
<td>mifepristone; progesterone receptor antagonist</td>
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<tr>
<td>σ1</td>
<td>sigma 1 receptor</td>
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<tr>
<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SGA</td>
<td>small for gestational age.</td>
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<td>SIDS</td>
<td>sudden infant death syndrome</td>
</tr>
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<td>T1</td>
<td>novel object recognition test, trial 1 (familiarisation)</td>
</tr>
<tr>
<td>T2</td>
<td>novel object recognition test, trial 2 (recognition)</td>
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<tr>
<td>TBPS</td>
<td>t-butylbicyclophosphorothionate</td>
</tr>
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<td>TBS-T</td>
<td>tris-buffered saline with tween</td>
</tr>
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<td>TC</td>
<td>total counts</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>THDOC</td>
<td>tetrahydrodeoxycorticosterone</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>UCO</td>
<td>umbilical cord occlusion</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<td>±</td>
<td>plus or minus</td>
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<tr>
<td>~</td>
<td>approximately</td>
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<tr>
<td>°C</td>
<td>degrees celsius</td>
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<td>/</td>
<td>per</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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<td>w/v</td>
<td>weight per volume</td>
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<td>hour</td>
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<td>kilodalton</td>
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<td>kg</td>
<td>kilogram</td>
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<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>mA</td>
<td>milliamp</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>millilitre</td>
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<td>millimolar</td>
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ng  nanogram
nm  nanometre
nmol nanomole
pH  scale of hydrogen ion activity
pmol picomole
rpm revolutions per minute
sec second
V  volts
W  watts
μg  microgram
μL  microlitre
μm  micrometre
μmol micromole
Chapter One

Introduction

Infant survival has improved markedly over the last decades with major advances in obstetric and neonatal care. One of the major goals of current fetal and neonatal research is the improvement of long-term outcomes for infants that experience complications during pregnancy and birth. The course of growth and development of the fetus during gestation has wide-ranging implications on health and disease throughout life. One of the major organ systems that undergoes critical periods of growth, development and differentiation during fetal life is the brain and central nervous system. Alterations in fetal growth, including those due to premature birth, coupled with the increased sensitivity of the immature brain to injury, are major causes of neurological disorders and cognitive deficits that are present in the neonatal period and in later life. The identification of therapies that protect the growing brain from injury and promote normal brain development in the presence of perinatal complications is therefore an important goal for the prevention of neurodevelopmental disorders in vulnerable infants.

Progesterone, as well as being an essential hormone for pregnancy, is also the major precursor of a group of “neuroactive” pregnane steroids that have marked effects within the nervous system. There is a significant body of evidence in adult systems and in vitro models for the actions of neuroactive steroids in regulating neural activity, providing neuroprotection and promoting cell survival. The specific actions of neuroactive steroids and regulation of their synthesis within the fetal
and neonatal brain, however, is less clearly understood. Evidence for changes in neuroactive steroid synthesis associated with complications during the perinatal period, along with the loss of neuroactive supply following birth, have raised the potential for therapeutic replacement of neuroactive steroids in order to improve neurodevelopmental outcomes in vulnerable infants.

In particular, this thesis will examine the actions of neuroactive steroids in the fetal and neonatal brain in relation to neuropathological and neurobehavioural changes. Importantly, the establishment of a preterm neonatal model in the guinea pig will allow for the assessment of the therapeutic potential and actions of postnatal progesterone replacement in resupplying neuroactive steroids within the preterm brain.

1.1 PRETERM BIRTH

Preterm birth is a significant health issue contributing to the global burden of disease and disability. Along with low birthweight, which is often associated with prematurity, preterm birth is the leading cause of poor neonatal outcomes, accounting for up to 70% of neonatal deaths and 75% of neonatal illness and disability (Wen, Smith et al. 2004). Neonatal outcomes following preterm birth are influenced by the relative immaturity of the neonatal organ systems at delivery, the pathophysiological causes that resulted in the preterm delivery of the infant, the instability of the neonatal transition to extrauterine life and complications associated with the medical management of premature neonates (Behrman and Butler 2007; Allen, Cristfalo et al. 2011). Gestational age at birth is a critical predictor of neonatal and infant health that can have wide-reaching and long-lasting effects on health in later life. The further development of therapeutic strategies that improve these long-term health outcomes is
therefore a critical goal for the management of those infants that are born preterm.

1.1.1 Rates of Preterm Birth

Births that occur before 37 weeks of gestation are classified as preterm and occur in 6-15% of live births (depending on demographics and regional location), involving approximately 13 million births globally in 2005 (Slattery and Morrison 2002; Beck, Wojdyla et al. 2009). In Australia in 2009, preterm births accounted for 8.2% of all babies born (Li, McNally et al. 2011), an increase since 1992 when 6.9% of births in Australia were preterm (Lancaster, Huang et al. 1995). Increases in preterm birth rates over the last few decades have also been reported in the United States, from 9.4% in 1981 to a peak of 12.8% of births in 2006 (Martin, Hamilton et al. 2008; Klebanoff and Keim 2011). The increased rates of preterm birth have been linked to the more widespread use of assisted reproductive technologies, leading to an increased risk of multiple births and other complications, increased maternal age at pregnancy and changes in patterns of obstetric management and medical technology (Behrman and Butler 2007). Recent data from the United States suggest that preterm birth rates have steadied and declined slightly with a rate in 2010 of 11.9% (Martin, Osterman et al. 2010; Hamilton, Martin et al. 2011).

Globally, preterm birth is a particular issue in developing nations. Africa has some of the highest rates of preterm birth with estimates of 11-12% (Beck, Wojdyla et al. 2009). In addition, over 85% of all preterm births, or nearly 11 million preterm births per annum, occur in Africa and Asia (Beck, Wojdyla et al. 2009). In comparison, preterm births make up 6% of live births in Europe, which corresponds to approximately 40000 births annually in the United Kingdom (Slattery and Morrison 2002; McParland, Jones et al. 2004). The large number of preterm births in developing nations can, in part, be attributed to high birth rates and large population
densities in these regions, but is also exacerbated by disparities in the provision of healthcare in these countries. Preterm births that occur in the developing world represent a major burden on public and global health and on medical spending worldwide.

Also relevant to the provision of care and subsequent outcomes of preterm infants are the rates of preterm births at different gestational ages. Gestational age at delivery is one of the best predictors of neonatal and infant health (Gilbert, Nesbitt et al. 2003) and has many implications in terms of choices of clinical management and the allocation of healthcare resources. Premature births can be categorised based on gestational age at the time of delivery, with large differences between the numbers of preterm births that occur at very early gestations or closer to term. In Australia, 79% of preterm births (6.5% of all births) occur between 32 and 37 weeks of gestation and are categorised as moderate-late preterm infants (Laws, Li et al. 2010). In contrast, a relatively small number of babies are born very preterm (10% of preterm births occur between 28 and 31 weeks gestation) or extremely preterm (11% of preterm births occur at less than 28 weeks gestation) (Li, McNally et al. 2011). Whilst a majority of preterm births occur at later gestations, extremely preterm infants have been a focus of much of the clinical research aimed at improving the health of preterm neonates. Later preterm infants are also more likely to be overlooked in terms of close monitoring and clinical interventions due to their relative health in early neonatal life. However, epidemiological studies have shown that even late preterm infants are at a greater risk of poor health outcomes, including neurodevelopmental outcomes, when compared to infants born at term (Dong and Yu 2011; Engle 2011; Teune, Bakhuizen et al. 2011).

1.1.2 Causes of Preterm Birth
Preterm birth encompasses a range of pathologies that ultimately results in the premature delivery of the neonate, with the mechanisms involved in
causing a premature birth having important ramifications on the subsequent health of the neonate. Preterm births can be broadly divided into those that are medically indicated due to maternal or fetal health concerns and those that occur spontaneously, due to maternal infection, preterm premature rupture of membranes (PPROM) or of unknown aetiology (Tucker and McGuire 2004).

Medically indicated (iatrogenic) preterm births include caesarean-section and induced deliveries that are carried out due to diagnosed medical conditions present in the mother and/or fetus. Maternal indications include severe pregnancy hypertension or pre-eclampsia, obstetric complications such as placental abruption or multiple pregnancy and other acute or chronic illnesses, including maternal or intra-uterine infection (Slattery and Morrison 2002). Fetal considerations include intra-uterine growth restriction (IUGR), non-reassuring fetal state or fetal distress, fetal anomaly and the presence of infection (Moutquin 2003). There has been an increase in the rate of medically indicated preterm deliveries, reflecting a shift of clinical management towards delivery of the infant. In 2003 in the US, caesarean rates reached nearly 50% in neonates delivered prior to 32 weeks and over 37% for infants born between 32 and 36 weeks gestation, compared to an overall rate of 26% (Behrman and Butler 2007). A study examining Norwegian birth data identified an increase in preterm caesarean sections from 22.8% in 1980 to 36.5% in 1996 (Thompson, Irgens et al. 2006). This increase in medically indicated preterm birth has also been associated with a concurrent increase in average maternal age (Behrman and Butler 2007). Preterm births that occur due to medical intervention contribute significantly to preterm neonates and their potential health problems.

Infection is a major clinical focus in relation to preterm birth. Maternal or intra-uterine infection is a medical indicator for preterm birth and is itself
implicated in the onset of labour mechanisms that result in preterm delivery (Goldenberg, Culhane et al. 2008; Bastek, Gomez et al. 2011). The presence of an infection stimulates the innate immune system to produce inflammatory mediators, including pro-inflammatory interleukins (IL-1α, IL-1β, IL-6, IL-8) and tumour necrosis factor-α (TNF-α), which are associated with the onset of preterm labour. These cytokines stimulate the local production of prostaglandins (PGs) and matrix metalloproteinases (MMPs) within uterus and fetal membranes. These factors then initiate myometrial contractility and dilatation of the cervix, resulting in parturition (Grigsby, Novy et al. 2010; Bastek, Gomez et al. 2011). Infection, particularly chorioamnionitis, is mostly associated with very early preterm deliveries, being present in only 10% of deliveries between 33 and 36 weeks gestation, compared to 85% of births that occur earlier than 28 weeks gestation (Romero and Mazor 1988; Goldenberg, Culhane et al. 2008). The likelihood of neonatal sepsis, which is relevant to fetal and neonatal health outcomes, is also highest in earlier gestations and with lower birthweights (McParland, Jones et al. 2004). Extra-uterine or systemic infections, such as malaria, pyelonephritis, pneumonia and appendicitis along with local, intra-uterine infection and bacterial vaginosis have a strong causal link to preterm birth (Bastek, Gomez et al. 2011). The occurrence of infection-associated preterm birth is also likely to be mediated by genetic susceptibility (Bastek, Gomez et al. 2011). Despite the well-studied mechanisms of infection-associated preterm birth, the diverse nature of the infectious agents implicated, the range of clinical severity of infection and the difficulties of diagnosis with standard screening techniques, infection remains a major cause of both preterm birth and fetal brain injury and poor outcomes.

Another cause of spontaneous preterm birth, PPROM, the premature rupture of membranes at less than 37 weeks gestation and at least 1 hour
prior to the onset of contractions (Bastek, Gomez et al. 2011), occurs in about 25% of all preterm births (Moutquin 2003). PPROM often progresses into preterm labour and medical management can involve the administration of tocolytics to delay delivery and antenatal steroids to aid neonatal outcomes if preterm delivery does occur. Infection is present in 25-40% of PPROM cases that do not progress to labour (Slattery and Morrison 2002; Simhan and Canavan 2005). However, even in these cases, the risk of intra-uterine infection and progression of the infection to involvement of the fetus increases markedly following PPROM and therefore premature membrane rupture can be an important factor in indicated preterm caesarean or induced deliveries (Simhan and Canavan 2005). Infection-mediated causes of preterm birth have important implications for later neonatal health and increase the risk of perinatal brain injury and neurodevelopmental disorders.

A high proportion (approximately 50%) of preterm births are of unknown spontaneous or idiopathic origin (Moutquin 2003). Whilst the aetiology and pathophysiological mechanisms by which this group of preterm births occur is unclear, a number of risk factors have been identified that increase the risk of preterm birth and have become the object of ongoing research as potential targets for therapies to reduce the burden of preterm birth. Understanding the mechanisms involved in preterm labour is important for the correct treatment and mitigation of poor fetal and neonatal outcomes in preterm infants.

1.1.3 Risk Factors for Preterm Birth

Epidemiological studies have identified many factors that convey an increased risk of preterm birth. One of the most well studied risk factors for preterm birth is the ethnic or racial background of the mother. In Australia in 2008, Aboriginal and Torres Strait Islander women had a preterm birth rate of 13.3% compared to 8.0% in non-indigenous women
In the United States, the rate of preterm birth is highest in Black/African-American women, which reached a peak rate of 18.9% in 1990 (Behrman and Butler 2007) but has declined to 17.2% in 2010 (Hamilton, Martin et al. 2011). These rates are significantly higher than those in other ethnic and racial groups in the US and are nearly double that for non-hispanic white women of similar age and socio-economic background (Slattery and Morrison 2002). Genetic predisposition, potential epigenetic or gene-environment interactions and relative social disadvantage may combine to contribute to the increased risk of preterm birth in some of these populations.

Social disadvantage, low educational attainment, single marital status, smoking, alcohol and drug use and unemployment have all been identified as factors associated with low socio-economic status that significantly increases the relative risk of preterm birth (Moutquin 2003; Thompson, Irgens et al. 2006). High levels of psychosocial stress and stressful life events, particularly in unmarried mothers with low social, family and partner support are associated with preterm birth (Kramer, Goulet et al. 2001; Dole, Savitz et al. 2003). Other forms of maternal stress, such as traumatic events and natural disasters, pregnancy-related anxiety and depression also nearly double the likelihood of preterm birth (Goldenberg, Culhane et al. 2008; Wadhwa, Entringer et al. 2011). Stress-related increases in glucocorticoid exposure have been suggested as a mediator of this increased risk. Elevated levels of cortisol due to stress can increase the placental expression of corticotrophin-releasing hormone (CRH) that can then act to enhance the effect of oxytocin on uterine activity (Challis, Lye et al. 2001; Slattery and Morrison 2002). The interaction between the stress response, immunity and inflammation may also be involved in the pathophysiology of stress-related preterm birth (Wadhwa, Entringer et al. 2011).
Reproductive history also has strong links to the occurrence of preterm birth. Previous preterm birth can double the risk of subsequent preterm births, with the risk being inversely proportional to the gestational age of the previous preterm birth (Goldenberg, Culhane et al. 2008). Previous low birthweight, short interpregnancy interval and previous pregnancy loss also increase the risk of preterm birth (Slattery and Morrison 2002). Multiple pregnancy is also a significant factor, with up to 60% of twin and nearly all higher order multiple pregnancies delivering preterm (Behrman and Butler 2007). Maternal depletion, uterine, genetic and epigenetic changes have been proposed as potential causes of this increased risk (Goldenberg, Culhane et al. 2008). Pregnancy pathologies that may compromise the health of the pregnancy, such as the presence of acute or chronic illness, pre-eclampsia, hypertension and diabetes, recurrent bleeding, placenta abrupta and placenta previa are also likely to lead to preterm birth (Slattery and Morrison 2002; Goldenberg, Culhane et al. 2008; Klebanoff and Keim 2011). Uterine abnormalities, excessive uterine stretch, cervical weakness and shortened cervix have also been associated with the incidence of preterm birth (Wen, Smith et al. 2004; Goldenberg, Culhane et al. 2008). These risk factors for preterm birth may also further compromise the health of the fetus and neonate, influencing postnatal and long-term outcomes.

1.1.5 Postnatal Preterm Outcomes

Preterm birth is a major contributor to neonatal and infant mortality. In 2008 in Australia, the overall neonatal mortality rate (within 28 days following delivery) was 2.8 deaths per 1000 births. Preterm births accounted for a high proportion of this neonatal mortality, with nearly 41% of infants born between 20 and 27 weeks gestation not surviving the neonatal period (Laws, Li et al. 2010). However, neonatal survival was significantly higher even in preterm neonates born between 28 and 31
weeks, with a survival rate of over 97% in neonates of that age (Laws, Li et al. 2010). Similar survival rates have also been reported in a study of New South Wales Neonatal Intensive Care Unit (NICU) admissions with survival of 30% for deliveries at 23 weeks and 98.8% at 31 weeks gestation (Abdel-Latif, Kecskes et al. 2011). This study also identified that male neonates were more likely to die than age-matched females and that a majority of preterm neonatal deaths occurred within the first three days of life (Abdel-Latif, Kecskes et al. 2011). Due to advances in medical technology and clinical management practices for preterm neonates, these survival rates are significantly higher than those seen as recently as the 1980s. Three decades ago, neonates born at 23 weeks gestation were unlikely to survive, in 2000, up to 50% of neonates at this age survived the neonatal period (Ward and Beachy 2003). Improved survival of preterm neonates, particularly those born at extremely preterm gestations, relies on access to high standards of neonatal intensive care, with some estimates suggesting that 15% of neonatal deaths that occur in lower level care centres may have been prevented if they had occurred in tertiary-care hospitals (Saigal and Doyle 2008). Preterm neonatal survival is also considerably lower in developing countries, where healthcare provision and accessibility is reduced (Beck, Wojdyla et al. 2009). Additionally, the survival rate of preterm neonates is adversely affected by the presence of other pathologies, such as IUGR (see section 1.2) or infection (Saigal and Doyle 2008).

Poor neonatal health in the immediate postnatal period, along with long-term morbidity, are also important considerations following preterm birth. Pathologies that are associated with preterm birth are related to the immaturity of the neonate at the time of delivery and the particular vulnerability to injury of organ systems and structures at different stages during development. The organ systems that are most vulnerable to injury
and complications of prematurity include the central nervous system and sensory organs, the respiratory system, cardiovascular system and gastrointestinal tract (Allen, Cristfalo et al. 2011). Some of the most severe problems associated with prematurity include periventricular leukomalacia (PVL), cerebral palsy (CP), intra-ventricular hemorrhage (IVH), retinopathy of prematurity (ROP), necrotising enterocolitis, respiratory distress syndrome (RDS) and patent ductus arteriosis (Saigal and Doyle 2008; The EXPRESS Group 2010; Allen, Cristfalo et al. 2011; Teune, van Wassenaer et al. 2011). Despite important advances in neonatal intensive care, illness and permanent disability in preterm infants are still significant issues. Indeed, in some health settings, disability rates have increased due to changes in the management of preterm neonates that have increased survival of even extremely preterm infants resulting in increased vulnerability and poorer health outcomes in some surviving infants (Allen, Cristfalo et al. 2011). The focus on morbidity has also begun to shift medical and research efforts towards identification and treatment of subtle developmental disorders that may not be apparent in early life but that place a significant burden on the healthcare system, social services and the education system. The mechanisms of perinatal brain injury and causes of neurodevelopmental deficits associated with preterm birth are described in sections 1.3 and 1.4 below.

1.2 INTRA-UTERINE GROWTH RESTRICTION

Normal fetal growth and development involve the specialised and regulated processes of cell proliferation, organisation and differentiation leading to the development and maturation of organ systems that are essential to life (Sankaran and Kyle 2009). All aspects of growth are determined by the genetic profile of the fetus, which is in turn influenced by maternal, placental and environmental factors. Pathologies or insults
during gestation may result in the inability of the fetus to reach its growth potential, causing intra-uterine growth restriction to occur. IUGR has profound effects on the health and development of the fetus, leading to neonatal morbidity, permanent disability later in life and potentially fetal or neonatal death (Bernstein, Horbar et al. 2000). The relationships between poor fetal growth, and preterm birth and the consequences of these pathologies on the brain, are important considerations for potential therapeutic interventions to aid normal neurodevelopment, neonatal health and brain function later in life.

1.2.1 Intra-Uterine Growth Restriction & Low Birth-Weight
The correct diagnosis of IUGR can be problematic due to the presence of confounding factors and other pathologies, different aetiologies and difficulties in determining individual fetal growth patterns in utero, (Harkness and Mari 2004). However, advances in imaging techniques and the use of ultrasound and Doppler measurements has aided in the diagnosis of and understanding of the causes of IUGR (Ott 2006; Falo 2009). Fetal or intra-uterine growth restriction is defined as a pathological reduction in the expected pattern of fetal growth caused by an insult in utero. For ease of diagnosis, population birth weight curves are often used to determine when fetal growth is below an expected range (Gardosi 1998). Infants with birthweights that fall below the 10th centile are commonly defined as either growth restricted or small for gestational age (SGA) (Bamberg and Kalache 2004) (Platz and Newman 2008). However, this definition can be ambiguous as infants that are appropriately grown but small, due to normal variability in genetic background or maternal physiology, may be defined as being growth restricted (Maulik 2006). The pathophysiology of IUGR that results in altered fetal growth patterns, can also cause asymmetric or abnormal growth without significantly reducing birthweight to below the 10th percentile (Bakketeig 1998). The potentially
ambiguous use of SGA (usually defined as symmetric growth restriction) and IUGR (asymmetrical) in defining reduced fetal growth complicates the identification, management, and research into IUGR. These distinctions, however, are important, as the pathological processes that result in IUGR can have a significant influence on neonatal health, including neurodevelopmental outcomes.

Other measures used to determine IUGR include use of the neonatal ponderal index (taking into account weight and length) and ultrasound measurement of growth parameters such as abdominal and head circumference, femur length and proportions of muscle and fat (Bhatia, Agarwal et al. 1984; Falo 2009; Sankaran and Kyle 2009). Changes in uterine and umbilical cord blood flow and placental perfusion are strong indicators of pathology that may lead to a diagnosis of IUGR (Marsal and Ley 1992; Yoshimura, Masuzaki et al. 1998; Lang, Baker et al. 2003; Baschat and Hecher 2004). A common feature of IUGR, seen in 70-80% of cases, is the asymmetrical reduction of growth that results in relative brain sparing and large head size, to the detriment of other organ growth, resulting in comparatively smaller abdominal growth (Brodsky and Christou 2004). Insults that occur early in gestation may result in an overall reduction in fetal size, whereas severe insults later in gestation may result in changes in the patterns of fetal growth and more asymmetric growth.

Low birthweight is, however, a good predictor of neonatal health and long-term outcomes and is used as an easily defined clinical measure. The WHO has defined categories of birthweight that also relate to the risk of neonatal morbidity and mortality. Infants with a birthweight of less than 2500g are low birthweight, which is further divided into very low birthweight (less than 1500g) and extremely low birthweight (less than 1000g) categories. Of live births in Australia in 2009, 6.2% of infants were classed as low birthweight, 1.0% had very low birthweights and in only 0.5% of live births
did infants weigh less than 1000g (Li, McNally et al. 2011). The rates of low birthweight include live births of all gestational ages, with preterm birth accounting for much of the incidence of low birthweight. However, the use of low birthweight cut-offs does not take into account the other significant pathophysiological changes that are present in the adaptations that cause IUGR. For this reason, detailed clinical diagnostic tools are important for the appropriate management and treatment of SGA, preterm and IUGR infants. Overall, fetal and neonatal health is influenced not only by size at birth but also by the accompanying physiological changes that can lead to IUGR or the developmental perturbation of fetal growth, which have serious consequences on morbidity and mortality.

1.2.2 Pathophysiology of IUGR

IUGR results from a pathological reduction in the supply of essential substrates, nutrients and oxygen so that the metabolic demands of the developing fetus are not met (Sankaran and Kyle 2009). In response to a reduction in substrate availability and in order to reduce metabolic demands, the fetus utilises a number of compensatory mechanisms. These physiological adaptations to adverse intra-uterine conditions result in changes in patterns of organ growth and maturation that may ultimately lead to altered growth and/or IUGR. Fetal adaptations associated with IUGR include changes in haemodynamic, metabolic and cardiovascular function; modified hormonal, growth factor and gene expression; and altered cell proliferation, differentiation and growth (Brodsky and Christou 2004). Compensatory fetal haemodynamic changes are mediated by signals from vasoactive factors, such as endothelin and nitric oxide metabolites, that respond to reduced fetal oxygen saturation and maternal conditions such as pre-eclampsia (Lyall, Greer et al. 1996; Napolitano, Miceli et al. 2000; Thaete, Kushner et al. 2005; Wang, Chang et al. 2010). Other changes that may accompany IUGR include reduced heart rate, myocardial
hypertrophy, fetal hypertension and increases in placental vascular resistance (Murotsuki, Challis et al. 1997). Deficits in substrate availability also cause the preferential redirection of blood flow to essential organs, such as the brain, heart and adrenal glands (Peeters, Sheldon et al. 1979; Yoshimura, Masuzaki et al. 1998). Animal models have shown increases in regional cerebral blood flow in response to reduced oxygen saturation and uteroplacental perfusion. However, despite this increase in blood flow, oxygenation in regions, such as the hippocampus and cerebellum may still be reduced, with accompanying changes in neuroendocrine metabolism and neurotransmitter expression (Jensen, Klonne et al. 1996). Additionally, hypoxic conditions can also reduce fetal autoregulatory control of vasodilatation within the cerebral vasculature, further altering cerebral blood flow (Salihagic, Georgescus et al. 2000). This suggests that responses in the growth-restricted fetus may be insufficient to protect the brain from hypoxic injury (see section 1.4), especially when faced with chronic or recurrent insults. A consequence of these haemodynamic changes to fetal blood flow is the pattern of asymmetric growth restriction (brain-sparing) that is a hallmark of IUGR. These vascular and haemodynamic changes also have important implications of the programming of cardiovascular disease in later life (McMillen and Robinson 2005). Redistribution of fetal blood flow and failure of fetal haemodynamic control change normal patterns of fetal growth and contribute to the brain injury that is associated with IUGR.

Another mechanism by which fetal growth is regulated is via the expression of growth factors and other hormones. Insulin-like growth factor (IGF), the expression of which is altered in some cases of IUGR, is central to the regulation of tissue specific growth and in response to alterations in glucose supply (Sorem and Siler-Khodr 1998). Abnormal levels of IGF are associated with poor fetal growth caused by
uteroplacental insufficiency (Langford, Blum et al. 1994) and congenital disorders (Gluckman, Gunn et al. 1992). The hormones cortisol, insulin, leptin and thyroxin, also regulate substrate use and fetal growth. The expression of these hormones is influenced by glucose and oxygen supply, which is reduced in IUGR caused by placental insufficiency or maternal calorie restriction.

These changes in blood flow, energy generation, and hormonal and growth factor signalling result in altered patterns of cell growth and differentiation, imbalance of cell types and altered gene expression. These changes ultimately lead to altered cell, tissue and organ growth. Much of the evidence for the pathophysiology of IUGR has been gathered from experimental animal models of reduced uteroplacental blood flow, placental insufficiency and umbilical cord embolisation (UCO), particularly in the sheep, non-human primate and rodent models such as the guinea pig (Robinson, Kingston et al. 1979; Rees, Stringer et al. 1997; Mallard, Rees et al. 1998; Rees, Mallard et al. 1998; Duncan, Cock et al. 2000; Moxon-Lester, Sinclair et al. 2007; Vuguin 2007; Westcott, Hirst et al. 2008; Turner and Trudinger 2009). Measurements of factors in cord blood, maternal blood and placental tissue have also provided direct information about the pathophysiological changes of IUGR in human infants.

1.2.3 Risk Factors and Causes of IUGR

Fetal growth restriction or IUGR does not have a single cause but rather results from a heterogeneous group of pathologies that have detrimental effects on fetal growth. Some common causes of IUGR include placental dysfunction, reduced nutrient and substrate supply from the maternal circulation or metabolic problems that mean the fetus is unable to utilise the nutrients supplied by the mother and placenta (Brodsky and Christou 2004).
The placenta, as the interface between the maternal and fetal circulations, is essential to normal fetal growth and is responsible for adequate gas exchange, the delivery of nutrients to the developing fetus and is the main metabolic resource available to the fetus (Pardi, Marconi et al. 2002). Abnormal placentation, placental vasculopathy, chronic placental inflammation, abnormal umbilical cord development and placental insufficiency are all pathologies that may cause IUGR, with reduced placental size also being a common feature of fetal growth restriction (Heinonen, Taipale et al. 2001; Pardi, Marconi et al. 2002; Baschat and Hecher 2004).

In addition to its metabolic roles, the placenta is also a major endocrine organ during pregnancy. The placenta is responsible for the production and transfer of steroid hormones, such as progesterone, estrogen, and peptide hormones to the fetus. These hormones, that are essential to the maintenance of pregnancy, also regulate fetal growth and provide essential developmental signals to the growing fetus (Everett and MacDonald 1979). Placental dysfunction can therefore effect the production and supply of critical hormones to the fetal and maternal circulations.

Another factor that influences the ability of the placenta to adequately supply the metabolic needs of the fetus is the maternal supply of substrates. Medical conditions that affect the mother such as hypertension, pre-eclampsia, diabetes, endocrine disorders and autoimmune diseases can contribute to IUGR by reducing uterine/placental perfusion, potentially resulting in reduced blood flow, impaired placental vascularisation and altered expression of important growth factors (Lang, Baker et al. 2003). Maternal malnutrition, low maternal pre-pregnancy weight and low caloric intake during pregnancy can also severely reduce the availability of essential substrates to the fetus and limit fetal growth (Belkacemi, Nelson et al. 2010).
Other important risk factors that influence the likelihood of IUGR include reproductive history, parity, previous pregnancies with IUGR, recurrent miscarriages, history of pre-eclampsia, presence of a multiple pregnancy and maternal age (teenage or >45yrs). Intrauterine infections and inflammation, chromosomal abnormalities and ingested teratogens, particularly maternal alcohol consumption during pregnancy, are also recognised as causes of fetal growth restriction (Ghidini 1996). Similarly to preterm birth, low maternal socio-economic status and drug use also increase the risk of IUGR pregnancies (Ong, Preece et al. 2002). Reduced oxygen concentrations also result in higher rates of IUGR in communities that live at high altitudes (Moore 2003). These factors and their affects on fetal growth convey an increased risk of fetal and neonatal mortality and morbidity and are therefore important considerations for the development of strategies to improve neonatal health and long-term outcomes.

1.2.4 Intra-Uterine Growth Restriction and Preterm Birth

There are strong associations between intrauterine growth restriction and the disruption of fetal growth patterns with the subsequent occurrence of preterm birth. Epidemiological studies have shown a 2- to 3-fold greater risk of spontaneous preterm labour amongst fetuses that are below the normal weight range for gestational age (Lackman, Capewell et al. 2001). Importantly, the increased incidence of preterm birth with IUGR was also associated with a 5-fold greater risk of perinatal death (Lackman, Capewell et al. 2001). Whilst there is difficulty in separating morbidity and mortality caused by either IUGR or preterm birth, as similar mechanisms can be involved in the aetiology of both problems, studies of infant outcomes have shown an increase in mortality in IUGR neonates even after data were corrected for gestational age (Bernstein, Horbar et al. 2000). Some mechanisms that may be responsible for the association between preterm birth and IUGR include IUGR-induced changes in prostaglandin signalling
that promote uterine activity and cervical ripening and increases in cortisol in response to fetal stress, however these and other mechanisms that link IUGR with preterm birth are not fully understood (Challis, Sloboda et al. 2002; Palliser, Welsh et al. 2011). The co-incidence of IUGR and preterm birth and their effects on neonatal health mean that these pathologies, along with other mediators of fetal and neonatal illness and disease, should not be considered in isolation, particularly for the identification of treatments that will improve long-term outcomes for vulnerable infants.

1.2.5 Postnatal Outcomes of IUGR Infants

Poor neonatal outcomes are strongly associated with altered fetal growth, low birth-weight and IUGR. The pathological insult that leads to IUGR, the fetal adaptations to that pathology and the increased vulnerability of low birthweight neonates to postnatal complications all contribute to the burden of neonatal morbidity and mortality present in IUGR infants. Much of the risk of postnatal complications associated with IUGR is related to the severity of the growth retardation, with an increase in the incidence of morbidity and permanent disability as birthweight decreases. In the neonatal period, infants born with a birthweight of 2000-2499g have a 10-times greater risk of mortality than infants born 1kg heavier (Ashworth 1998).

Neonatal outcomes associated with IUGR are closely linked to those seen in preterm infants and are difficult to isolate. When corrected for confounding factors, IUGR neonates have poorer health outcomes than non-IUGR infants of similar gestational age (Bernstein, Horbar et al. 2000) (Piper, Xenakis et al. 1996). Preterm IUGR neonates have higher rates of birth trauma and are more likely to be delivered by caesarean section (Piper, Xenakis et al. 1996). The incidence of intra-ventricular haemorrhage, necrotising enterocolitis and respiratory distress syndrome is also higher in IUGR neonates than appropriately grown infants, with
infants below the 3rd centile in weight most at risk (McIntire, Bloom et al. 1999).

Poor neurodevelopmental outcomes that are associated with IUGR are mediated by alterations in cellular composition and structural components of brain growth. IUGR also potentiates the vulnerability of the perinatal brain to injury caused by subsequent insults and comorbidities (Yanney and Marlow 2004; Wang, Liu et al. 2008). The structural changes in the brains of IUGR infants, which show reductions in grey matter volumes, as measured by magnetic resonance imaging (MRI) (Toft, Leth et al. 1995) and disrupted cortical folding, associated with functional neurological deficits (Dubois, Benders et al. 2008; Huppi 2010). At pre-school and school-age, both verbal intelligence quotient (IQ) and full-scale IQ are significantly lower in preterm-IUGR infants compared to appropriately grown preterm infants (Morsing, Asard et al. 2011). Some long-term effects of IUGR include poor childhood growth and short stature, increased risk of sudden infant death syndrome (SIDS), neurodevelopmental and cognitive deficits, and programming of adult disease, particularly metabolic syndrome disorders, hypertension, diabetes, renal disease and heart disease (Brodsky and Christou 2004). The presence of IUGR potentiates an increased vulnerability to developmental disorders, particularly in the presence of subsequent insults such as preterm birth, birth asphyxia and postnatal events that result from neonatal instability. The association of these poor functional outcomes with IUGR demonstrates the potential significance of fetal adaptations to compromises during pregnancy and alterations in fetal growth on the development of the immature brain and perinatal brain injury.
1.3 PERINATAL BRAIN INJURY

Neurodevelopmental disorders are associated with insults that occur during gestation and the postnatal period that cause injury to the developing brain or disrupt normal brain maturation. Perinatal brain injury is a term that encompasses a diverse range of pathologies that lead to brain damage resulting in neurological, cognitive, motor, and behavioural disorders and deficits. The developing immature brain is particularly vulnerable to injury stemming from pathologies present during gestation, complications during birth and the immaturity of brain structures and cells. The severity and long-term sequelae of perinatal brain injury is determined by the type of insult, the gestational age of the fetus/neonate at the time of injury and access to appropriate medical interventions (Inder and Volpe 2000; Volpe 2001; du Plessis and Volpe 2002). The success, however, of therapies for the prevention or treatment of perinatal brain injuries has been mixed, with the development of new therapies the focus of much research (Rees, Harding et al. 2011).

Complications during pregnancy such as chronic placental insufficiency leading to IUGR and preterm birth are important causes of perinatal brain injury and poor neurodevelopmental outcomes (Rees and Inder 2005; Rees, Harding et al. 2011). Pathologies during the perinatal period not only contribute directly to the pathogenesis of injury in the fetal and neonatal brain but also convey an increased vulnerability to injury in the event of a subsequent insult, such as preterm birth.

A number of mechanisms are responsible for perinatal brain injury, however, the breakdown of metabolic pathways, triggered by an initial insult or pathology, is a common factor that results in neuronal and glial cell damage and death. This section outlines some of the cellular and molecular mechanisms involved in the most common insults that lead to altered perinatal brain development and damage, including excitotoxicity,
oxidative stress and inflammatory processes (Dammann and Leviton 1997; Back, Gan et al. 1998; Johnston 2005). Whilst discussed separately, the complex nature of the central nervous system means that these processes are closely intertwined and that the aetiology of perinatal brain injury is multi-factorial. The interactions, some of which are outlined in Figure 1.1, are important considerations when examining the potential of therapeutic interventions to prevent or reduce the severity of brain injury in the perinatal period. Hypoxic-ischemic or asphyxial insults have been the focus of much of the research related to perinatal brain injury and have been closely examined using a number of experimental animal models (Yue, Mehmet et al. 1997; Hossman 1998; Nakajima, Ishida et al. 2000; Puka-Sundvall, Wallin et al. 2000; Tan, Drobyshevsky et al. 2005; Vannucci and Vannucci 2005; Hill and Fitch 2012). These insults include intrauterine events such as umbilical cord occlusion and chronic placental insufficiency that is associated with IUGR (Rees, Mallard et al. 1998). Postnatal insults such as intra-ventricular haemorrhage or transient ischemic events in the preterm neonatal brain have also been implicated along with maternal, intra-uterine and postnatal infection (Rees, Harding et al. 2008). Insults such as bilirubinaemia, exposure to toxins or teratogenic materials during gestation can also cause brain injury.

1.3.1 Excitotoxicity

Excitotoxic processes can be triggered by perinatal insults that lead to the breakdown of normal cellular respiration, inhibiting the ability of the cellular machinery to function (Vannucci 1990). Energy production within neurons and other cells is essential to the maintenance of the delicate ion balance, between the intracellular and extracellular compartments, that controls neural excitability and signalling. One of the main roles of
Perinatal brain injury is influenced by the relative immaturity of the brain (often associated with preterm birth), the presence of hypoxic/ischemic conditions, and the presence of insults such as IUGR and infection. The resulting metabolic disturbance from these pathologies leads to cellular energy failure, the malfunction of essential cellular processes, including ion transport, water balance, repair processes and normal gene transcription and translation. Dysregulation of ion balance can result in the excitotoxic propagation of action potentials throughout the neural network. The generation of toxic free radical species, due to mitochondrial failure, results in oxidative damage to DNA, lipid membranes and organelles. Cytokines released or recruited following cell damage or due to infection, activate immune and inflammatory pathways. Damage to cells also results in activation of cell death pathways. Short and long-term cell death occurs via both apoptotic and necrotic processes. ATP, adenosine triphosphate; IL-1β/6, interleukins; TNF-α, tumour necrosis factor-α.

Figure 1.1 - Mechanisms of Cell Death in Perinatal Brain Injury
adenosine triphosphate (ATP) as the major source of chemical energy within the brain is to maintain the correct concentration gradients of sodium, potassium and calcium ions across neuronal plasma membranes so that action potentials may be generated. This is achieved via action of energy-dependent Na\(^+\)/K\(^+\)-ATPase and Ca\(^{2+}\) receptor- and voltage-mediated exchange pumps (Erecinska and Silver 1989). Dysregulation of these ion control mechanisms due to energy failure can lead to reversal of Na\(^+\) ion gradients and intracellular influx and accumulation of calcium ions. As shown in Figure 1.1, this in turn causes further release of intracellular Ca\(^{2+}\) stores, rapid neuronal depolarisation, generation of unwanted action potentials and the uncontrolled release of signalling molecules, in particular, glutamate, an excitatory amino acid neurotransmitter (Szatkowski and Attwell 1994). Additionally, the uncontrolled changes in ionic concentrations due to breakdown of these mechanisms, also alters the osmotic balance of cells, causing mechanical damage due to swelling from excessive intracellular influx of water that may potentially lead to cell death (Inder and Volpe 2000).

Excitotoxicity, the pathological propagation of excitatory signals throughout the neural network, can lead to death of neuronal and glial cells via necrotic and apoptotic mechanisms (Portera-Cailliau, Price et al. 1997; Johnston 2005). During normal neuronal functioning, glutamate that is released into the synaptic cleft is rapidly taken up by transporters present on surrounding astroglia. However, as this process is energy-dependent, metabolic disruption causes high concentrations of glutamate to accumulate in the extracellular space and activate synaptic glutamate receptors, including the N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainic acid receptors. Activation of these receptors, which mediate ion flux, contribute to the toxic intracellular accumulation of calcium ions (Arundine and
Tyminski 2003), which further perpetuates excitatory signals throughout the neural network by triggering the additional uncontrolled release of glutamate. The excessive neural excitation that occurs due to excitotoxicity causes cell damage and contributes to other mechanisms of cell injury, leading to neuronal and glial cell death.

1.3.2 Oxidative Stress

Much of the cellular injury that results from metabolic disturbance in the perinatal brain is due to the production oxygen free radicals (OFRs)(Taylor, Edwards et al. 1999). These free radical species are highly reactive and cause chemical changes to essential biological molecules resulting in loss of function and cellular damage. The concentrations of these oxidative species increase when energy production fails, mitochondrial function is disrupted and inflammatory processes are triggered. Oxidative stress causes damage to cellular membranes, proteins, essential enzymes and nuclear DNA (see Figure 1.1). Lipid peroxidation damages the integrity of plasma lipid membranes, including those in the mitochondria, further affecting cellular osmotic and ionic balance and energy production (Delivoria-Papadopoulos and Mishra 2000). The accumulation and damage done by free radicals within the developing brain is further exacerbated by the relative immaturity of protective anti-oxidant systems. Both neuronal cells, glia and white matter are affected by lipid peroxidation (Ikeda, Murata et al. 1998; Castillo-Melendez, Chow et al. 2004) and there is evidence from studies in the guinea pig that the fetal brain, close to term, may be more susceptible to injury via this mechanism than earlier in gestation (Maulik, Zanelli et al. 1999). Indicating periods of particular sensitivity to this mode of injury throughout development. Cerebral reperfusion following an ischemic event also contributes to the generation of oxidative species and the severity of the resulting brain injury (Inder and Volpe 2000).
1.3.3 Inflammation

Inflammation, triggered by the above mechanisms and by intra-uterine and postnatal infections is also implicated in the aetiology of perinatal brain injury. The aetiology of inflammation-induced perinatal brain injury involves the induction of immune responses within the brain, proliferation of microglia in regions of the brain and production of pro-inflammatory cytokines (Rees and Harding 2004). The presence of cytokines such as IL-1β, IL-6 and TNF-α, within the uterine compartment, fetal circulation and fetal brain during maternal infection has strong associations with subsequent perinatal brain injury (Dammann and Leviton 1997) and microglia have been shown to accumulate in response to carotid artery ischemia in the fetal rat brain (Ivacko, Sun et al. 1996). Cytokine receptors that are present in the brain activate astrocytes and fetal microglia, triggering the local production of cytokines and soluble injury mediators, inducing leukocyte infiltration and activation of glial cells (Silverstein, Barks et al. 1997). Induction of pro-inflammatory cytokines also activates enzymes involved in the synthesis of oxygen free radical species, contributing to damage via oxidative stress pathways. In turn, the induction of inflammatory pathways also contributes to perinatal brain injury by increasing the risk of thrombosis, haemorrhage and vasoconstriction that leads to ischemic events.

