

# THE EFFECTS OF MATERNAL STRESS ON PERINATAL NEURODEVELOPMENT AND BEHAVIOUR

Greer Bennett

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FACULTY OF HEALTH  
UNIVERSITY OF NEWCASTLE  
AUSTRALIA

## Declaration

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Signed: Greer Bennett

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Date

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## **Abstract**

Maternal stress is associated with persistent changes in the development of offspring, which are thought to be 'programmed' in utero. Alterations in the brain development of prenatally stressed offspring has been linked with increased risks for behavioural and cognitive abnormalities later in life. There is evidence to suggest that the time at which a mother experiences stress during pregnancy may be critical in determining the specific outcomes in her offspring due to the effects on fetal ontogenesis at different times in gestation.

Neurosteroids including the progesterone metabolite, allopregnanolone, have been found to act as endogenous neuroprotective agents in the fetal brain during times of stress and pregnancy compromise. Due to a high placental secretion of progesterone throughout pregnancy, allopregnanolone levels are elevated in both the mother and the fetus. Allopregnanolone has important trophic actions through binding to the GABA<sub>A</sub> inhibitory receptor and is essential for normal neurodevelopment. Therefore, the aims of the studies described in this thesis were to assess the effects of maternal stress at different times in gestation on perinatal brain development and behavioural outcomes at juvenility and also to determine the role of allopregnanolone and the neurosteroid system in modulating the effects of stress on the perinatal brain.

The effect of prenatal maternal stress during pregnancy was assessed using an established guinea pig model. Pregnant dams were subjected to strobe light exposure to induce stress beginning from 'early' (0.5 gestation), 'mid' (0.7 gestation) or 'late' (0.8 gestation) pregnancy. Effects of prenatal stress were examined in the hippocampus, subcortical white matter and cerebellum of offspring as these regions are known to be vulnerable to the effects of stress and are also implicated in many of the behavioural outcomes observed in offspring. Assessments of perinatal brain development were used primarily with immunohistochemical markers for mature myelinating oligodendrocytes

and reactive astrocytes, which assess two key cell populations in the brain. Fetal tissues were collected at term (approximately 69 days gestation). In offspring that were delivered, behavioural testing was performed at the equivalent of childhood/juvenility (18 days postnatal age), which is an important time in postnatal development when many altered behaviours become apparent. Maternal and offspring cortisol concentrations were measured at the time of prenatal stress exposure and postnatal behavioural testing respectively.

Brain and plasma allopregnanolone were measured along with neurosteroidogenic enzymes and key GABA<sub>A</sub> receptor subunits to assess the role of the neurosteroid system in these offspring.

The major findings of the present studies were that maternal stress may lead to the development of marked changes in behaviour in juvenile offspring. Specifically, prenatally stressed offspring showed increased anxiety and neophobia at juvenility. These changes in behaviour were associated with reductions in markers for myelination and reactive astrocyte expression, primarily in the CA1 region of the hippocampus. In some brain regions, there were sexually dimorphic outcomes between male and female brain development however both sexes showed increased anxious behaviour and neophobic responses in juvenility, irrespective of the timing of maternal stress exposure. These studies further showed that offspring exposed to prenatal stress beginning earlier in gestation also showed sustained reductions in circulating allopregnanolone levels after birth, which suggests that the neurosteroid system may be altered by early stress exposure in utero and these changes may contribute to adverse behavioural outcomes in offspring.

These studies have demonstrated the marked effects of maternal stress on perinatal brain development that may contribute to the altered behavioural phenotypes observed in later life. Additionally, this work further indicates there may be an important role for

reduced allopregnanolone signalling in the development of these prenatally stressed offspring. The field of developmental origins of health and disease (DOHaD) is a rapidly growing one, and maternal stress is certainly an important factor for the programming of outcomes in offspring with the current studies suggesting that preventive approaches such as those that modulate neurosteroid processes warrant further attention.

## TABLE OF CONTENTS

|                                                                                                                                              |       |
|----------------------------------------------------------------------------------------------------------------------------------------------|-------|
| DECLARATION .....                                                                                                                            | I     |
| ACKNOWLEDGEMENTS .....                                                                                                                       | II    |
| ABSTRACT .....                                                                                                                               | V     |
| LIST OF FIGURES .....                                                                                                                        | X     |
| LIST OF TABLES .....                                                                                                                         | XI    |
| LIST OF ABBREVIATIONS .....                                                                                                                  | XII   |
| PUBLICATIONS FOR INCLUSION .....                                                                                                             | XVI   |
| ADDITIONAL PUBLICATIONS .....                                                                                                                | XVII  |
| CONFERENCE PRESENTATIONS .....                                                                                                               | XVIII |
| CHAPTER ONE: INTRODUCTION .....                                                                                                              | 1     |
| 1. A history of defining prenatal stress .....                                                                                               | 1     |
| 1.1 The Programming Hypothesis .....                                                                                                         | 2     |
| 1.2 The physiological stress response in pregnancy and fetal outcomes .....                                                                  | 3     |
| 1.2.1 The stress response .....                                                                                                              | 3     |
| 1.2.2 Fetal protection against prenatal stress .....                                                                                         | 8     |
| 1.2.3 Inappropriate levels of glucocorticoids .....                                                                                          | 10    |
| 1.2.4 Pregnancy outcomes with prenatal stress .....                                                                                          | 13    |
| 1.2.5 Prenatal Stress and Programming of the HPA Axis .....                                                                                  | 15    |
| 1.3 Structural and functional outcomes of prenatal stress .....                                                                              | 17    |
| 1.3.1 Normal neurodevelopment and the intrauterine environment .....                                                                         | 17    |
| 1.3.2 Prenatal stress and fetal neurodevelopment .....                                                                                       | 22    |
| 1.3.3 Sexually dimorphic brain development and outcomes .....                                                                                | 26    |
| 1.3.4 Functional outcomes with prenatal stress .....                                                                                         | 28    |
| 1.4 Neurosteroids and neuroprotection .....                                                                                                  | 31    |
| 1.4.1 Neurosteroids in pregnancy .....                                                                                                       | 35    |
| 1.5 Summary, Rationale and Hypotheses .....                                                                                                  | 38    |
| 1.5.1 Summary .....                                                                                                                          | 38    |
| 1.5.2 Model design .....                                                                                                                     | 39    |
| 1.5.3 Research aims .....                                                                                                                    | 40    |
| 1.5.4 Research design .....                                                                                                                  | 41    |
| CHAPTER TWO: EXTENDED METHODS .....                                                                                                          | 45    |
| 2.1 Animal Stress Protocol .....                                                                                                             | 45    |
| 2.2 Allopregnanolone Treatment .....                                                                                                         | 47    |
| 2.3 Tissue collection .....                                                                                                                  | 50    |
| 2.4 Behavioural Testing .....                                                                                                                | 53    |
| 2.5 Immunohistochemistry .....                                                                                                               | 54    |
| 2.6 Western Blotting .....                                                                                                                   | 57    |
| 2.7 Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) .....                                                                          | 61    |
| 2.8 Allopregnanolone radioimmunoassay .....                                                                                                  | 65    |
| 2.9 Cortisol and Progesterone Immunoassay .....                                                                                              | 67    |
| 2.10 Statistical Analysis .....                                                                                                              | 68    |
| CHAPTER THREE: EFFECTS OF PRENATAL STRESS ON FETAL NEURODEVELOPMENT AND<br>RESPONSES TO MATERNAL NEUROSTEROID TREATMENT IN GUINEA PIGS ..... | 70    |

|                                                                                                                                                                 |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 3.1 Abstract .....                                                                                                                                              | 72  |
| 3.2 Introduction .....                                                                                                                                          | 73  |
| 3.3 Methods.....                                                                                                                                                | 75  |
| 3.4 Results .....                                                                                                                                               | 79  |
| 3.5 Discussion .....                                                                                                                                            | 89  |
| 3.6 Acknowledgements.....                                                                                                                                       | 94  |
| CHAPTER FOUR: PRENATAL STRESS ALTERS HIPPOCAMPAL NEUROGLIA AND INCREASES ANXIETY IN CHILDHOOD. ....                                                             |     |
| 4.1 Abstract .....                                                                                                                                              | 95  |
| 4.2 Introduction .....                                                                                                                                          | 97  |
| 4.3 Methods.....                                                                                                                                                | 100 |
| 4.4 Results .....                                                                                                                                               | 109 |
| 4.5 Discussion .....                                                                                                                                            | 118 |
| 4.6 Acknowledgements.....                                                                                                                                       | 123 |
| CHAPTER FIVE: PRENATAL STRESS HAS PERSISTENT EFFECTS ON THE DEVELOPMENT OF THE CEREBELLUM AND GABA <sub>A</sub> RECEPTOR EXPRESSION. ....                       |     |
| 5.1 Abstract .....                                                                                                                                              | 124 |
| 5.2 Introduction .....                                                                                                                                          | 125 |
| 5.3 Methods.....                                                                                                                                                | 126 |
| 5.4 Results .....                                                                                                                                               | 128 |
| 5.5 Discussion .....                                                                                                                                            | 132 |
| CHAPTER SIX: TIMING MATTERS: THE EFFECT OF EARLY, LATE AND ACUTE PRENATAL STRESS ON NEURODEVELOPMENT, BEHAVIOURAL AND NEUROSTEROID RESPONSES IN OFFSPRING. .... |     |
| 6.1 Abstract .....                                                                                                                                              | 145 |
| 6.2 Introduction .....                                                                                                                                          | 147 |
| 6.3 Methods.....                                                                                                                                                | 149 |
| 6.4 Results .....                                                                                                                                               | 152 |
| 6.5 Discussion .....                                                                                                                                            | 156 |
| CHAPTER SEVEN: GENERAL DISCUSSION.....                                                                                                                          |     |
| 7.1 The effect of prenatal stress on perinatal brain development.....                                                                                           | 175 |
| 7.2 Prenatal stress results in increased anxious behaviour in juvenility .....                                                                                  | 175 |
| 7.3 Neurosteroids, endocrine development and prenatal stress .....                                                                                              | 183 |
| 7.4 Prenatal stress and maternal cortisol.....                                                                                                                  | 187 |
| 7.5 Conclusions and future directions.....                                                                                                                      | 191 |
| REFERENCES .....                                                                                                                                                | 194 |
| APPENDIX A: PUBLISHED MANUSCRIPTS.....                                                                                                                          | 199 |
| APPENDIX B: PERMISSIONS .....                                                                                                                                   | 228 |