Inflammatory processes also contribute to loss of integrity of the blood-brain barrier (BBB). Cytokines such as TNF-α and IL-1β, OFRs, and other inflammatory mediators, such as prostaglandins, which are elevated during inflammation and injury can directly influence the permeability of the BBB (Meqyeri, Abraham et al. 1992) and increase the uptake of compounds and entry of leukocytes into the injured tissue. These inflammatory immune responses also contribute to perinatal brain injury by causing vasoconstriction (Meqyeri, Abraham et al. 1992).
1.3.4 Apoptosis

Another process involved in perinatal brain injury is the activation apoptotic pathways that result in programmed cell death. Apoptosis in perinatal brain injury is usually involved in the delayed loss of cells up to a week after an insult occurs. Apoptosis mechanisms include the induction or activation phase, which may be reversible and the execution phase, when irreversible changes commit the cell to apoptosis and degradation, associated with the final disposal of the cell (Wilson 1998).

Developmentally regulated apoptosis occurs as a constitutive part of brain growth as brain signalling pathways form. Surpluses of cortical neurons are produced during brain development in gestation; with a pruning or reduction in neuronal number in the last quarter of gestation as neuronal connections develop (Rabiowicz, de Courten-Myers et al. 1996). Neuronal synaptogenesis across many regions of the brain also occurs rapidly in the third trimester of pregnancy and continues into the postnatal period (Rakic, Bourgeois et al. 1986). The correct formation of synaptic connections is a major mediator of brain developmental plasticity during this time. Metabolic stressors can interfere with this development, particularly via an aberrant increase in apoptosis, altering the neurodevelopmental path of remaining neuronal and glial cells (Bhutta and Anand 2002). Loss of injured cells and increases in apoptosis are evident in gross changes in grey matter volume, white matter volume and ventricle size.

A number of molecules regulate the activation of apoptosis. A family of cysteine proteases, known as caspases, that when activated result in activation of proteolytic enzyme cascades that are critical to the apoptosis process. Caspase-3 is an important member of the apoptotic signalling cascade, which is thought to be critical to apoptosis and which has developmentally regulated regional expression within the developing brain. Activation of apoptotic genes, such as Bel-2 and Bax and signalling via the
Fas death-receptor are also involved in the signalling and activation of apoptosis (Wilson 1998).

Apoptosis in perinatal brain injury as a result of hypoxic-ischemic insult or mitochondrial dysfunction has been closely examined in animal models. Even short periods of ischemia in the perinatal brain can result in increased caspase-3 and Bax activity and neuronal death (Bossenmeyer-Pourie, Koziel et al. 1999; Puka-Sundvall, Wallin et al. 2000). Evidence of elevated levels of apoptosis is present at 12-24hrs after the initial insult and remain for several days (Portera-Cailliau, Price et al. 1997; Yue, Mehmet et al. 1997; Nakajima, Ishida et al. 2000; Northington, Ferriero et al. 2001; Falkowski, Hammond et al. 2002). There is a potential therapeutic window for the reduction in the severity of perinatal brain injury if effective treatments can be administered before cells are committed to the apoptotic execution phase.

1.3.5 Preterm Brain Injury and Periventricular Leukomalacia

Perinatal brain injury can be defined by morphological and structural changes present in the brain, the pattern of which is strongly determined by the severity of insult and the developmental stage of the brain at the time of insult, with preterm and term brain injury differing greatly (Sie, van der Knapp et al. 2000). Neurodevelopmental disruption due to the premature transition from intra-uterine to extra-uterine life and the premature removal of maternal support are also important factors that mediate the long-term outcomes of perinatal brain injury. Whilst certain patterns of brain injury are more likely in preterm and term infants, the severity of perinatal brain injuries vary greatly with type of insult, resulting in a wide range of morphological changes and functional neurodevelopmental outcomes (du Plessis and Volpe 2002). Additionally, perinatal brain injury should be considered as a combination of direct injury to the immature brain, coupled with a disruption of critical
developmental processes, growth and differentiation that may result in lasting functional deficits (Volpe 2009).
Periventricular leukomalacia (PVL) is an important pathology in preterm brain injury that contributes to the cognitive, sensory-motor deficits, and incidence of cerebral palsy linked with preterm birth. PVL is most commonly associated with pathology between 23 and 32 weeks of gestation. This period coincides with the presence of high numbers of immature late oligodendrocyte progenitors in the periventricular white matter regions, prior to the commencement of myelination (Back, Luo et al. 2001). In addition, cerebral autoregulatory processes and the cerebrovasculature are also functionally immature at this stage in development, making the brain vulnerable to ischemic insults and haemorrhage. The neuropathology of PVL is associated with focal cystic lesions in the cerebral white matter and diffuse necrotic injury characterised by disseminated loss of oligodendrocyte precursor cells (Volpe 2001). The diffuse injury associated with PVL is suggested as being a postnatal event or due to less severe ischemic insults. As these injuries result in loss of pre-myelinating cells and disruption of oligodendrocyte lineage maturation, subsequent myelination is delayed and reduced (Leviton and Gilles 1996; Segovia, McClure et al. 2008). Both imaging studies in human preterm infants and animal models of infection and hypoxic-ischemic insults have identified hypomyelination, changes in white matter volume and increased ventricle size with PVL injury (Volpe 2001; Pang, Cai et al. 2003; Baud, Daire et al. 2004; Inder, Neil et al. 2005; Inder, Warfield et al. 2005).
The risk of PVL is greatest in extremely preterm infants and is further increased with the presence of IUGR (Rezaie and Dean 2002). PVL is most commonly associated with intra-ventricular haemorrhage, cardiac and respiratory disturbance, presence of infection and with postnatal survival for at least a few days. The aetiology of PVL involves a metabolic
disturbance that triggers excitotoxic, inflammatory, oxidative and apoptotic pathways (Volpe 2001; Volpe 2001; Back 2006). Fetal instability, hypotensive events, impaired cerebral vascular autoregulation and immature vascular structure contribute to periventricular ischemia and haemorrhage that are hallmark features in the aetiology of PVL. The patterns of PVL injury are associated with the terminal end regions of immature cerebral vessels, where ischemic and haemorrhagic events are most likely to occur.

The regions of cerebral vessel susceptibility correspond with the location of oligodendrocyte progenitors that are vulnerable to injury. Oligodendrocytes are the cells within the central nervous system responsible for the formation of myelin sheaths that facilitate the propagation of axonal action potentials. The developmental progress and differentiation of oligodendrocytes into mature myelinating cells has been established throughout gestation in a number of human and animal studies (Back, Gan et al. 1998; Back, Luo et al. 2001; Pang, Cai et al. 2003; Back, Riddle et al. 2006; Riddle, Luo et al. 2006; Segovia, McClure et al. 2008; Yang, Lewis et al. 2011). The differential expression of cell surface antigens (AB25, O4, O1) and myelin proteins (MBP, PLP, MOG) can be used to define stages of oligodendrocyte maturation, the accompanying vulnerability of these different cell populations to injury and the effect of perinatal insults on oligodendrocyte developmental profile (Back, Luo et al. 2001; Yang, Lewis et al. 2011).

The selective vulnerability of cells in the oligodendrocyte lineage to injury involves oxidative stress and OFR pathways (Tan, Zhou et al. 1999; Taylor, Edwards et al. 1999). The vulnerability of oligodendrocytes to oxidative injury from free radical attack, is mediated by anti-oxidant depletion, which is in turn regulated by the maturation stage of the cells (Back, Gan et al. 1998). More mature oligodendrocytes, are resistant to oxidative stress and
excitotoxic death due to the developmental expression of antioxidant enzymes, the most important of which are glutathione peroxidase and catalase that reduce the accumulation of toxic OFR species. Whilst there are fewer synapses in white matter, immature oligodendrocytes do express NMDA and AMPA/kainic acid receptors that can propagate excitotoxic signals (Salter and Fern 2005). In addition, AMPA/kainic acid receptor antagonists reduce injury in immature rodent models of PVL, suggesting that excitotoxicity contributes to its aetiology (Follett, Rosenberg et al. 2000). Excitatory glutamate receptors, activated by excess glutamate responses, also promote the production of damaging free radicals (Salter and Fern 2005).

PVL and loss of pre-myelinating cell populations is associated with reductions in cortical grey and white matter and increases in ventricle size and cerebro-spinal fluid (CSF) volumes. A significant reduction in the cross-sectional area and volume of the thalamus is associated with PVL injury (Teune, van Wassenaer et al. 2011). These structural changes that remain at term equivalent age in infants that are born preterm contribute to the neuromotor deficits and neurological disorders linked to PVL (Inder and Volpe 2000; Inder, Neil et al. 2005; Inder, Warfield et al. 2005).

Cells other than oligodendrocytes are also involved in PVL lesions. Activation of microglial cells can lead to activation of astrocytes, as detected by positive staining for glial fibrillary acidic protein (GFAP), at the borders of focal cystic PVL lesions (Tahraoui, Marret et al. 2001). Astrocyte activity is itself involved in the formation of gliotic scarring that is also present in regions of focal and diffuse PVL injury within the thalamic nuclei (Ligam, Haynes et al. 2010). Severe insults, ischemia and haemorrhage and PVL patterns of injury, are mostly associated with cerebral palsy (Johnston and Hoon 2006) and severe neurodevelopmental deficits. Importantly, white matter damage and PVL in preterm infants are
strong determinants of subsequent deficits in brain function and poor neurological and cognitive outcomes (Perlman 1998).

Whilst PVL is the predominant form of brain injury in preterm infants, it is not the only form of injury associated with preterm birth. Specific regional changes in white and grey matter volumes are also seen in preterm infants, with reduced cortical volumes in sensorimotor, midtemporal and occipital regions as well reduced cerebellum size (Peterson, Vohr et al. 2000). Neuronal injury and death particularly the developmentally important subplate neurons is also present in preterm infants (McQuillen, Sheldon et al. 2003). Late-preterm infants, that make up the largest proportion of all infants born preterm, are likely to develop less severe brain injury than those born extremely preterm (Dong and Yu 2011).

1.3.6 Term Brain Injury

Brain injury in term infants differs to that usually seen in preterm infants, with PVL-like patterns less common. Neuropathological studies have shown that term infants with acute, focal brain injury, tend to have neuronal cell necrosis in the thalamus, hypothalamus, basal ganglia, hippocampus and the brainstem (Roland, Poskitt et al. 1998). This type of injury is usually present in both hemispheres and associated with a postnatal rather than antenatal insult (Cowan, Rutherford et al. 2003). As well as other neurodevelopmental deficits, the involvement of the brainstem in perinatal brain injury can inhibit neonatal respiratory function and neonatal death. Injury to white matter and severe PVL are less commonly seen in term neonates compared to preterm infants, and are usually associated with chronic, in utero insults, resulting in more diffuse injury and neurodevelopmental disruption. The late developmental profile of the cerebellum also mediates its increased vulnerability to injury in the term neonatal brain (Diamond 2000). Severe or prolonged insults in the term brain can result in more widespread brain injury involving many
structures, white matter and myelinating cells. However, unlike the preterm neonatal brain where white matter is highly susceptible to damage, important processes of synaptogenesis, axon growth and migration, potentiate the selective vulnerability of neurons to injury in the term brain (Bhutta and Anand 2002).

Synaptogenesis is a particularly important process that occurs rapidly in late gestation and the neonatal period and can be affected by perinatal insults. Glutamate signalling is also involved in neuronal migration, proliferation and synapse formation. The formation of particular neurotransmitter systems and receptors is developmentally regulated, with the maturation of NMDA and AMPA receptors important in neuronal excitotoxicity (Jensen 2002). These processes place high energy demands on the fetus and are therefore extremely vulnerable to perturbations in metabolic pathways.

Less severe insults, whilst not causing gross structural or histological changes in the brain, may result in alterations and delays in normal maturation processes such as myelination and synapse formation, resulting in a number of different neurodevelopmental disorders. These less severe neurodevelopmental problems, whilst being more common than severe brain injuries, can be more difficult to diagnose and present a particular problem for educational attainment and cognitive function later in life.

1.4 NEURODEVELOPMENTAL SEQUELAE
- Consequences of Preterm Birth & IUGR

The complexity of the nervous system and its connections, the sensitivity of the immature nervous system to injury and the rapid rates of growth during the perinatal period mediate the many neurodevelopmental disorders associated with complications during pregnancy. The presence of pathology during gestation or the premature transition from intrauterine to extraterine life can greatly disrupt the highly regulated and important
neurodevelopmental processes that occur in perinatal life. These pathologies lead to neurodevelopmental disorders related to all the functions of the nervous system, including motor, sensory and cognitive deficits and serious neurological disorders. Subtle brain changes, often associated with mild IUGR and late-preterm birth, especially those that may go undiagnosed early in life, are also important in the aetiology of developmental, behavioural and learning problems that become apparent in low birthweight and preterm infants once they reach school age.

1.4.1 Sensory-Motor Disorders

Children born preterm or with low birthweight exhibit a spectrum of neuromotor disorders, with neuromotor abnormalities as sequelae of perinatal brain injury often evident early on in the neonatal period. Cerebral palsy is a serious non-progressive motor disorder, that results in muscle spasticity, impairment of voluntary muscle control and muscle contractures (Allen, Cristfalo et al. 2011). Rates of cerebral palsy increase with decreasing birthweight and gestational age, with an incidence of up to 20% in infants born at 25 or 26 weeks gestation (Doyle, Roberts et al. 2010). Along with preterm birth, other risk factors for the development of cerebral palsy include, IUGR, white matter injury and PVL, intraventricular haemorrhage and male sex (Allen, Cristfalo et al. 2011). For infants with perinatal insults that do not develop cerebral palsy, mild and moderate gross motor impairments are also common. Studies have shown that nearly 40% of preterm infants have at least mild motor dysfunction at school age (Williams, Lee et al. 2010), with fine motor dysfunction, poor graphomotor and handwriting skills, and impaired spatial processing common (Charkaluk, Truffert et al. 2010). Rates of motor disorders have not improved in recent decades, despite advances in obstetric and neonatal care (Foulder-Hughes and Cooke 2003).
As with cerebral palsy, the incidence of hearing and vision impairment is also greater in preterm than term infants, with an increased risk with earlier gestational age. Severe bilateral hearing impairment occurs in around 5% of preterm infants born at 25 weeks gestation (Wood, Costeloe et al. 2005). Gestational insults increase the risk of a spectrum of hearing disorders, which often require hearing aids or cochlear implants. Reductions in near and long-distance vision, contrast sensitivity and spatial awareness are also associated with preterm birth and IUGR.

### 1.4.2 Cognitive, Learning and Behavioural Disorders

Large epidemiological studies have outlined the relationship between gestational insults and cognitive and learning difficulties. The most common sequelae of perinatal brain injury are cognitive, learning or developmental delays. A meta-analysis of epidemiological studies found that these deficits are present in nearly 60% of infants that survived intra-uterine or neonatal insults (Mwaniki, Atieno et al. 2012). The coincidence of IUGR and preterm birth significantly increases the risk of poor cognitive performance, with preterm infants who also had retarded growth showing poorer performances in both verbal and full-scale IQ tests than gestational age-matched preterm infants without IUGR (Morsing, Asard et al. 2011). Aspects of cognitive function that may be affected by perinatal brain injury include reasoning, problem solving, comprehension of complex language, visual and auditory processing, abstract thinking and memory (Allen 2008). Higher order or executive functions involve many cognitive processes and are shown to be impaired with low birthweight (less than 1500g) and preterm birth (Mulder, Pitchford et al. 2009). Large cohort studies of preterm infants have also shown that decreasing gestational age at birth increases the risk of behavioural and attention problems, such as attention-deficit/hyperactivity disorder (ADHD), with the highest incidence in extremely preterm infants (Bhutta, Cleves et al.)
Environmental factors, such as socio-economic status modify the risk of ADHD only in cases of moderately preterm birth (Lindstrom, Lindblad et al. 2011).

One of the greatest problems related to poor cognitive, developmental and attention outcomes associated with perinatal brain injury, is the impact on learning ability, achievement at school and in the workforce and social interactions (Saigal and Doyle 2008). Preterm and low birthweight children demonstrate poorer performances in school-readiness tests at pre-school and school age, are less likely to attain secondary or tertiary education and on average have lower lifetime career earnings (Strauss 2000; Larroque, Bertrais et al. 2001; Kilbride, Thorstad et al. 2004) Social dysfunction has also been reported to be higher in infants that were preterm or low birthweight (Strauss 2000).

1.4.3 Neurological Disorders

Neurodevelopmental disruption has been implicated in the aetiology of a number of neurological disorders. There is some evidence that obstetric complications may increase the risk of autism spectrum disorders (Gardener, Spiegelman et al. 2009). Neonatal seizures and epileptic activity are commonly associated with perinatal brain injury and are implicated in permanent neurological disorders and epilepsy in later life (Lombroso 1996). Schizophrenia has a complex aetiology involving genetic, epigenetic and environmental factors. However, strong evidence suggests that neurodevelopmental disruption contributes to the development of schizophrenia in later life (Lewis and Levitt 2002). Evidence of increased cortical ventricle size in some cases of schizophrenia may be linked to perinatal brain injury that alters cerebral grey and white matter volume and causes ventriculomegaly (Li, Cheung et al. 2009). Perinatal animal models of inflammation (Nawa and Takei 2006) and placental insufficiency (Rehn,
Van Den Buuse et al. 2004) have also been developed that show schizophrenia-like symptoms in offspring after puberty.

1.5 NEUROPROTECTIVE STRATEGIES

Potential neuroprotective strategies involve therapies that target the mechanisms that result in perinatal brain injury. Some important considerations in the treatment of perinatal brain injury include the diverse and heterogeneous nature of insults involved, the length of available time for effective treatment to occur and the overall safety of any treatment on the immature or compromised neonate.

A number of different neuroprotective strategies have been investigated. Generally, these strategies are aimed at inhibiting excessive brain activity in order to reduce brain metabolic demands, inhibition of oxidative stress and free radical attack and the treatment of infection or inflammatory pathways. One of the most successful therapies for postnatal neuroprotection is the use of hypothermia or infant cooling for severe neonatal hypoxic-ischemic insults. Following success in animal models (Thoresen, Bagenholm et al. 1996), clinical trials have used either head cooling or systemic hypothermia to reduce infant core body temperature to between 33-34°C, within 6hrs of birth for at least 72hrs (Gunn and Bennet 2008; Edwards, Brocklehurst et al. 2010). These trials have shown reductions in severe neurodevelopmental disability at 18 months follow-up and significantly lower infant mortality rates (Edwards, Brocklehurst et al. 2010). The use of hypothermia is now clinically indicated with longer-term outcomes of these infants, particularly the assessment of less severe disability at school age is still continuing. The therapeutic window for effective treatment and the potential for extended periods of hypothermia are also under investigation.
Some established and experimental therapeutic drugs are also being examined for their neuroprotective properties and safety in neonatal brain injury. Erythropoietin is currently given to preterm infants as a treatment for anaemia. However, its neuroprotective, antioxidant, anti-inflammatory, neurogenic properties and positive effects on functional outcomes have also been described in neonatal rat models of brain injury (Kumral, Uysal et al. 2004; Yamada, Burke et al. 2011). Trials in preterm and term infants have suggested positive effects of erythropoietin treatment on neurodevelopmental outcomes that warrant further investigation (McPherson and Juul 2010). Melatonin and other anti-oxidant drugs are also emerging as viable therapeutic options. Melatonin has been shown to improve long-term behavioural and functional outcomes following perinatal ischemic brain injury in rats (Carloni, Perrone et al. 2008). The antibiotic treatment of neonatal infection is also an important potential therapy in cases of infection-induced perinatal brain injury in both preterm and term neonates. However, further investigation of the safety and effectiveness of neonatal antibiotic treatments is still required (Tsuji, Wilson et al. 2004; Buller, Carty et al. 2009). These drug therapies are aimed at reducing perinatal brain injury and rely on the timely and accurate diagnosis and identification that an injury has occurred. The timing of treatment and the effective therapeutic window of these neuroprotective strategies are an important consideration in terms of their efficacy. Neuroprotective approaches against perinatal brain injury also include the treatment and reduction of perinatal risk factors before birth that may potentiate subsequent injury. Whilst the early, antenatal diagnosis of intrauterine infection, IUGR and prediction of preterm birth present major challenges to healthcare personnel, advances in these areas present opportunities for substantial improvements in neonatal health outcomes and reductions in adverse neurodevelopmental sequelae. Obstetric care
also influences perinatal brain injury. The choice to deliver a fetus early, removing it from an inhospitable intrauterine environment, must be weighed against the benefits of maintaining gestation for as long as possible. Complex interactions between maternal, placental and fetal endocrine and nervous signalling mediate the intricate neurodevelopmental processes that are essential to normal brain growth and function. The development of effective neuroprotective therapies and strategies to manage perinatal insults and risk factors are essential to combating perinatal brain injury and the subsequent social, behavioural, cognitive and neurological disorders.

1.6 PROGESTERONE

Progesterone (pregn-4-ene-3,20-dione), named for its “pro-gestational” effects, was identified in the 1920s and 1930s as a hormone essential to the establishment and maintenance of pregnancy (see review (Davis and Plotz 1957)). Progesterone is most well known for its roles as a female sex steroid and its reproductive functions related to sexual maturation, the regulation of the female reproductive cycle and gestational effects during pregnancy. However, the identification of progesterone receptors and their distribution throughout the body have established many functions of progesterone as a regulator of growth and development, mediator of immune function and role in influencing homeostatic control (Savouret, Misrahi et al. 1990; Graham and Clarke 1997; Hansen 1998; Spencer and Bazer 2002). The identification of progesterone, progesterone synthetic enzymes and progesterone receptors within the brain (Brinton, Thompson et al. 2008; Wagner 2008) has also highlighted the role of progesterone as a neuroactive steroid, both as a mediator of neurodevelopment and also as a neuroprotective agent. Progesterone also influences brain function as a major precursor of the neuroactive pregnane steroids and through
involvement in the synthetic pathways of cortisol, estrogens and testosterone (Baulieu and Schumacher 2000). During pregnancy, in addition to its major gestational effects, progesterone therefore has potential to affect both the maternal brain and developing fetal brain.

1.6.1 Progesterone Synthesis

Progesterone is synthesised in a number of endocrine tissues, including the ovaries, adrenal glands and during pregnancy, the placenta (Tuckey 2005). Recent evidence has confirmed that the de novo synthesis of progesterone from cholesterol can also occur wholly within the brain and central nervous system (Tsutsui 2008; Schumacher, Hussain et al. 2012).

As outlined in Figure 1.2, progesterone synthesis begins with the conversion of cholesterol into pregnenolone by cytochrome P450 side-chain cleavage (P450scc) enzymes located in the mitochondria. This conversion of cholesterol into pregnenolone, which is dependent on cholesterol metabolism and the electron-supply-chain in the mitochondria, is the rate-limiting step in the production of progesterone. Progesterone is produced from pregnenolone via action of the enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD), which has two isoforms, types 1 and 2, and is also located in the mitochondria. Both P450scc and 3β-HSD are present in the placenta and have also been identified in the brain, in both neurons and glial cells (Compagnone and Mellon 2000). P450scc has been measured in the human placenta during early and mid-gestation, with expression increasing in the placenta throughout gestation in response to cyclic-AMP (cAMP) signalling (Chung, Matteson et al. 1986). The placenta has high concentrations of 3β-HSD type 1, whereas the type 2 isoform is associated with adrenal and gonadal expression (Mason 1993). Similarly to P450scc, 3β-HSD1 expression within the placenta is linked to cAMP effects on the placental syncitium (Mason, Keeney et al. 1997), as well as protein kinase A.
(PKA) signalling (Gomez-Concha, Flores-Herrera et al. 2011) and is relatively constant throughout gestation (Hill, Parizek et al. 2011).

1.6.2 Progesterone Receptors & Signalling

Progesterone signalling occurs via binding to cellular receptors located in target tissues. The classical progesterone receptor (PR) functions as a ligand-activated transcription factor. When progesterone binds to PR (as shown in Figure 1.3), the receptor becomes dimerised and interacts with progesterone response elements (PRE) that are present in the promoter regions of target genes, regulating gene transcription (Brinton, Thompson et al. 2008). PR is also able to interact with extranuclear signalling proteins, such as Src and mitogen-activated protein kinase (MAPK) pathways to regulate gene transcription and protein phosphorylation (Migliaccio, Piccolo et al. 1998; Boonyaratanakornkit, McGowan et al. 2007). PR has been identified in the uterus and other female reproductive tissues, fallopian tubes, pituitary gland, hypothalamus, cerebral cortex, testis, thymus and vascular smooth muscle (Savouret, Misrahi et al. 1990). PR expression is also associated with signalling in progesterone-sensitive cancers. Rapid onset effects of progesterone signalling can also be mediated by membrane bound progesterone receptors and signalling cascades that are shown in Figure 1.3. The membrane receptors of progesterone (mPR), progesterone receptor membrane component (PGRMC1) and the sigma 1 (σ1) receptors have been shown to interact with extracellular-signal regulated kinase (ERK), p38 MAPK, adenylate cyclase (AC), PKA and protein kinase G (PKG) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways (Schumacher, Sitruk-Ware et al. 2008; Thomas 2008; Zhu, Hanna et al. 2008; Ishrat, Sayeed et al. 2012). These non-classical membrane receptors also stimulate calcium
Figure 1.2 - Pathways of Steroid and Neuroactive Steroid Synthesis from Cholesterol

The production of steroids from cholesterol and pregnenolone is catalysed by a number of enzymes present within the brain, nervous system, adrenal glands, gonads and placenta. The developmental and tissue specific expression of these enzymes regulates steroid synthesis in these endocrine organs and the nervous system. Of particular interest is the importance of progesterone as a potential precursor in a number of steroid synthetic pathways, including those of testosterone, the estrogens, cortisol and corticosterone. Progesterone is also a major precursor of the neuroactive steroids, allopregnanolone and THDOC, which are potent positive modulators of the GABA<sub>A</sub> receptor. The neuroactive steroid, DHEAS is also shown. (Adapted from Mellon, et al. 2008)

P450scc, mitochondrial cholesterol side-chain cleavage enzyme; 3βHSD, 3β-hydroxysteroid dehydrogenase (located in the endoplasmic reticulum); 5αR, 5α-reductase (rate-limiting enzyme in the synthesis of allopregnanolone); 3αHSD, 3α-hydroxysteroid dehydrogenase; P450c11, mitochondrial 11 hydroxylase; P450c21, mitochondrial 21 hydroxylase; 11βHSD, 11β-hydroxysteroid dehydrogenase; 5α-DHP, 5α-dihydroprogesterone; 3α,5α-THP, 3α,5α-tetrahydroprogesterone; 5α-DHDOC, 5α-dihydrodeoxycorticosterone; 3α,5α-THDOC, 3α,5α-tetrahydrodeoxycorticosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate.
release from intracellular stores, affect voltage gates potassium channels and neurotransmitter receptors (Schumacher, Sitruk-Ware et al. 2008; Thomas 2008). The specific regulation and actions of these newly discovered receptors are still under investigation, particularly in nervous system tissues rather than the traditional reproductive targets of progesterone signalling. These non-genomic actions at membrane progesterone receptors may be important to the neurodevelopmental roles of progesterone within the fetal and neonatal brain (further outlined in sections 1.6.4 and 1.6.5). Further investigation of these actions with reference to perinatal brain injury may provide scope for the identification of new therapies for neuroprotection and the promotion of normal brain development.

1.6.3 Progesterone Functions & Concentrations During Gestation

Progesterone has a number of actions during gestation, which are essential for the successful implantation and maintenance of pregnancy in most species. Increased progesterone concentrations during the menstrual cycle are responsible for preparing the uterus and endometrium for implantation (Spencer and Bazer 2002). The maintenance of uterine quiescence and cervical integrity that prevents labour is mediated by progesterone signalling (Challis, Matthews et al. 2000). Progesterone is also important to the protection of the fetus from deleterious immune responses by regulating maternal immune tolerance during gestation (Siiteri and Stites 1982; Piccini, Giudizi et al. 1995).
**Figure 1.3 - Mechanisms of Progesterone and Allopregnanolone Signalling**

Progesterone acts classically at the intracellular progesterone receptor (PR). Progesterone enters the cell, binds to PR, causing the receptor to dimerise and move to the nucleus where it associates with progesterone response elements (PRE) in the promoter regions of target genes, regulating gene transcription and activity. PR can directly influence kinase activity. Progesterone can also bind to a number of membrane receptors (mPR, sigma-1 and PGRMC1) to activate second messenger systems, intracellular Ca\(^{2+}\) stores and protein kinases (MAPK, ERK) that influence gene transcription, translation, post-translational modifications and cellular activity. Allopregnanolone binds allosterically to the GABA\(_A\) receptor, a ligand-gated ion channel, positively modulating its activity. The classic action of the GABA\(_A\) receptor, as the main inhibitory neurotransmitter system in the nervous system, leads to the influx of negatively charged chloride ions into the cells, resulting in hyperpolarisation and the tonic inhibition of the generation of action potentials. Signalling of progesterone and allopregnanolone at these receptors mediate the neuroprotective and developmental actions of these steroids within the brain and nervous system (Adapted from Schumacher, et al. 2008). AC, adenylate cyclase; GABA; \(\gamma\)-aminobutyric acid; MAPK, mitogen-activated protein kinase; mPR, membrane progesterone receptor; PGRMC1, progesterone receptor membrane component 1; PKA, protein kinase A; PKG, protein kinase G; ERK, extracellular-signal regulated kinase.
Relatively high concentrations of progesterone are present in the maternal and fetal circulations throughout gestation, which fall rapidly following the removal of the placenta as a source of progesterone. During early gestation, progesterone is predominantly produced by the corpus luteum under the influence of human chorionic gonadotropin (hCG). However, at 6-8 weeks gestation, the placenta begins to take over as the main source of progesterone, continuing as the main site of synthesis throughout the remainder of pregnancy (Kallen 2004). The timing of increased placental production of progesterone, the luteal-placental shift, occurs when placental blood flow and oxygen supply rapidly increase and luteal hCG signalling is reduced.

Following the luteal-placental shift (~12th week of gestation), plasma progesterone concentrations progressively increase until the end of gestation (Ledoux, Genest et al. 1975). Mean progesterone concentration in maternal serum increased from 98nmol/L at 18 weeks gestation to nearly 800nmol/L at late gestation in pregnancies with fetuses appropriately grown for age (Donaldson, Nicolini et al. 1991). This represents a 7- to 9-fold increase from early gestation and requires a daily placental production rate of 250-350mg per day (Gilbert Evans, Ross et al. 2005; Pasqualini 2005). Fetal serum concentrations of progesterone range from approximately 200nmol/L up to 1500nmol/L (mean concentration of ~800nmol/L), and do not differ between male or female fetal sex (Donaldson, Nicolini et al. 1991). Within hours and days of birth and removal of the placenta as a source of progesterone, maternal and neonatal progesterone concentrations fall rapidly (Ledoux, Genest et al. 1975; Sippell, Becker et al. 1978). The transitional time between intra-uterine and extra-uterine life therefore involves a large change in endocrine milieu and removal from placental endocrine signals.
Fetal development occurs in an intra-uterine environment with high levels of placentally derived progesterone and other hormones that are essential to the regulation of normal developmental processes. Complications during gestation or birth may also disrupt the endocrine profile during perinatal life. Preterm birth results in the premature removal of placental steroid hormones and loss of developmental influence of these steroids on the immature neonate (Trotter and Pohlandt 2000; Hunt, Davis et al. 2004). IUGR, which is often associated with preterm birth has also been linked to an accelerated loss in neonatal placental hormone levels after birth (Trotter, Maier et al. 1999; Trotter, Maier et al. 2001).

Progesterone and other synthetic progestins have been trialled for the prevention of premature birth in women at high risk of preterm delivery (Schindler 2005). High doses of progesterone have short-term efficacy against threatened miscarriage early in pregnancy and in reducing the risk of pregnancy loss in women with a clinical history of recurrent miscarriage (Di Renzo, Mattei et al. 2005). Progesterone has also been proposed as a postnatal therapy to improve neonatal health and development following preterm birth, as discussed later in section 1.6.4. The use of progestogen treatments should be carefully considered for their potential influence on the developing fetal brain and endocrine system.

1.6.4 Progesterone Effects on Neurodevelopment

Progesterone directly influences brain development, function and activity, via signalling at nuclear and membrane progesterone receptors. Actions of progesterone in the nervous system include effects on myelination, oligodendrocyte maturation, neuronal growth and brain sexual differentiation (Wagner, Nakayama et al. 1998; Mellon 2007). Progesterone is known to influence neural development, including axonal and dendritic outgrowth (Mellon 2007) and myelin synthesis (Gago, Akwa et al. 2001).
As previously described, the main source of progesterone during gestation is the placenta. However, it has also been established that, in addition to neuroendocrine functions of progesterone within the central nervous system, the brain itself is a site of progesterone synthesis. The enzymes, P450scc and 3β-HSD, are expressed in neurons and glial cells across the brain during early development and later life. Studies in fetal and neonatal rats have identified the developmentally regulated, transient expression of PR in cortical neurons. PR was found to be present on neurons but not nestin-positive glial cells in the frontal, parietal, temporal and occipital lobes, the expression of which coincided with increased cortical progesterone synthesis (Lopez and Wagner 2009). In addition, the perinatal expression of PR is associated with regions with important cognitive, motor and visual functions, such as the hippocampus (Quadros, Pfau et al. 2007). These findings suggest a developmental role for progesterone in normal cortical development during fetal and neonatal life.

The roles of progesterone in the developing neuron have been well characterised in the immature mammalian Purkinje cell (Tsutsui 2008). The Purkinje cell expresses the enzymes necessary for de novo synthesis of progesterone, which acts via nuclear progesterone receptors, promoting dendritic growth, spinogenesis and synaptogenesis in the developing neuron (Ukena, Usui et al. 1998; Sakamoto, Ukena et al. 2001). Progesterone signalling in Purkinje cells has organising effects that may be important to cerebellar cognitive and motor development and functions.

Progesterone also has positive effects on myelination in immature oligodendrocytes in vitro. When treated with progesterone, slice cultures of 7-day-old rat and mouse cerebellums show enhanced expression of Ki67, a proliferation marker, microtubule-associated-protein-2 (MAP-2), a neuronal structural marker and myelin-basic protein (MBP), a marker of mature myelin (Ghoumari, Ibanez et al. 2003; Ghoumari, Baulieu et al.
Enhanced MBP expression with progesterone treatment was reversed with the administration of the PR antagonist, mifepristone and in PR knockout mice, indicating these effects are mediated by progesterone signalling at the classical nuclear PR (Ghoumari, Ibanez et al. 2003).

Steroid effects on cognitive function and development are also gaining more interest as exogenous progestins are more widely used. Children, whose mothers’ received progesterone for toxaemia during pregnancy, had enhanced development at 1 year-old and higher educational attainments at 10 years of age (Dalton 1968; Dalton 1976). Studies have also trialled the replacement of placental hormones, progesterone and estradiol, in female fetuses that have been prematurely removed from the hormonal influence of the placenta due to extremely preterm birth (Trotter, Maier et al. 1999; Trotter, Maier et al. 2001). These studies examined the importance of progesterone and estradiol on postnatal bone metabolism and accretion. Additional follow-up studies on neurodevelopmental outcomes in preterm neonates, who received postnatal progesterone and estradiol treatments, examining the risk of cerebral palsy and sensory deficits, have provided positive results that suggest postnatal steroid effects may be beneficial in the developing brain (Trotter, Bokelmann et al. 2001; Trotter, Steinmacher et al. 2012). However, larger studies are required to fully identify the effects of these postnatal steroid treatments. These results have highlighted the need for an increased understanding of the mechanisms and further studies on the effects of progesterone and steroid hormones on fetal and neonatal brain development, with potential for future therapeutic advances.

1.6.5 Neuroprotection by Progesterone

Steroid hormones are becoming widely recognised as neuroprotective agents, with effects of estradiol and progesterone being studied in clinical and animal studies of traumatic brain injury and stroke (Roof, Duvdevani et al. 1994; Singer, Rogers et al. 1996; Roof and Hall 2000; Garcia-Segura,
Azcitia et al. 2001; Stein 2001; Singh and Su 2012). However, the neuroprotective effects of progesterone have been examined most closely in models of adult brain injury and not in terms of perinatal brain development and injury. The first evidence for the neuroprotective effects of progesterone emerged with observations that brain injury, as a result of identical acute insults, was less severe in female rats, compared to males, and that this protection was mediated by the hormonal status of the female rat at the time of insult (Roof, Duvdevani et al. 1993; Stein, Wright et al. 2008). There is also evidence of progesterone and estrogen neuroprotection in clinical cases of brain injury in adult females (Stein 2001).

Progesterone neuroprotective effects involve the activation of a number of pathways that influence inflammatory and oxidative mechanisms, and the repair processes that follow in response to injury. In the adult rat brain, the upregulation of nitric oxide synthase-2 (NOS-2), involved in production of nitric oxide free radicals, and pro-inflammatory IL-1β is inhibited by progesterone treatment following ischemia caused by middle cerebral artery occlusion (Gibson, Constantin et al. 2005). Progesterone also reduces the proliferation of reactive astrocytes, reducing damage caused by the inflammatory response and gliotic scarring (Grossman, Goss et al. 2004). Additionally, progesterone reduces edema and blood-brain barrier leakage and TNF-α accumulation after cortical traumatic brain injury or stroke in adult rat brains (Roof, Hoffman et al. 1997; Jiang, Wang et al. 2009). Following this injury, progesterone treatment also reduces inflammatory prostaglandin synthesis, decreasing lipid peroxidation damage to the blood-brain barrier (Roof, Hoffman et al. 1997). The integrity of the blood-brain barrier is important in regulating the accumulation of inflammatory mediators and immune cells in response to
injury, mediating the extent of damage, scarring and potential for functional recovery.

Progesterone has also been linked to activation of ERK, MAPK and PI3K/Akt pathways that are neuroprotective against glutamate-induced cell death in mouse brain cell cultures (Kaur, Jodhka et al. 2007). This in vitro model and a model of spinal cord injury in the rat have also shown progesterone-mediated increases in the neurotrophin brain-derived neurotrophic factor (BDNF), a nerve growth factor that promotes neuron survival and synapse formation (Gonzalez, Labombarda et al. 2005; Kaur, Jodhka et al. 2007). The mechanisms by which progesterone promotes BDNF synthesis in different central nervous system (CNS) insults are not fully understood.

Neuroprotection by progesterone also involves positive effects on recovery following central nervous system injury. The application of trilostane, an inhibitor of progesterone synthesis, and the progesterone receptor antagonist, mifepristone (RU486), strongly inhibits myelin regeneration after injury to the spinal cord in rats (Schumacher, Akwa et al. 2000). Following injury, progesterone treatment itself is also able to increase MBP expression (Labombarda, Gonzalez et al. 2006). These pro-myelinating effects are potentially very important in protecting neurons from secondary axonal loss and degeneration following demyelinating injury to glial cells.

Studies of ischemic stroke and traumatic brain injury have provided extensive evidence for the neuroprotective effects of progesterone (Gibson, Gray et al. 2008; Stein 2008). Studies of administration of progesterone in experimental models of ischemia and middle cerebral artery occlusion in adult rats have shown preservation of neuron numbers, inhibition of inflammatory markers, reduction in infarct sizes and a lessening of functional deficits when compared to control animals (Gibson, Constantin et al. 2005; Morali, Letechipia-Vallejo et al. 2005; Sayeed, Wali
et al. 2007). Studies of models of traumatic brain injury in adult rats have also shown similar protective effects of progesterone, including reduced lesion size, decreased inflammation and cytokine production and significantly improved functional and behavioural recovery (Roof, Hoffman et al. 1997; Jones, Constantin et al. 2005). Clinical trials of progesterone therapy for the treatment of traumatic brain injuries have also been carried out, reporting potentially promising effects on outcomes at one month following injury in progesterone versus placebo treated patients (Wright, Kellermann et al. 2007). However, larger studies are required to confirm these effects.

As well as these actions, progesterone has been implicated in regulating a number of psychopharmacological effects. Either directly or via actions of its metabolites, progesterone influences mood and changes in progesterone levels are thought to be responsible for depressive disorders, such as post-natal depression (Rupprecht and Holsboer 1999; Luisi, Petraglia et al. 2000).

Importantly, as well as reducing neuropathological markers of brain injury, many studies have also provided evidence of improved functional and behavioural outcomes associated with progesterone treatment for traumatic and ischemic brain injury models in adult rats (Roof, Duvdevani et al. 1994; Goss, Hoffman et al. 2003; He, Evans et al. 2004). Evidence from models of experimental brain injury also indicate that some of the neuroprotective properties of progesterone are mediated by its neuroactive metabolites rather than solely by direct signalling by progesterone at its receptors.

1.7 NEUROACTIVE STEROIDS
Steroid hormones, including glucocorticoids, mineralocorticoids and the sex steroids, are essential mediators of growth and development,
homeostasis, the immune system and many other essential physiological processes throughout the body. All steroid hormones are derived from cholesterol, as shown in Figure 1.2 and synthesised in endocrine organs and throughout the body by specific enzymes, where they exert their actions via ligand-receptor interactions. The classically recognised mode of action of steroid hormones, involves binding of a steroid to its nuclear receptor, inducing a conformational change and leading to association of the receptor with high-affinity hormone response elements present in the promoter regions of target genes (Evans 1988). These events regulate the transcription and activation of different sets of genes, mediating the physiological effects of these steroids. Whilst sex steroids and glucocorticoids are generally associated with their primary functions and sites of synthesis, in reproductive tissues and adrenal glands respectively, their receptors and actions are widely distributed throughout the body. Neuroendocrine mechanisms of brain sexual differentiation and signalling of glucocorticoids within the nervous system are well established (Dorner 1977). More recently, however, it has been recognised that steroid hormones are able to interact with membrane receptors other than those of the classical steroid hormone receptor family. Of particular interest are the actions of a family of pregnane steroids, related to progesterone, that are able to mediate rapid, non-genomic effects within the nervous system. These compounds are known as neuroactive or neurosteroids (Baulieu 1997).

Neurosteroids (steroids synthesised within the nervous system) were first identified in animal experiments in which steroid concentrations in the brain remained high, despite the removal of peripheral glandular sources by adrenalectomy and gonadectomy (Corpechot, Robel et al. 1981; Corpechot, Synguelakis et al. 1983; Corpechot, Young et al. 1993; Baulieu 1997). These studies identified the de novo synthesis from cholesterol within
the brain of steroids such as pregnenolone, progesterone and dehydroepiandrosterone (DHEA), without the need of peripheral sites of production. Further identification and measurement of neurosteroidogenic enzymes within the brain and nervous system confirm the ability of the brain to directly produce neurosteroids (Mellon and Deschepper 1993; Celotti, Negri-Cesi et al. 1997; Patte-Mensah, Penning et al. 2004; Torres, Sanchez et al. 2004; Agis-Balboa, Pinna et al. 2006).