## List of Figures

|                                                                                                                                                                                     |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| FIGURE 1.1 THE STRESS RESPONSE PATHWAYS .....                                                                                                                                       | 7   |
| FIGURE 1.2 TIMELINE OF HUMAN BRAIN DEVELOPMENT. ....                                                                                                                                | 21  |
| FIGURE 1.3 PATHWAYS OF NEUROSTEROID SYNTHESIS.....                                                                                                                                  | 34  |
| FIGURE 2.1 STROBE LIGHT EXPOSURE APPARATUS. ....                                                                                                                                    | 47  |
| FIGURE 2.2 ANIMAL EXPERIMENTAL PROTOCOL. ....                                                                                                                                       | 49  |
| FIGURE 2.3 GUINEA PIG BRAIN DISSECTION. ....                                                                                                                                        | 52  |
| FIGURE 2.4 EXAMPLE OF GUINEA PIG BRAIN HISTOLOGY. ....                                                                                                                              | 56  |
| FIGURE 2.5 CEREBELLUM HISTOLOGY.....                                                                                                                                                | 57  |
| FIGURE 3.1 EFFECT OF STROBE LIGHT-INDUCED STRESS EPISODES ON MATERNAL SALIVARY CORTISOL<br>CONCENTRATIONS. ....                                                                     | 82  |
| FIGURE 3.2 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE<br>HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT.....             | 84  |
| FIGURE 3.3 EFFECTS OF PRENATAL STRESS ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1<br>REGION OF THE HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT..... | 85  |
| FIGURE 3.4 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN-2 (MAP-2) EXPRESSION IN THE CA1 REGION<br>OF THE HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT.....         | 87  |
| FIGURE 3.5 MATERNAL PLASMA ALLOPREGNANOLONE CONCENTRATIONS .....                                                                                                                    | 88  |
| FIGURE 3.6 FETAL PLASMA ALLOPREGNANOLONE CONCENTRATIONS .....                                                                                                                       | 89  |
| FIGURE 4.1 MATERNAL SALIVARY CORTISOL CONCENTRATIONS .....                                                                                                                          | 109 |
| FIGURE 4.2 GROWTH PARAMETERS IN OFFSPRING. ....                                                                                                                                     | 110 |
| FIGURE 4.3 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE<br>HIPPOCAMPUS IN PUPS AT 21 DAYS OF AGE.....                               | 112 |
| FIGURE 4.4 EFFECTS OF PRENATAL STRESS ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1<br>REGION OF THE HIPPOCAMPUS IN PUPS AT 21 DAYS OF AGE.....                   | 113 |
| FIGURE 4.5 HIPPOCAMPAL NEUROSTEROIDOGENIC CAPACITY OF PRENATALLY STRESSED ADOLESCENT OFFSPRING. .                                                                                   | 114 |
| FIGURE 4.6 BRAIN ALLOPREGNANOLONE CONCENTRATIONS IN ADOLESCENT OFFSPRING. ....                                                                                                      | 115 |
| FIGURE 4.7 RELATIVE PROTEIN EXPRESSION OF BDNF IN THE HIPPOCAMPUS OF 21-DAY-OLD GUINEA PIG PUPS. ..                                                                                 | 115 |
| FIGURE 4.8 GABA <sub>A</sub> RECEPTOR SUBUNIT EXPRESSION. ....                                                                                                                      | 116 |
| FIGURE 4.9 BEHAVIOURAL OUTCOMES IN ADOLESCENT OFFSPRING.....                                                                                                                        | 117 |
| FIGURE 4.10 STRESS RESPONSIVENESS OF OFFSPRING DURING BEHAVIOURAL TESTING.....                                                                                                      | 118 |
| FIGURE 5.1: EFFECTS OF PRENATAL STRESS ON EXPRESSION OF MYELIN BASIC PROTEIN (MBP) IN LOBE VIII (A) AND<br>LOBE X (B) OF THE CEREBELLUM. ....                                       | 136 |
| FIGURE 5.2: EFFECTS OF PRENATAL STRESS ON EXPRESSION OF GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) IN LOBE<br>VIII (A) AND THE DEEP WHITE MATTER (B) OF THE CEREBELLUM. ....            | 137 |
| FIGURE 5.3: RELATIVE mRNA EXPRESSION OF GABA <sub>A</sub> RECEPTOR SUBUNIT $\alpha 6$ (A) AND $\delta$ (B) IN THE CEREBELLUM<br>.....                                               | 138 |

|                                                                                                      |     |
|------------------------------------------------------------------------------------------------------|-----|
| FIGURE 5.4: RELATIVE mRNA EXPRESSION OF 5 $\alpha$ REDUCTASE TYPE 1 IN THE CEREBELLUM .....          | 139 |
| FIGURE 5.5: PLASMA ALLOPREGNANOLONE CONCENTRATIONS (NMOL/L) .....                                    | 139 |
| FIGURE 6.1. MATERNAL SALIVARY CORTISOL CONCENTRATIONS .....                                          | 157 |
| FIGURE 6.2. MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS .....         | 161 |
| FIGURE 6.3. MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE SUBCORTICAL WHITE MATTER.....               | 162 |
| FIGURE 6.4. GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS . | 164 |
| FIGURE 6.5. GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE SUBCORTICAL WHITE MATTER .....  | 165 |
| FIGURE 6.6. BEHAVIOURAL TESTING OUTCOMES .....                                                       | 166 |
| FIGURE 6.7. CIRCULATING ALLOPREGNANOLONE CONCENTRATIONS .....                                        | 167 |