The other aspect that determines steroid neuroactivity is the presence of receptors for these steroids within the brain. Signalling via the traditional steroid receptors mediates some of the well-known neuroendocrine function of steroid hormones related to actions of the hypothalamic-pituitary-adrenal and gonadal axes. However, other non-cognate receptors within the brain that interact with steroid ligands can have very different effects. In the mid-1980s, alfaxalone (5α-pregnane-3α-ol-11,20-one) was shown to enhance γ-aminobutyric acid (GABA) mediated inhibition of neural activity in a similar manner to that of the barbiturate, pentobarbitone (Harrison and Simmonds 1984). Alfaxalone, a steroid anaesthetic, and other endogenous neurosteroids that share structural similarities with alfaxalone, have now been shown to modulate γ-aminobutyric acid receptor type A (GABA_A) receptor-mediated hyperpolarisation of cells (Majewska, Harrison et al. 1986), exerting rapid non-genomic physiological effects on the nervous system. Many of these steroids that influence GABAergic function including the pregnanolones and deoxycorticosteroids, are products of progesterone metabolism. The effects of the steroids at the GABA_A receptor varies depending on slight variations in structure and conformation. The physiological effects of these neuroactive steroids, including anaesthetic, sedative and anti-seizure properties, have important implications on the roles of these compounds during neurodevelopment and as neuroprotective agents.
1.7.1 GABA<sub>A</sub> Receptor

The GABA<sub>A</sub> receptor is a ligand-gated chloride ion channel that is a major regulator of neural activity and signalling (MacDonald and Botzolakis 2009). Its endogenous ligand, GABA, is the major inhibitory neurotransmitter present in the adult nervous system. Upon activation of the GABA<sub>A</sub> receptor by its ligand, the ionotropic receptor mediates entry of chloride ions (Cl<sup>-</sup>) into the cell, reducing the resting potential of the neuron, leading to weak depolarisation, below threshold levels or hyperpolarisation, inhibiting the generation of action potentials within the cell (Chebib and Johnston 1999). This reduction in neural excitability can lead to a number of physiological effects, including sedative, hypnotic, anaesthetic, anxiolytic, anticonvulsant and sleep-modulatory actions (Rupprecht and Holsboer 1999). The balance between inhibitory and excitatory signals within a neural network is critical to normal brain function, the formation and pruning of synaptic connections and protection against negative consequences caused by uncontrolled signalling. The inhibitory action of the GABA<sub>A</sub> receptor is particularly important in relation to brain injury caused by excitotoxic processes and seizure disorders.

The GABA<sub>A</sub> receptor is a multimeric transmembrane receptor, comprised of five subunits arranged to form the central Cl<sup>-</sup> ion pore (MacDonald and Botzolakis 2009). A number of different subunit isoforms exist (α<sub>1-6</sub>, β<sub>1-3</sub>, γ<sub>1-3</sub>, δ, ε, π, θ), the arrangement of which modulate receptor activity and ligand binding. The endogenous ligand, GABA, interacts with the receptor via binding at an active site formed at the interface of α and β receptor subunits. The most common receptor subunit combination involves two α, two β and a γ subunit, forming two active GABA binding sites (MacDonald and Botzolakis 2009). Along with the GABA binding site, a number of regulatory, allosteric binding sites are also present on the
receptor, formed at the interfaces of different receptor subunits, which bind ligands such as barbiturates, benzodiazepines, ethanol, picrotoxin and neuroactive steroids. For example, the presence of the δ-subunit as part of a GABA_δ receptor conveys increased sensitivity to neuroactive steroid binding and action (Adkins, Pillai et al. 2001). The selective expression of δ-subunits at extrasynaptic sites also mediates the sensitivity and actions of neuroactive steroids within the brain (Stell, Brickley et al. 2003; Mitchell, Gentet et al. 2007). The regionally and temporally specific expression of GABA_δ receptor subunits therefore influences GABA_δ receptor action, including the modulation of receptor signalling by neuroactive steroids.

There is also evidence that mechanisms of GABA_δ receptor signalling are developmentally regulated. Measurements of brain activity in a number of experimental animal experiments have suggested that the GABA_δ receptor is excitatory in early life (Cherubini, Gaiarsa et al. 1991; Chen, Trombley et al. 1996; Owens, Boyce et al. 1996; Gao and van den Pol 2001; Tyzio, Cossart et al. 2006; Ben-Ari, Gaiarsa et al. 2007; Tyzio, Holmes et al. 2007). Microelectrode recordings from immature rat brain slice culture experiments have shown that activation of the GABA_δ receptor is excitatory until postnatal day 12-13, at which time a developmental switch occurs, resulting in the classic inhibitory profile of GABA present in adults (Ben-Ari, Cherubini et al. 1989; Ben-Ari, Gaiarsa et al. 2007). In contrast to recordings in the rat, it has been reported that signalling at the GABA_δ receptor is inhibitory immediately after birth in newborn guinea pigs (Rivera, Voipio et al. 1999). In addition, patch-clamp recordings in fetal guinea pig brain slices demonstrate that the inhibitory switch occurs in late-gestation at approximately 55-56 days gestation with term gestation ~70 days (unpublished data, H. Parkington, Monash University). This excitatory signalling at the GABA_δ receptor early in development may have important functions in the formation of synapses and network
development during brain maturation (Represa and Ben-Ari 2005). A recent review by Ben-Ari et al. (Ben-Ari, Woodin et al. 2012) summarises evidence for this excitatory role for the GABA<sub>A</sub> receptor during development,

Excitatory GABA signalling during early development has been linked to the developmental expression of ion transporters, including the sodium potassium chloride co-transporter NKCC1 and the potassium chloride co-transporter KCC2, that regulate transmembrane ion gradients (Rivera, Voipio et al. 1999; Payne, Rivera et al. 2003; Dzhala, Talos et al. 2005). The high expression of NKCC1 and low expression of KCC2 early in development, result in high intracellular concentrations of Cl<sup>-</sup> leading to depolarising (Cl<sup>-</sup> efflux) rather than hyperpolarising (Cl<sup>-</sup> influx) effects of GABA<sub>A</sub> receptor activation (Rivera, Voipio et al. 1999; Ganguly, Schinder et al. 2001). Increases in the expression of KCC2 during the maturation of the CNS result in reversal of these ion gradients leading to the switch to inhibitory GABA signalling. Measurement of KCC2 protein levels in human post-mortem tissues demonstrated increases in expression from late gestation, which continued during the first year of life (Dzhala, Talos et al. 2005). Similar changes in KCC2 and NKCC1 expression were noted in the immature rat brain and were associated with the switch in GABA<sub>A</sub> receptor signalling in this species (Dzhala, Talos et al. 2005; Jansen, Peugh et al. 2010). However, direct measurement of this switch in humans is problematic, with extrapolations made from receptor expression studies combined with in vitro activity recordings from rodents and other experimental animals. The reduced efficacy of anti-epileptic drugs, such as the GABA<sub>A</sub> receptor agonist, phenobarbital, in treating seizure activity in neonates has been suggested as evidence of reduced GABA inhibition in the immature human brain (Jansen, Peugh et al. 2010).
Whilst a number of studies have provided evidence for the excitatory action of the GABA_A receptor during early life, questions have been raised regarding the applicability of results from in vitro experiments in in vivo systems (Bregestovski and Bernard 2012). Slice culture systems, commonly used to record electrical activity of neurons, may intrinsically alter brain metabolism and ion concentrations, with damaged cells shown to accumulate Cl, resulting in readings that may not reflect physiological conditions in the intact brain (Dzhala, Valeeva et al. 2012). Depending on the experimental procedures used, GABA_A receptor-mediated excitation has also shown to be present in mature neurons (Marty and Llano 2005). Furthermore, traumatic injury to neurons may itself regulate the expression of KCC2 channels, altering cellular Cl homeostasis and inducing GABAergic excitotoxic cell death (Nabekura, Ueno et al. 2002). In animals with more advanced brain development in utero, such as the sheep, pharmacological studies in vivo have demonstrated that inhibitory GABAergic signalling is present from around mid-gestation (~85 days) (Crossley, Nitsos et al. 2003). Data from non-human primates has identified GABAergic inhibitory signalling from mid-gestation, with the GABA_A receptor antagonist bicuculline causing epileptiform activity at least one month prior to birth (Khazipov, Esclapez et al. 2001). A number of experiments in intact preparations or in vivo have also demonstrated inhibitory GABA signalling in immature rodent brains (Baram and Snead 1990; Khalilov, Khazipov et al. 1996; Juttner, Meier et al. 2001). The developmentally regulated, sexually dimorphic and regionally specific expression of ion transporters also suggests that sub-populations of both inhibitory and excitatory GABAergic neurons are likely to be present throughout development (Auger, Perrot-Sinal et al. 2001; Galanopoulou 2008). These differences along with changes in GABA_A receptor subunit expression may help to explain varied responses to GABA_A receptor
agonists and antagonists and their effects on the developing brain (Mtchedlishvili, Sun et al. 2003). Recombinant studies of GABA$_A$ receptor subunit expression have shown different responses to neurosteroid agonists with different receptor subunit combinations (Lambert, Belelli et al. 1995). The understanding of potential changes in GABA$_A$ receptor signalling during development is a complex matter, dependent on types of experimental models used and interpretation of the data produced. However, consideration of these factors is essential to the useful clinical translation of experimental data and therapeutic potential of GABAergic agents for the treatment of perinatal brain injury and neurodevelopmental deficits.

### 1.7.2 Pregnanolone Isomers

The pregnanolone steroids are an important family of neuroactive steroids due to their structural affinity for binding sites of the GABA$_A$ receptor and their ability to modulate its effects on neural activity (Hill, Cibula et al. 2007). Pregnanolone steroids include metabolites of progesterone produced by the action of the 5$\alpha$- and 5$\beta$-reductases, and 3$\alpha$- and 3$\beta$-hydroxysteroid dehydrogenase enzymes. The reductase enzymes reduce the A ring of progesterone at carbon 5, producing $\alpha$ or $\beta$ stereoisomers of dihydroprogesterone, depending on the active enzyme. Dihydroprogesterone is then converted into the pregnanolone steroids (tetrahydroprogesterones) by the hydroxysteroid dehydrogenase (also known as hydroxysteroid oxidoreductase) enzymes at the carbon-3 position. The basic structure of these GABA$_A$ receptor modulatory steroids is shown in Figure 1.4A. The reactions catalysed by these enzymes produce different stereoisomers of pregnanolone, that are synthesised from progesterone as shown in Fig 1.4B, producing pregnanolone (3$\alpha$-hydroxy-5$\beta$-pregnan-20-one), isopregnanolone (3$\beta$-hydroxy-5$\alpha$-pregnan-20-one),
epipregnanolone (3β-hydroxy-5β-pregnan-20-one) and allopregnanolone (3α-hydroxy-5α-pregnan-20-one) (Kancheva, Hill et al. 2007). Pharmacological studies have identified functions of these steroids at the GABA$_A$ receptor. Steroid isomers with the 3α rather than 3β conformation, and an electronegative atom at carbon 17 or 20 act as allosteric agonists of the GABA$_A$ receptor, positively modulating its action (Hill, Cibula et al. 2007).

The 3β-pregnanolone isomers inhibit the actions of the 3α-isomers by competing for the allosteric binding sites on the GABA$_A$ receptor. Additionally, the sulfated conjugates of the 3α- and 3β-isomers, as well as pregnenolone sulfate and DHEAS, antagonise GABA$_A$ action (Majewska, Harrison et al. 1986; Majewska 1992). The use of radio-labelled t-butylbicyclocophosphorothionate (TBPS), benzodiazepine and picrotoxin, ligands of the GABA$_A$ receptor, in combination with neuroactive steroids in binding assays have identified a number of different allosteric binding sites for the pregnanolone isomers and their conjugates, which mediate their different actions and do not coincide with the binding sites of benzodiazepine, barbiturates or GABA itself (Majewska, Harrison et al. 1986; Majewska 1992).

The actions of the pregnanolone isomers in modulating GABAergic neural activity may be particularly important during gestation when, along with high concentrations of progesterone, its pregnanolone metabolites are also highly expressed. In particular, the concentrations of 5α-reduced metabolites of progesterone (5α-dihydroprogesterone, pregnanolone and allopregnanolone) increase during late gestation in humans, whereas the 5β-pregnane steroids do not. Additionally, the ratio of the free to conjugated (sulfated) 5α-pregnanolone isomers also increases significantly after 30 weeks gestation (Hill, Cibula et al. 2007; Hill, Parizek et al. 2010;
Hill, Parizek et al. 2011). The increases in concentrations of free pregnanolone isomers with \( \text{GABA}_\lambda \) receptor agonist effects, such as allopregnanolone, are important in regulating aspects of maternal and fetal physiology during this time in late gestation and have potential effects on the regulation of parturition (Sheehan 2006). In addition to the pregnanolone isomers, the steroid metabolite of the adrenal steroid deoxycorticosterone, allotetrahydrodeoxycorticosterone (THDOC; \( \text{3\alpha,21-dihydroxy-5\alpha-pregnan-20-one} \)) is a potent, positive allosteric modulator of \( \text{GABA}_\lambda \) signalling. THDOC is synthesised from deoxycorticosterone by \( \text{5\alpha-reductase} \) and \( \text{3\alpha-hydroxysteroid dehydrogenase enzymes} \) and shares conformational and structural similarities to the other very potent \( \text{GABA}_\lambda \) receptor agonist, allopregnanolone. THDOC produces analogous sedative, anxiolytic and anticonvulsant effects within the nervous system as its \( \text{3\alpha,5\alpha-reduced progesterone metabolite counterpart} \). The actions of allopregnanolone and its known functions in the perinatal brain are detailed below.

1.8 ALLOPREGNANOLONE

Allopregnanolone, the \( \text{3\alpha,5\alpha-isomer of pregnanolone} \), is one of the most potent positive modulators of the \( \text{GABA}_\lambda \) receptor action (Harrison, Majewska et al. 1987). The neuroprotective properties of allopregnanolone in adult models of brain injury are becoming increasingly well recognised. However, there are fewer data available on the roles of allopregnanolone in the perinatal brain, in relation to neurodevelopment and neuroprotection. Fetal and neonatal life is a time of high endocrine activity and the important transition from intra-uterine to extra-uterine life. The high concentration of allopregnanolone during late gestation and evidence of allopregnanolone’s activity in the fetal CNS, has suggested the presence of an endogenous neuroprotective role for allopregnanolone within the
Figure 1.4 - Pregnane Isomer Synthesis and Structure

(A) The basic 4-ring steroid backbone of the pregnane isomers provides a rigid framework for the conformation of hydroxyl and methyl side-chains that determine the actions of the pregnane isomers within the nervous system. (B) The synthesis of the pregnane steroids from progesterone is catalysed first by the 5α- and 5β-reductases to form 5α- and 5β-dihydropregesterone, by reduction at C-5 on the A ring. The double bond at C-3 on the A ring is then reduced by the addition of a hydroxyl group by the 3α- and 3β-hydroxysteroid dehydrogenase (hydroxysteroid oxidoreductase) enzymes. The four different stereoisomers, produced by the action of these enzymes on progesterone, have varied potency and pharmacological action at the GABA_A receptor. Allopregnanolone (P3α,5α) positively modulates GABA_A receptor activity, whereas the 3α,5β-stereoisomer (pregnanolone) has antagonistic actions at the same receptor. (Reproduced with permission Hill, et al. 2007)
perinatal brain, particularly in response to intra-uterine stressors, during parturition and in the immediate postnatal period. The importance of the correct complement of inhibitory and excitatory inputs for the proper development of brain systems and for neuronal survival and plasticity (Galanopoulou 2008; Xu, Broadbelt et al. 2011), highlights the potential of allopregnanolone signalling to also have extensive and essential effects on normal brain development during the vulnerable perinatal period.

1.8.1 Allopregnanolone Synthesis

The production of the allopregnanolone de novo from cholesterol begins with the synthesis of progesterone by P450scc and 3β-HSD as previously described. As a pregnanolone isomer, allopregnanolone is then synthesised from progesterone in a two-step process by reduction (by 5αR) to form 5α-dihydroprogesterone (5α-DHP), followed 3α-HSD to produce allopregnanolone (3α,5α-tetrahydroprogesterone) (Compagnone and Mellon 2000). Due to the action of these synthetic enzymes, allopregnanolone therefore possesses the important 3α,5α-conformation that mediates its potent action at the GABA_A receptor. Expression of these steroidogenic enzymes has been shown to be both tissue-specific and developmentally determined. Over the course of gestation, neurosteroidogenic enzymes are highly expressed within the fetal adrenal glands, testes, fetal brain and placenta. Within the brain (Lauber and Lichtensteiger 1996), 5αR has been identified across all cell types, neurons, glia and astrocytes (Melcangi, Celotti et al. 1994; Lauber and Lichtensteiger 1996; Celotti, Negri-Cesi et al. 1997). The expression of 5αR, as the rate-limiting step in the synthesis of allopregnanolone, has an important influence over allopregnanolone concentrations within the fetal brain.
1.8.1.a 5α-Reductase

Steroid 5α-reductase (5αR; NAPDH-Δ¹-3-oxosteroid-5α-oxidoreductase) is a membrane-bound enzyme that catalyses the reduction of steroid hormones at the C-5 position, in an NADPH-dependent action (Li, Chen et al. 1995). 5αR is responsible for the conversion of progesterone into 5α-dihydroprogesterone, and analogous conversions of testosterone and deoxycorticosterone into their 5α-reduced metabolites. Two isoforms of 5αR have been identified and described in the human, rat, monkey and other mammalian species (Andersson, Bishop et al. 1989; Andersson and Russell 1990; Thigpen, Silver et al. 1993). These two isoenzymes, type 1 (5αR1) and type 2 (5αR2), are coded by two different genes and possess differing biochemical properties and tissue expression profiles. 5αR1 is widely expressed throughout the body, with high levels in the liver and skin outside of the genital regions. The type 2 isoform is highly expressed in fetal genital skin and is closely associated with male reproductive tissues and the adrenal glands (Thigpen, Silver et al. 1993). Studies have identified the presence of 5αR mRNA and protein within most structures of the brain, including the cortex, hippocampus, thalamus and cerebellum (Compagnone and Mellon 2000). The 5αR isoforms catalyse the metabolism of a number of substrates, however, both isoforms demonstrate the highest substrate affinity for progesterone (Celotti, Negri-Cesi et al. 1997; Poletti, Coscarello et al. 1998). The presence and activity of 5αR within the fetal brain, exposed to high concentrations of placental progesterone is therefore important in the regulation of allopregnanolone synthesis.

The expression of 5αR enzymes is associated with highly myelinated brain regions (Lauber and Lichtensteiger 1996; Poletti, Negri-Cesi et al. 1998). Along with the production of neurosteroids, the expression patterns of the
neurosteroidogenic enzymes is involved in the regulation of sex steroids within the brain, playing a role in the sexual differentiation of the brain (Compagnone and Mellon 2000). Comparisons of concentrations of progesterone metabolites in fetal and adult rat brains showed 20-times higher levels in the fetal brains, adding evidence for the importance of these metabolites during development (Kellogg and Frye 1999). Work in late gestation fetal sheep has also examined 5αR2 expression in the fetal and neonatal brain, with higher expression of 5αR before birth and a sharp decrease in both cerebellar 5αR expression and allopregnanolone levels soon after birth (Petratos, Hirst et al. 2000). Progesterone has been shown to influence the transcription of the 5αR gene. Experiments in the female mouse brain demonstrated an upregulation of 5αR2 mRNA in the presence of progesterone, with a progesterone regulatory element identified as part of the mouse 5αR2 gene promoter sequence (Matsui, Sakari et al. 2002). This may influence the expression of 5αR in the postnatal period when progesterone concentrations fall rapidly with removal of the placenta. The influence of progesterone on the regulation of 5αR enzymes may have important implications on the capacity of the neonatal brain to synthesise neuroactive steroids, particularly in preterm neonates, in which the removal of placental hormone production has occurred prematurely.

The regulatory mechanisms of 5αR expression during development are not, however, fully understood. Hypophysectomy and adrenalectomy in fetal sheep does not alter fetal brain allopregnanolone 5αR2 and P450scc expression, suggesting the main source of fetal brain allopregnanolone is local synthesis (Nguyen, Ross Young et al. 2004). However, a number of factors have been shown to influence both 5αR expression and allopregnanolone concentrations during development. In adult rats,
allopregnanolone levels increase in response to acute asphyxial insults (Barbaccia, Roscetti et al. 1996). In fetal sheep, intrauterine asphyxia, induced by brief occlusion of the umbilical cord, also results in a similar increase in brain allopregnanolone levels, along with the elevation of 5αR2 and P450scc (Nguyen, Yan et al. 2004). The response during gestation to a chronic insult such as placental insufficiency, however, differs to that of an acute hypoxic/asphyxial event. A reduction in fetal oxygen saturation, by umbilicoplacental embolisation, to approximately 40% of normal values, for a period of 20 days, was shown to increase the expression of 5αR2 in the brain tissue examined but did not lead to an elevation in brain allopregnanolone concentrations (Nguyen, Billiards et al. 2003). However, in the fetal rat, uteroplacental insufficiency and IUGR does lower fetal brain allopregnanolone concentrations (Westcott, Hirst et al. 2008). This may be due to the disruption in supply of neurosteroid precursors and substrate supply that also occurs with placental insufficiency. The lack of allopregnanolone response to chronic stress, in this case placental insufficiency, may increase the vulnerability of the fetus to brain injury and neurodevelopmental disruption, especially in the presence of chronic hypoxaemia and glucose deprivation that lead to IUGR adaptations.

1.8.1.b Finasteride

Finasteride (17β-(N-tert-butylcarbamoyl)-4-aza-5α-ansdrost-1-en-3-one) is a synthetic 4-azasteroid inhibitor of 5αR, inhibiting the action of both isoforms of the enzyme in converting steroid substrates to their 5α-reduced states (Rittmaster 1997). Figure 1.2 shows the multiple sites of 5αR action in the synthesis of a number of steroid hormones. Clinically, finasteride is most widely used as an anti-androgen drug, inhibiting the conversion of testosterone into the more potent androgen, dihydrotestosterone (DHT), for the treatment of benign prostatic
hyperplasia and androgenetic alopecia (Finn, Beadles-Bohling et al. 2006). Finasteride is also used experimentally to block the conversion of progesterone and deoxycorticosterone into their neuroactive metabolites (Lephart, Ladle et al. 1996; Melcangi, Riva et al. 1996; Kokate, Banks et al. 1999; Frye 2001; Rhodes, McCormick et al. 2004; Reddy and Apanites 2005; Mann 2006; Yawno, Yan et al. 2007).

Finasteride has a chemical structure that allows it to bind to the active site of the 5αR isoenzymes. Upon binding, 5αR reduces finasteride to dihydrofinasteride, forming a high-affinity complex between the enzyme, NADP and this finasteride metabolite (Bull, Garcia-Calvo et al. 1996; Azzolina, Ellsworth et al. 1997). This complex is very stable and inhibits the bound enzyme from reducing other steroid substrates. In clinical use in humans, finasteride treatment rapidly decreases circulating DHT concentrations by 60-80% (Rittmaster 1997; Finn, Beadles-Bohling et al. 2006). In vitro studies have shown that finasteride is more effective at inhibiting 5αR2 activity that 5αR1, with a100-fold greater selectivity for 5αR2. This has also been established in vivo with DHT concentrations even lower in the prostate, where 5αR2 is the predominant isoform, than those measured in peripheral blood (Geller 1990; Finn, Beadles-Bohling et al. 2006).

In rodent experimental models comparable doses to those used clinically have been shown to reduce 5αR activity by 50-80% in plasma, the brain and ovary (Finn, Beadles-Bohling et al. 2006). In stressed rats, a 10mg/kg dose of finasteride reduces brain allopregnanolone concentrations to approximately 10% of levels in vehicle treated animals (Mukai, Higashi et al. 2008). A single 25mg/kg dose in female rats can decrease the concentrations of 5α-reduced steroid metabolites (both THP and THDOC) by up to 75% in the cerebral cortex at 2hrs post-treatment (Maguire and Mody 2007). Long-term finasteride treatment effectively
maintains these reductions in 5αR activity steroid metabolite concentrations.

1.8.2 Allopregnanolone Actions
As described previously, allopregnanolone is a potent modulator of GABA_A receptor activity, being active at nanomolar concentrations within the CNS (Majewska, Harrison et al. 1986; Morrow, Suzdak et al. 1987). Activity at the GABA_A receptor leads to rapid non-genomic reductions of neural excitability resulting in the well-established sedative, anaesthetic, anxiolytic and anti-convulsant effects of allopregnanolone (Mellon, Griffin et al. 2001). These inhibitory actions are also neuroprotective in instances where excessive excitation causes brain injury and cell death related to excitotoxicity (Djebaili, Hoffman et al. 2004). Additionally, allopregnanolone has constitutive roles within the adult and developing CNS, with appropriate inhibitory signalling required for the normal processes of neuronal plasticity.

Evidence for the anesthetic and sedative effects of allopregnanolone has been gathered both in clinical and animal studies. In humans, many effects of allopregnanolone have been described in relation to sex steroid concentrations and contraceptive treatments. Increases in serum allopregnanolone as a result of administration of progesterone or direct allopregnanolone treatment increases ratings of sedation and reduces saccadic eye movements in both women and men (Timby, Balgard et al. 2006; van Broekhoven, Backstrom et al. 2006; van Broekhoven, Backstrom et al. 2007). Related to its sedative effects, is the ability of allopregnanolone to mediate mood, anxiety and affective disorders, particularly those related to female hormone status and pregnancy. The withdrawal of placental progesterone and the subsequent reduction in circulating allopregnanolone concentrations following birth has been implicated in the aetiology of post-natal depression and other psychiatric conditions related to pregnancy...
In pregnant rats, increases in anxiety and delayed maternal behaviours were observed when allopregnanolone concentrations were reduced by administration of finasteride during gestation (de Brito Faturi 2006; Mann 2006). Allopregnanolone concentrations also affect mood during the menstrual cycle with changes in serum progesterone and its metabolites being implicated in anxiety and panic disorders, as well as premenstrual dysphoric disorder (Nilini, Toufexis et al. 2011). Modulation of GABA\textsubscript{A} receptor-mediated anxiety by allopregnanolone has also been shown to be present in animal models of alcohol withdrawal (Finn, Gallaher et al. 2000; Hirani, Sharma et al. 2005). Due to their action at the GABA\textsubscript{A} receptor, neuroactive steroids may have potential as therapeutics for anxiety, mood disorders and in the treatment of drug addiction and withdrawal (Gasior, Carter et al. 1999).

Due to its effect on inhibiting neural excitability, allopregnanolone also reduces the frequency and severity of seizures in a number of models of epilepsy (Gasior, Carter et al. 1999; Reddy 2002; Salazar, Tapia et al. 2003; Hossain 2005; Mares, Mikulecka et al. 2006). Good evidence for the role of allopregnanolone in mediating seizure activity has been shown in models of hormone-sensitive catamenial epilepsy, in which allopregnanolone and its synthetic analogues have potential as anti-epileptic therapies for use clinically (Wojtal, Trojnar et al. 2006; Reddy and Rogawski 2009). Inhibition of allopregnanolone synthesis in animal models of induced epilepsy has provided strong evidence for the role of this neurosteroid in GABA\textsubscript{A} receptor-mediated inhibition of seizure activity (Kokate, Banks et al. 1999). Allopregnanolone pre-treatment in neonatal rats has also demonstrated a dose-dependent inhibition of seizure activity (Mares, Mikulecka et al. 2006). The incidence of seizures also influences neurosteroid synthesis, suggesting the presence of an endogenous neuroprotective response that regulates neurosteroid supply (Biagini,
Seizures are common in the aetiology of perinatal brain injury and in the neurological sequelae of preterm birth. The use of neuroactive steroids, in particular allopregnanolone, may be useful in treating these perinatal seizures and reducing subsequent brain injury.

Some of the most important and promising properties of allopregnanolone have been identified in its ability as a neuroprotective agent following brain injury in adult ischemic stroke and traumatic brain injury. Progesterone, as described earlier, conveys protection against adult brain injury and improves both histological and functional recovery. Some of these effects, however, are mediated by allopregnanolone, as a metabolite of progesterone, rather than by progesterone itself. Cell death, inflammatory cytokines and gliotic scarring are all reduced following contusion injuries in adult rat cerebral cortex with allopregnanolone administration (Djebaili, Hoffman et al. 2004; He, Evans et al. 2004; He, Hoffman et al. 2004; Djebaili, Guo et al. 2005). Impaired function and behavioural deficits arising from the traumatic brain injury were also improved by both progesterone and allopregnanolone treatment. The protective effects of progesterone in these models were mediated by allopregnanolone (Djebaili, Hoffman et al. 2004; He, Hoffman et al. 2004). Similar functional and histological benefits from treatment with allopregnanolone and progesterone are also seen in adult rat models of ischemic stroke (Sayeed, Guo et al. 2006). Allopregnanolone is emerging as a promising therapeutic agent for use as a neuroprotectant in these types of adult brain injuries.

Allopregnanolone also has potential as a therapeutic agent for use with neurodegenerative disorders such as Alzheimer’s disease. Positive growth, survival, regenerative, neurogenic and proliferative properties have been assigned to allopregnanolone (Wang, Yu et al. 2004; Charalampopoulos, Remboutsika et al. 2008; Wang, Liu et al. 2008), which has led to it and similar neurosteroids being proposed as potential therapies for Alzheimer’s
Disease, to improve memory and learning outcomes. Allopregnanolone also promotes neurogenesis and delays neurodegeneration in a neonatal mouse model of Neimann-Pick Type C, a degenerative neurological disease (Griffin, Gong et al. 2004; Mellon, Gong et al. 2008).

1.8.3 Allopregnanolone Actions in the Perinatal Brain

Allopregnanolone has also been proposed as having essential functions within the immature and developing brain. Gestation, birth and the perinatal period are times of both rapid growth and high vulnerability to injury of the immature brain. Allopregnanolone has constitutive signalling roles during this time, as well as potential protective functions against perinatal brain injury and neurodevelopmental disturbances.

Allopregnanolone, present at high concentrations during gestation (Luisi, Petraglia et al. 2000; Gilbert Evans, Ross et al. 2005; Hill, Cibula et al. 2007), has been shown to be active in the perinatal brain and CNS. A number of studies have examined the effects of allopregnanolone on CNS activity during gestation (Nicol, Hirst et al. 1997; Nicol, Hirst et al. 1998; Nicol, Hirst et al. 2001; Yawno, Yan et al. 2011). Administration of progesterone to fetal sheep, suppresses fetal arousal as measured by electrocortical, electro-ocular and electromyograph recordings (Crossley, Nicol et al. 1997; Nicol, Hirst et al. 1997). Additionally, inhibition of allopregnanolone synthesis by finasteride treatment increases fetal sheep arousal, decreases inhibition of activity and reduces fetal sleep-like behaviour (Nicol, Hirst et al. 2001). The co-administration of finasteride and the allopregnanolone analogue, alfaxalone, re-establishes normal patterns of fetal sleep and activity (Yawno, Yan et al. 2011). These studies demonstrate the influence of allopregnanolone on activity in the fetal CNS. The ontogeny of fetal sleep and the regulation of inhibitory/excitatory signalling within the fetal brain are important processes during
development, disruption of which may lead to abnormal neuronal apoptosis, pruning and proliferation. Constitutive, regulatory roles of allopregnanolone in mediating neuronal plasticity, migration, synapse formation and pruning have also been examined within the fetal brain. Normal levels of apoptosis are required in the developing brain for refinement of signalling pathways and synapse formation. In *in vitro* studies, allopregnanolone has been shown to enhance myelination, neuronal proliferation and axonal growth (Charalampopoulos, Remboutsika et al. 2008). In the fetal sheep, inhibition of allopregnanolone synthesis late in gestation, increases the number of apoptotic, caspase-3 positive cells, and the pyknotic cells in both the hippocampus and cerebellum (Yawno, Hirst et al. 2009). Inhibition of allopregnanolone synthesis has an influence on proliferation that is reversed following alfaxalone administration. These findings suggest an important regulatory role for allopregnanolone in balancing neuronal apoptosis and proliferation as part of normal brain development. The role of allopregnanolone in modulating GABA$_A$ receptor action during early gestation when the GABA$_A$ receptor has excitatory signalling effects (Galanopoulou 2008; MacDonald and Botzolakis 2009) may also play an important constitutive role in correct neuronal and synaptic development and growth. Insults, such as placental insufficiency that can be present early in gestation and also effect neurosteroid supply may be detrimental to this normal signalling necessary for brain development.

The sensitivity of the fetal brain to allopregnanolone action is also affected by GABA$_A$ receptor expression, with functional GABA$_A$ receptors present in the fetal brain from early gestation, with the highest density of expression in the fetal sheep brain being the frontal cortex (Crossley, Walker et al. 2000). The regionally specific and developmentally regulated expression of GABA$_A$ receptors in the fetal sheep brain, indicate potential
functions of the receptor during brain development. Neurosteroids have also been shown to directly influence the expression of \( \text{GABA}_A \) receptors in the immature cerebral cortex of late gestation guinea pig fetuses and potentiating the actions of other pharmacological modulators of \( \text{GABA}_A \) function (Bailey, Brien et al. 1999).

Allopregnanolone synthesis and actions have also been examined in the perinatal brain exposed to insults during gestation, delivery and postnatally. A model of birth asphyxia in the near-term spiny mouse, a precocial rodent species, shows that pre-treatment with allopregnanolone partially ameliorates the signalling changes and altered calcium metabolism in spiny-mouse pup brains that occurred as a result of the asphyxial insult (Fleiss, Parkington et al. 2012). In the fetal sheep, inhibition of allopregnanolone synthesis in combination with umbilical cord occlusion causes an increase in cell death compared to asphyxia alone (Yawno, Yan et al. 2007). The intravenous administration of endotoxin (lipopolysaccharide; LPS) as a model of infection and inflammation in newborn lambs results in increased concentrations of brain and plasma allopregnanolone. This increase in allopregnanolone concentrations coincided with behavioural changes, increased sleep and reduced activity compared to control lambs (Billiards, Walker et al. 2002). Interestingly, the combination of repeated LPS administrations and an acute hypoxic event further increased allopregnanolone concentrations in the neonatal sheep brain (Billiards, Nguyen et al. 2006). This increase in brain allopregnanolone suggests the potential induction of an endogenous neuroprotective response in the perinatal brain following noxious stimuli.

1.9 RATIONALE, HYPOTHESIS & AIMS

There is significant evidence for the neuroprotective role of neuroactive steroids in the adult brain exposed to trauma or ischemic stroke. A number
of animal studies have also identified the activity of neuroactive pregnane steroids in regulating perinatal brain activity and fetal sleep states. However, the protective roles of neurosteroids in the perinatal brain are newly emerging. This study aims to examine the effects of neuroactive progesterone metabolites on brain development during gestation and in the immediate postnatal period. Additionally, due to the major influence of the placenta in supplying progesterone, it is a further aim of this study to examine the effects of pathologies such as IUGR and preterm birth that may disrupt this supply. In order to elucidate the functions of neuroactive steroids in the presence of IUGR and following preterm birth, this study also establishes fetal and neonatal models in the guinea pig for the study of these effects on the perinatal brain.

1.9.1 The Guinea Pig as an Animal Model for the Study of Neurosteroid Actions in the Immature Brain

The complex interactions between biological systems that occur during gestation and fetal and neonatal development means that the use of animal models to study pregnancy, under both normal and pathological conditions, is particularly valuable. The action of neuroactive steroids on the developing brain and the regulation of their synthesis could not be fully explored using in vitro methods or in clinical studies that do not allow for manipulation of experimental conditions. The use of animal models with physiology that is relevant to the systems being studied is essential to improving our knowledge of human physiology and disease. The guinea pig is a useful model for the study of certain aspects of reproductive, fetal and neonatal physiology particularly with reference to perinatal brain development, neuroendocrine function during pregnancy and the neuroactive steroid system. The guinea pig (Cavia porcellus), is a small domesticated rodent, originally from South America, with an average adult size of ~1000g, that gives birth to precocial young that, unlike other
commonly used rodent species, are born fully furred, with eyes open and fully developed teeth (Sachser, Kunzl et al. 2004). Female guinea pigs have a post-partum estrous and periodical 16-day estrous cycle that allows for easy control and timing of mating. Gestation length varies between particular colonies and strains of guinea pigs but is reported as being between 65-70 days. The average litter size for guinea pigs is 1-4. However, up to 7 or 8 pups can be present in a single litter (Peaker and Taylor 1996). An important advantage of the guinea pig for its use in studying neuroactive steroids is the endocrine profile of guinea pigs during gestation. Unlike other commonly used models, such as the sheep, in which progesterone concentrations fall precipitously just prior to parturition, it has been shown that the guinea pig maintains concentrations of progesterone during late gestation (Challis, Heap et al. 1971). This is similar to the maintenance of progesterone concentrations and the proposed functional withdrawal of progesterone signalling that triggers parturition in humans (Palliser, Zakar et al. 2010). Unlike species such as the sheep, guinea pigs also have haemomonochorial placentation, with the placenta the major site of progesterone synthesis throughout gestation (Heap and Deanesly 1966). The maintenance of progesterone concentrations throughout gestation is important for the continued supply of neuroactive steroid precursors to the fetus and fetal brain.

Guinea pigs have also proved useful for the study of fetal growth and the pathologies associated with intrauterine growth restriction. Similarly to humans, size at birth is variable in guinea pigs neonates, with spontaneous growth restriction occurring in some pups, particularly, but not solely those with larger numbers of littermates (Saintonge and Rosso 1981). Experimental models of IUGR are well-established in guinea pigs, using caloric restriction, uterine artery ligation and modifications of this technique to produce significant reductions in newborn size, weight and
asymmetrical reduction in brain weights (Byrne, Smart et al. 1978; Carter 1993; Haugaard and Bauer 2001; Tolcos, Rees et al. 2002; Briscoe, Rehn et al. 2004; Loeliger, Briscoe et al. 2004; Turner and Trudinger 2009). The relative timing of perinatal brain growth and differentiation during gestation is another factor important to the use of animal models for the study of the perinatal brain and the processes that we can infer may be present in humans (Dobbing and Sands 1979). In contrast to the rat and mouse, in which a considerable amount of brain development occurs postnatally, the guinea pig, which is precocial at birth, has a high degree of brain growth that occurs in utero (Dobbing and Sands 1970). Importantly, this means that brain development in the guinea pig predominantly occurs under the influence of maternal and placental factors that may not be present in the ex utero environment. Of particular interest in relation to the present studies, is the effect of this placental steroid supply on this brain development.

Studies of the perinatal guinea pig brain have examined the morphological development and ontogeny of neuroendocrine and signalling systems (Peters and Flexner 1950; Dobbing and Sands 1970; Connolly, Roselli et al. 1994; Burdge and Postle 1995; Resko and Roselli 1997). Developmental programming mechanisms of fetal exposure to glucocorticoids and prenatal stress have also been studied in guinea pigs with changes in behavioural outcomes and marked effects on brain expression of glucocorticoid and mineralocorticoid receptor (Matthews 1998; Dean, Yu et al. 2001; Kapoor and Matthews 2005; Dean and McCarthy 2008). Evidence of perinatal brain injury, lipid peroxidation and NMDA receptor-mediated excitotoxicity following hypoxia have also been gathered in fetal guinea pigs (Mishra and Delivoria-Papadopoulos 1988; Mishra and Delivoria-Papadopoulos 1989; Mishra and Delivoria-Papadopoulos 1992; Maulik, Zanelli et al. 1999; Maulik, Qayyum et al. 2001). Other causes of
perinatal brain injury and developmental disruption/programming, such as prenatal ethanol exposure, maternal anaesthesia and postnatal isolation stress have been examined in fetal and neonatal guinea pigs, with altered behavioural outcomes, reduced neuronal proliferation and neurotransmitter signalling observed (Byrnes, Reynolds et al. 2001; Rizzi, Bianchi et al. 2007; Rizzi, Carter et al. 2008). These studies demonstrate the usefulness of the guinea pig for the study of a wide range of pathologies during the perinatal period and their effects on the developing brain. The use of the guinea pig for the study of mechanisms of parturition has gained some recent support (Mitchell and Taggart 2009), and the examination of neuroactive steroids in this species is a novel use for this eponymous experimental animal model. The development and establishment of a preterm neonatal paradigm in this species is therefore an important aspect of this thesis.

1.9.2 Hypotheses and Specific Aims
We hypothesise that the perturbation of placental progesterone production and availability disrupt the supply of neuroactive steroids to the perinatal brain. The disruption of neuroactive pregnane steroid systems within the fetal and neonatal brain contributes to the increased incidence of brain injury and poor neurodevelopmental outcomes associated with placental pathologies and preterm birth. Progesterone and neurosteroid replacement during the perinatal period will provide neuroprotection and restore normal neurodevelopmental processes.

1.9.2.a Low Neurosteroid Environment and IUGR
We hypothesised that neuroactive steroids have important trophic and protective roles in the fetal brain and that complications during pregnancy, such as IUGR that may disrupt fetal neurosteroids, contribute to poor
neurodevelopmental outcomes in fetuses and neonates with these pregnancy complications.

The specific aims of this study were to examine late gestation fetal guinea pig brain developmental processes, with reference to the fetal neuroactive steroid system, in the presence of IUGR and following the inhibition of allopregnanolone synthesis (Figure 1.5A). We also aimed to examine the combined vulnerability of the IUGR fetal brain to reduced neuroactive steroid synthesis.

1.9.2b Preterm Neonatal Loss of Progesterone

We hypothesised that disruption of neuroactive steroid concentrations, which occurs with the premature removal of placental steroid supply at preterm birth, contributes to poor neurodevelopmental outcomes and vulnerability to perinatal brain injury observed in premature infants. The specific aims of this study were to establish a model of preterm birth in the guinea pig, in which the effect of preterm birth and the premature removal of placental progesterone supply on the neurosteroid system and brain development could be examined (Figure 1.5B).

1.9.2c Preterm Neonatal Progesterone Replacement

We hypothesised that postnatal progesterone therapy during the preterm period, as a replacement of normal placental supply, would augment endogenous allopregnanolone synthesis, improving postnatal brain development and outcomes in preterm infants. Specifically, we aimed to evaluate the effectiveness of postnatal progesterone replacement to increase neonatal allopregnanolone concentrations and to examine markers of brain growth and morphology, along with functional/behavioural effects of this hormone replacement in the preterm neonatal guinea pig at term-equivalent age (Figure 1.5C).
Figure 1.5 - Schematic Diagram of Specific Aims
This thesis examines the roles of progesterone and allopregnanolone in the fetal and neonatal brain and effects on perinatal brain injury and developmental outcomes. The disruption of neuroactive steroid concentrations in relation to perinatal complications such as (A) intrauterine growth restriction (IUGR) or finasteride treatment and (B) preterm birth are also assessed, along with the potential for (C) postnatal progesterone replacement therapy in restoring neuroactive steroid concentrations.
Chapter Two

MATERIALS & METHODS

2.1 ANIMAL ETHICS
All animal experiments and procedures were approved by the University of Newcastle Animal Care and Ethics Committee (approvals 1029, 144 & 151) and carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals were monitored daily for signs and symptoms of distress or illness. All pregnant animals were weighed daily to monitor weight gain as an indication of fetal growth and closely monitored for signs of unexpected or early labour. Neonatal animals were monitored in relation to activity, posture, respiration, feeding, urination and temperature for any indication of poor or declining health. Specialist veterinary advice was sought if any abnormal symptoms were observed and according to ethics guidelines, were humanely euthanised if necessary.