## List of Tables

|                                                                                                                                             |     |
|---------------------------------------------------------------------------------------------------------------------------------------------|-----|
| TABLE 2.1: COMPLETE GUINEA PIG SPECIFIC PRIMER SEQUENCES FOR QUANTITATIVE REAL-TIME REVERSE<br>TRANSCRIPTASE-POLYMERASE CHAIN REACTION..... | 64  |
| TABLE 3.1: FETAL PHYSICAL CHARACTERISTICS.....                                                                                              | 81  |
| TABLE 4.1: GUINEA PIG SPECIFIC PRIMER SEQUENCES FOR QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-<br>POLYMERASE CHAIN REACTION.....         | 107 |
| TABLE 4.2: NEONATAL PHYSICAL GROWTH PARAMETERS AT 21 DAYS .....                                                                             | 111 |
| TABLE 5.1: CEREBELLAR LOBES VIII AND X LAYER WIDTHS.....                                                                                    | 134 |
| TABLE 6.1: FETAL PHYSICAL CHARACTERISTICS .....                                                                                             | 158 |
| TABLE 6.2: POSTNATAL (JUVENILE) PHYSICAL CHARACTERISTICS.....                                                                               | 159 |

## List of Abbreviations

|                  |                                                                         |
|------------------|-------------------------------------------------------------------------|
| $\beta$ -ME      | $\beta$ -Mercaptoethanol                                                |
| 5 $\alpha$ R1    | 5 $\alpha$ -Reductase Enzyme type 1                                     |
| 5 $\alpha$ R2    | 5 $\alpha$ -Reductase Enzyme type 2                                     |
| 11 $\beta$ -HSD2 | 11 $\beta$ - Hydroxysteroid dehydrogenase type 2                        |
| AC               | Abdominal Circumference                                                 |
| ACTH             | Adrenocorticotrophin releasing hormone                                  |
| ADHD             | Attention Deficit Hyperactivity Disorder                                |
| ANOVA            | Analysis of Variance                                                    |
| AVP              | Arginine vasopressin                                                    |
| BCA              | Bicinchoninic Acid                                                      |
| BDNF             | Brain Derived Neurotrophic Factor                                       |
| BLR              | Brain-to-Liver Ratio                                                    |
| BSA              | Bovine Serum Albumin                                                    |
| CA1              | Cornus Ammonis region 1                                                 |
| CBG              | Corticosteroid Binding Globulin                                         |
| CC               | Corpus Callosum                                                         |
| cDNA             | Complimentary Deoxyribonucleic Acid                                     |
| CG               | Cingulum                                                                |
| CNS              | Central Nervous System                                                  |
| CO <sub>2</sub>  | Carbon Dioxide                                                          |
| CReDITTS         | Clinical Research Design, Information Technology and Statistical Design |
| CRH              | Corticotrophin releasing hormone                                        |
| CRH-BP           | Corticotrophin releasing hormone Binding Protein                        |
| CSF              | Cerebrospinal Fluid                                                     |
| CX               | Cortex                                                                  |

|                               |                                             |
|-------------------------------|---------------------------------------------|
| DAB                           | 3,3'-diaminobenzidine                       |
| DOHaD                         | Developmental Origins of Health and Disease |
| DG                            | Dentate Gyrus                               |
| DHEA                          | Dehydroepiandrosterone                      |
| DHEAS                         | Dehydroepiandrosterone sulphate             |
| DNA                           | Deoxyribonucleic Acid                       |
| dNTP                          | Deoxynucleotide Triphosphate                |
| EDTA                          | Ethylenediaminetetraacetic Acid             |
| EGL                           | External Granule Layer                      |
| EtOH                          | Ethanol                                     |
| GA                            | Gestational Age                             |
| GABA                          | $\gamma$ -aminobutyric acid                 |
| GABA <sub>A</sub>             | $\gamma$ -aminobutyric acid type A receptor |
| GCMS                          | Gas Chromatography Mass Spectrometry        |
| GFAP                          | Glial Fibrillary Acidic Protein             |
| GR                            | Glucocorticoid receptor                     |
| H <sub>2</sub> O              | Water                                       |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen Peroxide                           |
| HAPS                          | Hunter Area Pathology Service               |
| HC                            | Head Circumference                          |
| HiF                           | Hippocampal Formation                       |
| HL                            | Head Length                                 |
| HPA                           | Hypothalamic Pituitary Adrenal axis         |
| IGL                           | Internal Granule Layer                      |
| IUGR                          | Intrauterine Growth Restriction             |
| LCMS                          | Liquid Chromatography Mass Spectrometry     |