2.2 ANIMAL HOUSING
Pregnant, outbred, tricolour guinea pigs were obtained from the University of Newcastle Research Support Unit. Guinea pigs were time-mated during the post-partum estrous and housed separately with their litters until weaning at 21 days, at which time pregnancy was confirmed by gentle palpitation of the abdomen. All animals were housed indoors under a 12hour light/dark cycle and provided with commercial guinea pig feed pellets, lucerne hay and water fortified with ascorbic acid ad libitum. Neonatal guinea pigs were housed in a separate, dedicated room in a small
animal incubator, kept at temperatures between 30-35°C and humidity between 20 and 30%.

2.3 IUGR SURGERY

As part of a study to examine the effect of IUGR on neurosteroids and late gestation fetal brain development, an experimental model of IUGR was used. Abdominal laparotomy surgery was performed at mid-gestation to surgically restrict blood flow to the placenta in order to induce intra-uterine growth restriction. To allow for comparison across studies, IUGR or sham surgeries, as detailed below, were performed on all pregnant animals used in each experiment.

2.3.1 Surgical Preparation

Surgery was performed at mid-gestation, between 32 and 36 days gestation (term=~70 days). Animals were starved for approximately 4 hours prior to surgery, with drinking water remaining unrestricted. Pregnant guinea pigs then received subcutaneous injections of atropine sulfate (0.05mg/kg) and buprenorphine analgesia (0.5mg/kg, Temgesic, 324μg Buprenorphine HCl and Glucose anhydrous/mL, Reckitt Benckiser Healthcare, Hull, UK) approximately 20min before anaesthetic induction. Anaesthesia was performed using an isoflurane vapouriser with a flow rate of 6L/min oxygen (Medical Grade O₂, Coregas, Yennora, NSW, Australia) and 4% isoflurane (Laser Animal Health, Salisbury, Qld, Australia) chamber induction. Guinea pigs were then placed in a supine position with head raised to reduce the potential of fluid aspiration and anaesthesia was maintained by mask on 2% isoflurane with 2L/min oxygen. Animals were placed on heat pads to maintain body temperature during anaesthesia. A section along the midline of the ventral abdomen was shaved and prepared for surgery using 4 washes, alternating between Microshield 4 Surgical
Scrub (4% w/v Chlorhexidine gluconate, Johnson & Johnson Medical, North Ryde, NSW, Australia) and Betadine surgical scrub (7.5% w/v povidone-iodine, Faulding Pharmaceuticals, Australia). The surgical site was then covered with Betadine antiseptic solution (10% povidone-iodine in 70% ethanol) and rinsed with 70% ethanol prior to the incision being made. Aseptic technique was observed throughout the surgery, with surgeons wearing masks, caps, autoclaved surgical gowns and sterile gloves (Ansell Healthcare, Australia). All drapes and instruments were also autoclave sterilised and surgeons scrubbed hands with Microshield 4 surgical scrub prior to surgery.

2.3.2 Surgical Procedure

A skin incision was made, extending approximately 5cm along the midline from the umbilicus to the pubic symphysis, exposing the subcutaneous and muscle layers. An incision was then made along the *linea alba*, in order to open the peritoneal cavity and expose the uterus. The position of each uterine horn was determined and the uterine arteries (shown in Figure 2.1) located via manipulation of the uterine fat pads. The mesometrial (radial)/uteroplacental (spiral) arteries branching from the uterine-ovarian arcade artery and supplying each placenta were then identified and approximately 50% of these branches ablated using a diathermy tool (Birtcher, El Monte, CA, USA). This was repeated for each fetus in one of the uterine horns. Exposed tissue was moistened with sterile saline (0.9% NaCl, Baxter Healthcare, Toongabbie, NSW, Australia) as necessary. The uterine horns were replaced in the peritoneal cavity and the muscle layer incision closed with a simple interrupted suture pattern using Biosyn monofilament absorbable sutures (Syneture, Norwalk, CT, USA). The number, position and viability of each fetus were recorded at the time of surgery.
Figure 2.1 - Uterine Blood Supply in the Guinea Pig and Site of Radial Artery Ablation for IUGR Surgery

(A) Left uterine horn of a pregnant guinea pig at mid-gestation with three fetuses. The uterine-ovarian vein, mesometrial veins, spiral arteries and uterine fat pad are visible. (B) Diagram of blood supply in a pregnant guinea pig uterine horn. The guinea pig possesses a bicornuate uterus that is supplied with oxygenated blood from the uterine-ovarian arcade artery, which branches into mesometrial (radial) and uteroplacental (spiral) arteries to supply the placenta and fetus. Mid-gestation IUGR surgery induces placental insufficiency by the cauterisation of ~50% of the radial/spiral arteries supplying each placenta. Adapted from (Kaufmann and Davidoff 1977)
The subcutaneous and skin layers were closed using Daclon blue nylon suture (Duke Veterinary Supplies, St Peters, NSW, Australia) in a continuous horizontal mattress stitch pattern. Incision sites were treated with Chloromide Antiseptic Spray (Troy Laboratories, Smithfield, NSW, Australia) and antibiotic Terramycin wound surface spray (Oxytetracycline HCl; Pfizer, West Ryde, NSW, Australia) following surgery.

2.3.3 Sham Surgery
Control animals received sham surgery in which all steps, including location and manipulation of the uterus and uterine vessels, were carried out as outlined in section 2.3.2, with the exception of cauterisation of the radial artery branches.

2.3.4 Post-Surgery Recovery
Following surgery, animals were placed in a prone position until fully recovered from the anaesthetic, at which time they were returned to their home cages, where they remained on heat pads and were monitored closely until normal posture and activity returned. Animals received a second dose of buprenorphine analgesia (0.5mg/kg) at 8 hours post-surgery. Twice daily monitoring was carried out for the first 48 hours following surgery and daily monitoring continued for the duration of the experiment. Skin sutures were removed 10 days after surgery.

2.4 FINASTERIDE TREATMENT
Finasteride, a synthetic 4-azasteroid inhibitor of 5αR (Finn, Beadles-Bohling et al. 2006), was administered to pregnant guinea pigs to reduce supply of allopregnanolone in the late gestation fetal guinea pig. Animals used for experiments in Chapter 3 were randomly assigned to control (vehicle treated, sham surgery), IUGR (vehicle treated, IUGR surgery), FIN (finasteride treated, sham surgery) and IUGR+FIN (finasteride
treated, IUGR surgery) groups. Finasteride powder (Steraloids Inc, Newport, RI, USA) was dissolved in vehicle (16% v/v ethanol in peanut oil (Steric Trading Pty Ltd, Villawood, NSW, Australia)) at a concentration of 40mg per mL. Pregnant guinea pigs received finasteride at a dosage of 25mg/kg (600μL/kg) daily, which has previously been used in pregnant rats (de Brito Faturi 2006). Control and IUGR animals received 600μL/kg vehicle only. As part of this study, sub-cutaneous injections commenced at 55 days gestation and proceeded daily until tissue collection at 65 days gestation.

2.5 PRETERM NEONATAL MODEL

A model of preterm delivery was developed in the guinea pig for the study of the effect of preterm birth on neurosteroids in the preterm brain and the resultant consequences on neonatal brain development. These protocols were used in studies for Chapters 4, 5 and 6.

2.5.1 Experimental Groups

Pregnant guinea pigs were divided into three main treatment groups based on gestational age of pups at delivery and post-natal age of pups at tissue collection. Neonates allocated to the term group were delivered just prior to term at either 69 days gestation or on the second day of pubic symphysis separation being 1.5cm or greater (an indicator of imminent delivery), whichever occurred first. All term neonates were killed and tissues collected at 24hours post-delivery/post-natal day 1 (PND1).

Preterm neonates were delivered at approximately 0.87 of the average total gestation at gestational age 62-63 days. Preterm neonates were then further divided into animals that were killed and tissues collected at 24hours and a second group of “term-equivalent” aged animals that were delivered
preterm and sacrificed at corrected term age (i.e. equivalent of 70 days gestation, PND8).
The three main treatment groups based on gestational age were classified as Term, Preterm (Pre-T) and Preterm term-equivalent (Pre-T8). In addition, the preterm animals in both postnatal age groups were further divided by drug treatment (see section 2.5.6 for details). The preterm animals that received progesterone treatment were classified as +Prog (PND1) and +Prog8 (PND8).

2.5.2 Caesarean-Section Deliveries
Pregnant guinea pigs received antenatal steroid treatment prior to scheduled caesarean-section delivery of neonates. A course of Celestone Chronodose (1mg/kg betamethasone sodium phosphate/betamethasone acetate, Schering-Plough, North Ryde, NSW, Australia) was given subcutaneously consisting of injections at 24hrs and 12hrs prior to delivery. At the time of c-section delivery, pregnant guinea pigs were anaesthetised by chamber induction in 4% isoflurane in medical grade oxygen. Animals were then placed in a supine position with an elevated head, with anaesthesia maintained by mask isoflurane at 2% for the duration of the procedure. Incisions were made through the skin and muscle layers along the midline of the ventral abdomen from above the umbilicus to the pubic symphysis, exposing the uterus. Each fetus was then located within the uterus and an incision was made through the myometrium, being careful not to rupture the amnion. Amniotic fluid was collected using a syringe carefully inserted into the amniotic sac. The amnion was then rapidly removed from the nose and mouth of the fetus, the umbilical cord was tied using surgical silk and cut. Neonates were then transferred for resuscitation (section 2.5.3). Each subsequent neonate was monitored for signs of distress during the delivery and was delivered within 2 minutes of the previous animal. Following delivery of the neonates, maternal guinea pigs
were killed via cardiac administration of Lethabarb (344mg/mL Sodium pentobarbitone, Virbac, Regents Park, NSW, Australia).

2.5.3 Neonatal Resuscitation

Neonates delivered at term or preterm age received airway support and positive pressure ventilation at birth. Immediately following delivery, neonates were rubbed vigorously to stimulate respiration and briskly inverted to aid in removal of fluid from the lungs and upper respiratory tract. Each neonatal guinea pig then received a dose of 50uL surfactant (Curosurf, 80mg/mL Poractant alfa, Douglas Pharmaceuticals, Baulkham Hills, NSW, Australia) delivered into the oropharynx using a modified neonatal feeding tube attached to a luer lock syringe (Terumo Medical Corporations, Somerset, NJ, USA). Continuous positive airway pressure (CPAP) was then administered using a small animal anaesthesia mask (Harvard Apparatus, Holliston, MA, USA) attached to a Neopuff infant T-piece resuscitator (Fisher & Paykel Healthcare, Melbourne, Australia). Once a seal was made over the mouth and nose of the guinea pig pup, a positive end expiratory pressure (PEEP) of 7cmH₂O and peak inspiratory pressure (PIP) of 20cmH₂O at a flow rate of 8L/min was applied. An initial sustained PIP of 20sec duration was administered, with PIP ventilation used only in the absence of spontaneous respiration and only until normal respiration was established. Over the course of the experiment, animals received an additional dose of 50uL surfactant and CPAP at 3hours post-delivery. CPAP was repeated if respiration became unstable until normal breathing was re-established. Animals that were not able to maintain normal respiration over an extended period of time were humanely euthanised.
2.5.4 Neonatal Care and Monitoring

In order to maintain animal temperatures, all resuscitations were carried out under animal heat lamps and using heat pads. Following delivery and establishment of stable respiration, neonates were dried and placed in a humidified incubator (Small animal intensive care incubator, Thermocare, Incline Village, NV, USA) maintained at ~34°C. Guinea pig pups were monitored closely for respiration, posture/muscle tone, activity (using a scale shown in Table 2.1) and normal urination. Body weight, body and incubator temperatures and humidity were also recorded. Following each feed, urination was stimulated using a moistened cotton tip. Animals that demonstrated signs or symptoms of illness or distress were humanely euthanised.

Table 2.1 - Neonatal Scoring

<table>
<thead>
<tr>
<th>Score</th>
<th>Respiration</th>
<th>Posture</th>
<th>Alertness/Movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No breathing</td>
<td>Lying on side with severe weakness and little muscle tone</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Gasping only</td>
<td>Lying down; severe spasticity of neck and front paws</td>
<td>Only when stimulated</td>
</tr>
<tr>
<td>2</td>
<td>Gasping with some normal breathing</td>
<td>Can sit up, but difficulty holding head up; some spasticity.</td>
<td>Some spontaneous movement</td>
</tr>
<tr>
<td>3</td>
<td>No Gasping but irregular or shallow breaths</td>
<td>Upright, sitting, can walk but wobbly and uncoordinated</td>
<td>Some activity</td>
</tr>
<tr>
<td>4</td>
<td>Normal, fast, and regular breaths</td>
<td>No spasticity, can stand and walk easily</td>
<td>Very active and alert.</td>
</tr>
</tbody>
</table>

Neonatal Condition:
0 - 3 Very poor; 4 - 6 Poor; 7 - 9 Good; 10 - 12 Very Good
2.5.5 Feeding

The guinea pig neonates were fed at 2 hourly intervals using commercial guinea pig milk replacement formula (Wombaroo Food Products, Adelaide, SA, Australia), at 100μL/g/24hrs, made up in 50% v/v water and glucose solution (5% glucose solution, Baxter Healthcare) for the first 4 feeds and with no additional glucose for subsequent feeds. Neonates were fed using a modified feeding tube made from a 20cm length of polyvinyl tubing (internal diameter 0.8mm, external diameter 1.2mm, Microtube Extrusions, North Rocks, NSW, Australia) attached to a 1mL syringe (Terumo). The open end of each tube was blunted 1cm from the end. Markings were made at 6, 7 and 8cm from the blunted end. In order to feed neonates that did not have strong swallowing reflexes, the tube was gently fed into the oesophagus to ~7cm and the syringe plunger slowly depressed, ensuring excess air bubbles were not present in the syringe or tubing. Neonates with developed swallowing reflexes were fed slowly into the mouth with care taken to reduce the risk of aspiration of the milk into the lungs of the pups. The volume of each feed was carefully recorded.

2.5.6 Drug Treatments

Preterm neonates were randomly assigned to receive either progesterone (16mg/1.6mL/kg) treatment or “control” vehicle (22.5% w/v 2-Hydroxypropyl-β-cyclodextrin) injections. All preterm animals received injections at 1 hour post-delivery (IP), with an additional injection at 6 hours post-delivery (SC). Preterm animals that continued until term-equivalent age also received a single SC injection on each subsequent day. Progesterone dose used in this study was based on that used by Goss et al (Goss, Hoffman et al. 2003).
2.5.7 Behavioural Testing

Open-field and novel object recognition testing (NORT) were carried out on preterm term-equivalent aged (PND8) guinea pig neonates to measure general activity levels and non-spatial memory (Kapoor and Matthews 2005) following progesterone treatment, compared to vehicle treated controls. The testing procedure, as detailed below, was performed on post-natal day 7 in a quiet room, away from other animals under standard fluorescent lighting conditions. All trials were recorded using a digital video recorder for later analysis.

The testing procedure involved a 15min open-field trial (OF), followed by a 15min sample/familiarisation trial (T1) in which the addition of 2 identical objects were placed in the open-field arena, a retention/rest period of 80min and a final 15min recognition phase (T2), in which a novel object replaced one of the objects from the familiarisation trial. The open-field arena consisted of a 43x43cm box with 20cm tall sides, made from rigid polypropylene plastic board. The objects (golf balls and small plastic cylinders mounted and fixed so as not to be moveable) were placed 14cm from each wall at adjacent corners. The arena and objects were carefully washed between trials and between animals. The recordings from each trial were analysed for a number of parameters over the length of the trials and for shorter periods during each trial, by researchers blinded to treatment groups. Analysis of general activity, number of sector visits, time spent in each sector, time spent in the inner zone (inner 14x14cm square), wall-climbs, rearing, vocalisation and grooming behaviours was performed for the OF trials. During the NORT T1 and T2 trials, the general activity, exploration time and total time the subject interacted with each object was recorded. Object exploration was defined as the animal handling, sniffing or facing the object (within 2cm).
2.6 TISSUE COLLECTION

Tissues were collected from fetal animals (Chapter 3), pregnant maternal guinea pigs and from neonatal animals (Chapters 4, 5 & 6) following euthanasia by inhalation of carbon dioxide (BOC gases) for a period of 10 minutes. The absence of maternal and neonatal pedal and palpebral reflexes was confirmed prior to any incisions being made. Confirmation of death was determined by excision of the diaphragm and the absence of respiration and heartbeat in all animals.

Fetal animals were removed from the uterus by an initial midline incision down to the ventral abdomen of the pregnant guinea pig dam and a careful incision through the myometrium and fetal membranes. The absence of reflexes, respiration and heartbeat was then confirmed in the fetal animals. The number of fetuses for each dam, fetal sex, position and fetal body weight were then recorded.

The following procedures apply to both fetal and neonatal animals. Fetal and neonatal blood was collected via cardiac puncture with 23G luer lock needle and 1mL syringe (Terumo) and added to 3mL ethylenediaminetetracetic acid (EDTA) blood collection Vacuette tubes (Greiner Bio-One, Monroe, NC, USA), refrigerated and centrifuged to separate plasma and red blood cells. Plasma was then collected, added to sterile 1.5mL eppendorf tubes and snap frozen in liquid nitrogen.

Brains were collected via a midline incision through the scalp to expose the skull, which was then carefully removed so as not to damage the underlying tissue. Following the excision of the spinal cord and cranial nerves the whole brain was removed and weighed, before the hemispheres were separated by a midline cut through the sagittal plane. Each hemisphere was then either fixed or frozen prior to further processing. Before fixing (left hemisphere only collected for immunohistochemistry) the brain was cut into three coronal portions. The ventral (containing mainly cortex), middle...
(containing cortex, hippocampus and thalamus) and dorsal (containing the cerebellum) sections were then placed in tissue cassettes and fixed by immersion in 10% neutral buffered formalin for 24 hours at 4°C and stored in 0.1M Phosphate Buffer with 0.05% sodium azide until ready for embedding. If not fixed, the hemispheres were either cut into the same 3 coronal sections and frozen in liquid nitrogen or the hippocampus and a portion of the frontal cortex was carefully dissected from the brain and frozen. The adrenal glands and placentae of each animal was also collected, and heart, liver and kidney weights were recorded. Maternal uterine tissues, fetal membranes, amniotic fluid and maternal blood was also collected as part of other ongoing studies (data not presented in this thesis).

2.7 MEASUREMENT OF ALLOPREGNANOLONE

The measurement of the neurosteroid, allopregnanolone, as outlined in this protocol, involves the use of a competitive-binding radio-immunoassay (RIA). RIA is a useful method for the quantification of a wide variety of biological substances and has been widely used for the measurement of steroid concentrations in a number of sample types since their development in the late 1960s (Yalow and Berson 1960; Jeffcoate 1977). RIA binding assay methods differ based on the type of analyte to be measured but usually involve the same basic steps, including: a) the extraction and/or purification of the analyte from the biological matrix; b) addition of a ‘binder’ that will form a complex with the analyte (ligand); c) addition of a radio-labelled ‘tracer’ that will compete with the ligand for binding sites on the ‘binder’; d) separation of bound and free fractions of the tracer; and e) quantification of the remaining tracer (bound fraction) to determine analyte concentration. The ratio of bound to unbound (free) tracer is the basis by which this type of assay is able to detect changes in concentration of the analyte. Because a radio-labelled tracer has been used,
the relative radioactivity of the sample is used as the detection method, with the concentration of the analyte in a given sample being inversely proportional to the amount of bound tracer detected. Quantification of the analyte is achieved by comparing unknown samples to known concentrations that form a standard curve.

The method used here has been modified from previously published protocols used to measure allopregnanolone concentrations in human plasma, ovine plasma and brain samples (Barbaccia, Roscetti et al. 1992; Bicikova, Lapeik et al. 1995; Billiards, Walker et al. 2002). An extra purification step has also been added to reduce potential cross-reactivity from high concentrations of progesterone present in samples, particularly those augmented by progesterone administration.

2.7.1 Steroid Extraction

2.7.1.a Brain Homogenate Preparation

Frozen brain tissue from the middle coronal section of the brain (containing cortical, hippocampal and thalamic tissue) was crushed on dry ice with a mortar and pestle. Approximately 80mg tissue was weighed into a screw-top plastic tube and 1mL ice-cold 50% acidified methanol (50% v/v methanol containing 1% acetic acid) added to each sample. This mixture was then homogenised using an Ultra-Turrax T-25 homogeniser (IKA Labortechnik, Staufen, Germany) for 3 x 30sec bursts at the maximum setting. The homogenate was then centrifuged in a J-6 M/E centrifuge (Beckman Coulter, Gladesville, NSW, Australia) at 2500rpm for 25min at 4°C. The supernatant was decanted an additional 1mL of 50% methanol, 1% acetic acid added to the precipitated pellet and the homogenisation and centrifuge steps repeated. The supernatants were then pooled in glass tubes (12 x 75mm, Kimble Chase, Vineland, NJ, USA) and stored at -80°C until required for further extraction.
2.7.1.b Brain and Plasma Sep-Pak Extraction

Brain homogenates and neonatal plasma was thawed. The plasma was spun down and 60uL added to glass tubes. 600uL of radioactive allopregnanolone tracer (see 2.7.2 for details) at ~2000cpm/600uL and 2mL 50% acidified methanol was then added to each tube. Solid-phase extraction was carried at room temperature out for both plasma and brain samples, in a similar manner, using Sep-Pak Classic C\textsubscript{18} cartridges (360mg, 55-105um, Waters Corporation, Milford, MA, USA) that were primed with 2.5mL 100% methanol, 2.5mL 50% methanol and 2.5mL 50% acidified methanol. Samples were added to the cartridges, followed by washes with 2.5mL of 50% acetic acid and 2.5mL of 50% acidified methanol. Steroids were eluted from the cartridge by the addition of 3mL 100% methanol. The eluent was then dried using a dry block evaporator (Ratek, Boronia, Vic, Australia) at 50°C under a stream of nitrogen gas (Coregas).

2.7.1.c Progesterone Oxidation

Dried, extracted samples were reconstituted in 450uL of milliQ water, followed by the addition of 50uL of 5% potassium permanganate (KMnO\textsubscript{4}) solution. Samples were then incubated for 30min at room temperature to allow for the oxidation of non-saturated steroids. In order to extract the steroids from this aqueous solution, 2mL of a 50% n-hexane and 50% diethyl-ether mixture was added to the samples. Samples were mixed vigorously, aqueous and organic layers allowed to separate and the aqueous layer frozen for 1min on dry ice and the n-hexane/diethyl-ether layer decanted into a fresh tube. The addition of n-hexane/diethyl-ether was repeated a total of three times to improve the recovery of steroids from the aqueous segment. Samples were then dried under nitrogen at 50°C on a dry block evaporator.
2.7.1.d Extraction Recoveries

Samples were reconstituted in 600μL of assay buffer (0.05M phosphate buffered saline (PBS) with 0.025M EDTA, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN₃)). Recovery was determined by adding 50μL of each sample, to 5mL of BCS Liquid Scintillation Cocktail (GE Healthcare, Rydalmere, NSW, Australia), in duplicate, to mini Poly-Q polyethylene scintillation vials (Beckman Coulter, Gladesville, NSW, Australia). Samples were then counted on a LS3801 liquid scintillation counter (Beckman Coulter) to determine β-radiation emissions. The recovery of allopregnanolone was determined for each individual sample and extraction loss accounted for in final calculations of allopregnanolone concentrations (section 2.7.4). Average extraction recoveries for specific experiments are presented in each chapter.

2.7.2 Allopregnanolone Radio-Immunoassay

2.7.2.a Allopregnanolone Standards

Allopregnanolone standards were prepared from a primary stock (5α-pregnane-3α-ol-20-one, Steraloids, New Port, RI, USA) dissolved in 100% ethanol at a concentration of 200μg/mL. Secondary stock dilutions were also prepared in 100% ethanol. All standard stock dilutions were stored at -20°C. A standard curve ranging from 25 to 8000pg/mL was prepared by serial dilution of allopregnanolone stock in assay buffer, immediately prior to use. Quality controls were also prepared at high, mid and low allopregnanolone concentrations and used to calculate assay co-efficients of variance that are reported in each results chapter.

2.7.2.b Allopregnanolone Tracer

Allopregnanolone extraction recovery and the measurement of allopregnanolone concentration by RIA were carried out using tritium-
labelled allopregnanolone tracer (5α-pregnan-3α-ol-20-one, 5α-[9,11,12,3H(N)], PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Tracer stock was diluted in 100% ethanol and stored at -20°C, which, on the day of the assay, was further diluted in assay buffer and concentration determined by counting 250uL of tracer in 5mL scintillation fluid using a β-counter. For the allopregnanolone RIA, ~7800cpm allopregnanolone tracer was added to each tube.

2.7.2.c Allopregnanolone Antisera

A polyclonal antibody, raised against the carboxymethyl ether group of allopregnanolone conjugated to BSA was obtained from Dr R. H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA). Lyophilised antibody was reconstituted in assay buffer and stored in small aliquots at -20°C. On the day of the assay, the antibody was further diluted in assay buffer (working dilution 1:1250, final assay concentration of 1:3750). The steroid cross-reactivities of the allopregnanolone antisera have previously been characterised (Bernardi, Salvestroni et al. 1998) and are shown in Table 2.2. In addition, due to unavailability of the Purdy antisera, measurement of brain allopregnanolone in Chapter 5, was performed using an egg-yolk polyclonal anti-allopregnanolone antibody manufactured by Agrisera (Vannas, Sweden), raised against the same antigen (3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether coupled to BSA) used for the production of the sheep antisera. The Agrisera antibody has previously been validated against the Purdy antibody for use following steroid extraction (Timby, Balgard et al. 2006).

2.7.2.d Assay Procedure

A volume of 250uL of prepared allopregnanolone standards, unknown samples (diluted if necessary in assay buffer) and quality controls were
added, in duplicate, to glass tubes, kept at 4°C for the duration of the protocol. Antisera (250uL, 1:1250) was added to each tube and incubated for 15min, followed by the addition of 250uL (~7800cpm) of tracer. The samples mixed and incubated overnight at 4°C. The next day, 200uL of a 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 each assay tube, which were then centrifuged at 2500rpm for 10min at 4°C (J-6M/E Refrigerated Centrifuge, Beckman Coulter). This step separates the free (unbound) tracer from the antisera-bound fraction, so that the concentration of bound tracer remaining in the supernatant can be determined. In order to measure the allopregnanolone concentration, 500uL of the supernatant was added to scintillation vials along with 5mL scintillation cocktail and vials counted using a liquid scintillation β-counter.

**Table 2.2 - Cross Reactivity of Sheep Allopregnanolone Antisera with Related Steroid Compounds** (Adapted from Bernardi, et al. 1998)

<table>
<thead>
<tr>
<th>Steroid Compound</th>
<th>Cross Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopregnanolone (3α-Hydroxy-5α-pregnane-20-one)</td>
<td>100</td>
</tr>
<tr>
<td>Pregnanolone (3α-Hydroxy-5β-pregnane-20-one)</td>
<td>6.5</td>
</tr>
<tr>
<td>Progesterone (Pregn-4-ene-3,20-dione)</td>
<td>0.7</td>
</tr>
<tr>
<td>5α-dihydroprogesterone (5α-Pregnane-3,20-dione)</td>
<td>0.1</td>
</tr>
<tr>
<td>5β-dihydroprogesterone (5β-Pregnane-3,20-dione)</td>
<td>0.1</td>
</tr>
<tr>
<td>20β-Hydroxy-5α-pregnane-3-one</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-Pregnane-3α,20α-diol</td>
<td>0.1</td>
</tr>
<tr>
<td>Isopregnanolone (3β-Hydroxy-5α-pregnane-20-one)</td>
<td>0.05</td>
</tr>
<tr>
<td>5β-Pregnane-3α,20α-diol</td>
<td>0.03</td>
</tr>
<tr>
<td>Epipregnanolone (3β-Hydroxy-5β-pregnane-20-one)</td>
<td>0.01</td>
</tr>
<tr>
<td>5β-Pregnane-3β,20α-diol</td>
<td>0.01</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dehydroepiandrosterone (DHEA)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5α-dihydrotestosterone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
The scintillation counts for the known allopregnanolone standards were used to generate a standard curve from which the unknown sample concentrations of allopregnanolone were calculated. Each assay also contained additional tubes for the determination of total counts (TC), non-specific binding (NSB) and tracer-antisera binding (Bₒ).

2.8 STEROID IMMUNOASSAYS
Steroid immunoassays were performed to quantify progesterone and cortisol concentrations in samples of neonatal plasma, collected at the time of euthanasia. Salivary progesterone concentrations were also measured to determine postnatal levels of this steroid throughout the experimental period.

2.8.1 Salivary Progesterone Enzyme Immunoassay
Saliva samples were collected from neonatal animals prior to each feed (approximately 2 hourly) by placing a cotton bud into the mouth of each guinea pig, stimulating chewing and saliva secretion. Cotton buds were then centrifuged in microfuge tubes to collect saliva samples, which were then frozen and stored at -20°C until use.
Saliva samples were first thawed and briefly centrifuged to remove solid contaminants and mucins. Salivary progesterone concentrations were measured in competitive-binding assays, carried out according to the manufacturer's instructions, using Salivary Progesterone Enzyme Immunoassay (EIA) kits (Salimetrics LLC, State College, PA, USA). Unknown samples, standards and controls were incubated in microtitre plates in wells coated with rabbit anti-progesterone antibody, to which a solution containing progesterone linked to horseradish peroxidase (HRP) was also added. The concentration of progesterone in each sample determines how much of the labelled progesterone is able to bind to the
antibody binding sites. In order to quantify the amount of bound enzyme complex, an HRP substrate, tetramethylbenzidine was added to each well, forming a coloured reaction product. Sulfuric acid was then added to stop the reaction and the optical density of each well was read using a Fluostar Optima plate-reader (BMG Labtech, Ortenberg, Germany) at 450nm (corrected at 490nm). The resulting values were then analysed using GraphPad Prism software (version 4.0, Graphpad Software Inc., La Jolla, CA, USA) and unknown concentrations of progesterone calculated using results from the standard curve, with the amount of bound progesterone-HRP being inversely proportional to the concentration progesterone in each unknown sample. Samples were diluted in assay buffer prior to addition to each well and the dilution factor taken into account in the final calculations. Control samples were used to calculate assay variance, which is reported in each result chapters.

2.8.2 Plasma Progesterone & Cortisol Enzyme Immunoassays
Progesterone and cortisol concentrations in neonatal plasma were determined by immunoassay performed by Hunter Area Pathology Service (John Hunter Hospital, Newcastle, NSW, Australia). These assays were carried out using the UniCel Dxi 800 Access Immunoassay System (Beckman Coulter). Using this system, steroid concentrations were quantified by competitive binding assay. Co-efficients of variance are reported in the results chapters below.

2.9 PROTEIN WESTERN BLOT IMMUNODETECTION
The relative expression of proteins in tissue sample homogenates can be quantified using the process of Western blotting (Burnette 1981). Following the separation, based on size, of protein components by electrophoresis, proteins are transferred to a membrane on which
antibodies against specific protein antigens are used to label proteins of interest. These proteins are then detected using enzyme colourimetric reactions, the density of which can then be analysed to quantify relative protein abundance.

### 2.9.1 Protein Extraction

Frozen brain samples were crushed using dry ice and liquid nitrogen to prevent thawing and 50-80mg weighed into sterile tubes for protein extraction. A volume of 300-500μL (depending on mass of tissue sample) of RIPA protein extraction buffer (50mM Tris-HCl, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with Complete Protease Inhibitor and PhosStop Phosphatase Inhibitor Cocktails (Roche Diagnostics, Castle Hill, NSW, Australia) was then added to each tube. Samples were homogenised for 3 x 30sec (Ultra-Turrax T-25 homogeniser, IKA Labortecnik). Samples were incubated on ice for 30min followed by centrifugation at 13000 rpm for 10min. The pellet, containing unwanted cellular debris was discarded and aliquots of the supernatant frozen at -80°C.

### 2.9.2 Estimation of Protein Concentration

Following extraction, protein concentration for each sample was determined using a colourimetric assay based on the reaction between the protein, Cu²⁺ ions and bicinehoninic acid using the Peirce BCA Protein Assay Kit (Peirce, Rockford, IL, USA). Colour change for the reaction has a direct relationship to the concentration of protein in the sample. Samples were added in duplicate to a multisorb 96-well plate (NUNC, Roskilde, Denmark) and assayed according to manufacturers instructions, with samples diluted to fall within the range of the standard curve (2000-25μg/mL). Optical density for each sample and standards was read at 570nm using a Fluostar Optima plate-reader (BMG Labtech).
2.9.3 Electrophoresis

Unless otherwise stated, all reagents and apparatus used in this section were supplied by Invitrogen (Life Technologies, Mulgrave, Vic, Australia). Samples were prepared for electrophoresis with 25-50μg of protein per sample diluted in milliQ water as necessary, with NuPAGE 4xLDS sample buffer and 10x NuPAGE reducing agent followed by 10min incubation at 70°C. NuPAGE MOPS SDS running buffer was prepared prior to assembly of Xcell SureLock Mini-Cell system with NuPAGE Novex 12% Bis/Tris, 15 well, 1.0mm gels. Running buffer was then added to the inner chamber and samples pipetted, in duplicate, into the wells, along with 1uL of MagicMark XP protein standard and internal control samples. Purified human placental protein was used as an internal control for 5αR1 and crude guinea pig brain protein extract was used as a control for 5αR2. Electrophoresis was performed at 200V, 120mA and 25W for 55min, using a Zoom Dual Power Supply.

2.9.4 Western Blot Transfer

Following electrophoresis, Hybond-P PVDF membrane (GE Life Sciences) was activated in 100% methanol and transfer buffer (NuPAGE transfer buffer, 20% v/v methanol and milliQ water) for at least 10min prior to transfer. Filter paper and sponge pads were cut to size and soaked in transfer buffer immediately before use and assembled for transfer with PVDF membranes and gels. Transfer sandwiches were placed in Xcell II Blot modules (Invitrogen), the inner chamber filled with buffer and Western transfer performed at 30V, 160mA, and 17W for 45min.

2.9.5 Immunodetection

Following transfer, PVDF membranes were dried and blocked for 1hr at room temperature in BSA/skim-milk blocking solution (5%w/v BSA, 5% w/v skim-milk powder in TBS-T (25mM Tris-HCl, 15mM NaCl, 0.1% v/v
Membranes were then incubated overnight at 4°C blocking solution with the addition of appropriate antibodies against the proteins of interest (details below). Following primary antibody incubation, membranes were washed 4x5min in 0.1M TBS-T. Membranes were then incubated in secondary antibody and again washed prior to detection of the immunoreactive protein using ECL Western Blotting Detection kit (GE Healthcare) on the LAS-3000 Imaging System (Fuji Photo, Tokyo, Japan). The relative expression of the protein bands of interest, as determined by blocking peptide and IgG only controls were then measured using Multigauge v2.4 Software (Fuji Photo) and relative protein densities normalised to β-actin loading control and internal controls (see section 2.7.7).

2.9.5.a 5α-Reductase Types 1 and 2

The relative expression of 5αR1 (~26kDa band) and 5αR2 (~29kDa band), in protein extracts from the middle coronal brain section or hippocampus, was determined using goat polyclonal antibodies against 5αR1 (NB100-1491, 0.5mg/mL; Novus Biologicals, Littleton, CO, USA) or 5αR2 (Ab27469; AbCam, Cambridge, UK), diluted at 1:1000 in 5% BSA/5% skim-milk blocking solution. Membranes were then incubated in anti-goat secondary antibody conjugated to horseradish peroxidase (P0449; DakoCytomation, Glostrup, Denmark), diluted to 1:3000 in 3% skim-milk in TBS-T.

2.9.6 Western Blot Controls

Results were normalised to β-actin loading control and internal controls. β-actin was chosen as a control as other commonly used housekeeping loading control proteins such as GAPDH have been shown to be altered after hypoxial insult (Zhong and Simons 1999). In order to account for non-specific binding of the primary antibody, representative samples from
guinea pig placenta, hippocampus and adrenal gland protein extracts were treated with Normal Goat IgG (sc-2028; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) pre-immune serum, at a concentration of 1:1000 in 1% skim milk blotto made up in TBS-T. Secondary antibody only was also applied to a membrane with representative protein samples so as to control to non-specific binding of the secondary antibody.

2.10 BRAIN IMMUNOHISTOCHEMISTRY

Immunohistochemistry allows for the identification and location of the regional expression of different proteins within specific tissues. The \textit{in situ} identification of protein expression is particularly important within the different regions of the brain. The immunodetection of a number of proteins that are markers for different aspects of brain development, growth and injury was performed as part of the studies described here.

2.10.1 Fixation & Embedding

The middle coronal section and cerebellum of the left hemisphere of the fetal and neonatal brains were immersion fixed in neutral-buffered formalin for 24hrs, at 4°C on an orbital rocker. Sections were then stored at room temperature in 0.1M phosphate buffer (PB) with 0.05% sodium azide until ready for embedding. Brains were then sent for processing by Debbie Pepperall (Newcastle TAFE). Fixed sections dehydrated, paraffin-embedded and mounted.

2.10.2 Tissue Preparation

Paraffin-embedded blocks were cut into 8μm sections using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia), which were mounted on SuperFrost plus slides (Menzel-Glaser, Braunschweig, Germany) and allowed to air-dry. Mounted sections were
dewaxed in serial washes in xylene and ethanol. Prior to antigen retrieval, slides were finally incubated in methanol with 3% hydrogen peroxide (to inhibit endogenous peroxidases) and washed in 70% ethanol. Antigen retrieval was performed in Reveal It solution (ImmunoSolutions Pt Ltd, Australia) as per manufacturers’ instructions.

2.10.3 Immunodetection

Slides were rinsed in 0.1M phosphate buffered saline (PBS) before blocking in BSA Blocking Solution (0.5% w/v BSA, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1M PBS) for a period of 30 minutes at room temperature. Slides were then placed in slide mailers in which antibodies had been diluted in blocking solution (details below). The tertiary reagent (streptavidin-biotin-horseradish peroxidase complex (RPN1051V; Amersham)) used for immunodetection with all antibodies used, was diluted to a concentration of 1:300 in blocking solution (with 0.05% v/v thimerasol added instead of the sodium azide).

Two protocols with different incubation times were used in these studies. For the short protocol, primary antibody incubation occurred overnight, with secondary antibody and tertiary reagent incubated for 2hrs each prior to diaminobenzidine (DAB) chromagen development. In the longer protocol, incubation times were extended to 3 days, 5hrs and overnight for the primary, secondary and tertiary reagent, respectively. All incubations were carried out at room temperature on a continuous orbital rocker. Both protocols were used for the detection of proteins outlined below. Immunolabelling was revealed using 3,3’-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce) as a chromagen. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

Sections were mounted with DEPX (Merck, Kilsyth, Vic, Australia) and examined using a Zeiss Axioskop Microscope. Images were acquired using
a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Densitometry analysis was performed on these images using ImageJ 1.40 software (National Institutes of Health, Bethesda, MD, USA). The photomicrographs were converted into binary images by adjusting the threshold manually, with the percent area of coverage recorded for four fields of view per region on two sections per animal. For cell counts, the number of cells positive for the antibody of interest was also determined in four fields of view per region on two sections per animal.

2.10.3.a Myelin Basic Protein

Myelin basic protein (MBP) is a protein component of myelin membranes that are essential for neuronal signalling and an important part of brain development. MBP is associated with mature myelin and differentiated oligodendrocytes (Boggs 2006). A rat monoclonal anti-myelin basic protein antibody (M9434; Sigma-Aldrich) at a dilution of 1:4000 was used to detect MBP. Biotinylated anti-rat IgG (B7139; Sigma-Aldrich) at 1:300 was used as the secondary antibody for MBP immunodetection. The density of immunostaining of MBP in guinea pig brains was examined in the hippocampal CA1 subcortical white matter regions. In addition, the percent density of MBP staining in the cerebellum, in lobules X and VIII, was also measured.

2.10.3.b Glial Fibrillary Acidic Protein

Glial fibrillary acidic protein (GFAP) is expressed in and used as a marker of reactive or proliferating astrocytes. Astrocytes are important support cells that help to maintain the blood brain barrier and also form gliotic scars in response to injury (Eng, Ghirnikar et al. 2000). Immunodetection of GFAP was performed using a mouse monoclonal anti-glial fibrillary acidic protein primary antibody (1:4000 dilution; G3893, Sigma-Aldrich) and a biotinylated secondary polyclonal rabbit anti-mouse antibody (E0354; Dako) at 1:300 dilution. GFAP staining density was analysed in the
hippocampal dentate gyrus and CA1 regions, along with the sub-cortical white matter region of fetal and neonatal guinea pig brains.

2.10.3.c Microtubule-Associated Protein 2

Microtubule-associated protein type 2 (MAP-2) is a neuronal structural protein that has roles in neuronal growth, signalling and plasticity (Johnson and Jope 2004). MAP-2 expression was determined in the cerebral cortex, adjacent to the pial surface and in the hippocampal CA1 region using a mouse monoclonal anti-MAP-2 primary antibody (1:30000 dilution; M9942, Sigma-Aldrich) and biotinylated secondary anti-mouse antibody (E0354; Dako) at 1:300 dilution.

2.10.3.d Activated Caspase-3

Caspase-3 is a member of the cysteine-aspartic acid protease family of proteins that is involved in the execution phase of the apoptosis cascade (Wilson 1998). The cleavage of caspase-3 to form its active form is a marker of cell death. Staining for activated caspase-3 was performed using a rabbit polyclonal antibody (anti-human/mouse caspase-3 active, 1:3000 (AF835; R&D Systems, Minneapolis, MN, USA)) and anti-rabbit IgG (1:300; BA1000, Vector Labs, CA, USA) secondary antibody. Cells positive for activated caspase-3 staining and that showed apoptotic morphology were counted as terminal apoptotic cells. Apoptotic cells were counted in the CA1 region of the hippocampus.

2.10.3.e Calbindin

Calbindin is protein marker that can be used to identify Purkinje cells (Legrand, Thomasset et al. 1983). Immunostaining of calbindin was carried out using a monoclonal mouse antibody (Calbindin [CL-300], ab 9481, AbCam) diluted at 1:1000, with anti-mouse biotinylated secondary antibody (RPN1001, Amersham). Purkinje cells in mid-sagittal cerebellar
sections were stained for calbindin for morphometric analysis and cell counting in lobules VIII and X. Purkinje cells were counted manually and length of Purkinje cell layer measured using ImageJ software (photomicrographs at 20x magnification taken from serial sections) to determine overall number of Purkinje cells and the density of packing of cells within the Purkinje cell layer. Purkinje cell soma area and perimeters were also calculated for a subset of animals using ImageJ software analysis on serial photomicrographs at 40x magnification.

2.11 STATISTICAL ANALYSES
Statistical analyses were carried out using PASW Statistics Package v18.0 for Mac (SPSS Inc., Chicago, IL, USA) and Prism v4.0b for MacOSX (GraphPad Software Inc., La Jolla, CA, USA). Data were analysed firstly by treatment/age separated by neonatal sex and then by treatment only if no effect was identified. Individual subjects were from separate litters, except where a male and female animal were used from a single litter. Data is presented as group mean ± SEM unless indicated otherwise. For normally distributed data with two categorical predictors, data was analysed by two-way ANOVA with Bonferroni post-hoc comparison between groups. Where a single variable was present for more than two treatment groups, data was analyzed by one-way ANOVA with Bonferroni post-hoc analysis. Multiple samples over time were analysed by repeated measure two-way ANOVA. Results comparing two treatment groups with a single variable were analysed by Student’s T-test. Analysis of survival data was performed using Fisher’s exact test contingency table. Data that exhibited unequal variance (Bartlett’s test for equal variance p<0.05) were log or square root transformed so that assumptions of normality were met. Data that was not normally distributed was analyzed by Kruskal-Wallis non-parametric test with post-hoc Dunn’s multiple comparison. In cases with more than two
variables, where multiple comparisons were necessary, multiple regression analysis was performed. Models were produced that compared the measured, continuous outcome variables against the categorical predictors. Two-way interactions were tested and a two-tail significance level of 5% was used throughout. The dependent variables were transformed as required in order to satisfy model assumptions. Sub-group analysis of means was carried out using two-tailed Students’ T-test where appropriate. The limit of statistical significance was set at p<0.05 and is indicated in figure legends and on graphs by asterisks (*p<0.05, **p<0.01 and ***p<0.001) where appropriate.
Chapter Three

SEX-SPECIFIC EFFECT OF INHIBITION OF NEUROSTEROID SYNTHESIS AND INTRA-UTERINE GROWTH RESTRICTION ON FETAL GUINEA PIG BRAIN DEVELOPMENT

The studies presented in this chapter examine the effects of reduced neuroactive steroid concentrations and the pregnancy complication, IUGR, on fetal brain development and the neuroactive steroid system in the late gestation guinea pig. This chapter has been published in the Journal of Endocrinology (2011; 208(3):301-309). For consistency, it is presented with minor changes to the original manuscript.

3.1 ABSTRACT

Progesterone and its neuroactive metabolite, allopregnanolone, are present in high concentrations during pregnancy, but drop significantly following birth. Allopregnanolone influences fetal arousal and enhances cognitive and behavioural recovery following traumatic brain injury. Inhibition of allopregnanolone synthesis increases cell death in fetal animal brains with experimental hypoxia. We hypothesised that complications during pregnancy, such as premature removal of placental steroids with preterm birth and intra-uterine growth restriction (IUGR) would disrupt the fetal
neurosteroid system, contributing to poor neurodevelopmental outcomes. The present study aimed to investigate the effects of chronic inhibition of allopregnanolone synthesis before term and IUGR on developmental processes in the fetal brain.

Guinea pig fetuses were experimentally growth restricted at mid-gestation and treated with finasteride, an inhibitor of allopregnanolone synthesis. Finasteride treatment reduced fetal brain allopregnanolone concentrations by up to 75% and was associated with a reduction in MBP (p=0.001) and an increase in GFAP expression in the sub-cortical white matter brain region (p<0.001). IUGR resulted in decreased MBP expression (p<0.01) and was associated with a reduction in the expression of steroidogenic enzyme 5αR2 in the fetal brain (p=0.061). Brain levels of 5αR1 were higher in male fetuses (p=0.008).

Both IUGR and reduced fetal brain concentrations of allopregnanolone were associated with altered expression of myelination and glial cell markers within the developing fetal brain. The potential role of neurosteroids in protecting and regulating neurodevelopmental processes in the fetal brain may provide new directions for the treatment of neurodevelopmental disorders in infants that are exposed to perinatal insults and pathologies.

3.2 INTRODUCTION

Fetuses that are born small for gestational age due to intrauterine growth restriction are at higher risk for perinatal morbidity, mortality and long-term disability (Larroque, Bertrais et al. 2001). Abnormal fetal growth is associated with a high risk of fetal brain injury leading to postnatal motor disorders, neurodevelopmental delay and long-term cognitive impairments. The risk of preterm birth is also higher in infants that are growth restricted or small for gestational age (Lackman, Capewell et al. 2001). Placental
insufficiency and IUGR have many implications for fetal brain development. Along with clinical observations, animal studies have also revealed the morphological changes and neurological impairments associated with fetal IUGR. In fetal sheep, experimental chronic placental insufficiency late in gestation impairs neural development and results in white matter damage and a reduction in the density of pyramidal cells within the hippocampus (Rees, Mallard et al. 1998). In pregnant guinea pigs, IUGR induced by experimentally limiting uterine blood flow to the placenta results in reduced hippocampal volume and fewer hippocampal pyramidal neurons in the fetal brain at the end of gestation (Mallard, Loeliger et al. 2000).

Progesterone is present in high concentrations throughout pregnancy, and plasma concentrations increase in both the maternal and fetal circulations during late gestation in humans, non-human primates and guinea pigs (Gilbert Evans, Ross et al. 2005; Mitchell and Taggart 2009). Levels of allopregnanolone, a major metabolite of progesterone, increase concurrently with progesterone over the course of gestation in both tissues and plasma (Gilbert Evans, Ross et al. 2005). In the brain, allopregnanolone has positive modulatory actions at the γ-aminobutyric acid type A (GABA_A) receptor, causing neuronal hyperpolarisation and inhibition of neural activity, resulting in anxiolytic (de Brito Faturi 2006), sedative (Paul and Purdy 1992) and anticonvulsant (Kokate, Banks et al. 1999) effects. Allopregnanolone has also been shown to influence fetal arousal and sleep-wake activity in utero (Nicol, Hirst et al. 1997), demonstrating that synthesis and release of this steroid has a physiological and regulatory role within the central nervous system (CNS).

Allopregnanolone is converted from progesterone by the enzymes, 5α-reductase types 1 and 2 (5αR1; 5αR2) and 3α-hydroxysteroid oxidoreductase (3α-HSOR) (Compagnone and Mellon 2000). These
Enzymes are present in the adrenal gland, ovary, brain, and the placenta. ‘De novo’ synthesis of allopregnanolone from cholesterol wholly within the CNS is also possible (Poletti, Coscarello et al. 1998). Endogenous neurosteroids, such as allopregnanolone have been implicated in the regulation of normal brain development and may be particularly important to the fetal brain exposed to sub-optimal or adverse conditions in utero, including pregnancies complicated by placental insufficiency and IUGR. For example, in a fetal sheep model of chronic placental insufficiency, expression of 5αR2 increased in many regions of the fetal brain following late-gestation umbilicoplacental embolisation, a procedure that reduces the area of the placental vasculature available for nutrient exchange (Nguyen, Billiards et al. 2003).

There is increasing evidence that allopregnanolone has a neuroprotective function in both the adult (Djebaili, Hoffman et al. 2004; He, Hoffman et al. 2004; Schumacher, Guennoun et al. 2007) and fetal brain (Yawno, Yan et al. 2007; Yawno, Hirst et al. 2009). Inhibition of allopregnanolone synthesis, using finasteride (a 5αR inhibitor) in fetal sheep increased the number of cells undergoing programmed cell death within the brain, and accentuated the cell death caused by asphyxia arising from transient umbilical cord occlusion (Yawno, Yan et al. 2007). Inhibition of 5αR also appears to influence cell proliferation in the fetal sheep brain, with an increase in proliferating astrocytes observed in the hippocampus and cerebellum after finasteride treatment (Yawno, Hirst et al. 2009).

We have previously shown that 5αR1 and 5αR2 mRNA levels are altered within the fetal guinea pig brain in a sex-specific manner in response to uterine artery ablation, a procedure that produces IUGR in this species (McKendry, Palliser et al. 2010). This disruption of the neurosteroid synthesis in pregnancies with perturbed placental function may result in vulnerability to fetal brain injury. In human pregnancies, IUGR often
occurs in association with preterm birth (Lackman, Capewell et al. 2001), and prematurity is itself a risk factor for perinatal brain damage (Saigal and Doyle 2008). It is also known that fetal sex affects susceptibility to brain injury, with perinatal brain injury occurring more frequently in male infants (Di Renzo, Rosati et al. 2007). A reduction in fetal allopregnanolone concentrations in late gestation may mimic the removal of progesterone and neurosteroid support with birth, including premature birth. We hypothesised that complications during pregnancy, such as premature birth or IUGR would disrupt the fetal neurosteroid system, contributing to poor neurodevelopmental outcomes in fetuses and neonates with these pregnancy complications. The present study aimed to investigate the effects of IUGR, the chronic inhibition of allopregnanolone synthesis and the combination of these insults on late developmental processes within the fetal brain.

3.3 MATERIALS AND METHODS

3.3.1 Animals
All animal procedures were approved by the University of Newcastle Animal Care and Ethics Committee and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Time-mated outbred tricolour guinea pigs, supplied by the University of Newcastle Research Support Unit, were housed indoors under a 12hr light/dark cycle. Animals were provided with commercial guinea pig pellets and water fortified with ascorbic acid ad libitum.

Surgery was performed under strict aseptic conditions between 33 and 35 days gestation (term is approx. 70 days) to induce placental insufficiency and fetal IUGR. Food was removed 4hrs prior to surgery and the pregnant guinea pig dams then received 0.5mg/kg Temgesic® (324μg
Buprenorphine HCl and Glucose anhydrous/mL, Reckitt Benckiser Healthcare Ltd., Hull, UK) prior to induction of general anaesthesia with 4% isoflurane in oxygen and maintenance of anaesthesia with 2% isoflurane in oxygen. A midline abdominal incision was made, the uterus exposed and approximately 50% of the radial arteries supplying each placenta were ablated using diathermy (Turner and Trudinger 2009). The uterus was then replaced and the incision closed. Animals were returned to individual cages and received a second dose of Temgesic at 8hrs postsurgery. A control group of animals received sham surgery in which the same procedures were followed but no ablation of radial arteries was performed.

Animals were divided into groups based on surgery (sham/normally grown or IUGR) and daily drug treatment (vehicle or finasteride). This resulted in four fetal groups: Control (sham, vehicle treated), IUGR (growth restricted, vehicle treated), FIN (sham, finasteride treated), IUGR + FIN (growth restricted, finasteride treated). The pregnant dams received daily subcutaneous injections of vehicle (600μL/kg; 16% v/v ethanol in peanut oil) or finasteride (25mg/kg; Steraloids, New York, NY, USA) from day 55 until they were euthanised at 65 days of gestation by carbon dioxide inhalation. Term in this colony of guinea pigs is 71 ± 0.5 days.

3.3.2 Tissue Collection

Fetuses were removed from the uterus and fetal sex, body weight and organ weights recorded. The fetal brain was removed from the skull within 2-3 minutes of maternal death before being weighed, hemisected and divided coronally into rostral, middle and caudal blocks. Blocks from the right hemisphere were snap frozen in liquid nitrogen and stored at -80°C before being finely crushed for protein and steroid extraction. Tissues from the left hemisphere were fixed by immersion in PFA (4% w/v
paraformaldehyde in 0.1M phosphate buffer). The blocks used in this study (middle block) contained cerebral cortex, sub-cortical white matter, corpus callosum, thalamus and the hippocampus.

3.3.3 Radio-Immunoassay
Allopregnanolone was extracted from brain tissue and measured by radioimmunoassay using previously described methods (McKendry, Palliser et al. 2010). Briefly, crushed frozen brain tissue was treated with 50% methanol with 1% acetic acid and added to Sep-Pak C18 cartridges (Waters, Milford, MA, USA) for separation of steroid components using methanol gradients. Residual methanol was removed by vacuum drying. In order to minimise cross-reactivity, the concentration of progesterone was reduced by treating samples with potassium permanganate to oxidise non-saturated steroids. Recovery was measured by the addition of tritium-labelled allopregnanolone (1000-1500cpm, 5α-[9, 11, 12, 3H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to each sample prior to extraction. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. The average recovery was 57.8 ± 2.6%. Allopregnanolone was quantified by radioimmunoassay using a polyclonal antibody supplied by Dr R. H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA). The limit of detection for allopregnanolone was 35.0 ± 2.5 pg/tube. Intra-assay coefficient was 10.9%.

3.3.4 Western Blot Analysis
The protein expression of the neurosteroidogenic enzymes, 5αR1 and 5αR2 were determined by Western blot analyses. Brain tissue was homogenised in RIPA protein extraction buffer (50mM Tris HCl, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with
Complete Protease Inhibitor Cocktail (Roche Diagnostics Australia Pty. Ltd. Castle Hill, NSW, Australia) and PhosStop Phosphatase Inhibitor Cocktail Protein (Roche Diagnostics). The protein concentration of each sample was then determined using BCA \textsuperscript{TM} Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (20μg total protein) were separated by electrophoresis on precast NuPAGE Novex 12% Bis/Tris gels (Invitrogen, Mt Waverley, Australia) before transfer to Hybond-P PVDF membrane (GE Healthcare Sydney, NSW, Australia). Membranes were blocked in BSA Blocking Solution (5% w/v bovine serum albumin, 5% w/v skim milk in 1xTBS-T (25mM Tris-HCl, 15mM NaCl, 0.1% v/v Tween-20) for 1hr before incubation overnight in primary antibody. Goat polyclonal antibody against 5αR1 (NB100-1491; Novus Biologicals, Littleton, CO, USA) and goat polyclonal antibody to 5αR2 (Ab27469; AbCam, Cambridge, UK) were used at a dilution of 1:1000. The immunoreactive protein was detected using the ECL Western Blotting Detection Kit (GE Healthcare) and LAS-3000 Imaging System (Fuji Photo Film, Japan) following incubation with anti-goat secondary antibody conjugated to horseradish peroxidase (P0449; DakoCytomation, Glostrup, Denmark) at 1:3000 dilution for 1hr at room temperature. The relative amounts of 5αR1 (~26kDa) and 5αR2 (~29kDa) protein was quantified using Multigauge v2.4 software (Fuji Photo Film, Tokyo, Japan), adjusted to β-actin loading Control (ab8227-50; AbCam, Cambridge, UK) and an internal control sample (human placental protein extract) present on each gel. Control membranes in which the 5αR1 primary antibody was omitted and where the primary antibody was pre-incubated with the blocking peptide (NB100-1491PEP, Novus Biologicals) confirmed the specificity of the 26kDa band. For 5αR2, control membranes in which the primary antibody was omitted and replaced by goat IgG (sc-2028, Santa Cruz
Biotechnology, Santa Cruz, CA, USA) confirmed specificity of the 29kDa band.

3.3.5 Immunohistochemistry

Eight-micron (8μm) coronal sections were cut from paraffin-embedded brain tissue blocks using a rotary microtome. Sections were dewaxed in xylene and rehydrated through a graded series of ethanol/water washes. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in methanol. Antigen recovery was performed with Reveal It Solution (ImmunoSolution Pty Ltd, Australia) as per manufacturer instructions, before being blocked with BSA in PBS (0.1M phosphate-buffered saline pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin, 0.05% v/v sodium azide). Sections were then incubated in 1:5000 dilution of either GFAP (monoclonal anti-glial fibrillary acidic protein antibody (G3893; Sigma-Aldrich)), MBP (monoclonal anti-myelin basic protein antibody (M9434; Sigma-Aldrich)) or activated caspase-3 (anti-h/m caspase-3 active (AF835; R&D Systems, Minneapolis, MN, USA)) primary antibodies for 3 days. Sections were washed in PBS, followed by incubation in the appropriate biotinylated secondary antibodies: polyclonal rabbit anti-mouse (E0354; DakoCytomation, Glostrup, Denmark), anti-rat IgG (B7139; Sigma-Aldrich) and anti-rabbit IgG (BA1000; Vector Labs, CA, USA), respectively. Sections were then washed and incubated overnight in streptavidin-biotin-horseradish peroxidase complex (RPN1051V; Amersham). Labeling was revealed using 3,3’-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce) as a chromagen. Sections were mounted with DEPX (Merck, Kilsyth, Vic, Australia) and examined using a Zeiss Axioskop Microscope. Images were acquired using a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). GFAP, MBP and caspase-3 immunostaining were
examined in subcortical white matter and hippocampal CA1; additionally, GFAP stained sections from the dentate region of the hippocampus were also analysed. The GFAP 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, with the percent area of coverage recorded for four fields of view per region on two sections per animal. Cells positive for activated caspase-3 that showed apoptotic morphology were counted in four fields of view per region (cortex and hippocampal CA1) on two sections per animal. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

3.3.6 Statistical Analyses
Statistical analysis was carried out using PASW Statistics Package v18.0 for Mac (SPSS Inc., Chicago, IL, USA). Multiple regression was used to model measured, continuous, outcome variables against the three categorical predictors; finasteride, IUGR and sex, using backwards stepwise mode. Two-way interactions were tested. A two-tail significance level of 5% was used throughout. The dependent variables were transformed as required in order to satisfy model assumptions. Sub-group analysis of means was carried out using two-tailed Students’ T-test where appropriate. Significance level of p<0.05 are represented with asterisks’ on Figures 3.1-3.4, where applicable.

3.4 RESULTS
3.4.1 Fetal Characteristics
The mean body and organ weights for fetal guinea pigs are shown in Table 3.1. A brain to liver weight ratio (BLR) of greater than 90% was used to
define asymmetric growth restriction in fetuses that received IUGR surgery. Animals that received sham surgeries had BLRs of approximately 55%, both with and without finasteride treatment. Regression analyses found that fetal guinea pigs with IUGR had significantly reduced placenta weight (p<0.001), liver weight (p<0.001) and higher BLRs (p<0.001). Finasteride treatment was not associated with fetal body weight change. Following adjustment for other variables, fetal body weight was significantly reduced in IUGR fetuses (p<0.001), with the overall body weight of female fetuses, across all treatment groups, reduced in comparison to male animals (p=0.040). Mean fetal brain weight was significantly reduced in IUGR fetuses (p=0.005) and a marginally significant reduction in brain weights was observed in female fetuses compared with that in males (p=0.051). Finasteride treatment alone was not associated with fetal body weight change. Interestingly, however, there was a significant positive interaction effect (p=0.020) between finasteride treatment and IUGR.

3.4.2 Fetal Brain Allopregnanolone Concentrations

Figure 3.1 presents the mean fetal brain concentrations of allopregnanolone with and without IUGR and finasteride treatment. Control animals (sham + vehicle) had average brain allopregnanolone of 11.63 ±2.33 ng/g, which was reduced by up to 75% in growth restricted animals with finasteride treatment to 3.09 ±0.57 ng/g. Finasteride treatment in normally grown animals reduced allopregnanolone concentrations to 4.20 ±0.51 ng/g. IUGR, vehicle treated fetuses, had a mean brain concentration of allopregnanolone of 7.72 ±1.43 ng/g. In regression modelling of the fetal brain allopregnanolone concentrations, a highly significant reduction was identified, associated with finasteride
treatment (p=0.001). IUGR and fetal sex (not shown) were not found to have a significant effect on fetal brain concentrations of allopregnanolone.

**Figure 3.1 - Fetal Brain Allopregnanolone Concentrations**

Finasteride treatment reduces brain concentrations of allopregnanolone in male and female fetal guinea pigs with and without IUGR. Relative expression measured by radio-immunoassay and expressed as ng/g of wet weight tissue; n= 4-6. * P<0.05 compared to controls of same sex. Statistical analysis by one-way ANOVA followed by LSD post hoc testing for multiple comparisons. No differences between sexes (within treatment groups) were present as determined by Student T-test.

**Table 3.1 - Fetal Animal Body and Organ Weights**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body Wt</th>
<th>Placenta Wgt</th>
<th>Brain Wgt</th>
<th>Liver Wgt</th>
<th>BLR</th>
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<tr>
<td>Female</td>
<td>6</td>
<td>85.9 ± 3.3</td>
<td>6.3 ± 0.2</td>
<td>2.3 ± 0.04</td>
<td>4.1 ± 0.3</td>
<td>0.57 ± 0.04</td>
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<tr>
<td>Male</td>
<td>9</td>
<td>91.1 ± 2.6</td>
<td>6.0 ± 0.3</td>
<td>2.4 ± 0.02</td>
<td>4.6 ± 0.2</td>
<td>0.54 ± 0.03</td>
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<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>89.0 ± 2.1</td>
<td>6.1 ± 0.2</td>
<td>2.4 ± 0.03</td>
<td>4.5 ± 0.2</td>
<td>0.55 ± 0.03</td>
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<td></td>
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<tr>
<td>Female</td>
<td>8</td>
<td>57.9 ± 4.2</td>
<td>4.3 ± 0.7</td>
<td>2.2 ± 0.03</td>
<td>2.5 ± 0.3</td>
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<tr>
<td>Male</td>
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<td>59.5 ± 5.3</td>
<td>4.4 ± 0.5</td>
<td>2.3 ± 0.08</td>
<td>2.5 ± 0.3</td>
<td>1.03 ± 0.17</td>
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<td><strong>Total</strong></td>
<td>16</td>
<td>58.7 ± 3.4</td>
<td>4.3 ± 0.4</td>
<td>2.3 ± 0.04</td>
<td>2.5 ± 0.2</td>
<td>1.04 ± 0.13</td>
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<td><strong>Fin</strong></td>
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<tr>
<td>Female</td>
<td>4</td>
<td>84.3 ± 7.0</td>
<td>5.0 ± 0.3</td>
<td>2.3 ± 0.03</td>
<td>4.1 ± 0.6</td>
<td>0.60 ± 0.08</td>
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<td>Male</td>
<td>7</td>
<td>90.4 ± 3.1</td>
<td>6.2 ± 0.5</td>
<td>2.3 ± 0.04</td>
<td>4.6 ± 0.4</td>
<td>0.52 ± 0.05</td>
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<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td>87.4 ± 3.0</td>
<td>5.8 ± 0.4</td>
<td>2.3 ± 0.03</td>
<td>4.4 ± 0.3</td>
<td>0.56 ± 0.04</td>
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<tr>
<td><strong>IUGR + Fin</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>55.4 ± 3.5</td>
<td>3.2 ± 0.5</td>
<td>2.0 ± 0.29</td>
<td>2.4 ± 0.1</td>
<td>0.95 ± 0.08</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>68.0 ± 6.4</td>
<td>4.1 ± 0.3</td>
<td>2.4 ± 0.08</td>
<td>2.8 ± 0.2</td>
<td>0.86 ± 0.04</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
<td>57.1 ± 2.4</td>
<td>3.6 ± 0.2</td>
<td>2.2 ± 0.12</td>
<td>2.5 ± 0.1</td>
<td>0.93 ± 0.05</td>
</tr>
</tbody>
</table>
3.4.3 Brain $5\alpha$-Reductase Enzyme Expression

The mean abundances of $5\alpha$R1 and $5\alpha$R2 enzymes, expressed relative to internal control and $\beta$-actin loading control, are shown in Figures 3.2C and D, respectively. Representative Western blots for both isoforms (Figures 3.2A and B) with results for negative controls shown for blots incubated in the presence of $5\alpha$R1 antibody blocking peptide and anti-goat pre-immune serum for $5\alpha$R2, demonstrating specificity of antibody staining. When regression analysis was carried out, fetal sex was significantly associated with the expression of the $5\alpha$R1 enzyme isoform ($p=0.008$), with female fetuses having lower expression than males. Finasteride and IUGR had no significant effect on $5\alpha$R1 expression. IUGR was marginally associated with a reduction in the expression of $5\alpha$R2 in the fetal brain ($p=0.060$, Figure 3.2B & D). Finasteride and fetal sex were not associated with changes in $5\alpha$R2 expression.

3.4.4 GFAP Expression

Figures 3.3A and C, show GFAP immunostaining in the subcortical white matter in male and female fetuses, respectively, with fetuses that received sham or IUGR surgery and subsequently administered with vehicle or finasteride. The greatest density of GFAP staining appeared to be present in finasteride treated IUGR male fetuses and finasteride treated females (Fig 3.3A(i) and 3.3C(i)). Animals that were not administered finasteride and received either IUGR or sham surgery showed no qualitative increase in GFAP staining in this region. The mean densities of GFAP staining, by proportion of area coverage, for treatment groups with sham and IUGR surgery and vehicle and finasteride treatments are shown in Figures 3.3E. Data for the dentate region is not shown.
Figure 3.2 - Fetal Brain 5α-Reductase Enzyme Expression

Representative Western blots (A: 5αR1 26kDa band and B: 5αR2 29kDa band) showing two paired guinea pig brain tissue samples and human placenta internal control (20 μg of total protein per lane) and corresponding β-actin loading controls. Relative protein expression of 5α-reductase type 1 (C: 5αR1) and 2 (D: 5αR2) was determined in control (sham) and IUGR male and female fetal guinea pig brains following vehicle (open bars) and finasteride treatment (black bars). Preincubation of primary 5αR1 antibody with blocking peptide blocked specific binding at 26kDa in pooled guinea pig brain sample. Goat normal IgG showed no binding at 29kDa in pooled guinea pig brain sample.
Regression analysis of GFAP coverage showed that in male and female fetuses, finasteride treatment was associated with a significant increase in GFAP expression in the sub-cortical white matter (p<0.001) and dentate (p=0.009). No significant interactions with or effects of IUGR or sex were observed in these regions. This was also confirmed by sub-group analysis of means which indicated that in both sham and IUGR sub-groups finasteride treated fetuses had a significant increase in GFAP expression (Figure 3.3E, p<0.05).

In the CA1 region of the hippocampus male fetuses with finasteride treatment (Figure 3.3B(iv)) and female IUGR fetuses with finasteride treatment (Figure 3.3D(iii)) appeared to have the highest degrees of GFAP expression. As an effect of fetal sex was identified (see regression model below), Figure 3.3F presents the mean proportion of GFAP staining in the CA1 region divided by treatment groups and fetal sex. Regression analysis of density of GFAP staining in the hippocampal CA1 region found that IUGR was not associated with a change in GFAP expression. A marginally significant increase in overall GFAP expression in this region was observed in female guinea pigs compared to males (p=0.051) and whilst finasteride treatment alone did not have a significant effect on GFAP in the CA1 (p=0.625), there was a significant positive interaction between finasteride treatment and sex (p=0.021). A sub-group analysis of means indicated that in the CA1 region, both male and female IUGR fetuses significantly increased GFAP expression compared to IUGR vehicle fetuses of the same sex (Figure 3.3F, p<0.05).

### 3.4.5 MBP Expression

In both the sub-cortical white matter (Figure 3.4A and C) and CA1 region of the hippocampus (Figure 3.4B and D), MBP expression was highest in control male and control female fetuses. There appeared to be a large
Figure 3.3 - GFAP Immunostaining in the Fetal Guinea Pig Brain

Glial Fibrillary Acidic Protein (GFAP) immunostaining in the sub-cortical white matter (A, C, E) and the hippocampal CA1 region (B, D, F). Representative micrographs show staining in male (A, B) and female (C, D) fetal guinea pig brain regions. Fetuses were normally grown and vehicle treated (i), growth restricted and vehicle treated (ii), normally grown and finasteride treated (iii) or growth restricted and finasteride treated (iv). Density of staining was measured and is expressed as percentage coverage of total area (E, F) for vehicle (open bars) and finasteride treated (black bars) animals in sham and IUGR groups. Asterisks indicate significant differences between groups (p<0.05, E: n=10-12; F: n=6-7 males, n=5-6 female fetuses). Horizontal scale bars represent 0.1mm.
reduction in MBP immunostaining in animals that were both growth restricted and received finasteride treatment. The mean per-cent coverage of MBP for each treatment group is presented in Figures 3.4E and F. Multiple regression analysis identified a significant reduction in MBP expression in the sub-cortical white matter associated with finasteride treatment (p=0.001) and IUGR (p=0.003). A significant positive interaction between finasteride and IUGR was also identified (p=0.044). Additional sub-group analysis of means showed a significant reduction in MBP expression between sham vehicle and finasteride treated fetuses (Figure 3.4E, p<0.05) but not in IUGR fetuses with the different drug treatments, indicating a mediation of the effect of finasteride in the presence of IUGR. Fetal sex did not affect MBP expression in this region. Multiple regression identified an association between IUGR and reduced MBP expression in the CA1 region of the hippocampus (p<0.001). The different pattern of expression of vehicle and finasteride animals between the sham and IUGR groups (Figure 3.4F) suggests an interplay between, finasteride treatment and IUGR. The regression model identified a significant interaction effect on the expression of MBP in the CA1 in the presence of both treatments (p=0.016), despite finasteride treatment alone not having a significant effect (p=0.116). Fetal sex did not have a significant effect on MBP expression in this region. Sub-group analysis identified a significant difference in the means of IUGR and sham animals (Figure 3.4F, p<0.05), but no significant effect of finasteride treatment within sham or IUGR sub-groups.

3.4.6 Activated Caspase-3 Expression

There was no significant effect of finasteride treatment, IUGR or fetal sex on the number of activated caspase-3 positive cells in the CA1 region of the hippocampus of the fetal guinea pigs in this study (data not shown).
Figure 3.4 - MBP Immunostaining in the Fetal Guinea Pig Brain

Myelin Basic Protein (MBP) immunostaining in the sub-cortical white matter (A, C, E) and the hippocampal CA1 region (B, D, F). Representative micrographs show staining in male (A, B) and female (C, D) fetal guinea pig brain regions. Fetuses were normally grown and vehicle treated (i), growth restricted and vehicle treated (ii), normally grown and finasteride treated (iii) or growth restricted and finasteride treated (iv). Density of staining was measured and is expressed as percentage coverage of total area (E, F) for vehicle (open bars) and finasteride treated (black bars) animals in sham and IUGR groups. Asterisks indicate significant differences between groups (p<0.05, n=10-12). Horizontal scale bars represent 0.1mm.
3.5 DISCUSSION

The neurosteroid, allopregnanolone has many associated neuroprotective, developmental and regulatory effects. This study examined late gestation brain development in fetal guinea pigs with the chronic gestational insult, IUGR, in the presence and absence of high late gestation allopregnanolone concentrations. The current model involves chronic placental insufficiency resulting in significantly lower birth weights, with relative brain sparing, typical of IUGR pregnancies. IUGR can be thought of as a chronic adaptation in the fetus that becomes maladaptive, potentially disrupting the neurodevelopmental actions of neurosteroids and leading to strong associations between IUGR, poor neonatal outcome and perinatal brain injury.

In the present study, the chronic administration of finasteride during late gestation was successful in markedly reducing allopregnanolone concentrations in the fetal guinea pig brain. It has been proposed that this late gestation reduction in fetal brain allopregnanolone may mimic the change in brain neurosteroid concentrations that occurs when a fetus is born preterm and the placenta is prematurely removed as a major source of progesterone as a precursor of allopregnanolone (Hirst, Yawno et al. 2006). This suggests that the ability of the preterm infant to synthesise important neurosteroids independently of placentally-derived precursors may be limited and the effect of this decline in endogenous steroids on preterm *ex utero* brain development may influence the vulnerability of the preterm neonatal brain to injury.

In the present study, IUGR did not affect fetal brain concentrations of allopregnanolone. Male fetuses did, however, have higher levels of expression of 5αR1 than female fetuses. This may be related to the action
of $5\alpha R2$ in androgen and testosterone synthesis as, along with allopregnanolone, $5\alpha R$-action is also involved in the synthetic pathways of other steroid hormones. IUGR fetuses had a marginal reduction in the type 2 $5\alpha R$ isoform of the enzyme. A previous study carried out by members of our laboratory showed an upregulation of $5\alpha$-reductase type 2 in the fetal sheep brain following late gestation chronic umbilicoplacental embolisation (Nguyen, Billiards et al. 2003). This may be explained by the different types of chronic insults in the two models. Both models showed no change in brain allopregnanolone concentrations despite the changes noted in enzyme expression. Local reductions in allopregnanolone may have been present at the initiation of the chronic-type insults, however, may not persist to the end of gestation. Acute or transient hypoxic insults during pregnancy have been shown to cause an increase in fetal brain concentrations of allopregnanolone, for a period immediately following the insult (Nguyen, Yan et al. 2004). This raises the relative importance of the placenta and other glandular sources for the supply of allopregnanolone and its precursors and the ability for this system to compensate for chronic disruptions of supply, particularly when, as often occurs with IUGR, placental growth or function is compromised. The finding of reduced $5\alpha R2$ expression in the brain of growth-restricted fetuses suggests that IUGR may further limit their neurosteroid synthetic capacity following birth. Such a disruption of neurosteroid synthesis or supply potentially predisposes the vulnerability of the IUGR fetus to neurodevelopmental disorders. The relative importance or otherwise of the various sources of neurosteroids is still unclear and the elucidation of these mechanisms may be useful for the understanding of neurosteroid function in both normal pregnancies and when chronic pathological changes are present.
A key finding of this study is the effect of finasteride treatment and the resultant low concentrations of allopregnanolone on myelination. Correct myelination is essential for the conduction of brain signals and disruption of brain myelination processes during development can lead to lasting neurological, cognitive and motor effects. In the sub-cortical white matter region, finasteride was associated with significantly reduced myelination and in addition, a significant interaction was also identified between finasteride treatment and IUGR in both regions examined. This interaction indicates that the effect of chronically low fetal brain allopregnanolone concentrations on myelination is altered in fetuses with a chronic perturbation of growth. This is suggestive of a protective mechanism acting in the presence of chronic IUGR, however the mechanism is unclear. These processes may involve the IUGR-induced reduction in 5αR2 enzyme and consequently a chronic reduction in the conversion of progesterone into other steroids, prior to finasteride treatment. Progesterone has been shown to have positive effects on myelination in the developing CNS, potentially by the enhancement of maturation processes of oligodendrocyte precursors into myelinating oligodendrocytes (Ghoumari, Ibanez et al. 2003; Ghoumari, Baulieu et al. 2005). Whilst allopregnanolone has been shown to have positive effects on MBP expression, progesterone appears to be a more potent positive modulator of myelination. The investigation of direct progesterone-progesterone receptor effects in this guinea pig model of IUGR may therefore help to explain the changes observed in fetal brain MBP expression.

Together the present observations of changes in MBP expression are consistent with studies that have previously suggested a trophic effect of neurosteroids on the late gestation myelination (Schumacher, Akwa et al. 2000) and support the concept that the low neurosteroid levels may
negatively impact myelination when endogenous neurosteroid supply is disrupted due to impaired placental function or preterm birth.

The finding in this study that GFAP expression was increased when allopregnanolone synthesis was inhibited supports a key regulatory role for this steroid. GFAP is expressed in astrocytes throughout development and when activated by pathological processes (Eng, Ghirnikar et al. 2000). The increase in GFAP expression may be a consequence of the loss of allopregnanolone-mediated inhibition of neural excitability and hence an increase in excitotoxic cellular processes, cell death and damage. The present study identified no significant associations between IUGR and GFAP expression. This is consistent with a previous study by Nitsos et al, which demonstrated no marked difference in GFAP positive cells in growth restricted fetal guinea pig brains, (Nitsos and Rees 1990). In vitro studies in organotypic brain slice cultures have previously shown that pre-treatment with allopregnanolone reduces astrogliosis following a hypoxic insult (Kruse, Rey et al. 2009). Studies of traumatic brain injury in adult rats have also shown that both progesterone and allopregnanolone administration reduces the size of GFAP positive astrocytes (Djebaili, Guo et al. 2005). Despite the increase in GFAP expression when allopregnanolone synthesis was inhibited, qualitative analysis identified no overt areas of glial scarring or obvious signs of damage related to GFAP staining.

We have previously demonstrated the neuroprotective role of allopregnanolone in the fetal sheep brain, when allopregnanolone synthesis was inhibited transiently and acutely. The suppression of allopregnanolone synthesis increases markers of cell proliferation, an effect that is ameliorated when the synthetic neurosteroid, alfaxalone is used to replace the loss of the endogenous neurosteroid (Yawno, Hirst et al. 2009). In adult rat models of traumatic brain injury, allopregnanolone administration
has also reduced the size of GFAP positive astrocytes at the site of the experimental lesions (Djebaili, Guo et al. 2005), and there is increasing evidence for the effectiveness of allopregnanolone in reducing lesion size and enhancing functional recovery in animal models of traumatic brain injury (Djebaili, Hoffman et al. 2004; Djebaili, Guo et al. 2005; Schumacher, Guennoun et al. 2007). In addition to the previously reported neuroprotective actions of allopregnanolone, the current study provides evidence of delayed neurodevelopment in the presence of a low neurosteroid environment and suggests an important role for allopregnanolone in the regulation of developmental processes in the late gestation fetal brain. These findings support the concept that the loss of endogenous neurosteroids and long-term physiological stressors in utero may contribute to the vulnerability of the prematurely born IUGR neonate to abnormal brain development and function. We have also shown that the neurosteroid response of the fetus to an in utero insult is highly dependent on the type and length of insult. The potentially important role of neurosteroids in the regulation of developmental processes and neuroprotection in the fetal brain may provide new directions for the prevention and treatment of neurological disorders that are associated with perinatal insults and pathologies.
The fetal brain is adversely affected by the loss of neuroactive steroids during gestation, reductions of which may mimic the postnatal loss of steroids that occurs after birth. The potential effects of the premature loss of neuroactive steroids following preterm birth are presented in this chapter. These studies establish a novel model of preterm delivery in the guinea pig for the examination of the postnatal neuroactive steroid environment. The efficacy of progesterone replacement therapy on restoring postnatal neuroactive steroid concentrations is also investigated.

4.1 ABSTRACT
Preterm birth is a major cause of neurodevelopmental disorders. Allopregnanolone, a key metabolite of progesterone, has neuroprotective and developmental effects in the brain. The objectives of this study were to describe neurodevelopment and neuroactive steroid concentrations in a preterm neonatal model in the guinea pig and to examine the efficacy of postnatal progesterone therapy on replacing neuroactive steroid concentrations in the neonatal plasma and brain. Preterm (62-63 days) and term (69 days) guinea pig pups were delivered by c-section and tissue collected at 24 hours. Myelination and glial cells were assessed by immunohistochemistry and brain 5α-reductase expression
determined by Western blot. Plasma progesterone, cortisol, allopregnanolone and brain allopregnanolone concentrations were measured by immunoassay.

Preterm neonates had reduced myelination, low birth weight and high mortality compared to term. Brain allopregnanolone concentrations and 5α-reductase expression were significantly reduced after preterm and term delivery. Postnatal progesterone treatment increased brain and plasma allopregnanolone concentrations.

Preterm neonates were neurodevelopmentally immature and experienced a premature reduction of brain allopregnanolone. Postnatal progesterone therapy reestablished neuroactive steroid levels in preterm brains, a finding that has implications for postnatal neurodevelopment following preterm birth, particularly effects on myelination, a key process affected by preterm delivery in this model.

4.2 INTRODUCTION

Preterm birth (birth at <37 weeks gestation) is a major contributor to the burden of disease and mortality in newborn infants. Due to the vulnerability of immature organ systems to injury and the disruption of normal developmental processes, preterm infants are at a greater risk of poor short and long-term health outcomes (Behrman and Butler 2007). A physiological consequence of preterm birth is the premature removal of the fetus from the influence of placentally derived hormones, including progesterone. Progesterone is essential for the establishment and maintenance of pregnancy, with high concentrations of progesterone and its metabolites present during gestation, which fall rapidly following birth and removal of the placenta (Dolling and Seamark 1979; Gilbert Evans, Ross et al. 2005). The neuroactive steroid allopregnanolone (3α,5α-
tetrahydroprogesterone) is synthesised from progesterone by the enzymes 5α-reductase types 1 and 2 (5αR1 and 5αR2) and 3α-hydroxysteroid dehydrogenase, which are present in the placenta and within the brain as well as other endocrine organs (Compagnone and Mellon 2000). The expression of allopregnanolone synthetic enzymes is developmentally regulated and altered by stressors during gestation and the postnatal period. Expression of 5αR2 and concentrations of allopregnanolone increase in the fetal brain in response to hypoxic/ischemic events in utero (Nguyen, Yan et al. 2004) and in the neonate (Billiards, Walker et al. 2002). Allopregnanolone binds to the γ-aminobutyric acid receptor type A (GABA_A), reducing neural excitability and exerting neuroprotective and anti-seizure effects within the brain (Mellon 1994). Allopregnanolone administration is also able to improve morphological and functional recovery in adult animal models of hypoxic/ischemic and traumatic brain injury (He, Hoffman et al. 2004). Allopregnanolone also enhances myelination and myelinating oligodendrocytes in brain slice culture systems (Ghoumari, Baulieu et al. 2005). This neuroactive steroid has important roles in the developing brain. Inhibition of allopregnanolone synthesis in the late-gestation guinea pig reduces myelination and enhances the expression of glial fibrillary acidic protein (GFAP)-positive astrocytes, a marker of brain insult (Kelleher, Palliser et al. 2011). Low allopregnanolone concentrations increase apoptosis in fetal sheep brains, an increase that is ameliorated by the administration of a synthetic allopregnanolone analogue (Yawno, Hirst et al. 2009). Additionally, stress during gestation in rats, reduces brain allopregnanolone turnover from progesterone and results in offspring with poor cognitive functioning (Paris and Frye 2011). Progesterone and its metabolites, influence neural development, axonal and dendritic outgrowth, myelin synthesis and inhibits the excessive gliosis that
impairs recovery following injury (Mellon 2007). The effect of neuroactive steroids and their actions in the preterm brain has not been studied. We hypothesise that the loss of placental progesterone at the time of premature birth disrupts the levels of neuroactive steroids and may contribute to poor neurodevelopmental outcomes in preterm infants. The objectives of this study were to examine the effect of preterm birth on neuroactive steroid levels in the brain of neonatal guinea pigs, an animal with relatively high concentrations of progesterone that are derived from the placenta (Heap and Deanesly 1966). The specific aims were to identify gestational age and sex-specific changes in brain allopregnanolone concentrations in immature preterm guinea pig neonates and establish the efficacy of postnatal progesterone therapy for the re-supply of allopregnanolone concentrations in the preterm neonatal brain.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Animals
Animal procedures were conducted with approval from the University of Newcastle Animal Care and Ethics Committee, in accordance with Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The University of Newcastle Research Support Unit provided time-mated outbred tricolor guinea pigs that were housed indoors under controlled 12-hour light/dark cycles. Pregnant animals were provided with ascorbic acid fortified water and commercial guinea pig pellets *ad libitum.*
4.3.2 Preterm and Term C-Section Delivery

Pregnant guinea pigs were randomly allocated to preterm and term groups for delivery at gestational day 62-63 (Pre-T) and gestational day 69 or after 2 days of the pubic symphysis being greater than 1.5cm open (Term). Spontaneous term delivery occurs at 71 ± 0.5 days in this colony (Palliser, Zakar et al. 2010). To aid neonatal respiratory function, pregnant guinea pigs received Celestone chronodose (1mg/kg betamethasone sodium phosphate/ betamethasone acetate s.c.; Schering-Plough, North Ryde, NSW, Australia) at 24 and 12hours prior to c-section delivery. Pregnant guinea pigs were anesthetized under 1-3% isoflurane in oxygen, the uterus was exposed via midline abdominal incision, fetuses removed and umbilical cord tied off and cut. The maternal guinea pig was then killed by intracardiac sodium pentobarbital (200mg/kg). Neonates were dried and suction applied to remove excess fluid from the upper respiratory tract. Surfactant (50uL Curosurf, 80mg/mL Poractant Alfa; Douglas Pharmaceuticals, Baulkham Hills, NSW, Australia) was administered into the oropharynx and continuous positive airway pressure applied using a modified mask and Neopuff Infant T-Piece Resuscitator (Fisher & Paykel Healthcare Australia, Melbourne, Australia) with a positive end expiratory pressure (PEEP) of 7cmH$_2$O, peak inspiratory pressure (PIP) of 20cmH$_2$O and inspired oxygen concentration of 50%. With the establishment of stable respiration, neonates were placed in a humidified incubator (Thermocare, NV, USA) maintained at ~34°C. Neonatal respiration, posture/muscle tone and activity was monitored and recorded. Neonates were fed at 2hourly intervals using commercial milk formula (Wombaroo Food Products, Adelaide, SA, Australia).
4.3.3 Progesterone Treatment

Preterm neonates were randomly assigned to receive vehicle (22.5% w/v (2-Hydroxypropyl)-β-cyclodextrin) or progesterone (16mg/kg) injections at 1 (i.p.) and 6 hours post-delivery (s.c.). Progesterone dose used in this study was based on that used by Goss et al (Goss, Hoffman et al. 2003).