|          |                                              |
|----------|----------------------------------------------|
| LV       | Lateral Ventricle                            |
| MAP2     | Microtubule Associated Protein 2             |
| MB       | Midbrain                                     |
| MBP      | Myelin Basic Protein                         |
| ML       | Molecular Layer                              |
| MOPS     | 3-(N-morpholino)propanesulfonic acid         |
| MR       | Mineralocorticoid receptor                   |
| mRNA     | messenger Ribonucleic Acid                   |
| mPFC     | medial Prefrontal Cortex                     |
| NHMRC    | National Health and Medical Research Council |
| NRL      | Nose-Rump Length                             |
| PCR      | Polymerase Chain Reaction                    |
| PFC      | Prefrontal Cortex                            |
| PND      | Postnatal day                                |
| PVDF     | Polyvinylidene Fluoride                      |
| QC       | Quality Control                              |
| REM      | Rapid Eye Movement                           |
| RIPA     | Radioimmunoprecipitation assay               |
| RM-ANOVA | Repeated Measures Analysis of Variance       |
| RNA      | Ribonucleic Acid                             |
| RT       | Reverse Transcription                        |
| RT-PCR   | Real-Time Polymerase Chain Reaction          |
| SEM      | Standard Error of the Mean                   |
| SGA      | Small-for-gestational-age                    |
| TMB      | Tetramethylbenzidine                         |
| TBS      | Tris-buffered Saline                         |

|       |                                   |
|-------|-----------------------------------|
| TBS-T | Tris-buffered Saline with Tween   |
| THDOC | Allotetrahydrodeoxycorticosterone |
| WM    | White Matter                      |

## **Publications for Inclusion**

*The work in this thesis describes the effects of prenatal stress exposure at varying times in gestation on perinatal brain development and behavioural outcomes, with a focus on the effects of stress on the developing neurosteroid system. As such, this thesis is divided into four publications beginning with the effect of prenatal stress on fetal brain development, then assessing postnatal brain development and behaviour and finally investigating additional brain regions and prenatal stress timings on outcomes:*

**Bennett GA**, Palliser HK, Saxby B, Walker DW, Hirst JJ. Effects of prenatal stress on fetal neurodevelopment and responses to maternal neurosteroid treatment in guinea pigs. *Developmental Neuroscience* 2013; 35:416-426.

I warrant that I have obtained, where necessary, permission from the copyright owners to use my own published work in which the copyright is held by another party for the manuscript listed above DOI: 10.1159/000354176 by the journal of Developmental Neuroscience, Karger Publishers licence number: 3595720198860.

**Bennett GA**, Palliser HK, Shaw JC, Walker DW, Hirst JJ. Prenatal stress alters hippocampal neuroglia and increases anxiety in childhood. *Developmental Neuroscience* 2015;37:533-545. I warrant that I have obtained, where necessary, permission from the copyright owners to use my own published work in which the copyright is held by another party for the manuscript listed above DOI: 10.1159/000437302 by the journal of Developmental Neuroscience, Karger Publishers licence number: 3745730055895.

**Bennett GA**, Palliser HK, Shaw JC, Walker DW, Hirst JJ. Neurosteroidogenesis, GABA<sub>A</sub> receptor subunit expression and perinatal neurodevelopment of the cerebellum following prenatal stress. Prepared for submission to the Journal of Stress.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. The effect of the timing of prenatal stress exposure on neurodevelopmental, behavioural and neurosteroid responses in guinea pig offspring. Prepared for submission to the Journal of Psychoneuroendocrinology.

#### **Additional Publications**

Palliser HK, **Bennett GA**, Kelleher MA, Cumberland AL, Walker DW, Hirst JJ. Models of Perinatal Compromises in the Guinea Pig: Their Use in Showing the Role of Neurosteroids in Pregnancy and the Newborn. Book Chapter. Chapter 10, Neuromethods. *Springer Science*. In Press.

Hirst JJ, Kelleher MA, Walker DW, **Bennett GA**, Cumberland AL, Palliser HK. Loss of neurosteroid-mediated neuroprotection following stress during fetal life. *J Steroid Biochem Mol Biol*. Review. Submitted.

## Conference Presentations

Bennett GA, Shaw JC, Palliser HK, Walker DW, **Hirst JJ**. GABA<sub>A</sub> receptor subunit expression following prenatal stress in the perinatal guinea pig hippocampus and cerebellum. Perinatal Society of Australia and New Zealand (PSANZ), April 2015, Melbourne, Australia. *Oral presentation*.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Prenatal stress causes sex specific persistent reductions in myelination and reactive astrocytes in adolescent guinea pig offspring. International Conference for Neuroendocrinology (ICN), August 2014, Sydney, Australia. *Poster presentation*.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Sex differences in the effects of prenatal stress exposure on early life cortisol profiles and behaviour in guinea pigs. Perinatal Society of Australia and New Zealand (PSANZ), April 2014, Perth, Australia. *Oral presentation*.

**Bennett GA**, Palliser HK, Hirst JJ. Stressed out? A model of stress in pregnancy and the role of neurosteroids. Australian Society for Medical Research (ASMR), March 2014, Newcastle, Australia. *Poster presentation*.

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**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Ongoing effects of prenatal stress on perinatal brain development and function. Fetal and Neonatal Physiological Society (FNPS), September 2013, Puerto Varas, Chile. *Oral presentation*.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Growth patterns of offspring exposed to prenatal stress. Australian Society for Medical Research (ASMR), June 2013, Sydney, Australia. *Poster presentation*.