4.3.4 Tissue and Plasma Collection

At 24 hours after delivery, neonates were euthanised by carbon dioxide inhalation. Plasma was collected and stored at -80°C for steroid analysis. Brains were removed from the skull within 2-3 minutes of euthanasia, weighed, hemisected and divided coronally into 3 regions (rostral, middle, caudal). The middle region, used in this study, contained portions of the cerebral cortex, corpus callosum, thalamus and hippocampus. The right hemisphere was snap frozen and stored at -80°C and left hemisphere immersion fixed in PFA (4% w/v paraformaldehyde in 0.1M phosphate buffer) before paraffin embedding. Neonatal sex, body and organ weights were recorded at the time of tissue collection. Samples for fetal steroid concentrations and brain immunoblotting were obtained from a study of gestational neuroactive steroids (Kelleher, Palliser et al. 2011) and are presented for comparison between fetal and neonatal values. Fetal animals used for these studies did not receive betamethasone treatment, with tissue collected, as above, from animals killed in utero at 65-68 days of gestation.

4.3.5 Western Blot Analysis

The expression of 5αR, types 1 and 2, was determined in the brain using Western blot (Kelleher, Palliser et al. 2011). Brain protein was extracted from crushed tissue containing regions of the cortex, hippocampus and thalamus by homogenization in protein extraction buffer (50 mM Tris HCl,
150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibitors). SDS-PAGE electrophoresis was performed using NuPAGE Novex 12% Bis/Tris gels (Invitrogen, Mt Waverley, Australia) before transfer to polyvinylidene fluoride (PVDF) membranes, which were then blocked in BSA/skim-milk, incubated overnight in primary antibodies (5αR1, NB100-1491, Novus Biologicals, Littleton, CO, USA; 5αR2, Ab27469, AbCam, Cambridge, UK), followed by secondary antibody incubation (P0449; DakoCytomation, Glostrup, Denmark) and chemiluminescence detection. Protein expression of 5αR1 (~26kDa) and 5αR2 (~29kDa) was quantified using Multigauge v2.4 software (Fuji Photo Film, Tokyo, Japan), normalized to β-actin and internal controls.

4.3.6 Immunohistochemistry

Immunohistochemistry was performed on 8μm sections from paraffin-embedded brains that were dewaxed, incubated in Reveal It Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia), blocked (0.1M phosphate-buffered saline pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin, 0.05% v/v sodium azide) and incubated in primary antibody (GFAP at 1:4000, G3893; myelin basic protein (MBP) at 1:4000, M9434; microtubule-associated protein (MAP-2) at 1:30000, M9942; Sigma-Aldrich). Incubations in secondary antibodies (E0354, DakoCytomation; B7139, Sigma-Aldrich) and streptavidin-biotin-horseradish peroxidase complex (RPN1051V; Amersham) with 3,3’-diaminobenzidine tetrahydrochloride chromagen were then performed. GFAP and MBP immunostaining was examined in the hippocampal CA1 and sub-cortical white matter regions. MAP-2 was measured in the CA1, subgranular zone and cortex, adjacent to the pial surface. Staining density was analyzed using ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA) on binary
8-bit images captured at 40x magnification, with percent area coverage recorded for four fields of view per region on two consecutive sections per animal.

4.3.7 Steroid Radioimmunoassay and Enzyme Immunoassay

Allopregnanolone concentrations in plasma and brain were determined via radioimmunoassay (Barbaccia, Roscetti et al. 1992). Brain and plasma samples were treated with acidified 50% methanol and brain samples homogenized. Brain and plasma samples were then added to Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) for steroid extraction using graded methanol washes. Allopregnanolone antibody cross-reactivity to progesterone was minimized by oxidation of non-saturated steroids using 5% potassium permanganate (Bicikova, Lapcik et al. 1995). Steroids were then re-extracted using diethyl-ether/n-hexane (50% v/v). Allopregnanolone concentration in each sample was quantified by radioimmunoassay using an antibody supplied by Dr R. H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA) and tritium-labelled allopregnanolone (5α-[9, 11, 12, 3H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Extraction efficiency was determined by addition of tritiated allopregnanolone prior to extraction and used to correct for extraction loss. The limit of detection for allopregnanolone was 35.0 ± 2.5 pg/tube. The average recovery was 57.7 ± 1.1% for plasma samples and 57.8 ± 2.6% in brain samples. The intra-assay coefficient for plasma was 10.6% and 10.9% in brain assays.

Progesterone and cortisol were measured in guinea pig plasma by immunoassay carried out by Hunter Area Pathology Service using the Beckman Coulter UniCel Dx1800 Access Immunoassay System. The intra-assay coefficients of variance were 4.3% and 7.9% for cortisol and progesterone assays, respectively.
4.3.8 Statistical Analyses
Statistical analyses were performed using Prism v4.0b for MacOSX (GraphPad Software Inc., La Jolla, CA, USA). Data is presented as group mean ± SEM. Data were analyzed by two-way ANOVA for neonatal sex and gestational age at delivery, with Bonferroni post-hoc comparison between groups. Where no sex differences were identified data were analyzed by one-way ANOVA with Bonferroni post-hoc analysis. Data that exhibited unequal variance (Bartlett’s test for equal variance p<0.05) were log or square root transformed. Plasma cortisol data were not normally distributed and was analyzed by Kruskal-Wallis with post-hoc Dunn’s multiple comparison. Survival data was analysed by Fisher’s exact test. P<0.05 was considered statistically significant and indicated on graphs by asterisks (*p<0.05, **p<0.01 and ***p<0.001).

4.4 RESULTS

4.4.1 Neonatal Animals
The gestational age at delivery, mean body and organ weights for preterm and term neonates that survived the initial 24hour period are shown in Table 4.1. There were no significant differences in gestational age between male and female or preterm vehicle and progesterone treated neonates. Preterm animals had a significantly greater mortality rate of 60% at 24hours following delivery, compared to a 3% mortality rate in term neonates (p<0.001). Male neonates made up a higher proportion of those animals that did not reach 24hours (67%). When examined by neonatal sex and progesterone treatment, male neonates had survival rates of 40% with vehicle and 62% with progesterone treatment. Preterm female neonatal survival at 24hours was 50% with vehicle and 38% with progesterone administration. However, these values for male and female preterm
### Table 4.1 - Animal Characteristics and Organ Weights of Preterm and Term Neonatal Guinea Pigs

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Litters</th>
<th>GA Delivery</th>
<th>Birth Weight</th>
<th>PM Weight</th>
<th>Brain Weight</th>
<th>Liver Weight</th>
<th>BLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term</td>
<td>22</td>
<td>11</td>
<td>68.2 ± 0.16*</td>
<td>87.3 ± 2.50*</td>
<td>83.3 ± 2.58*</td>
<td>2.3 ± 0.03</td>
<td>3.6 ± 0.15</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>8</td>
<td>68.3 ± 0.21*</td>
<td>86.3 ± 3.32*</td>
<td>82.9 ± 3.37*</td>
<td>2.3 ± 0.03</td>
<td>3.5 ± 0.16</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>9</td>
<td>68.2 ± 0.24*</td>
<td>88.2 ± 3.76*</td>
<td>83.7 ± 3.95*</td>
<td>2.3 ± 0.04</td>
<td>3.7 ± 0.24</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Preterm</td>
<td>20</td>
<td>19</td>
<td>62.5 ± 0.15</td>
<td>69.0 ± 1.92</td>
<td>64.1 ± 1.70</td>
<td>2.1 ± 0.03</td>
<td>3.0 ± 0.11</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>9</td>
<td>62.4 ± 0.22</td>
<td>67.3 ± 2.88</td>
<td>62.3 ± 2.52</td>
<td>2.1 ± 0.04</td>
<td>2.9 ± 0.13</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>10</td>
<td>62.6 ± 0.22</td>
<td>70.7 ± 2.58</td>
<td>66.0 ± 2.26</td>
<td>2.2 ± 0.03</td>
<td>3.1 ± 0.03*</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>Preterm Prog</td>
<td>16</td>
<td>15</td>
<td>62.6 ± 0.16</td>
<td>67.1 ± 2.06</td>
<td>63.2 ± 1.97</td>
<td>2.1 ± 0.04</td>
<td>2.9 ± 0.14</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>8</td>
<td>62.7 ± 0.29</td>
<td>69.9 ± 3.59</td>
<td>66.2 ± 3.54</td>
<td>2.1 ± 0.05</td>
<td>3.1 ± 0.26</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>7</td>
<td>62.5 ± 0.20</td>
<td>64.3 ± 1.73</td>
<td>60.3 ± 1.24</td>
<td>2.1 ± 0.06</td>
<td>2.8 ± 0.12*</td>
<td>0.76 ± 0.04</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM; weight (g); GA Delivery, gestational age at delivery; PM, post-mortem; BLR, brain to liver weight ratio; Prog, progesterone replacement (* p<0.05 term significantly different from preterm groups; lower case letters indicate significant difference in male liver weights)
survival with progesterone treatment were not significantly different between groups. Preterm neonates had significantly lower body weights than term animals at birth and one-day postnatal age (p<0.05). There were no differences in preterm body weights with progesterone treatment. Brain weights were significantly lower in preterm and preterm progesterone treated groups when compared to term (p<0.001). No differences in brain to liver weight ratio (BLR) were identified with gestational age or progesterone treatment. Male preterm progesterone treated animals had significantly lower liver weights than those in term males. No significant differences in organ or body weights were present between male and female neonates of the same gestational ages. Regular observations of preterm neonates showed periods of apnea, forelimb spasticity and irregular respiration, not present in term neonates. In addition, relative activity and motility was markedly lower in preterm animals.

4.4.2 MBP Expression

Figure 4.1 shows the expression of MBP in the CA1 region of the hippocampus (A, C) and sub-cortical white matter region (B, D) in preterm and term neonatal brains. Qualitatively, immunostaining for MBP appeared to be reduced in preterm neonates compared to term (Fig 4.1A & B). When the percent coverage of this staining was quantified, both male and female preterm neonatal guinea pigs had significantly lower expression of MBP in the CA1 region of the hippocampus when compared to term neonatal brains in the same region (shown in Fig 4.1C). In the sub-cortical white matter region (Fig 4.1D), female but not male preterm neonates, had significantly less positive immunostaining for MBP than term neonates of the same sex (p<0.01). Progesterone treatment of preterm neonates had no significant effect on MBP expression at 24hours age (data not shown).
Figure 4.1 - Myelin Basic Protein Expression in Neonatal Guinea Pigs

(A) Regions of analysis in the cortex and hippocampus (Scale bar represents 2.0mm; C, cortex; H, hippocampus; T, thalamus). Representative micrographs at 40x magnification, in (B) the hippocampal CA1 and (C) the subcortical white matter brain regions show immunostaining in term female (i), term male (ii), preterm female (iii) and preterm male (iv) brains (Scale bars represent 0.1mm). Density of staining was quantified in the (D) CA1 region of the hippocampus and the (E) subcortical white matter tract and is expressed as mean percent area coverage ± S.E.M (n=6; term, grey bars; preterm, open bars). Asterisks indicate significant differences between groups (** p<0.01, *** p<0.001).
4.4.3 GFAP Expression

No marked differences were identified in the staining of GFAP between term and preterm neonates (representative micrographs shown in Fig 4.2A & B). When quantified, GFAP expression did not differ between preterm and term neonates or by neonatal sex in the sub-cortical white matter region (Fig 4.2D). However, a significant interaction effect between neonatal sex and age of gestation was identified in the hippocampal CA1 region (p=0.0185; Fig 4.2C), suggesting a potential sex difference in astrocyte development. No effect of progesterone treatment in preterm neonates was identified (data not shown).

4.4.4 MAP-2 Expression

In the regions examined, the cortex, subgranular zone of the dentate gyrus and the CA1 region of the hippocampus, immunostaining for MAP-2 did not differ with gestational age at delivery, progesterone treatment or with neonatal sex (data not shown).

4.4.5 Plasma Steroid Concentrations

Figure 4.3 presents plasma progesterone, allopregnanolone and cortisol concentrations for fetal, term, preterm and progesterone treated preterm neonates. Analysis of these data showed no sex difference (two-way ANOVA) and therefore the data is presented with combined neonatal sex. As shown in Figure 4.3A, plasma concentrations of progesterone in term neonates at 24 hours after birth were significantly lower than those measured in late gestation fetal plasma (p<0.01). Preterm neonates had similar plasma progesterone concentrations as fetal guinea pigs. Postnatal progesterone treatment significantly increased plasma progesterone concentrations compared to vehicle treated preterm animals (p<0.01; Fig 4.3A, right panel).
Figure 4.2 Glial Fibrillary Acidic Protein in Neonatal Guinea Pig Brains

Expression of glial fibrillary acidic protein (GFAP) in the (A, C) hippocampal CA1 and (B, D) the subcortical white matter brain regions of term and preterm neonatal animals. (A, B) Representative micrographs at 40x magnification, show immunostaining in term female (i), term male (ii), preterm female (iii) and preterm male (iv) brains (Scale bars represent 0.1mm). Density of staining was quantified in the (C) the CA1 region of the hippocampus and the (D) sub-cortical white matter tract and is expressed as mean percent area coverage ± S.E.M (n=6; term, grey bars; preterm, open bars). 2-way ANOVA identified significant interaction between neonatal sex and age in CA1 region (p=0.0185, D).
Figure 4.3 - Plasma Steroid Concentrations in Fetal and Neonatal Guinea Pigs
Plasma concentrations of (A) progesterone, (B) allopregnanolone and (C) cortisol in fetuses (n=9, black bars), term neonates (n=12, grey bars), preterm neonates (n=9, open bars) and preterm progesterone treated neonatal (n=9, hashed bars). In A, note different scale for right panel comparing preterm to progesterone treated preterm neonates. Each bar represents mean ± SEM. Asterisks indicate significant differences between groups (*p<0.05, **p<0.01, ***p<0.001).
Plasma allopregnanolone concentrations were significantly lower in term neonates compared to late-gestation fetal concentrations (p<0.01; Fig 4.3B). Preterm plasma allopregnanolone concentrations were not significantly reduced from fetal levels. Following postnatal progesterone treatment, preterm neonatal plasma concentrations of allopregnanolone rose significantly above term (p<0.001) and preterm (p<0.05) levels. Plasma concentrations of cortisol are shown in Figure 4.3C. Term neonatal concentrations at 24 hours following birth were unchanged from fetal levels. Cortisol concentrations were markedly higher in preterm neonates compared to fetal (p<0.001) and term (p<0.01) animals. Preterm neonates that received progesterone treatment had similar circulating cortisol concentrations to vehicle treated preterm neonates, which were significantly higher than fetal concentrations (p<0.01).

4.4.6 Allopregnanolone Concentrations in the Brain

The mean concentration of allopregnanolone in the brains of late gestation fetal guinea pigs (shown in Fig 4.4) was significantly higher than allopregnanolone concentrations in the brains of preterm and term neonatal guinea pigs at 24 hour following birth (p<0.001). There was no difference in allopregnanolone concentrations between term and preterm neonatal brains or between male and female neonates in these groups. Postnatal progesterone administration in preterm neonates resulted in significantly increased brain allopregnanolone concentrations compared to vehicle treated preterm neonates (p<0.001; Fig 4.4, right panel).
Figure 4.4 - Fetal and Neonatal Brain Allopregnanolone Concentrations
Allopregnanolone concentrations were measured in the brains (coronal section containing cortical, hippocampal and thalamic tissue) of fetal (n=12, black), term neonatal (n=9, grey) and preterm neonatal animals (n= 11, open; left panel). Preterm neonates (open) compared with progesterone treated preterm neonates (n=10, hashed; right panel, different scale). Each bar represents mean ± SEM. Asterisks indicate significant differences between groups (**p<0.001).

4.4.7 Brain 5α-reductase Expression
No differences in the expression of the 5αR1 (Fig 4.5A) were identified between fetal, term or preterm groups. In contrast, 5αR2 expression was significantly higher in fetal animals when compared to term, preterm and preterm progesterone treated animals (p<0.05; Fig 4.5B). No sex differences were identified for either isoform.
Figure 4.5 - Expression of 5α-reductase in Fetal and Neonatal Guinea Pig Brains

Relative expression and representative Western blots for 5α-reductase (A) type 1 and (B) type 2 in the brains (coronal section containing cortical, hippocampal and thalamic tissue) of fetuses (n=16, black bars), term neonates (n=11, grey bars), preterm neonates (n=12, open bars) and preterm progesterone treated neonates (n=8, hashed bars). Bars represent mean ± SEM. Asterisks indicate significant differences between groups (*p<0.05). β-actin loading controls are shown in the lower panels of A and B.
4.5 DISCUSSION

This study describes characteristics of prematurely delivered guinea pig neonates and the changes in neuroactive steroid concentrations that result from preterm delivery. The key findings of the study were a reduction in brain allopregnanolone concentrations at 24 hours after term and preterm birth that likely results from the removal of the placenta. Preterm neonates also showed evidence of neurodevelopmental immaturity, with reduced myelination compared to term controls. In these neonates we have also demonstrated the short-term efficacy of progesterone treatment in reestablishing brain and plasma allopregnanolone concentrations following preterm delivery.

A principal finding of this study was the reduction in myelination seen at preterm age when compared to neonates delivered at term. Previous studies have shown reduced myelination as a result of IUGR (Tolcos et al. 2011) in fetal guinea pigs; however, this is the first study looking at the preterm postnatal period in this species. Myelination is less advanced at earlier gestation with disruption of myelination processes due to preterm delivery and the potential irreversible loss of myelinating oligodendrocytes can result in permanent perturbation of myelin development, severe white matter damage and lasting neurological effects (Kinney 2006). Evidence from imaging studies in human preterm infants has shown that white matter injury and myelination disorders in the early preterm postnatal period, are likely to persist to term equivalent age and are not rectified by catch up growth (Inder, Warfield et al. 2005). Additionally, myelin formation and maturation is influenced by both progesterone and its neuroactive steroid metabolites (Baulieu and Schumacher 2000; Ghomari, Ibanez et al. 2003; Kelleher, Palliser et al. 2011), the supply of which was disrupted by preterm delivery in this model. This may further disrupt the
progression of normal myelination in the preterm neonatal brain. Postnatal loss of myelination and white matter injury should be assessed in to determine whether there is catch-up myelination or long-term deficit in this model.

Neurodevelopmental processes associated with preterm delivery were further examined by assessing MAP-2 and GFAP expression. The observation that MAP-2 staining, a structural marker of neuronal differentiation, did not differ between preterm and term neonates suggests that a loss of neurons or reduced dendritic branching is not present at one-day postnatal age in this model of preterm delivery. This finding may be due to the relatively precocial nature of guinea pigs at term and a high degree of brain and neural development having occurred early in gestation (Dobbing and Sands 1979) with the insult of premature birth in this model potentially not sufficient to cause severe deficits. Additional studies of markers of proliferating neurons may provide further information regarding neural development in this preterm guinea pig model. Expression of GFAP is used as a marker of reactive gliosis and is altered in late gestation fetal guinea pig brains with reduced allopregnanolone concentrations (Kelleher, Palliser et al. 2011). The observation in this study that GFAP expression was influenced by neonatal sex and the apparent increase in GFAP in male preterm neonatal brains may suggest increased vulnerability to injury in this group or the presence of protective mechanisms in female neonatal brains. Such endogenous protective processes have been reported in cultured astrocytes from female neonatal mice, which are more resistant to injury and cell death than male astrocytes (Liu, Hurn et al. 2007). Qualitative observations of the preterm neonatal animals suggest considerable immaturity and the trend towards an increased male mortality rate, is relevant to clinical findings that male preterm neonates have poorer outcomes than female preterm neonates.
Increased plasma cortisol concentrations in the preterm neonates may also suggest increased stress responses due to poorer health in these animals (Hughes, Murphy et al. 1987). In addition, we cannot exclude the possibility that some neuropathological findings may be masked by the considerably higher mortality rate of preterm neonates, as animals that did not survive to 24 hours were excluded from this study. Long-term assessments will be valuable in identifying the progression of neurodevelopmental changes observed in the preterm neonatal brain.

The transition from fetal to neonatal life in this guinea pig model of preterm delivery is associated with a loss of placental progesterone and a decrease in allopregnanolone concentrations. This observation is consistent with previous findings of a loss of allopregnanolone supply following term delivery in neonatal sheep (Nguyen, Billiards et al. 2003). Surprisingly, compared to fetal levels, plasma progesterone concentrations, in the current study, declined after birth in term neonates but not preterm neonatal animals. The mechanisms involved in maintaining these concentrations at 24 hours after birth, despite the removal of the placenta as a source of progesterone, remain unclear. We speculate that variations in the metabolism of progesterone and its precursor pregnenolone (Riepe, Mahler et al. 2002), mediated by gestational age, or contribution of adrenal steroidogenesis, may be responsible for the maintenance of progesterone concentrations in preterm neonates at 24 hours of age. In addition, pregnenolone sulfate concentrations are relatively high in the fetal circulation and may function as a reserve of pregnenolone in preterm neonates (Klak, Hill et al. 2003). The trend towards lower plasma allopregnanolone concentrations in preterm neonates also suggests altered or lower levels of steroid metabolism in the neonatal guinea pig at this age.
Without the continued supply of placentally derived steroids, concentrations of progesterone are likely to decline in the neonatal period. Importantly, higher plasma concentrations of progesterone at 24 hours did not result in maintenance of brain allopregnanolone concentrations. The finding that 5αR2 expression was also reduced within the brain, suggests that both the endogenous supply of progesterone and the capacity of the brain to synthesise neuroactive progesterone metabolites is compromised in the preterm neonate. This supports the potential use of exogenous progesterone replacement to increase allopregnanolone precursor supply. Additionally, previous studies of allopregnanolone production in fetal sheep have identified 5αR2 as the most important isoform in regulating neuroactive steroid levels within the fetal brain and in controlling responses to stress (Nguyen, Yan et al. 2004). It cannot be excluded that betamethasone treatment may also influence 5αR expression. Repeated betamethasone doses over five days reduced 5αR2 mRNA in a sex-specific manner in male fetal guinea pig brains (McKendry, Palliser et al. 2010), suggesting the potential for betamethasone to influence neurosteroid synthetic capacity of preterm infants, a high proportion of which receive antenatal steroid treatment.

Given these changes in progesterone supply and expression of synthetic enzymes, a major objective of this study was therefore to determine the efficacy of progesterone replacement therapy in increasing preterm brain allopregnanolone concentrations. The present finding demonstrates that administration of progesterone to preterm neonates elevated plasma progesterone concentrations and consequently increased the concentration of its metabolite, allopregnanolone. Despite the reduced expression of 5αR2, the exogenous administration of progesterone was sufficient to raise brain allopregnanolone concentrations. The synthesis of allopregnanolone
may also be influenced by compensatory mechanisms involving 5αR1 and other synthetic enzymes of allopregnanolone such as P450scc and 3α-HSD. This finding provides support for the augmentation of allopregnanolone levels in the brain indirectly via endogenous synthetic mechanisms when sufficient substrate is available. Short-term administration of progesterone to preterm neonates over 24 hours did not have an effect on neuropathological findings in this study. Longer-term progesterone replacement, however, during the preterm postnatal period, warrants further examination of effects on neuroactive steroid concentrations and potentially brain development and functional neurological outcomes.

The present findings are also of interest in view of the existing use of perinatal progesterone treatments. Progesterone and synthetic progestins have been investigated for use as preventative treatments for preterm birth (Mackenzie, Walker et al. 2006). There is currently, however, little information on the effect of such treatments on fetal and neonatal allopregnanolone concentrations. The finding that neuroactive steroid levels in the preterm neonate are at least partially dependent on progesterone concentrations suggest some caution is needed over the potential effect of treatments on brain development. Additionally, synthetic progestins may inhibit endogenous neurosteroid synthesis (Belelli and Herd 2003), whilst being unable to be metabolized into neuroactive steroids themselves (Giriza, Azcoitia et al. 2004). Pilot data from clinical trials in extremely preterm neonates, examining effects of postnatal replacement of placental hormones on bone mineralization (Trotter, Maier et al. 1999) and antenatal progesterone therapy on postnatal lung function (Dodd, Crowther et al. 2009) supports the investigation of these steroid hormones for use in preterm neonates. However, assessments of
neurodevelopmental outcomes or effects on neurosteroid supply were not objectives of these studies.

This study establishes a model of preterm delivery in the guinea pig, which demonstrates a premature reduction in brain concentrations of the key neuroactive steroid, allopregnanolone. Current findings suggest that this reduction can be reversed by postnatal treatment with progesterone. The development of this model, with immature neurodevelopment at delivery, will enable future investigations into progesterone administration during the immediate preterm postnatal period. Progesterone and its neuroactive steroid metabolites may have important actions on postnatal processes of myelination and perturbation of other key neurodevelopmental pathways associated with preterm birth.
Chapter Five

NEUROACTIVE STEROIDS IN PRETERM GUINEA PIGS FOLLOWING POSTNATAL PROGESTERONE THERAPY

Chapter five extends the studies from the previous chapter to examine postnatal effects of neuroactive steroids in preterm neonates that have reached term-equivalent age. This builds on the novel preterm neonatal guinea pig model, previously established, to investigate the efficacy of progesterone administration in restoring allopregnanolone concentrations following preterm birth and the effect of this treatment on markers of brain development and the functional neurobehavioural outcomes in the preterm guinea pig neonate.

5.1 ABSTRACT

Neurodevelopmental disorders are severe consequences of preterm birth. The identification of new, effective and safe therapies is an important goal in improving developmental outcomes in vulnerable preterm infants. Neuroactive steroids, particularly the progesterone metabolite, allopregnanolone, have been proposed as important protective and trophic agents within the developing brain. At the time of preterm birth, the placental steroid influence is lost, resulting in disruption of progesterone supply, affecting neonatal neuroactive steroid concentrations. The aims of this study were to determine the effect of postnatal replacement of progesterone on allopregnanolone concentrations in the preterm brain during the postnatal period and the effects of this
neuroactive steroid replacement on myelination, brain injury, developmental markers and functional behavioural outcomes in neonates at term-equivalent age.

Preterm guinea pig pups were delivered at 62-63 days gestation by c-section and received daily injections of progesterone (16mg/kg; +Prog8) or vehicle ((2-Hydroxypropyl)-β-cyclodextrin; Pre-T8). Tissues were collected at term-equivalent age (GA70; postnatal day 8), following behavioural open field (OF) and novel object recognition testing (NORT). A control group of term neonates were also delivered at GA69, with tissues collected at 24hours postnatal age. Neonatal condition was scored and salivary progesterone concentrations were sampled over the postnatal period. Neonatal brain and plasma allopregnanolone concentrations were measured by RIA and plasma cortisol and progesterone measured by EIA. Brain myelination, astrocyte activation and neuronal maturation and structure were determined by immunohistochemistry, staining for MBP, GFAP and MAP-2, respectively. Hippocampal expression of 5α-reductase (the rate-limiting enzyme in the synthesis of allopregnanolone) was also determined by Western blot.

Postnatal survival was significantly reduced in both Pre-T8 and +Prog8 groups, with neonatal condition similar to term scores only after postnatal day 4. Progesterone treatment significantly increased salivary progesterone concentrations over the postnatal period but only male +Prog8 neonates had significantly elevated plasma progesterone at the time of tissue collection at postnatal day 8. Additionally, female but not male +Prog8 animals had elevated plasma allopregnanolone concentrations. Female +Prog8 neonates also demonstrated reduced MBP staining in the external capsule adjacent to the CA1 region of the hippocampus. Both male and female neonates that received progesterone treatment had increased brain allopregnanolone concentrations and higher concentrations of plasma
The expression of 5αR1 in the hippocampus was reduced in all preterm animals compared to term, with no differences identified in the expression of 5αR2. Both Pre-T8 and +Prog8 groups had similar levels of activity in the OF test and demonstrated preferential exploration of the novel object in the NORT behaviour trial. However, +Prog8 animals spent more time exploring the familiar object in the NORT trial than the Pre-T8 animals.

This study has established the ability of the postnatal preterm guinea pig brain to endogenously synthesise allopregnanolone from exogenous progesterone sources with important implications on the use of steroid therapies in the perinatal period. Sex-specific endocrine patterns and potential differences in steroid metabolism following postnatal progesterone treatment should be further investigated. Long-term follow up is needed to identify neurodevelopmental and neurobehavioural effects of postnatal neuroactive steroid changes and replacement therapy following preterm birth.

5.2 INTRODUCTION

Preterm birth is the leading cause of neonatal mortality and morbidity. Perinatal brain injury and developmental delay are severe consequences of preterm birth. The severity and incidence of neurodevelopmental disorders increases with decreasing gestational age (Behrman and Butler 2007). However, even late preterm neonates are at an increased risk with preterm infants born between 34 and 36 weeks gestation, over three times more likely to develop cerebral palsy than term infants (Petrini, Dias et al. 2009). Other neurodevelopmental disorders such as behavioural, attention and learning deficits are also more common following preterm birth but may not become apparent until school age (Saigal and Doyle 2008). Along with
immaturity at birth, postnatal brain development, in the extra-uterine environment is also affected by preterm birth. Structural, biochemical and functional deficits are present in preterm infants at term-age when compared to infants delivered at full-term, with impaired maturation of myelin, reduced differentiation and volumes of grey and white matter, and deficits in neurobehavioural functions (Huppi, Schuknecht et al. 1996; Inder, Warfield et al. 2005). A recent systematic review of the literature also identified that, at 6 months of age, up to 30% of all preterm infants have some degree of neurodevelopmental impairment (Mwaniki, Atieno et al. 2012). These studies demonstrate that whilst advances in diagnostic techniques and therapeutic interventions have improved the survival of even extremely preterm infants, the development of effective and safe therapies to treat preterm brain injury and improve developmental outcomes should remain a priority.

Progesterone is an essential hormone in pregnancy (Davis and Plotz 1957) that maintains uterine quiescence, modulates maternal immune responses and is involved in the signalling mechanisms of labour (Zakar and Hertelendy 2007; Challis, Lockwood et al. 2009; Mendelson 2009). Progesterone is produced by the placenta in high concentrations, which increase over the course of gestation, reaching levels over 600nmol/L, which also results in high concentrations of progesterone metabolites in the maternal and fetal circulation (Gilbert Evans, Ross et al. 2005).

Allopregnanolone, a neuroactive metabolite of progesterone, is a potent positive allosteric modulator of $\gamma$-aminobutyric acid type A (GABA$_A$) receptor function, increasing GABAergic inhibition of excitability and producing anxiolytic, sedative, anticonvulsant and neuroprotective effects (Paul and Purdy 1992). Maternal plasma concentrations of allopregnanolone increase up to nine-fold between 10 and 36 weeks gestation to over 40nmol/L (Gilbert Evans, Ross et al. 2005). Importantly
these levels are below 2nmol/L in maternal plasma at 4-6 weeks after delivery (Gilbert Evans, Ross et al. 2005), indicating a marked reduction in steroid supply with the removal of the placental influence at birth. These high concentrations of progesterone and allopregnanolone during gestation have important implications for fetal and neonatal development following preterm birth. The immature fetal brain has been shown to express the enzymes for the de novo synthesis of allopregnanolone from progesterone. This two-step process involves the rate-limiting 5α-reductase (5αR) conversion of progesterone into 5α-dihydropregesterone, which is then further reduced by 3α-hydroxysteroid dehydrogenase to allopregnanolone (3α,5α-tetrahydroprogesterone) (Mellon and Deschepper 1993). The expression of the 5αR2 can be influenced by stress, with upregulation of this isoform in response to asphyxial insult in the fetal sheep brain (Nguyen, Billiards et al. 2003). These studies demonstrate the ability of the fetal brain to directly synthesise neuroactive steroids and suggests the potential regulation of this synthesis in response to physiological stressors. Actions of allopregnanolone within the fetal brain include effects on fetal sleep cycles, with experimentally reduced allopregnanolone concentrations causing altered periods of arousal and sleep in fetal sheep (Nicol, Hirst et al. 2001). Inhibition of allopregnanolone synthesis has also been shown to increase cell death in the brains of fetal sheep (Yawno, Yan et al. 2007) and reduce myelination in the fetal guinea pig brain (Kelleher, Palliser et al. 2011). Progesterone itself, as well as being a precursor of allopregnanolone, has also been shown to have positive effects on myelin growth and oligodendrocyte development (Ghoumari, Baulieu et al. 2005). These effects provide evidence for the direct action of allopregnanolone and progesterone within the fetal brain and suggest that neuroactive steroids may provide important
regulatory, neuroprotective and developmental signals in the immature brain. Disruption of progesterone and allopregnanolone supply/synthesis may contribute to the poor developmental outcomes associated with complications during pregnancy and birth and related to postnatal brain development.

We hypothesise that the premature loss of supply of placental progesterone at preterm birth will result in a persistent deficiency in allopregnanolone and neuroactive steroid supply, compared to in utero levels, contributing to the vulnerability of preterm neonates to altered developmental processes in the preterm brain. The purpose of this study was to examine the postnatal replacement of progesterone to augment allopregnanolone concentrations in the preterm brain during the postnatal period and the effects of progesterone and allopregnanolone replacement on myelination, brain injury, developmental markers and functional behavioural outcomes.

5.3 MATERIALS AND METHODS

5.3.1 Animals
All animal procedures were carried out with approval from the University of Newcastle Animal Care and Ethics Committee according to guidelines from the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Pregnant, outbred, tricolour guinea pigs were supplied by the University of Newcastle Research Support Unit and housed indoors with a 12hr light/dark cycle, commercial guinea pig pellets and water (fortified with ascorbic acid) provided ad libitum.

5.3.2 Preterm C-section Delivery
As part of this study, preterm neonates were delivered at 62-63 days gestation by c-section and maintained until term-equivalent age at postnatal
day 8 (GA equivalent 70-71 days). C-section delivery was performed following maternal anaesthesia with 1-3% isoflurane and 24hrs after a course of prenatal steroid treatment (1mg/kg betamethasone sodium phosphate/betamethasone acetate, s.c.). The uterus was exposed via a midline abdominal incision, fetuses removed from the fetal membranes and the umbilical cord tied and cut. Following delivery maternal guinea pigs were euthanised by intracardiac sodium pentobarbital (200mg/kg). Preterm neonatal guinea pigs received surfactant treatment (50uL Curosurf, 80mg/mL Poractant Alfa, Douglas Pharmaceuticals) and continuous positive airway pressure via a Neopuff Infant T-Piece Resuscitator (Fisher & Paykel Healthcare Australia) fitted with a modified small animal mask, 7cmH₂O PEEP and 20cmH₂O PIP. Once stabilised, neonates were transferred to a small animal incubator and fed at 2hrly intervals using commercial milk formula (Wombaroo Food Products) and mixed pellet food after postnatal day 4-5. On each day, prior to each feed at 8am, 4pm and midnight, guinea pig neonates were given cotton buds to chew on for 3min, to allow collection of passive salivary secretions.

5.3.3 Treatment Groups
In order to assess postnatal progesterone replacement therapy, preterm neonates were randomly assigned to progesterone treatment (+Prog8) or control (Pre-T8) groups. Neonates received an initial i.p. injection at 1hr post-delivery of vehicle (22.5% w/v (2-Hydroxypropyl)-β-cyclodextrin)) or progesterone (16mg/kg in vehicle), followed by a second injection s.c. at 6hrs and daily injections until term-equivalent age. These Pre-T8 and +Prog8 animals were euthanised at term-equivalent age (postnatal day 8) and tissues collected (plasma frozen and brain dissected for fixation and freezing) for analysis. In addition, a group of neonates were also delivered by c-section at ~69 days gestation (Term) and tissues collected at 24hrs
after delivery for comparison to preterm delivered animals. Results for term neonates included in this study have previously been described in Chapter 4.

5.3.4 Neonatal Scoring & Behavioural Testing

Neonatal condition was monitored and recorded using a scale of neonatal activity, muscle tone and respiration as outlined in Chapter 2, Table 2.1; with a maximum score of 12, indicating good condition. On PND7, prior to tissue collection on PND8, open-field and novel object recognition testing was performed to assess activity and short-term memory function. All trials were carried out in a 43x43cm box, with 20cm tall walls, made from rigid, opaque polypropylene board and was carefully washed between trials. The testing procedure involved an initial 15min open-field trial (OF), a 15min familiarisation trial (T1) in which two identical objects were added to the arena, a retention period of 80min and a final 15min object recognition trial (T2) in which a novel object replaced one of the identical ones. All trials were recorded using a digital video recorder for later analysis by researchers blinded to treatment group. In the OF trial the first 5min was analysed for time active, number of gridlines crossed, number of zone visits and time spent in the inner zone (inner 14x14cm square). For the novel object T1 and T2 trials, the general activity, exploration time and total time the subject interacted with each object was recorded. Object exploration was defined as the animal handling, sniffing or facing the object (within 2cm).

5.3.5 Plasma, Brain & Salivary Steroid Analysis

Allopregnanolone concentrations were measured in the neonatal brain and plasma by RIA as detailed in section 2.7.1 & 2.7.2. Briefly, plasma and brain steroids were isolated using solid-phase extraction with graded acidified methanol washes. To reduce cross-reactivity, the extracts were
then treated with potassium permanganate to oxidise excess progesterone. Allopregnanolone concentrations were then determined by RIA, as previously described (McKendry, Palliser et al. 2010; Kelleher, Palliser et al. 2011). Plasma assays were performed using a polyclonal anti-allopregnanolone antibody supplied by Dr R.H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA). Due to supply problems, all brain assays of allopregnanolone presented in this chapter were performed using an egg-yolk polyclonal anti-allopregnanolone antibody supplied by Agrisera (Vannas, Sweden) and raised against the same antigen (3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether coupled to BSA (Purdy, Moore et al. 1990). The Agrisera antibody has previously been validated against the Purdy antibody for use following steroid extraction (Timby, Balgard et al. 2006). The average extraction recovery was 53.4 ± 1.4% for plasma samples, with an intra-assay coefficient of variance of 15.6%. Brain extractions had 60.3 ± 1.3% average recovery and an intra-assay coefficient 8.1%.

Plasma progesterone and cortisol concentrations were assayed in neonatal guinea pig plasma collected at the termination of the experiments by enzyme immunoassays carried out by Hunter Area Pathology Service (John Hunter Hospital, Newcastle, NSW, Australia) using the Beckman Coulter UniCel Dx1800 Access Immunoassay system. The intra-assay coefficients of variance were 7.9% for progesterone assays and 4.3% for cortisol.

Salivary progesterone concentrations were measured over the postnatal period using Salivary Progesterone EIA kits (Salimetrics LLC, State College, PA, USA). Assays were performed according to manufacturers’ instructions, with samples diluted in assay buffer as needed and an intra-assay coefficient of variance of 16.5%.
5.3.6 Protein Analysis

Immediately after neonates were euthanised, the brain was removed and the hippocampus was isolated from the cortical tissue prior to freezing. Frozen tissue from the hippocampus was then crushed and protein extracted in protein extraction buffer (50mM Tris HCl, 150mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibitors). SDS-PAGE electrophoresis was performed using Novex 12% Bis/Tris gels (Invitrogen), followed by Western transfer to polyvinylidene fluoride membranes. Expression of 5αR types 1 and 2 was then determined in the hippocampal protein extract by immunodetection with polyclonal primary antibodies to the 5αR isoforms (5αR1, NB100-1491, Novus Biologicals; 5αR2, Ab27469, AbCam) and detection using hrp-conjugated secondary antibody (anti-goat polyclonal, P0449, DakoCytomation) and chemiluminescence, quantified using Multigauge v2.4 software (Fuji), normalised to β-actin and internal controls.

5.3.7 Immunohistochemistry

As detailed in section 2.9, immunohistochemistry was performed on sections from formalin fixed, paraffin-embedded brains collected at term-equivalent age in Pre-T8 and +Prog8 neonatal groups and 24hrs in Term animals. Using a rotary microtome, 8μm sections were cut, containing the hippocampus, dentate gyrus and CA1 regions. Sections were dewaxed, antigen retrieval carried out (RevealIt Solutions, ImmunoSolution Pty Ltd) and sections incubated in primary antibody overnight (GFAP, G3893; MBP, M9434; MAP-2, M9942; Sigma-Aldrich). Sections were then incubated in biotinylated secondary antibodies, streptavidin-biotin-horseradish peroxidase complex (RPN1051V; Amersham) and immunostaining detected using diaminobenzidine tetrahydrochloride chromagen. MBP and GFAP immunostaining was quantified in the
external capsule, adjacent to the CA1 region of the hippocampus and in the sub-cortical white matter region. MAP-2 coverage was determined in the cortex, adjacent to the pial surface and in the CA1 region of the hippocampus. Staining density was analysed using ImageJ software for four fields of view per region, on two serial sections per animal, at x40 magnification.

5.3.8 Statistical Analysis

All data is presented as the mean ± SEM for each treatment group, Pre-T8, +Prog8 and Term. Variance per group was analysed by Bartlett’s test for equal variance and data were log or square root transformed if p<0.05. In order to identify the presence of sex-differences, data were first analysed by two-way ANOVA by neonatal sex and treatment group. Post-hoc Bonferroni analysis was then performed if p<0.05 by two-way ANOVA. Where no sex differences were identified, neonatal sexes were combined and analysed by one-way ANOVA, with Bonferroni post-hoc analysis. Where data were not normally distributed, non-parametric Kruskal-Wallis analysis was performed with post-hoc multiple comparison using Dunn’s test. Salivary progesterone and neonatal scoring data were analysed by repeated measures two-way ANOVA. Survival data were analysed by Fisher’s exact test. Familiar and novel object exploration was analysed by paired Wilcoxon signed rank test. P<0.05 was considered statistically significant and indicated on graphs by asterisks (*p<0.05, **p<0.01 and ***p<0.001). All analyses were performed using Prism v4.0b for MacOSX (GraphPad Software Inc., La Jolla, CA, USA).
5.4 RESULTS

5.4.1 Neonatal Animals, Survival & Scoring
Of all the neonates delivered in this study, 56.8% of preterm vehicle treated and 47.6% of progesterone treated neonates survived until post-mortem age at PND8. Overall survival of preterm male neonates was 45.7%, with survival of 52.4% in Pre-T8 males and 40% in +Prog8 males. Female neonatal survival was 60.6%, with 62.5% in Pre-T8 and 58.8% in +Prog8 female neonates. However, survival data did not differ significantly in male and female neonates or between Pre-T8 and +Prog8 treatment groups. Results presented for this study for preterm neonates refer only to animals that survived to term-equivalent age, with those that died prior to this age excluded from analysis.