Bennett GA, Palliser HK, Walker DW, **Hirst JJ**. The lasting effects of prenatal stress on neonatal behavioural outcomes in guinea pigs. Perinatal Society of Australia and New Zealand (PSANZ), April 2013, Adelaide, Australia. *Oral presentation*.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Sex specific effects of prenatal stress on myelination in the hippocampus, cerebral cortex and cerebellum. Australian Neuroscience Society (ANS), February 2013, Melbourne, Australia. *Oral presentation*.

Bennett GA, Palliser HK, Kelleher MA, Saxby B, Walker DW, **Hirst JJ**. Prenatal maternal psychosomatic stress: effects on fetal brain development following maternal neurosteroid treatment in guinea pigs. Fetal and Neonatal Physiological Society (FNPS), September 2012, Utrecht, The Netherlands. *Oral presentation*.

**Bennett GA**, Palliser HK, Walker DW, Hirst JJ. Sexually dimorphic responses to prenatal stress in guinea pigs. Australian Society for Medical Research (ASMR), June 2012, Sydney, Australia. *Poster presentation*.

**Bennett GA**, Palliser HK, Kelleher MA, Saxby B, Walker DW, Hirst JJ. Prenatal stress and effect of maternal neurosteroid treatment on fetal brain development in guinea pigs. Perinatal Society of Australia and New Zealand (PSANZ), April 2012, Sydney, Australian. *Oral presentation.*

## **Chapter One: Introduction**

### **1. A history of defining prenatal stress**

The effect of the maternal psychological state on fetal development has been studied for centuries. In 1889, “maternal impressions” were reported to impact on fetal outcomes, where the outcome of the offspring was specific to the gestational age of insult (1) and in the 1940s, maternal stress was specifically shown to be linked with increased in-utero fetal movements (2). Many of these earlier studies however, struggle to agree on a definition of stress that now satisfies both traditional and modern understandings of the term.

One of the first definitions of stress originates from the Latin term *stringere*- to draw tight (3). It was in the early 20<sup>th</sup> century that stress was first associated with physiological conditions, where as early as 1910, Sir William Osler described a relationship between disease and stress, noting that chest pains were associated with a strenuous lifestyle (3, 4). However, Walter Cannon was the first to link emotional responses to stimuli with physiological changes, implicating particular regions of the brain in orchestrating this response and resulting in a ‘fight or flight’ reaction (5).

Typically, many studies have found it difficult to effectively define stress based on the idiosyncratic nature of both the experience of, and the response to, ‘stressful’ stimuli. Broadly however, stress can be classified as the physiological response to stimuli in order to return to or maintain homeostasis. In this way, stress acts as the adaptive response of an organism to ready itself for a threat to survival (both perceived and real threats). The stress response is often classified by endocrine and physiological changes, in particular the increased release of adrenal cortisol, and the downstream effects this glucocorticoid produces. Interestingly, despite the long history of investigation into the

maternal psyche and influences on fetal development, the precise mechanisms by which prenatal stress exposure affects perinatal development still remain somewhat elusive.

### **1.1 The Programming Hypothesis**

Beginning in 1989 and throughout the 1990s, David Barker proposed that in utero conditions which shape a baby's development, can have permanent changes on the function of systems and organs, thereby increasing the risk for development of disease after birth (6-8) and this is commonly referred to as the Development Origins of Health and Disease (DOHaD) hypothesis. Albeit Barker was referring to under nutrition during pregnancy and the later risk for diabetes, hypertension and coronary heart disease in offspring, however these findings have shaped a new field of maternal and fetal medicine where 'programming' is now an accepted and increasingly studied phenomena. Today, prenatal stress and over exposure of the fetus to glucocorticoids is widely accepted as one of the most critical programming factors in mediating postnatal health of offspring. However, as Barker describes, the timing at which the development of the fetus is perturbed by external factors is key in determining what developing systems are affected and thus, the extent to which postnatal functioning may also be affected. Therefore, the pathogenesis of many outcomes associated with prenatal stress exposure may be attributable to time-dependent perturbations in fetal development, which may confer increased vulnerability for altered health outcomes throughout the life of the offspring.

Prenatal stress has been linked with a variety of altered perinatal outcomes including shortened gestational length and reduced birthweight (9) however some of the most striking associations are those relating to the cognitive, emotional and behavioural development of offspring (10-12). Therefore these outcomes and the potential

alterations in perinatal brain development that may underpin them will be the focus of this thesis.

## **1.2 The physiological stress response in pregnancy and fetal outcomes**

### **1.2.1 The stress response**

Prenatal stress is a broad term that encompasses many different types of maternal psychological conditions, the classification of which differs from study to study.

Conditions such as anxiety, pregnancy-specific anxiety (e.g. the fear of giving birth or of an unhealthy baby), depression, major life events and even more common classifications of stress such as work-related stress, financial stress and relationship problems are often included in the broad classification of prenatal stress. In 2014, the prevalence of non-psychotic mental disorders in pregnancy were reviewed in the United States and the rates of anxiety were as high as 13% in pregnant women and depression was shown to affect up to 11% of pregnant women (13). Self-reported affective symptoms in some populations have been reported to be as high as 51% (14) with the highest rates seen in younger mothers and those from a low socioeconomic background (15). Thus, the classification of what constitutes stress itself can be a major confounding factor between studies with many different types of maternal experiences all considered to represent prenatal stress, yet each measure potentially yielding different outcomes. Therefore, there are several key barriers to studying the effects of prenatal stress, which may contribute to the inconsistent results seen in the literature today. As well as controversy over what is considered prenatal stress, there is also a lack of reliable, standardized measurements to assess the stress response and this contributes to the variance in reporting of prenatal stress. In line with this, there is inherent subjectivity in reporting the levels of stress that possibly confound many human studies and finally, it

is very difficult to control for the multitude of other factors that may contribute to the health of the fetus such as maternal nutrition, the level of perinatal care and the quality of the emotional support system to the mother during pregnancy. Regardless of the stressor itself however, any attempt to study prenatal stress must first consider the physiological stress response systems and how these may affect the developing fetus. Much of what is known currently about the maternal stress response and the interactions it has with the developing fetus are inferred from the actions of the adult stress response.