The gestational age, mean body weights and organ weights for Term, Pre-T8 and +Prog8 animals, that survived the full experimental period, are presented in Table 5.1. Gestational age at delivery and body, brain, heart and kidney weights at post-mortem were significantly lower in preterm neonates when compared to neonates delivered at term (p<0.05). These differences were also present when data were analysed by neonatal sex, but no differences were identified between preterm groups that received postnatal vehicle (Pre-T8) or progesterone treatment (+Prog8). When analysed by sex, male Pre-T neonates had significantly reduced liver weights compared to term male neonates (p<0.05), however, this difference was not present in females or when sexes were combined. Overall, +Prog8 neonates had significantly reduced liver weights compared to term. Birth weights in all preterm animals were significantly reduced compared to those neonates delivered at term (p<0.001). However, when birth weights were analysed by neonatal sex, only female Pre-T8 neonates had significantly lower birth weights compared to term. Progesterone
treated (+Prog8) females had birth weights that were similar to term female neonates, potentially indicating a survival bias in this group and increased loss of smaller female preterm neonates. Percent change in body weight from birth to postnatal day 8 were similar across preterm and term groups as were brain to liver weight ratios.

Preterm neonatal condition, as shown in Figure 5.1, improved significantly over the study period in both Pre-T8 and +Prog8 groups (p<0.05). However, no differences were identified in condition or wellbeing of preterm neonates by neonatal sex or with progesterone treatment. Animals in both Pre-T8 and +Prog8 treatment groups did not reach the higher scores achieved by term neonates until PND4 (Fig 5.1).

### 5.4.2 Salivary Progesterone

Progesterone concentrations over the experimental period were determined by serial measures of salivary progesterone in Pre-T8, +Prog8 and Term neonates are shown in Figure 5.2. Pooled daily salivary progesterone (Fig 5.2A) concentrations were significantly higher overall in animals that received progesterone treatment (+Prog8) when compared to Pre-T8, vehicle treated neonates (p<0.0001). In addition, salivary progesterone concentrations were lower in term neonates at delivery and remained reduced at 24hrs after birth compared to both preterm groups (p<0.05). Concentrations of progesterone were also compared across each sampling time (Fig 5.2B), with +Prog8 animals having elevated salivary progesterone levels compared to the Pre-T8 neonates at each time-point (p<0.05). However, within the Pre-T8 group, salivary progesterone concentrations were different between sampling times, with significantly higher concentrations at the 4pm sample compared to 8am (p<0.05).

Salivary progesterone concentrations did not differ with neonatal sex, in either Pre-T8 or +Prog8 groups.
Table 5.1 - Animal Characteristics and Organ Weights of Term, Preterm and Progesterone Treated Neonates

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Litters</th>
<th>GA Delivery</th>
<th>Birth Weight</th>
<th>PM Weight</th>
<th>% Weight Change</th>
<th>Brain Weight</th>
<th>Liver Weight</th>
<th>Brain:Liver Ratio</th>
<th>Heart Weight</th>
<th>Kidney Weight</th>
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<td>Term</td>
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<td>87.5 ± 4.84</td>
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<td>0.5 ± 0.02</td>
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<tr>
<td></td>
<td>Female</td>
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<td>68.2 ± 0.22</td>
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<td>75.6 ± 3.36</td>
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<td>0.70 ± 0.03</td>
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<tr>
<td>Pre-T8</td>
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<td>68.4 ± 2.39</td>
<td>65.1 ± 2.38</td>
<td>95.7 ± 2.70</td>
<td>2.1 ± 0.04</td>
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<td>66.3 ± 3.32</td>
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<td>62.8 ± 0.20</td>
<td>66.2 ± 4.59</td>
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<td>96.3 ± 4.17</td>
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<td>2.1 ± 0.04</td>
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<td>0.74 ± 0.05</td>
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<td>1.3 ± 0.10</td>
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<td>72.2 ± 2.53</td>
<td>65.3 ± 4.05</td>
<td>90.3 ± 3.60</td>
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<td>0.70 ± 0.04</td>
<td>0.4 ± 0.03</td>
<td>1.2 ± 0.05</td>
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</table>

Pre-T8, preterm control; +Prog8, preterm progesterone treatment. Values expressed as mean ± SEM, weight (g); GA, gestational age; PM, post-mortem. Letters indicate significant differences between Term, Pre-T and +Prog8 treatments (p<0.05).
Figure 5.1 - Daily Condition Scores in Preterm Guinea Pigs with Postnatal Progesterone Treatment

Mean daily scores in term (open; postnatal day 1 only as term neonates were euthanased at this age), preterm (black; Pre-T8, postnatal day 8, term-equivalent age) and preterm progesterone treated neonates (grey; +Prog8, postnatal day 8). Neonates were scored based on respiration, activity and posture, with a maximum score of 12 indicating very good condition. Preterm neonatal scores were lower than term scores and increased significantly over the postnatal period (p<0.05). No differences were identified between Pre-T8 and +Prog8 animals at any time point. Each point represents daily group mean ± SEM.
Figure 5.2 - Neonatal Salivary Progesterone Concentrations in Preterm Guinea Pigs with Postnatal Progesterone Treatment

(A) Mean daily salivary progesterone concentrations in term (open; term neonates were euthanased at postnatal day 1), preterm (Pre-T8; black) and preterm progesterone treated neonates (+Prog8; grey). Preterm neonates were sampled until term-equivalent age (postnatal day 8). By repeated measures 2-way ANOVA, +Prog8 animals had higher progesterone concentrations overall compared to Pre-T8 animals (p<0.05) and on day 6 (post-hoc Bonferroni). (B) Over the postnatal period, the mean salivary progesterone concentrations were significantly elevated at each of the three sampling times in +Prog8 (hashed) compared to Pre-T8 (grey) animals. Salivary progesterone was elevated within the Pre-T8 group at the 4pm sample. Each bar/symbol represents mean ± SEM. Asterisks indicate significant differences between groups (*p<0.05, **p<0.01, ***p<0.001). Letters indicate significant difference between samples within the Pre-T8 groups (p<0.05).
5.4.3 Plasma Steroids

Plasma progesterone concentrations were significantly higher in +Prog8 male neonates compared to both Term and Pre-T8 male animals (Fig 5.3A, p<0.05). However, in female neonates, plasma progesterone concentrations were similar across Pre-T8, +Prog8 and Term groups, with no significant differences identified, despite progesterone administration to +Prog8 animals and elevated salivary progesterone concentrations.

Plasma allopregnanolone concentrations are presented in Figure 5.3B. Overall, female neonates had significantly higher plasma concentrations of allopregnanolone than male neonates, regardless of treatment group (p<0.0412). When neonatal treatment was assessed by neonatal sex, +Prog8 female neonates had significantly higher plasma allopregnanolone concentrations compared to levels in the plasma of Term female neonates (p<0.01), but not females in the Pre-T8 group. Additionally, there were no significant differences in plasma allopregnanolone concentrations between Term, Pre-T8 or +Prog8 male neonates, despite increases in plasma concentrations of progesterone, the major steroid precursor of allopregnanolone, in +Prog8 animals.

Plasma cortisol concentrations were also assessed in preterm neonatal guinea pigs (Figure 5.3C). Animals in the +Prog group had significantly higher plasma concentrations of cortisol compared to Pre-T8 animals (p<0.0169). However, this increase was only present when neonatal sexes were combined. No differences were identified between Term plasma cortisol concentrations and cortisol levels in either of the preterm groups.
Figure 5.3 - Plasma Steroid Concentrations in Preterm Guinea Pigs following Postnatal Progesterone Treatment

Plasma steroid concentrations of (A) progesterone, (B) allopregnanolone and (C) cortisol in term (open bars), preterm (Pre-T8; grey bars) and preterm progesterone treated neonates (+Prog8; hashed bars). Pre-T8 and +Prog8 samples collected at term-equivalent age at postnatal day 8. Each bar represents mean ± SEM for each group. (A, B, n=4-6 per group; C, n=8-10) as neonatal sexes combined. Asterisks indicate significant differences between groups or with neonatal sex (p<0.05). Lower case letters indicate differences within female neonates in B (p<0.05).
5.4.4 Brain Allopregnanolone
Brain concentrations of allopregnanolone, in a coronal brain section containing cortical, hippocampal and thalamic tissue, were similar in preterm control and term animals, with no significant differences between Pre-T8 (at term equivalent age) and term levels (Fig 5.4). Progesterone treatment in preterm neonates increased brain allopregnanolone concentrations, with +Prog8 animals having significantly higher concentrations than Pre-T8 and term animals (p<0.05).

5.4.5 Hippocampal 5α-Reductase Enzyme Expression
Western blot analysis of the expression of the neurosteroidogenic enzymes, 5αR types 1 and 2 in hippocampal protein extracts are shown in Figure 5.5. The expression of 5αR1 was significantly higher in the Term hippocampus compared to both Pre-T8 and +Prog8 preterm groups. The type 2 isozyme had similar expression across all three groups. Hippocampal expression of both isoenzymes did not differ with neonatal sex.

5.4.6 Immunohistochemistry
MBP immunostaining in the CA1 region of the hippocampus, is mostly present in the external capsule region, with representative photomicrographs of female MBP staining in Term, Pre-T8 and +Prog8 neonates shown in Figure 5.6A. The average percent coverage of MBP immunostaining in this region (Fig 5.6B) was analysed by treatment group and neonatal sex, with treatment having a significant effect (p<0.0193). Comparison between groups identified a significant reduction in MBP CA1 staining in +Prog8 female neonates compared to staining in Term female neonatal brains (p<0.05) but was not different from staining in Pre-T8 female neonates. No differences in MBP immunoreactivity were identified between groups in this region in male neonates. MBP staining was also
Allopregnanolone concentrations were measured in brain homogenates from male and female neonates delivered at term (open bars), preterm (Pre-T8; grey bars) and preterm with postnatal progesterone treatment (+Prog8; hashed bars). Results show similar effects of progesterone treatment in both male and female neonates. Pre-T8 and +Prog8 samples were collected at term-equivalent age at postnatal day 8; Term at postnatal day 1. Each bar represents mean ± SEM for each treatment group (n=5). Asterisks indicate significant differences between groups (*p<0.05).

The expression of 5α-reductase types 1 and 2 was measured in extracted protein homogenates from the hippocampus of term (open bars), preterm (Pre-T8; grey bars) and preterm progesterone treated (+Prog8; hashed bars) neonates. +Prog8 samples collected at term-equivalent age at postnatal day 8. Each bar represents mean ± SEM for each treatment group (n=5). Asterisks indicate significant differences between groups (*p<0.05).
Figure 5.6 - Myelin Basic Protein Immunoreactivity in Preterm Guinea Pig Brains following Postnatal Progesterone Treatment

Representative photomicrographs of myelin basic protein (MBP) immunoreactivity in the external capsule adjacent to the CA1 region of the hippocampus in (A) female and (B) male neonates delivered at (i) term or (ii) preterm gestations and in (iii) preterm neonates that received progesterone treatment (scale bar = 0.1mm). Density of staining was quantified in the (C) CA1 region of the hippocampus and (D) the subcortical white matter in term (open bars), preterm (Pre-T8; grey bars) and preterm progesterone treated (+Prog8; hashed bars). Columns represent mean percent area coverage ± S.E.M (n=4-6). Asterisks indicate significant differences between groups (* p<0.05).
analysed in the sub-cortical white matter tract (Fig 5.6C & D); however, no significant differences were present between treatment groups or with neonatal sex. The same regions, hippocampal CA1 and sub-cortical white matter tract were also analysed for GFAP expression, with no differences identified in immunostaining between male and female neonates or Pre-T8, +Prog8 or Term groups (Fig 5.7). Analysis of cortical and hippocampal expression of MAP-2 also showed no significant changes with treatment or neonatal sex (Fig 5.8).

5.4.7 Behavioural Analysis

Results for locomotor activity in the open-filed task (OF) and novel object recognition task for working memory are shown in Table 5.2. Overall, 2-way ANOVA identified no significant effect of progesterone treatment (Pre-T8 vs +Prog8) or neonatal sex on locomotor activity or distance travelled during the OF test. There was also no difference present in the time spent in the inner zone of the arena between preterm treatment groups or male and female neonates.

During the object recognition retention trial (T2, Figure 5.9), there was a significant difference in novel object exploration, compared to exploration of the familiar object, in both Pre-T8 and +Prog8 groups of animals. All animals exhibited preferential exploration of the novel object as determined by Wilcoxon matched pairs tests (Pre-T8, p<0.014; +Prog8, p<0.027). However, the exploration time of the familiar object was increased significantly in +Prog8 animals, when compared to familiar object exploration in the Pre-T8 group (p<0.035).
Figure 5.7 - Glial Fibrillary Acidic Protein Expression in Preterm Neonatal Guinea Pig Brains following Postnatal Progesterone Treatment
Density of glial fibrillary acidic protein (GFAP) immunoreactivity was measured in the (A) external capsule/CA1 region of the hippocampus and (B) sub-cortical white matter in male and female neonates. Density of staining was quantified in term (open bars), preterm (Pre-T8; grey bars) and preterm progesterone treated (+Prog8; hashed bars) and expressed as mean percent area coverage ± S.E.M (n=4-6). No significant differences in immunoreactivity were identified between treatment groups or neonatal sexes.

Figure 5.8 - Microtubule Associated Protein 2 Expression in Preterm Neonatal Guinea Pig Brains following Postnatal Progesterone Treatment
Density of microtubule associated protein-2 (MAP2) immunoreactivity was measured in the (A) CA1 region of the hippocampus and (B) cortex at the pial surface in male and female neonates. Density of staining was quantified in term (open bars), preterm (Pre-T8; grey bars) and preterm progesterone treated (+Prog8; hashed bars) and expressed as mean percent area coverage ± S.E.M (n=4-6). No significant differences in immunoreactivity were identified between treatment groups or neonatal sexes.
Figure 5.9 - Novel Object Recognition by Preterm Progesterone Treated Neonates

The time spent (in seconds) by preterm (Pre-T8) and preterm progesterone treated (+Prog8) neonates spent exploring the familiar (black bars) and novel (grey hashed bar) objects in the novel objection recognition trial T2. Animals in the Pre-T8 and +Prog8 groups spent greater time exploring the novel object when compared to the familiar object (p<0.05). The exploration of the familiar object only was significantly greater in +Prog8 animals compared to familiar object exploration by Pre-T8 animals. Asterisks indicate significant differences (p<0.05).

Table 5.2 - Open Field Activity and Novel Object Recognition in Preterm Neonates with Progesterone Treatment

<table>
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<tr>
<th></th>
<th>Litters</th>
<th>Time Active (sec)</th>
<th>Squares Transited</th>
<th>Inner Zone Entries</th>
<th>Time Active (sec)</th>
<th>Familiar Exploration (sec)</th>
<th>Novel Exploration (sec)</th>
<th>Discrimination Ratio</th>
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<td>Pre-T8</td>
<td>10</td>
<td>36.9 ± 5.93</td>
<td>115.0 ± 13.75</td>
<td>3.1 ± 0.54</td>
<td>22.2 ± 3.30</td>
<td>2.4 ± 0.60</td>
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<td>130.6 ± 19.66</td>
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<td>0.6 ± 0.17</td>
</tr>
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<td>+Prog8</td>
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<td>105.3 ± 16.53</td>
<td>3.5 ± 0.67</td>
<td>22.1 ± 3.39</td>
<td>5.9 ± 1.52</td>
<td>9.3 ± 1.27</td>
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<td>23.4 ± 5.39</td>
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<td>0.4 ± 0.15</td>
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<tr>
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<td>112.0 ± 29.45</td>
<td>4.4 ± 1.08</td>
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<td>5.6 ± 1.12</td>
<td>8.0 ± 1.30</td>
<td>0.2 ± 0.09</td>
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</table>

Pre-T8, Preterm control vehicle treated; +Prog8, Preterm progesterone treated; postnatal day 8. Discrimination Ratio= (novel-familiar)/time.
5.5 DISCUSSION

A key finding of this study was the ability of postnatal administration of progesterone to enhance the endogenous synthesis of allopregnanolone in the preterm guinea pig brain. This finding demonstrates the capacity of the preterm brain to synthesise neuroactive steroids when sufficient precursors are made available. Allopregnanolone concentrations were augmented in the preterm brain despite the finding that hippocampal expression of the allopregnanolone synthetic enzyme 5αR1 was reduced. This finding suggests that precursor availability may be the rate-limiting step for the synthesis of neuroactive steroids in the preterm brain rather than reduced 5αR expression. However, the type 2 isoform of 5αR has previously been reported to be the developmentally regulated isoform of the enzyme, with increasing expression in the fetal brain with advancing gestation (Poletti, Negri-Cesi et al. 1998). The type 2 5αR isoform has also been shown to be upregulated in the mid-late gestation fetal sheep brain in response to reduced umbilical cord blood flow (Nguyen, Billiards et al. 2003). In addition, results in preterm guinea pigs, presented in Chapter 4, identified a significant reduction in the brain expression of 5αR2 at 24 hours after delivery, compared to fetal animals (see Fig 4.5), with no differences in 5αR1 expression across any of the groups. 5αR2 has therefore been suggested as having greater importance than the type 1 isoform in relation to the synthesis of allopregnanolone in the immature brain. However, in the current study, the changes in 5αR1 expression in the hippocampus, suggest that this isoform may also be affected by perinatal events including birth and the neuroendocrine changes accompanying it. This may indicate the potential importance of both enzymes in synthesising allopregnanolone in the developing preterm brain in the absence of progesterone replacement therapy. This study also shows that, along with changes in
steroidogenic enzyme expression, the supply of progesterone remains an essential regulator of allopregnanolone concentrations within the immature brain following preterm birth.

In contrast to the finding in the brain, plasma steroid concentrations showed sex-specific changes following progesterone treatment. It was found that progesterone concentrations, measured in plasma collected at PND8, were significantly elevated in male +Prog8 animals. However, in female neonates, plasma progesterone concentrations were increased in the +Prog8 group, despite the direct administration of progesterone. One potential explanation for these sex-specific changes in plasma progesterone concentrations may be differences in steroid metabolism between male and female neonates. Adding weight to this explanation is the finding that plasma concentrations of allopregnanolone also differed in male and female neonates, with +Prog8 females but not males having elevated plasma allopregnanolone concentrations. Sex differences in adult neurosteroid concentrations have been reported only in relation to increases in female sex steroids during the menstrual cycle, with no differences in serum progesterone or allopregnanolone between adult males and women in the follicular phase of the menstrual cycle (Genazzini, Petraglia et al. 1998). In the rat, cerebral cortex concentrations of allopregnanolone demonstrate sexual dimorphism at PND25 and not in the early postnatal period (Grobin and Morrow 2001). Additionally, as outlined above, unlike concentrations in the plasma, brain concentrations of allopregnanolone in the current study showed no sex-specific differences when measured at PND8 and previously at 24hrs (Fig 4.4). The finding that allopregnanolone concentrations were elevated in the plasma of female neonates that received progesterone but not in males supports the sex-specific metabolism of progesterone in preterm animals. This does not discount the presence of differences in the expression or activity of
other enzymes involved in the conversion of progesterone into steroid hormone metabolites other than allopregnanolone. The mechanisms involved in the regulation of neuroactive steroid synthesis in the preterm infant and contribution of plasma steroids to brain neurosteroid supply are still to be elucidated. These potential sex-differences in the conversion or elimination of circulating neuroactive steroids have important implications on the use of exogenous steroid replacement in preterm infants and their mothers during pregnancy, particularly given the increased risk of poor outcomes present in male preterm neonates. This finding also confirms that the ability of the neonatal brain to synthesise allopregnanolone is not dependent on neonatal adrenal supply even after preterm birth. Supporting previous studies of neurosteroids in fetal sheep following adrenalectomy and hypophysectomy that showed independent regulation of neurosteroid synthesis and steroidogenic enzyme expression between the fetal brain and adrenals (Nguyen, Ross Young et al. 2004).

An additional endocrine effect of postnatal progesterone replacement identified in the current study was significantly elevated concentrations of plasma cortisol in the +Prog8 animals compared to both the term and the control preterm (Pre-T8) groups. It is known that the neonatal adrenal is able to synthesise cortisol from progesterone (Villee and Loring 1965), the increased supply of progesterone therefore is likely to have contributed to enhanced cortisol production by the neonatal adrenal in progesterone treated animals. The finding of elevated cortisol concentrations in these animals, however, raises important questions about the effects of glucocorticoids during early life on brain development and neuroendocrine programming. In addition, to aid fetal lung maturation, pregnant guinea pigs in this study received antenatal betamethasone treatment prior to scheduled c-section delivery, which also has the potential to influence postnatal steroid synthesis with known effects of repeated glucocorticoid
exposure on reducing the expression of neurosteroidogenic enzymes in the fetal guinea pig brain (McKendry, Palliser et al. 2010). It has also been shown that antenatal steroids can transiently suppress cortisol production in the postnatal period in preterm infants (Poggi Davis, Townsend et al. 2004), although this suppression does not persist long-term (Ballard, Gluckman et al. 1980; Ng, Wong et al. 1997). Data from our laboratory also suggests that exposure to prenatal stress, resulting in increased endogenous production of cortisol in the fetus, may also affect neurosteroid concentrations in the fetal brain (Bennett, Palliser et al. 2012). The previous study, presented in Chapter 4, also identified elevated plasma cortisol concentrations in preterm animals compared to their term-delivered counterparts, both with and without progesterone treatment (see Fig 4.3C). Taken together, the relatively modest (approximately 25%) increase in cortisol following progesterone treatment may be less likely to influence lung or brain function than other stressors or exogenous glucocorticoid sources in the perinatal period. Potential increases in the synthesis of neuroactive steroid metabolites of cortisol, such as THDOC (Dodds, Taylor et al. 1997) may also have protective and trophic effects on the developing brain (Reddy and Rogawski 2002).

As well as altered neuroendocrine patterns, functional effects of progesterone treatment were also identified in this study. Whilst allopregnanolone is known to have sedative affects (Barbaccia 2004; van Broekhoven, Backstrom et al. 2007), no changes in overall activity were associated with the increases in allopregnanolone concentrations that resulted from progesterone treatment in preterm guinea pigs. In the novel object recognition trial, both Pre-T8 and +Prog8 animals demonstrated a significant preference in exploration time of the novel object. However, the time +Prog8 animals spent exploring the familiar object was still significantly higher than the time spent by Pre-T8 animals. This finding
suggests that memory and learning behaviours may be altered by this
treatment, potentially by the inhibitory effects of progesterone and
allopregnanolone on short-term memory formation in the presence of high
concentrations of these steroids. The neonatal administration of
neuroactive steroids can alter programmed responses to anxiety and stress
and alters drug actions in adolescent and adult rats. The effects of
neurosteroid administration, however, differ greatly with the models used,
the timing of administration and the age at behavioural testing. For
example, postnatal neurosteroid treatment potentiates the affects of later
neurosteroid treatment in reducing activity in adult rats, but has no direct
effects on behaviour (Darbra and Pallares 2011). Neurosteroid effects are
also influenced by programming of neuronal signalling and organisation,
with localisation of GABAergic neurons altered in the prefrontal cortex by
postnatal allopregnanolone treatment (Grobin, Heenan et al. 2003; Grobin,
Gizerian et al. 2006). However, these previous studies examining early
neonatal neurosteroid treatment have mostly been performed in neonatal
rat models. The developmental course of the rat brain varies greatly to the
timing of brain maturation in the guinea pig, with particularly immature
brain development in the rat at birth compared to the precociality and
prenatal brain development in guinea pigs (Dobbing and Sands 1979).
This is an important distinction that should be recognised in the
interpretation of neurosteroid effects in the immature brain in different
animals models. In addition to this, there is evidence that the GABA\textsubscript{\lambda}
receptor can have excitatory properties in the immature brain, prior to a
developmentally regulated switch to inhibitory actions, which is thought to
occur in the rat brain from around postnatal days 12-13 (Ben-Ari, Gaiarsa
et al. 2007). In contrast to this, the GABAergic switch occurs prenatally in
the guinea pig, with inhibitory signalling in the fetal guinea pig from mid-
late gestation at approximately 55 days gestational age (unpublished data,
personal correspondence H. Parkington, Monash University). The relative expression of GABA<sub>A</sub> receptor subunits may also influence the sensitivity of the immature brain to neuroactive steroid actions, particularly related to steroid-mediated feedback that can influence this expression (Smith, Shen et al. 2007). Studies performed in our laboratory also demonstrated marked differences in the effects of the postnatal withdrawal of progesterone via preterm birth and the reduction allopregnanolone concentrations in utero caused by administration of finasteride. Inhibiton of allopregnanolone synthesis by finasteride significantly reduced MBP expression in the late gestation fetal guinea pig brain (Kelleher, Palliser et al. 2011). However, deficits in MBP expression following preterm birth are less evident at term-equivalent age suggesting the presence of compensatory or catch-up growth after preterm birth that were not present in utero or with the secondary insult of IUGR. Differences in the gestational age of treatment and effects of finasteride on the degree of withdrawal of progesterone and allopregnanolone concentrations may also account for differences in effects between the fetal and preterm neonatal studies. The relative gestational age and timing of postnatal neurosteroid treatment (or disruption of steroid concentrations following preterm birth) may therefore modulate the effects these steroids on brain development and vulnerability to brain injury of these neonates. Neonatal health was monitored and scored during the postnatal period, with preterm neonates showing reduced scores compared to term neonates. Preterm neonates also exhibited episodes of apnea, irregular respiration, muscle spasticity and reduced activity that were not present in term animals. Despite previous observations of potentially positive effects of progesterone treatment on the survival of preterm neonates at 24hrs in this guinea pig model (discussed in chapter 4), no differences in survival or neonatal scoring were identified in preterm neonates at PND8 that
received either progesterone or vehicle treatments. However, early clinical studies, examining the replacement of placental steroids in human preterm neonates have reported some promising results of progesterone treatment on respiratory outcomes and reduced severity of RDS in preterm human infants (Dodd, Crowther et al. 2009; Trotter, Kipp et al. 2009). Whilst the effect of postnatal progesterone treatment on lung development was not a focus of the current study (representative micrographs of lung histology from term and preterm neonates are shown in Appendix B), closer examination of lung histology and function in these neonates may elucidate mechanisms of progesterone action in the neonatal lung. These actions may also be important in identifying factors that influence survival in this model, the significance of progesterone treatment on survival and potential differences present in neonates at 24 hours and PND8.

In addition to changes in steroid concentrations in preterm neonates, neuropathological changes were also examined. Previous studies of in vitro cerebellar slice cultures from neonatal rats showed enhanced expression of MBP with progesterone treatment and its metabolism into allopregnanolone via both PR and GABA_A receptor mediated effects (Ghoumari, Ibanez et al. 2003). The current study found that, in female neonates only, progesterone treatment was associated with reduced MBP staining in the CA1 region of the hippocampus compared to term females. However, MBP immunoreactivity in this region was similar in both preterm progesterone (+Prog8) and preterm vehicle treated (Pre-T8) animals. Additionally, whilst blocking allopregnanolone synthesis, has been shown to increase brain injury and cell death in fetal models, particularly in the presence of insults such as asphyxia/hypoxia (UCO in the fetal sheep), the trophic effects of increasing allopregnanolone-mediated inhibitory signalling may alter the pathways of some processes such as myelination, as
well as affecting the signals that control developmental plasticity and normal brain growth.

A degree of postnatal growth restriction may however, have confounded these findings, as percent change in body weight in preterm neonates from birth to PND8 was minimal. This was despite intensive care and regular feeding. Organ weights also remained significantly reduced in both Pre-T8 and +Prog8 groups compared to term organ weights at 24 hours age, indicating that extensive postnatal catch-up growth had not occurred following preterm delivery, at term-equivalent age. In addition, unlike Pre-T8 female neonates, +Prog8 female neonates (excluding those that did not survive the experimental period) had similar body weights compared to female neonates delivered at term. This raises the potential effect of survivor bias on neonatal outcomes measured in this study, in which those neonates that were potentially more likely to demonstrate changes in the measured outcomes were excluded from the study. The lack of major differences in the neuropathological markers examined may also have been influenced by the exclusion of neonates that did not survive the experimental period and were therefore more severely affected by the preterm birth experimental paradigm used. Alternatively, neuropathological changes may be less likely to be present in animals delivered at this relatively late gestation. Further examination of the non-surviving animals, with reference to brain neuropathology and neurosteroid concentrations and synthesis may provide valuable information about the role of neurosteroids in vulnerable preterm infants.

This study has established the ability of the preterm guinea pig brain to endogenously synthesise allopregnanolone from exogenous progesterone sources with important implications on the use of steroid therapies in the perinatal period, particularly the use of progesterone and neuroactive
steroids in preterm infants and how this may influence long-term neurobehavioural outcomes and neurodevelopment.
Chapter Six

CEREBELLAR DEVELOPMENT IN THE NEONATAL GUINEA PIG FOLLOWING PRETERM BIRTH & PROGESTERONE REPLACEMENT THERAPY

The cerebellum is an important synthetic site of progesterone and its neuroactive steroid metabolites. In turn, these steroids have key actions on cerebellar and Purkinje cell development. This chapter examines postnatal effects of progesterone replacement on aspects of cerebellar development, myelination and Purkinje cells following preterm birth.

6.1 ABSTRACT

The effect of preterm birth on the development of the cerebellum and the consequences of this altered development on sensorimotor outcomes in premature infants is becoming more widely recognised. The cerebellum and Purkinje cells in particular, are major sites of neuroactive steroid synthesis, with the production of progesterone and allopregnanolone shown to occur within the cerebellum during fetal and neonatal life. There is evidence for progesterone's role in promoting dendrite and synapse formation and myelination within the developing cerebellum.

The effects of preterm birth and postnatal progesterone replacement therapy on the cerebellum were examined using the preterm neonatal guinea pig model as described in Chapters 4 and 5. Morphological differences were assessed in term and preterm (Pre-T) cerebella at 24hrs after delivery to determine cerebellar maturity at birth. Postnatal
development and effects of progesterone replacement were examined in preterm delivered neonates at term-equivalent age with vehicle (Pre-T8) or progesterone treatment (+Prog8). External granular layer thickness showed immaturity in Pre-T animals with evidence of maturation by term-equivalent age (Pre-T8 and +Prog8). Myelination was significantly reduced in Pre-T animals compared to term and remained lower in Pre-T8 neonates but not +Prog8 animals. Both Pre-T8 and +Prog8 animals showed evidence of reduced Purkinje cell area and soma perimeter, potentially indicating a detrimental postnatal effects as these findings were not present in Pre-T animals. The preterm neonatal model used in these studies demonstrates immature cerebellar development at birth, with altered postnatal growth. Initial findings of progesterone replacement in this study warrant further investigation to elucidate the actions and synthetic mechanisms of postnatal steroid replacement in the preterm cerebellum.

6.2 INTRODUCTION

Preterm birth is a significant factor that contributes to perinatal brain injury, including damage and developmental deficits in the cerebellum (for review, see (Biran, Verney et al. 2012). The cerebellum undergoes a large degree of growth and maturation during mid-late gestation with the highest rates of cell proliferation occurring in the external granular layer between 28 and 34 weeks gestation (Abraham, Tornoczky et al. 2001). Postnatal growth is also of great importance in the cerebellum, with increases in volume of 240% in the first year of life (Knickmeyer, Gouttard et al. 2008). Perinatal insults that occur at this time of rapid cerebellar growth may convey particular vulnerability of the cerebellum to injury and disrupted development. Imaging studies in preterm infants has shown reduced cerebellum volumes when compared to neonates delivered at term
These reductions in cerebellar hemispheric volumes, loss of white matter, increased ventricle size and changes to cerebellar morphology that occur symmetrically are suggested to result from disruptions in normal cerebellar development following preterm birth (Messerschmidt, Brugger et al. 2005). Histopathological studies in human preterm brains has also identified decreased thickness of the external granular layer, disruption of internal granular layer growth, reduced expression of Bergmann glial cells and a reduction in the expression of the important developmental signalling protein, sonic hedgehog, in the cerebellum with postnatal development after preterm birth (Haldipur, Bharti et al. 2011). Cerebellar malformations and injury have also been associated with damage in the contralateral cerebral hemisphere, in regions such as the prefrontal cortex and sensorimotor cortex, indicating the importance of neuronal activation and signalling between the cerebrum and cerebellum to normal brain development (Limperopoulos, Chilingaryan et al. 2010; Boldoc, Du Plessis et al. 2011). Models of in utero asphyxia in fetal sheep have also demonstrated the vulnerability of the immature cerebellum. Lipid peroxidation products and pyknosis are increased in Purkinje cells and other regions of the cerebellum at 24hrs after umbilical cord occlusion (Castillo-Melendez, Chow et al. 2004).

An important aspect of cerebellar function is the synthesis of neuroactive steroids, including allopregnanolone, which occurs in Purkinje as well as other cerebellar cells. This neurosteroid synthetic capacity of the cerebellum has been assessed in a number of in vitro and in animal studies. Adult rat cerebellar granule cells have been shown to synthesise allopregnanolone from progesterone in culture, with effects on GABA<sub>A</sub> receptor subtype expression (Follesa, Serra et al. 2000). Allopregnanolone has also been shown to be present within the fetal cerebellum and the
enzymes responsible for its synthesis are present in Purkinje cells during fetal life (Ukena, Usui et al. 1998; Petratos, Hirst et al. 2000; Tsutsui 2006). Evidence that allopregnanolone concentrations increase in the cerebellum following brief fetal asphyxia (Nguyen, Yan et al. 2004) also suggests the involvement of allopregnanolone in a cerebellar response to this acute insult during gestation. The cerebellum is therefore an important site of neuroactive steroid synthesis, which can be affected by insults during gestation and early life.

There is also evidence for specific neuroprotective and developmental actions of allopregnanolone in the cerebellum. Allopregnanolone administration reduces cell death in cultured adult rat Purkinje cells following ischemic insult and oxygen-glucose deprivation (Ardeshiri, Kelley et al. 2006; Kelley, Taguchi et al. 2008). Allopregnanolone also influences cell death in the immature brain. In late-gestation fetal sheep, finasteride administration, which blocks allopregnanolone synthesis, increases the basal level of apoptosis within the molecular and granule cell layers of the cerebellum (Yawno, Yan et al. 2007). Finasteride treatment also increases the expression of reactive astrocytes in the cerebellum (Yawno, Hirst et al. 2009), a potential response to injury associates with disrupted neurosteroid supply. Following UCO, finasteride treatment further exacerbates cell death in the granular layer of the cerebellum (Yawno, Yan et al. 2007).

In addition to the action of allopregnanolone, its major precursor, progesterone, also has potentially important trophic effects within the developing cerebellum. Progesterone was shown to promote dendritic growth of Purkinje cells in neonatal rat slice cultures, with these positive effects abolished by the progesterone receptor antagonist, mifepristone (Sakamoto, Ukena et al. 2001). In vivo studies in the neonatal rat demonstrated that progesterone administration, from postnatal days 3 to 7, into reticulospinal fluid around the posterior vermal lobule of the
cerebellum, resulted in increases in dendrite area and differentiation, increases in synapse density, as well as increases in perimeter of Purkinje cells (Sakamoto, Ukena et al. 2001). These previous studies demonstrate the organising and developmental actions of progesterone, at this stage of maturity, in the neonatal rat cerebellum.

We hypothesise that the premature loss of placental steroids may contribute to developmental changes in the cerebellum that adversely affect cerebellar function, including the subsequent synthesis of neuroactive steroids within cerebellar cells. The main objective of this study was to assess cerebellar maturity in the neonatal guinea pig after preterm delivery and to determine potential effects of postnatal progesterone replacement on cerebellar development. In order to assess these changes, immunohistochemical analyses Purkinje cells and cerebellar morphometry were performed on cerebellar sections from term and preterm (Pre-T) neonates at 24 hours after delivery, along with postnatal development in preterm neonates at term-equivalent age that received either vehicle injections (Pre-T8) or postnatal progesterone therapy (+Prog8).

6.3 METHODS

Tissues collected for analysis in this chapter were obtained from animal experiments previously described in Chapters 4 and 5. Refer to these previous chapters for details of preterm and term deliveries, neonatal care, drug administration, animal weights and measurement of steroid concentrations.

6.3.1 Tissue Collection

Neonates were killed, brains rapidly removed and cerebellums dissected from the cerebrum. Cerebellums were halved sagittally and one half
immersion fixed in formalin before paraffin embedding, sectioning and immunohistochemical staining.

All measurements were performed on mid-sagittal sections of the cerebellum in lobules X and VIII (Fig 6.1A), chosen for their different developmental profiles, with lobule X developing earlier in gestation and maturation of lobule VIII occurring later (Tolcos, Bateman et al. 2011). Photomicrographs were coded and researchers were blinded to treatment groups until analysis was completed.

Figure 6.1 - Lobules and Layers in the Guinea Pig Cerebellum
(A) Sagittal, thionin-stained section through the vermis of the cerebellum, with the lobules analysed, VIII and X, indicated. (B) Thionin-stained section, illustrating layers of the cerebellum (scale bar = 200 \( \mu \text{m} \)). EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; PCL, Purkinje cell layer; WM, white matter; dWM, deep white matter. Reproduced from Tolcos, et al. 2011, with permission.
6.3.2 Determination of Cerebellar Lobule Thickness

In each of lobules VIII and X, cerebellar morphometric measurements were performed on cresyl violet stained mid-sagittal sections. Cerebellar cell layers are shown in Figure 6.1B, with the internal granular layer (IGL), molecular cell layer (ML) and external granular layer (EGL) measured for each animal on micrographs, taken at 20x magnification, from two serial sections. The widths of the cell layers were determined using ImageJ v1.40 software and the proportion of the total lobule thickness calculated. The mean and SEM were calculated for each treatment group and analysed by one-way ANOVA.

6.3.3 MBP Staining of Cerebellar White Matter Tracts

In order to quantify mature myelin, immunohistochemical staining for MBP (as outlined in detail in section 2.10.3.a) was performed on mid-sagittal sections of perinatal guinea pig cerebellums. Analysis of per-cent MBP staining in the deep white matter of cerebellar lobules X and VIII was carried out, for each animal, on two serial sections across two fields of view for each section at 4x magnification. The mean and SEM were calculated for each treatment group and analysed by one-way ANOVA.

6.3.4 Calbindin Staining for Purkinje Cell Measurements

Purkinje cells in mid-sagittal cerebellar sections were stained for calbindin (see section 2.10.3.e) for morphometric analysis and cell counting in lobules VIII and X. Purkinje cells were counted manually and length of Purkinje cell layer measured using ImageJ software (photomicrographs at 20x magnification taken from two serial sections) to determine overall number and density of cells within the Purkinje cell layer. Purkinje cell soma area and perimeters were also calculated for a subset of animals using ImageJ software analysis on photomicrographs at 40x magnification.
6.4 RESULTS

6.4.1 Cerebellar Growth and External Granular Layer Thickness
All ten lobules were present in preterm and term cerebella. As EGL thickness reduces with continuing cerebellar development, examination of this cell layer was carried out as a measure of maturation in Term, Pre-T, Pre-T8 and +Prog8 animals. As expected, Pre-T animals had significantly greater EGL thicknesses when compared to term animals, as measured in lobule VIII (p <0.05; Figure 6.2A & B). Following a period of postnatal development (PND8), animals in the Pre-T8 and +Prog8 groups, showed significant reductions, in EGL thicknesses in both lobules VIII (Fig 6.2B) and X (Fig 6.2C), compared to those in Pre-T animals at only 24hrs after delivery (p<0.05). At term-equivalent age, preterm EGL thickness was similar to that in term delivered animals. In contrast to these differences in EGL thickness with preterm delivery, commensurate increases in the thickness of the IGL (Fig 6.2A) were not present in these groups either by gestational age at birth or postnatal age (data not shown).

6.4.2 MBP in Cerebellar White Matter Tracts
MBP immunoreactivity was determined in the deep white matter tracts of lobules VIII and X, as shown in representative photomicrographs for lobule X in Figure 6.3A. Qualitatively, the micrographs show less MBP staining in the white matter tract and reduced projection of myelin fibres into the IGL in preterm cerebella, compared with term. When quantified, this MBP staining was significantly reduced between Term and Pre-T8 animals in lobule VIII (p<0.05; Fig 6.3B) and between Term and both the Pre-T and Pre-T8 groups in lobule X (p<0.05; Fig 6.3C). Differences in MBP staining in the +Prog8 group did not reach statistical significance when compared to term expression levels across both lobules.
Figure 6.2 - Cerebellar Lobule Development and Extracellular Granular Layer Thickness in Preterm and Progesterone Treated Preterm Guinea Pigs

(A) Representative photomicrographs of lobule VIII in (i) term, (ii) preterm, (iii) preterm term-equivalent and (iv) preterm term-equivalent progesterone treated neonates showing lobule organisation and cerebellar layers (cresyl violet and MBP stained sections; x4 magnification; scale bar =500μm). The thickness of the external granular layer in (B) lobule VIII and (C) lobule X is indicated as a proportion of total lobule width in term (open bars), preterm (Pre-T; grey bars), preterm term-equivalent (Pre-T8; black bars) and preterm term-equivalent progesterone treated (+Prog8; hashed bars) neonates. Bars express group means ± S.E.M (n=6-8). # indicates significant difference of Pre-T group and all other groups (p<0.05, B). Asterisks indicate significant differences between groups as indicated (* p<0.05, ** p<0.01, C).
Figure 6.3 - Myelin Basic Protein (MBP) Immunoreactivity in Cerebella of Preterm and Progesterone Treated Preterm Guinea Pigs

(A) Representative photomicrographs of MBP immunoreactivity in lobule X in (i) term, (ii) preterm, (iii) preterm term-equivalent and (iv) preterm term-equivalent progesterone treated neonates (MBP stained sections; x20 magnification; scale bar =100 µm). The density of MBP immunoreactivity in the white matter tract of (B) lobule VIII and (C) lobule X was measured in term (open bars), preterm (Pre-T; grey bars), preterm term-equivalent (Pre-T8; black bars) and preterm term-equivalent progesterone treated (+Prog8; hashed bars) neonates. Bars express group means ± S.E.M (n=6-8). Asterisks indicate significant differences between groups (* p<0.05, ** p<0.01).
6.4.3 Purkinje Cells

The numbers, linear density, somal area and perimeters of Purkinje cells in lobules VIII and X were also determined in sections stained with the Purkinje cell marker, Calbindin (shown in Fig 6.4A & B). There were no differences in Purkinje cell number or the linear density of cells in the Purkinje cell layer in either of lobules VIII or X between any of the groups (data not shown). In lobule X only, Purkinje cell area was significantly reduced in Pre-T8 and +Prog8 groups compared to Term (p<0.05; Fig 6.4D). Additionally, Purkinje cell somal areas in lobe X were also significantly reduced in +Prog8 compared to Pre-T animals (p<0.05). No differences were present in lobule VIII (Fig 6.4C). The perimeters of Purkinje cell soma in these lobules were also measured, with reduced perimeters in lobule VIII in both Pre-T8 and +Prog8 animals compared to Term (p<0.05; 6.4E). A similar pattern was seen in lobule X, with the Purkinje cell perimeters were significantly reduced in animals at term-equivalent age in the Pre-T8 and +Prog8 groups compared to both Term and Pre-T groups (p<0.05; 6.4F).

6.5 DISCUSSION

The major findings of this study of the cerebella of preterm neonatal guinea pigs were the changes in morphometric markers of cerebellar development that indicate immaturity at preterm delivery in this model and altered development in the postnatal period. The areas and perimeter lengths of Purkinje cell soma in lobule X were found to be reduced in Pre-T8 and +Prog8 animals, compared to those soon after birth in preterm animals, suggesting an effect of postnatal development on Purkinje cell growth and development in the preterm guinea pig. As somal size has been correlated with dendritic arborisation in Purkinje cells and other neurons.
Figure 6.4 - Calbindin Staining in Purkinje Cells in Cerebella of Preterm and Progesterone Treated Preterm Guinea Pigs

Photomicrographs of calbindin staining of Purkinje cells in lobule VIII at (A) low magnification showing Purkinje cell layer organisation (cresyl violet counterstain; x4 magnification; scale bar =500µm) (B) At high magnification, calbindin positive Purkinje cell soma and dendrites are shown (x40 magnification; scale bar =50µm). Purkinje cell soma area in (C) lobule VIII and (D) lobule X and Purkinje cell soma perimeters in (E) lobule VIII and (F) lobule X were measured in calbindin-positive cells. Group means ± S.E.M (n=6-8) are shown for term (open bars), preterm (Pre-T; grey bars), preterm term-equivalent (Pre-T8; black bars) and preterm term-equivalent progesterone treated (+Prog8; hashed bars) neonates. Asterisks indicate significant differences between groups (* p<0.05, ** p<0.01).
(Andrews, Li et al. 1994; Jacobs, Driscoll et al. 1997; Rees, Loeliger et al. 2009), the reduction in somal size observed in the current study may have important implications on cerebellar function and signalling. This finding may also be significant to postnatal neuroactive steroid supply, as the Purkinje cell is an important site of progesterone and allopregnanolone synthesis (Agis-Balboa, Pinna et al. 2006; Tsutsui 2006). In fetal sheep, expression of the neurosteroidogenic enzyme 5αR2 was localised to the Purkinje cell soma, indicating that a reduction in the size of cell bodies of Purkinje cells may impact on enzyme expression and therefore synthetic capacity (Petratos, Hirst et al. 2000). Examination of the cerebellar expression of 5αR and other steroidogenic enzymes in this model may be useful in providing new information regarding steroid synthesis in the immature cerebella of preterm neonates. Previous studies have demonstrated postnatal changes in 5αR enzyme expression within Purkinje cells (Petratos, Hirst et al. 2000), which have the potential to be influenced by endocrine changes after birth and the replacement of placental steroids in the postnatal period.

Purkinje cell perimeters were also measured as a marker of development in the cerebellum. The finding that somal perimeter was reduced in term-equivalent preterm neonates with progesterone treatment, is in contrast to findings in the neonatal rat in which Purkinje cell somal perimeters were increased with progesterone treatment (Sakamoto, Ukena et al. 2001). As discussed previously, the development of the rat brain, in particular the mechanisms of neuroactive steroid signalling, may be quite dissimilar to that in the guinea pig at the developmental ages in this study. This may account for the potentially different actions of progesterone therapy on cerebellar development in the two species. Additionally, in the studies performed by Sakamoto et al, the direct administration of progesterone into the reticulospinal fluid and effects this may have had on steroid
metabolism and dose may explain the different effects noted in comparison to peripheral administration in the current study. Evidence that allopregnanolone concentrations in the cerebrum were elevated following postnatal progesterone administration (Figures 4.4 and 5.4) suggests a similar increase may be present in the cerebellum, which is known to be a region with some of the highest concentrations of progesterone and its metabolites in the adult brain (Bixo, Andersson et al. 1997). However, the changes in Purkinje cell size that were identified in the current study have potential to affect steroid synthesis and concentrations within the cerebellum. As mentioned above, synthesis of neuroactive steroids may be regulated differently in the cerebellum compared to other regions of the brain. The administration of endotoxin in fetal sheep increases allopregnanolone concentrations in a number of brain regions; however, increased concentrations were not present in the cerebellum (Billiards, Walker et al. 2002). The direct measurement of progesterone and allopregnanolone concentrations in the cerebellum may therefore be an important consideration for the development of neuroactive steroid replacement therapies that are likely to influence this region, given the developmental changes identified in this study and as an important site of synthesis. The contribution of altered cerebellar development in the aetiology of life-long cognitive and sensorimotor deficits is also gaining more clinical attention (Alin, Matsumoto et al. 2001). The functional effects of progesterone treatment on exploratory behaviours in preterm neonates, identified in Chapter 5 (Fig 5.9) may also be influenced by actions of progesterone in the cerebellum. Progesterone administration has previously been shown to suppress patterns of individual Purkinje cell firing associated with locomotion in adult rats (Smith, Woodward et al. 1989).
This study also demonstrated altered myelination in the preterm guinea pig cerebellum, with reduced myelination in preterm animals at 24 hours after birth that remained lower at term-equivalent age. Further studies of oligodendrocyte markers and neuroactive steroid receptors within these preterm cerebella may identify a role for progesterone in promoting myelin maturation or the outgrowth of myelinated axons. Studies in slice cultures of neonatal rat cerebellar have shown increases in MBP expression mediated by both progesterone and allopregnanolone signalling at the PR and GABA_\_ receptor, respectively (Ghoumari, Ibanez et al. 2003). The findings in Chapters 4 and 5, that identified reduced myelination in the hippocampus of preterm neonates and with progesterone treatment, suggests that myelination processes are affected by premature birth in this preterm model and that changes in progesterone and neuroactive steroid concentrations have the potential to influence these processes. More detailed examination of cerebellar myelination with reference to progesterone treatment is needed to elucidate the effects of neurosteroid replacement on the cerebellum in this in vivo model. Potential effects of endogenous and synthetic steroids on myelination in the cerebellum should be considered particularly when being used clinically during the perinatal period.

Along with being a major site of neuroactive steroid synthesis, the cerebellum is an important region in the aetiology of neuromotor disorders and developmental deficits associated with preterm birth. Initial findings of this study do not demonstrate strong effects of progesterone replacement therapy on guinea pig cerebella at the ages examined. However, given the cerebellum’s role as a site of synthesis of neuroactive steroids, the effects of neuroactive steroids within the developing cerebellum may warrant further investigation to elucidate the actions and synthetic mechanisms of postnatal steroid replacement within the preterm
brain. The developmental changes in the cerebellum of preterm guinea pigs in this model highlight the importance of the development of new and effective therapies that target perinatal brain injury.
Chapter Seven

DISCUSSION & CONCLUSIONS

7.1 DISCUSSION
The perinatal period is a time of extraordinary growth and involves a number of critical stages in the development and organisation of the immature brain. Alterations to normal developmental processes during this time can result in permanent neurological disability and developmental deficits. The studies outlined in this thesis examined the role of neuroactive steroids in the developing brain and the effects of disruptions in their supply during the perinatal period. The findings of these studies validate the guinea pig as an animal model for the study of neuroactive steroid actions in the perinatal brain. In particular, it has been demonstrated that reduced allopregnanolone concentrations during late gestation in the guinea pig, is associated with impaired myelination and increases in markers of brain injury. In addition, we have established a preterm neonatal animal model in the guinea pig that displays altered steroid and allopregnanolone concentrations and an immature stage of brain development, supporting the need for examination of the role of neuroactive steroids in postnatal brain development in this group vulnerable to perinatal brain injury. Finally, we have shown that postnatal replacement of progesterone augments brain allopregnanolone concentrations in neonates delivered preterm, which may result in functional changes in preterm neonatal behaviour at term-equivalent age.
7.1.1 Allopregnanolone in the Late Gestation Fetal and Preterm Neonatal Guinea Pig

The studies presented in this thesis demonstrate alterations in fetal and neonatal allopregnanolone concentrations in the guinea pig following birth and with complications during pregnancy. As hypothesised, birth resulted in a postnatal loss of allopregnanolone supply in the term neonatal guinea pig. Interestingly, at 24hrs after birth, neonates delivered prematurely did not display the same rapid fall in plasma allopregnanolone that was observed in term neonates. Significantly, however, at 24hr after birth, it was found that the concentration of allopregnanolone in the brains of both preterm and term neonates was significantly reduced. This finding demonstrates the disruption to neonatal allopregnanolone and steroid concentrations that occurs with birth and in the case of preterm delivery, the potentially premature loss of neuroactive steroids in the developing brain. Additionally, in preterm guinea pigs at 8 days postnatal age (term-equivalent gestational age), both plasma and brain concentrations of allopregnanolone remain lower than in utero concentrations at this gestational age. This demonstrates the prolonged reduction in neurosteroid supply that occurs following preterm birth, indicating the premature disruption of neurosteroid supply in the postnatal period when high in utero levels of neuroactive steroids would normally be present.

The guinea pig is a useful model for the study of neuroendocrine changes related to perinatal progesterone supply. A number of important changes occur in the endocrine system of mother and infant during the transition from pregnancy and fetal life to neonatal life. Both maternal and fetal concentrations of progesterone increase over gestation due to increases in supply from the placenta. Many species experience a decline in progesterone concentrations just prior to birth; however, both humans and guinea pigs maintain progesterone concentrations until birth (Challis, Heap
et al. 1971). Instead birth occurs despite elevated progesterone, suggesting a mechanism for functional withdrawal of progesterone. This is caused by changes in progesterone receptor expression and related to the onset of labour in women (Zakar and Hertelendy 2007).

The finding that allopregnanolone concentrations were lower in the brain but were not significantly lower in the plasma of preterm neonates at 24hrs after birth suggests that allopregnanolone concentrations in the preterm brain may not be directly dependent on supply of allopregnanolone from the plasma but potentially on peripheral precursors, pregnenolone or progesterone. It has been shown in previous studies that the central supply of allopregnanolone can be maintained independent of the direct supply of peripheral allopregnanolone or neuroactive steroid concentrations. For example, adrenalectomy and ovariectomy in adult rats results in reduced plasma allopregnanolone but does affect cerebral cortex allopregnanolone concentrations (Purdy, Morrow et al. 1991). Treatment with the $5\alpha$R inhibitor finasteride in pregnant rats also results in reduced maternal concentrations of allopregnanolone, with much greater effects on brain concentrations than those in the plasma (Concas, Mostallino et al. 1998). These previous studies demonstrate the differential regulation and supply of allopregnanolone in the brain and periphery. The current study is the first to show that these differences are present in preterm neonatal animals during the postnatal period. The postnatal regulation or maintenance of neurosteroid synthesis may also be influenced by maturity, as term neonates demonstrated a loss of plasma allopregnanolone at 24hours after delivery compared to neonates delivered preterm. As with the maintenance of plasma allopregnanolone concentrations at 24hrs in preterm neonates, progesterone concentrations were also maintained compared to term animals at the same postnatal age. One explanation may be that steroid precursors, such as pregnenolone, persist in the neonatal circulation after
birth and contribute to continued substrate supply for the synthesis of progesterone and allopregnanolone. In particular, steroid conjugates, such as pregnenolone sulfate, are present in relatively high concentrations during fetal life (Riepe, Mahler et al. 2002) and may make a greater contribution to the maintenance of neurosteroid precursors at preterm age compared to those at term. Adrenal synthesis of neurosteroids may also contribute to the postnatal concentrations of allopregnanolone in the plasma, affecting peripheral levels but not necessarily contributing to neurosteroid concentrations within the brain itself (Nguyen, Ross Young et al. 2004).

The role of peripheral steroidogenesis in contributing to brain concentrations of allopregnanolone is an important question as, in addition to altered steroid concentrations following birth, the expression of the essential rate-limiting enzyme in allopregnanolone synthesis, was also found to be altered in both term and preterm neonatal brains. The finding that the expression of the type 2 isoform of 5αR was reduced postnatally, compared to the expression in fetal brains, suggests that the capacity of the neonatal brain to synthesise allopregnanolone de novo may be reduced. The postnatal withdrawal of progesterone supply from the placenta itself may directly affect the expression of 5αR in the neonatal brain as there is evidence for the presence of feedback mechanisms regulated by changes in progesterone concentration (Matsui, Sakari et al. 2002). Preterm neonates may therefore experience both a reduced substrate supply and reduced capacity to synthesise allopregnanolone, enhancing the negative effects of preterm birth on continued neurosteroid concentrations. The regulation of 5αR expression may also be affected by the presence of stressors during the perinatal period. Evidence from studies in fetal sheep that umbilical cord occlusion or umbilicoplacental embolisation increase 5αR2 expression within the fetal brain (Nguyen, Billiards et al. 2003; Nguyen, Yan et al. 2004) has been proposed as a component of an endogenous
neuroprotective response. Mechanisms within the fetal brain may enhance
the synthesis of protective neurosteroids, including allopregnanolone, in
response to injurious stimuli (Hirst, Palliser et al. 2008). Such a mechanism
and the role of allopregnanolone in it have not previously been examined
in the preterm postnatal brain. The postnatal regulation of
allopregnanolone synthesis and the differences identified in the current
studies therefore provide the first evidence for reduction in both the supply
and synthetic capacity of allopregnanolone within the preterm brain.
These findings in the postnatal preterm guinea pig brain were an extension
of the fetal study (Chapter 3), which examined aspects of neuroactive
steroid production within the fetal guinea pig brain in pregnancies
complicated by IUGR as well as the pharmacological inhibition of 5αR
activity. As expected, the chronic administration of finasteride reduced
both fetal guinea pig plasma concentrations and brain levels of
allopregnanolone, potentially mimicking some of the effects of the
postnatal withdrawal of allopregnanolone following birth. In growth
restricted fetuses, the concentrations of allopregnanolone in the brain were
similar to those measured in sham-operated control animals. However, it
was found that IUGR did result in reduced plasma concentrations of
allopregnanolone (Appendix A). This finding adds weight to the
importance of direct synthesis of allopregnanolone within the fetal brain in
maintaining allopregnanolone concentrations independently of circulating
levels. In addition, unlike studies in fetal sheep, that showed an increase in
plasma concentrations of allopregnanolone following acute periods of
hypoxia, this study, in the presence of a chronic insult (chronic placental
insufficiency resulting in IUGR) showed the opposite effect on circulating
allopregnanolone concentrations. Furthermore, in contrast to the effects of
acute UCO in fetal sheep, which increased 5αR2 expression in the cerebral
cortex (Nguyen, Yan et al. 2004) and chronic umbilicoplacental
embolisation which increased 5αR2 across all brain regions, including the hippocampus and cerebellum (Nguyen, Billiards et al. 2003), no changes in 5αR expression were identified in the brains of growth-restricted guinea pig fetuses. Interestingly, in the fetal sheep brain, allopregnanolone concentrations were elevated in association with the acute insult but not the umbilicoplacental embolisation, potentially suggesting an exhaustion of substrate availability within the brain following the chronic insult. Taken together with the current findings in the compromised fetal guinea pig, substrate supply and enzyme expression, as important regulators of fetal brain allopregnanolone concentrations, may respond differently in acute versus chronic insults during gestation and in different regions of the brain. Both the placental insufficiency model of IUGR in the guinea pig and the umbilicoplacental embolisation in the fetal sheep have the potential to impair the placental supply of pregnenolone and progesterone to the fetal brain, reducing their availability for the synthesis of allopregnanolone from these precursors. However, other mechanisms may also influence fetal brain neuroactive steroid content, as allopregnanolone concentrations can increase in the presence of chronic insults, with increased concentrations in the brains of growth-restricted fetal rats (induced by uteroplacental insufficiency) when compared to normally grown littermates (Westcott, Hirst et al. 2008). Moreover, in the current IUGR study, the potential for a transient increase in allopregnanolone, as seen after acute insults (Nguyen, Yan et al. 2004), over the latter half of gestation, cannot be ruled out. This thesis adds evidence to the effects that complications during pregnancy and endocrine changes at birth may have on neuroactive steroid concentrations in the developing brain. The complex regulation of neurosteroid responses to insults in utero and the potential for changes in the concentrations of neuroactive steroids to influence brain function and
development are potential new targets for therapeutic interventions to improve outcomes in vulnerable infants.

7.1.2 Progesterone and Allopregnanolone Replacement in Preterm Guinea Pigs

The major aim of the study presented in Chapter 5 was the examination of postnatal progesterone treatment on the restoration of brain allopregnanolone concentrations within the preterm brain for an extended period after birth. As discussed above, concentrations of allopregnanolone fall rapidly following birth, and in the case of preterm neonates, results in both the premature loss of neuroactive steroid concentrations in the neonatal brain and potentially, a reduced capacity of the preterm neonatal brain to directly synthesise allopregnanolone from its steroid precursors. The studies in Chapters 4 & 5, demonstrated the efficacy of postnatal progesterone treatment in increasing both plasma and brain concentrations in the preterm neonates. Importantly, this increase in allopregnanolone synthesis was achieved by increasing substrate availability of progesterone, despite the reduced expression of 5αR2 in the neonatal brain. Additionally, treatment with exogenous progesterone was able to increase preterm brain allopregnanolone concentrations when preterm plasma concentrations of progesterone, which remained at fetal concentrations at 24hrs after birth, were apparently insufficient. Brain allopregnanolone concentrations remained elevated with extended progesterone treatment until term-equivalent age. These findings verify the potential of progesterone replacement therapy for the augmentation of postnatal allopregnanolone levels in these animals, without the need for direct administration of allopregnanolone.

There are a number of potential advantages to the administration of progesterone rather than allopregnanolone in this model.
Allopregnanolone has a relatively short half-life of approximately 5 to 6 hrs (Timby, Balgard et al. 2006) and has potent sedative and anaesthetic effects even at doses in the nanogram range (van Broekhoven, Backstrom et al. 2007). Treatment with progesterone has the potential to avoid these issues by augmenting allopregnanolone concentrations via increased substrate supply and endogenous synthesis of allopregnanolone. Clinically this may be important as it allows neonatal factors to regulate synthesis.

Progesterone itself also has the potential to have direct effects on developmental processes in preterm neonatal brains and other organ systems. Early studies into the potential benefit of progesterone and estradiol replacement in preterm infants have shown some promising findings that may benefit neonatal respiratory function (Dodd, Crowther et al. 2009), bone mineralisation (Trotter, Maier et al. 1999) and have positive effects on preterm neurodevelopmental outcomes (Trotter, Steinmacher et al. 2012). Importantly, no adverse effects were identified in these trials of postnatal steroid replacement therapy, with long-term wide scale studies needed to address the use of these therapies in premature infants. Current trials have not assessed the effects of these therapies on neuroactive steroid concentrations or actions in the perinatal brain. Additionally, given the potential importance of peripheral supply on fetal and neonatal neurosteroid synthesis, the use of progesterone therapies in some high-risk pregnancies may be of significance to neuroactive steroid concentrations in the perinatal brain. It has been shown that commonly used progestin, medroxyprogesterone acetate (MPA) is not able to be metabolised into neuroactive steroids as progesterone is (Ciriza, Carrero et al. 2006). Importantly, there is evidence that MPA may inhibit the endogenous synthesis of neuroactive steroids (Belelli and Herd 2003). These treatments may therefore have feedback effects on endogenous progesterone concentrations, whilst influencing further synthesis of neuroactive steroids.
Endocrine changes related to cortisol concentrations in animals from the current study that were administered progesterone, may also have important effects on the developing brain. Fetal progesterone and cortisol concentrations show strong positive correlations (Donaldson, Nicolini et al. 1991) and progesterone can act as a precursor for cortisol production in the human neonatal adrenal (Villee and Loring 1965). The neonatal adrenal is functional in terms of steroidogenesis at term delivery in the newborn guinea pig (Dalle and Delost 1976). The elevated cortisol concentrations, compared to term values, in all preterm animals at 24 hours (Fig 4.3C) and in progesterone but not vehicle treated preterm animals at term-equivalent age (+Prog8 group, Fig 5.3C), does however, highlight the potential for wide-reaching endocrine effects in the preterm neonate following progesterone treatment. In agreement with the current study, assays in human preterm infants have also shown increased plasma cortisol concentrations with earlier gestational ages at birth (Scott and Watterberg 1995). Exogenous glucocorticoid treatment and increases in perinatal cortisol concentrations have been associated with altered stress responses, neurobehavioural changes, programming of the HPA axis and increased risk of metabolic diseases in later life (Matthews 1998). Other endocrine considerations of postnatal progesterone treatment are the effects on cortisol and exogenous glucocorticoid exposure on the developing brain. Work carried out in our research group has identified negative effects of repeated doses of betamethasone on placental expression of 5αR2, with reduced fetal plasma allopregnanolone in IUGR fetal guinea pigs with repeated glucocorticoid exposure (McKendry, Palliser et al. 2010). Exogenous glucocorticoid treatment has been shown to transiently suppress postnatal steroidogenesis (Poggi Davis, Townsend et al. 2004). In addition, exposure of guinea pig fetuses to prenatal maternal stress, resulting in elevated concentrations of endogenous glucocorticoids, is
associated with reduced myelination and inhibition of maternal transfer of
neuroactive steroids to the fetal brain (Bennett, Palliser et al. 2012). In
contrast to these potential negative neuroendocrine and developmental
effects of glucocorticoid exposure, elevated concentrations of these
steroids may also result in increased synthesis of neuroactive steroid
metabolites (Dodds, Taylor et al. 1997). THDOC, which has a similar
mode of action at the GABA$_A$ receptor as allopregnanolone and is a
corticosterone metabolite (see Fig 1.2), may have beneficial,
neuroprotective actions (Reddy 2002) in the developing brain. The
interactions between stress hormones and the neuroactive progesterone
metabolites may have important implications on the use of neuroactive
steroid therapies, particularly in vulnerable and high-risk fetuses and
neonates.

The mode of drug delivery is also an important consideration in the use of
potential steroid replacement therapies. It was found that salivary
progesterone concentrations fluctuated following each daily subcutaneous
progesterone injection and that these fluctuations were independent of
normal changes in concentration identified in vehicle treated animals. The
use of implants or minipump systems for the constant infusion of
progesterone may lead to a more stable delivery of allopregnanolone
precursor to the neonate. An alternative option, may be the use of
synthetic neuroactive agents that have similar actions to allopregnanolone
at the GABA$_A$ receptor. Alfaxalone, a synthetic allopregnanolone analogue
that is can be used as an anaesthetic agent, has been shown to ameliorate
brain injury associated with inhibition of allopregnanolone synthesis in fetal
sheep after asphyxia (Yawno, Hirst et al. 2009) However, alfaxalone has a
very short half-life of approximately 20min (Ferre, Pasloske et al. 2006).
The use of synthetic neuroactive steroids that have been developed to have
longer half-lives may therefore be useful for the replacement of
neuroactive steroids following preterm birth. Ganaxolone, a recently
developed synthetic allopregnanolone analogue, being tested for the
treatment of epilepsy and seizure disorders, has a longer plasma half-life of
approximately 20hrs (Nohria and Giller 2007). Ganaxolone may therefore
provide a therapeutic option for the replacement of neuroactive steroids
following preterm birth if current trials of this agent demonstrate safety
and efficacy. The action of these agents at the GABA₃ receptor which may
have excitatory effects at earlier gestational ages is also an important
consideration for the development of neuroactive steroid replacement as
future therapies.

There has previously been examination of allopregnanolone treatment
effects on stress responses and the later development of anxiety disorders.
These studies have identified changes in brain structure, function and
responses to psychoactive drugs following altered exposure to neuroactive
steroids. For example, early neonatal administration of allopregnanolone to
rat pups has anxiolytic effects in adulthood, however, after postnatal stress,
neonatal allopregnanolone treatment potentiates increases in anxiety
behaviours that are also sex-specific (Zimmerberg, Rackow et al. 1999).
Altered neonatal allopregnanolone concentrations have also been proposed
to potentiate the development of psychiatric disorders, such as
schizophrenia in adolescence and adulthood (Gizerian, Ritsner et al. 2008).
Studies such as this, demonstrate the complex neuroendocrine interactions
that involve neuroactive steroid actions and that can lead to programming
of later life stress responses. This further supports the proposal that
alterations in perinatal neurosteroid concentrations, such as those that
occur with preterm birth, are likely to exert long-term influence on brain
function and highlights the need for further examination of the actions of
these steroids in the perinatal period.
Potentially confounding some studies of neuroactive steroid actions is the use of animal models that have different developmental profiles with regard to GABA\textsubscript{A} receptor function during the perinatal period (Cherubini, Gaiarsa et al. 1991; Ben-Ari, Gaiarsa et al. 2007). GABA\textsubscript{A} receptor function is dependent on the generation of chloride ion gradients that drive ion movement through the activated GABA\textsubscript{A} receptor ion channel. The factors that control the relative intracellular and extracellular concentrations of ions are developmentally regulated, with the maturation dependent expression of the ion transporters NKCC1 and KCC2 important to the regulation of changes in cellular chloride ion concentrations (Rivera, Voipio et al. 1999; Ganguly, Schinder et al. 2001; Ludwig, Li et al. 2003). These changes in chloride ion concentrations result in a developmental switch from excitatory to inhibitory signalling by the GABA\textsubscript{A} receptor based on the direction of the ion gradient (Miles 1999; Rivera, Voipio et al. 1999). This raises the potential for the complex interactions and lasting effects of postnatal allopregnanolone administration in some studies in which neuroactive steroids were administered before and/or after the developmental switch in GABA signalling. The neuroprotective, anxiolytic and anti-seizure effects of allopregnanolone may therefore be reversed at these times in the developing brain. This was recently demonstrated in neonatal rats, where progesterone and allopregnanolone administration were shown to exacerbate brain injury in rats up to 2 weeks after birth (Tsuji, Taguchi et al. 2012). As the pharmacological inhibition of the GABA\textsubscript{A} receptor partially blocked these increases in injury, the negative effects of progesterone and allopregnanolone were associated with excitatory GABA\textsubscript{A} receptor signalling at these postnatal ages. However, these results may be further complicated by the relative selectivity of agents that act at the GABA\textsubscript{A} receptor to preferentially block depolarising or hyperpolarising currents (Mueller, Taube et al. 1984). The developmental
switch in GABA \(_A\) receptor action is clearly an essential consideration when assessing the actions of neuroactive steroid agonists of this receptor in the immature brain. The presence of a pre- or postnatal switch is particularly relevant when studying placentally derived neuroactive steroids, which have altered concentrations after birth. Conflicting evidence from *in vitro* and *in vivo* studies and from different experimental paradigms and species further complicates our understanding of these factors. In developing a novel small animal model of neurosteroid replacement in the guinea pig, a species with precocial brain development and a suggested prenatal switch (Rivera, Voipio et al. 1999), we may be able to delineate the perinatal effects of allopregnanolone on GABAergic inhibitory signalling, in a system where this inhibitory signalling emerges prior to birth and under the influence of placental neuroactive steroid precursors. The clinical translation of the use of neuroactive steroids in human fetuses and neonates should therefore be considered in relation to both potential excitatory developmental signalling, proposed inhibitory neuroprotective actions and the likely combination of these processes in different regions within the brain, across individual cells and at different stages during development. The potential for sex-specific differences in the development of these GABA \(_A\) receptor-signalling pathways (Galanopoulou 2008) may also provide a significant therapeutic avenue for the targeted treatments, given the increased risk of poor neurodevelopmental outcomes in male fetuses and neonates (Wood, Costeloe et al. 2005).

The replacement of neuroactive steroids in the perinatal period may also influence the developmentally-regulated expression of GABA \(_A\) receptor subunits, receptor actions and sensitivity of the receptor to other GABAergic agents in the immature brain. The presence of a \(\delta\)-subunit in a GABA \(_A\) receptor increases the binding affinity of allopregnanolone at the receptor and is important to the potentiation of direct GABA effects.
(Smith, Shen et al. 2007; Hosie, Clarke et al. 2009). Additionally, progesterone and allopregnanolone exposure and withdrawal can increase the expression of the α4 subunit of the GABA<sub>A</sub> receptor, the upregulation of which is shown to reduce the sensitivity of action of benzodiazepines (Gulinello, Gong et al. 2002). The postnatal expression of the α4 subunit, which is higher than other α subunits (Mtchedlishvili, Sun et al. 2003), may be influenced by the postnatal withdrawal of progesterone and neuroactive steroid concentrations, affecting the efficacy of anti-epileptic drugs in the neonatal period. Co-administration of progesterone with finasteride, to block allopregnanolone synthesis, also has regulatory effects on the expression of other γ and α subunits (Follesa, Serra et al. 2000), demonstrating potential feedback mechanisms on GABA<sub>A</sub> receptor conformations by progesterone itself, independent of allopregnanolone. Whilst the regulation of GABA<sub>A</sub> receptor subunit expression was not a focus of the current studies, the influence of neuroactive steroid concentrations that are altered due to fetal compromise or preterm birth, may be of particular importance, not only to neuroactive steroid actions in the perinatal brain but also to the efficacy of other agents that act at the GABA<sub>A</sub> receptor. This is particularly important given the regional and developmentally specific expression of GABA<sub>A</sub> receptor subunits.

Examination of the regulatory effects of postnatal progesterone replacement on GABA<sub>A</sub> receptor conformation may reveal new insights into the maturation of GABAergic signalling on neurodevelopmental processes and brain function.

7.1.3 Neurosteroids and Perinatal Myelination in the Guinea Pig

The studies presented in this thesis in fetal and neonatal guinea pigs have shown that changes in allopregnanolone concentration are associated with
alterations in myelination in the developing brain. There is also evidence that allopregnanolone influences myelination in a sex-specific manner and relative to normal growth processes. The use of finasteride in fetal guinea pigs identified that the chronic reduction of allopregnanolone concentrations during late gestation was associated with reduced myelination in the sub-cortical white matter tract. Interestingly, reductions in myelination were not identified in this region when finasteride treatment was combined with experimental growth restriction. This observation may point to mechanisms that are protective in the presence of these dual insults. However, the finding that IUGR resulted in significant reductions in MBP immunostaining in the hippocampus, suggests that fetal growth restriction is an important mediator of myelin maturation or growth and that effects may be region-specific. This finding is consistent with previous findings in which growth-restricted guinea pigs have reduced expression of myelin proteins, including MBP, myelin-associated glycoprotein (MAG) and proteolipid protein (PLP), with a degree of postnatal catch-up maturation indicating a delay but not necessarily a permanent loss of myelination (Tolcos, Bateman et al. 2011). Given the developmental changes associated with IUGR, the future examination of IUGR combined with preterm birth in the guinea pig may provide insight into the effects of additional or exacerbating insults on the actions of neurosteroids in the immature postnatal brain.

The development of a preterm neonatal model in the guinea pig, whilst demonstrating reduced myelination after premature birth, also showed more mature brain development at the time of delivery when compared to more altricial species, such as the mouse or rat. Thus, brain development is relatively well advanced at this age in gestation (day 62) in the guinea pig. Growth curves calculated on the velocity of brain weight change have identified the brain growth spurt as occurring during mid-late gestation in
the guinea pig (Dobbing and Sands 1979). The timing of brain growth in the guinea pig is therefore predominantly prenatal and is an important aspect of understanding changes in brain development seen in this animal model, in addition to the potential effects of neurosteroids on myelination in this species as related to preterm birth.

7.1.4 Neurosteroids, Neuropathology and Behaviour in the Perinatal Guinea Pig

Along with altered myelination, the studies in this thesis identified changes in other neuropathological markers in association with altered neuroactive steroid concentrations. The most notable changes were associated with reduced allopregnanolone concentrations in the late gestation fetal brain, due to the inhibition of allopregnanolone synthesis. GFAP immunostaining was measured as a marker of astrocytic activation, an important process in the brain that occurs in response to many forms of brain injury, particularly hypoxic-ischemic and excitotoxic damage (Roessmann and Gambetti 1986; Burtrum and Silverstein 1994; Eng and Ghirnikar 1994; Cai, Pan et al. 2000). The finding that GFAP immunostaining was increased in the cortical white matter of guinea pig fetuses treated with finasteride, suggests the presence of brain responses to injury in association with reduced brain allopregnanolone concentrations. Finasteride has previously been shown to abolish the stimulatory effect of progesterone on GFAP mRNA expression in isolated cultured astrocytes from adult rats (Melcangi, Riva et al. 1996). Increases in GFAP staining were also identified in the CA1 region of hippocampus of IUGR fetal guinea pigs following finasteride treatment. In contrast to previous studies that observed marked increases in GFAP staining around cortical blood vessels in growth-restricted fetal guinea pigs (Nitsos and Rees 1990), IUGR without finasteride treatment, did not result in any changes in GFAP
expression. One point of difference between the two studies, however, was the experimental procedure undertaken to induce IUGR. Nitsos et al., employed the technique of bilateral uterine artery ligation to reduced uteroplacental bloody supply to the developing fetus. Both the radial artery diathermy technique used in the current studies, and the uterine artery ligation technique result in a similar degree of body weight reduction, however, uterine artery ligation results in a higher proportion of fetal deaths (Turner and Trudinger 2009), suggesting an increase in the severity of insult with this technique. This may explain potential increases in markers of brain injury in these previous studies.

As part of studies presented in this thesis that examined preterm neonates (Chapters 4 and 5), no differences in GFAP expression were identified, except for a significant interaction between neonatal sex and gestational age on GFAP in the hippocampus. This may indicate the action of sex-dependent maturational processes in the preterm brain and potentially, a vulnerability associated with the premature neuroendocrine alterations following preterm birth.

The increases in GFAP staining observed in the current studies, were not associated with a concurrent increase in cell death as measured by the number of apoptotic cells. Finasteride infusion in fetal sheep has previously shown an increase in apoptosis associated with lower concentrations of allopregnanolone, which was further exacerbated by fetal asphyxia (Yawno, Yan et al. 2007). This previous study, however, did not examine changes in astrocytes. In the current study, changes in brain markers were examined in relation to two chronic insults (IUGR and prolonged finasteride exposure) that may have affected the type and extent of brain changes identified. Transient increases in apoptosis following IUGR and finasteride treatment that did not persist to the time of tissue collection cannot be excluded.
MAP-2 immunostaining was determined in the preterm brains, as an indicator of neuron development and density in relation to neurosteroids and brain maturation in the preterm guinea pig. The observation that MAP-2 staining was similar in preterm and term brains, in the subcortical white matter, hippocampus and sub-germinial zone, suggests that neuronal populations have reached birth levels at the time of preterm delivery in the guinea pig. Previous studies have demonstrated elevated MAP-2 expression in the hippocampus of adult rats following the administration of estradiol and progesterone to investigate synaptic plasticity related to menstrual hormones (Reyna-Neyra, Camacho-Arroyo et al. 2002). Similar findings in rats with spinal cord lesions also indicate a protective effect of progesterone on the maintenance of MAP-2 expression and reduction in cytoskeletal abnormalities (Gonzalez, Lopez-Costa et al. 2009). In contrast to these findings, progesterone treatment did not alter the staining of MAP-2 positive neurons in preterm guinea pig brains. The transient expression of the cognate progesterone receptor in the perinatal rat brain is also associated with MAP-2 positive neurons but not other types of cells (Lopez and Wagner 2009), suggesting a signalling mechanism via which progesterone may influence neuronal structure. Neurodevelopmental effects of progesterone and allopregnanolone on neuronal proliferation, synaptic plasticity and dendritic growth in the immature and preterm brain require further investigation.

It was also observed, in the current studies, that preterm neonatal guinea pigs had differences in the cerebellar morphology, particularly in the EGL that indicated reduced maturity at the time of birth. There was evidence that cerebellar maturation continued postnatally in the preterm cerebellum, with no difference in the external granular layer thickness at term-equivalent age, in neonates delivered preterm, compared to term animals. However, other markers in the cerebellum were remained altered in the
term-equivalent group despite a period of postnatal development. The finding that both Purkinje cell area and perimeter length were reduced in lobules X and VIII in preterm cerebella at PND8, suggests a postnatal loss or reduction in Purkinje cell size following preterm birth, as no differences in Purkinje cell area or perimeter were present between term and preterm neonates at 24hrs. This finding may be significant to postnatal neuroactive steroid supply, as the Purkinje cell is an important site of progesterone and allopregnanolone synthesis (Agis-Balboa, Pinna et al. 2006; Tsutsui 2006). However, the actions on the cerebellum of progesterone in this in vivo preterm guinea pig model, at this developmental stage, were not significant. Progesterone, either directly or by the action of allopregnanolone has previously been shown to have positive effects on the neuroprotection, differentiation of dendritic processes and migration of Purkinje cells within the cerebellum (Sakamoto, Ukena et al. 2001; Ardesiri, Kelley et al. 2006; Tsutsui 2006). The positive effects of allopregnanolone in these experiments may be related to differences in GABA_\_ receptor activity or function at this developmental stage in rats in relation to proposed effects on the development of neural networks (Ben-Ari, Gaiarsa et al. 2007). Direct measurement of progesterone and allopregnanolone concentrations within the cerebellum following the use of neurosteroid replacement therapies is important to the elucidation of these mechanisms in relation to GABA_\_ receptor signalling.

Progesterone treatment did, however, affect functional outcomes in preterm neonates. Novel object recognition testing is used to assess short-term spatial memory. Whilst it was observed that both groups of preterm animals preferentially explored the novel object, signs of the potentially inhibitory effects of allopregnanolone on brain function were noted in the increased exploration of the familiar object by progesterone treated neonates. This finding is significant as it indicates a neurobehavioural
action of progesterone treatment, with potential implications on later behavioral outcomes.

The developmental stage of the guinea pig brain in these preterm studies is also an important consideration for the interpretation of the results, particularly with regard to the neuropathological findings. The advanced brain development of the guinea pig at birth (Dobbing and Sands 1970) makes it an advantageous model in many respects, particularly in terms of examining brain development under the influence of placental steroid supply, which is confounded in species such as the rat in which a large degree of brain maturation occurs postnatally. However, maturational events also influence the susceptibility of the perinatal brain to injury and potentiate the type of injury or developmental disorder that is likely to occur. The less striking patterns of injury noted in the current studies may be related to the relative maturity of the guinea pig brain at the age of preterm delivery, which may limit the usefulness of this model for these type of assessments. It is therefore essential, when investigating developmental and injury processes in the immature brain, to recognise the differences in the animal models being used and critically examine the important findings as they relate to maturation, particularly when interpreting data in relation to human perinatal brain development. The delivery of preterm neonatal guinea pigs at earlier ages would potentially show increased signs of injury and neurobehavioural changes at postnatal follow-up. However, whilst a precocial species with regard to brain development, lung development in the fetal guinea pig occurs relatively later in gestation (Sosenko and Frank 1987; Sosenko and Frank 1987; Kelly, Rickett et al. 1991), with respiratory problems noted in some of the preterm neonates in the current studies. This can also be seen, qualitatively, in haemotoxylin and eosin stained lung sections from preterm and term guinea pigs in this study, micrographs of which are shown in Appendix B.
Postnatal survival of preterm neonates until term was significantly reduced, suggesting those neonates that were most severely affected by preterm birth did not survive the experimental period. Elucidation of potential steroid effects on lung development in the preterm guinea pig may provide further information regarding the factors that influenced survival of neonates in these studies. Some of the differences in animals organ and body weights in preterm neonates when compared to term may also indicate variations in vulnerability, mediated by interactions between neonatal sex and treatment.

The investigation of the effects of neuroactive steroids on perinatal brain injury following preterm birth should also be considered in the presence of secondary insults such as intrauterine infection or growth-restriction that are known to exacerbate or potentiate brain injury and poor neurodevelopmental outcomes following preterm birth (Dammann and Leviton 1997; Tolsa, Zimine et al. 2004). The development of the preterm neonatal guinea pig model for the study of these insults, and other such as maternal stress, in combination with preterm birth may therefore provide further valuable information for the role of neuroactive steroids in the perinatal brain.

7.2 CONCLUSIONS

In conclusion, the studies presented in this thesis have provided new evidence for the importance of neuroactive steroids for the normal development of the fetal brain. These studies have also established some of the neuroendocrine changes, particularly related to neuroactive steroid concentrations and synthesis that occur following preterm birth in a novel preterm neonatal model in the guinea pig. Additionally, postnatal progesterone therapy has been shown to be effective in augmenting the endogenous synthesis of allopregnanolone within the preterm brain,
despite reductions in the expression of neurosteroidogenic enzymes following preterm birth. Neuropathological markers of brain maturation and injury were also found to be altered following preterm birth and perturbation of fetal and neonatal neuroactive steroid concentrations. However, these relatively mild neuropathological deficits may have limited the ability of these studies to fully elucidate the potential neuroprotective and neurodevelopmental actions of neuroactive steroid replacement in this model. Future studies to examine the effects of allopregnanolone and other neuroactive steroids in pregnancies complicated by more severe insults will be essential for the development of therapeutic strategies using neuroactive steroids to protect the vulnerable immature brain from injury and promote normal brain development.
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Appendix A

PLASMA ALLOPREGNANOLONE CONCENTRATIONS IN FETAL GUINEA PIGS WITH IUGR AND FINASTERIDE TREATMENT

Figure A.1 shows the plasma concentrations of allopregnanolone, measured in fetal guinea pigs with finasteride treatment and experimentally induced IUGR. In the animals that received the control, vehicle injections, plasma allopregnanolone concentrations were significantly reduced in growth restricted fetuses. As in the brain (Fig 3.1), finasteride treatment, in both sham control and IUGR fetuses, resulted in a significant reduction in fetal plasma allopregnanolone concentrations.

![Figure A.1 - Fetal Plasma Allopregnanolone Concentrations](image)

Plasma allopregnanolone concentrations were significantly reduced in fetal plasma following finasteride treatment. In vehicle treated guinea pigs, IUGR (filled bars) also significantly reduced plasma allopregnanolone concentrations. Relative expression measured by radio-immunoassay and expressed as ng/mL of plasma (n= 4-6; *p<0.05; **p<0.001).
Appendix B

PRETERM AND TERM GUINEA PIG LUNGS

Figure B.1 - Term and Preterm Neonatal Guinea Pig Lung Sections
Lung sections at 24hrs after delivery from (A) term (delivered at GA69 days), (B) preterm (GA62 days) and (C) preterm (GA62 days) progesterone treated neonates were stained with haemotoxylin and eosin. Alveolar size appeared to be reduced, with increased septal width, in preterm neonatal lungs compared to term neonates. Progesterone treatment may alter alveolar expansion and lung development in preterm neonates (scale bar = 100μm).