Upon exposure to stressful stimuli, the sympathetic nervous system activates a rapid stress response (via direct ganglionic nerve connections) where epinephrine and norepinephrine are released primarily from the adrenal medulla (5). Concurrently, when stress is experienced, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular nucleus of the hypothalamus and the amygdala and subsequently stimulate the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (5). This in turn prompts endogenous release of the glucocorticoids, largely cortisol (cortisol in humans, mammals and guinea pigs; corticosterone in rats and mice), from the adrenal cortex (5). Cortisol then acts back via a negative feedback loop to inhibit the continual release of CRH from the hypothalamus (Figure 1).

The stress response systems are remarkably effective in causing activation of physiological processes that aid in both immediate and sustained survival from the stressful stimuli and either preserve, or return the body to homeostasis. The catecholamines epinephrine and norepinephrine trigger the immediate so called 'fight or flight' response: increasing heart rate, blood pressure and blood glucose levels. Cortisol then acts to mobilise energy stores and cause vasoconstriction of peripheral vessels to redirect blood flow to the core and to vital organs. Another action of cortisol

is immune modification, which is thought to be a function involved in energy redirection to cardiac and skeletal tissues, however stress has also been implicated in the production of pro-inflammatory cytokines (16). Furthermore, stress exposure results in increases in glutamatergic neurotransmission (17) and CRH receptor expression in the brain (18) indicating an overall increase in excitatory brain neurotransmission to produce a more excitable and alert neurological state.

During pregnancy, these stress response systems must also cope with the fetoplacental unit and the contribution that this has on regulating Hypothalamic Pituitary Adrenal (HPA) axis functioning (Figure 1). In humans, there is evidence that placental CRH may act in a positive feed forward loop, increasing HPA axis activity at the level of the maternal pituitary, releasing ACTH and downstream cortisol until term (19) however this appears to be a primate specific function and has not been shown in other species such as rats or guinea pigs (20). A considerable amount of human placental CRH is inactivated by a concurrent increase in CRH binding proteins (CRH-BP) during early pregnancy however these levels of these binding protein decline at term, allowing active CRH to circulate in high concentrations (21), potentially as a regulator of gestational length (22). In other species such as guinea pigs, cortisol production during pregnancy is increased through normal HPA axis activation (23). Endogenous circulating levels of cortisol increase exponentially throughout gestation, particularly in the last 10 days, and this has been shown in humans, sheep, guinea pigs, horses and pigs (24). Indeed in humans, increases in cortisol throughout pregnancy have been shown to be an interaction between increased placental CRH levels and cortisol binding globulin (CBG) displacement by progesterone (25) as well as maternal and fetal adrenal production of cortisol. CBG levels rise in many species, with a 2-3 fold increase during human pregnancy and 3-4 fold increase in guinea pig pregnancy (26). In humans, this is primarily due to a large increase in circulating progesterone levels which have a high

affinity for CBG. In the guinea pig however, excess progesterone is bound by a specific progesterone binding globulin (27), which explains why guinea pig CBG levels rise so dramatically during pregnancy. The site of action of cortisol also increases throughout gestation with glucocorticoid receptor (GR) expression being shown to increase in the fetal brain of guinea pigs, particularly after 50 days of gestation (term ~69 days) to reach peak levels at term (24), suggesting an important role for glucocorticoids in the fetal brain at this time. Due to this exponential rise in circulating cortisol concentrations with increasing gestation, maternal HPA axis responsiveness has been reported to be somewhat dampened as pregnancy progresses (28, 29) however very few studies have assessed the direct relationship between maternal stress, cortisol concentrations and resultant outcomes.

Evolutionarily, the physiological response to stress/stressors is critical for adaptation to threats to survival, therefore stress-induced effects may function to effectively 'program' the fetus for a stressful ex utero life, however when there are discrepancies between what is experienced prenatally and postnally, prenatal stress may be considered to be a maladaptive process. Thus, when stress is experienced either inappropriately or in excess during pregnancy, it can lead to detrimental programming effects on the developing fetus leading to disorders and these may depend on the severity and the time at which it is experienced during pregnancy. Thus, the stress response and HPA axis regulation during pregnancy does have major effects on the offspring and these relationships warrant further investigation.

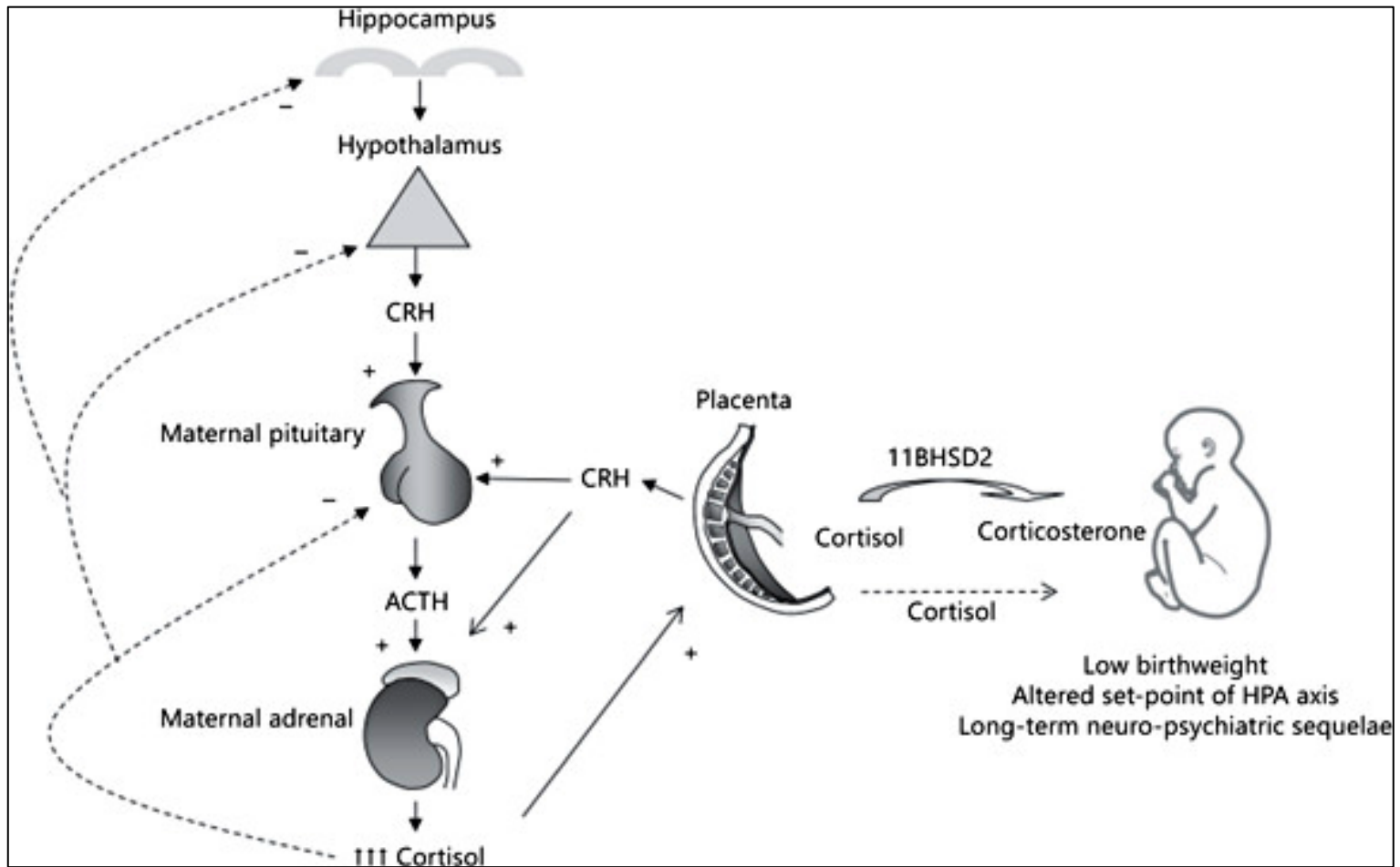


FIGURE 1.1 THE STRESS RESPONSE PATHWAYS. 11B-HSD2= 11B- HYDROXYSTEROID DEHYDROGENASE TYPE 2, ACTH = ADRENOCORTICOTROPHIN RELEASING HORMONE, CRH= CORTICOTROPHIN RELEASING HORMONE, HPA AXIS= HYPOTHALAMIC-PITUITARY-ADRENAL AXIS. ADAPTED FROM DUTHIE ET AL, 2013, WITH PERMISSION (30).

### **1.2.2 Fetal protection against prenatal stress**

During gestation, both maternal and fetal baseline catecholamine levels do not rise significantly above adult levels (31-33). Norepinephrine transporters and catecholamine metabolising enzymes are produced in placental cells and act as a barrier against transfer to the fetus, thereby protecting against excessive exposure (34). The fetus also has a high relative basal secretion rate of catecholamines which may prevent damage against high exposure when travelling through the catecholamine rich birth canal (35, 36).

In contrast to the catecholamines, cortisol, a lipophilic steroid hormone, is able to semi-readily cross the placenta during pregnancy (37). However the substantial expression of the enzyme 11 $\beta$ -Hydroxysteroid Dehydrogenase Type 2 (11 $\beta$ -HSD2) acts to oxidise cortisol into its inactive form cortisone in the placenta. This creates an enzymatic barrier to allow appropriate glucocorticoid levels to reach fetal circulation during development and minimize the transfer of peak levels of maternal cortisol to the fetus. 11 $\beta$ -HSD2 is also present in other tissues that express mineralocorticoid receptors (MR), particularly in the brain, to prevent overstimulation by glucocorticoids that have a high affinity to these receptors and circulate in higher concentrations than mineralocorticoids themselves. Typically, maternal levels of cortisol are over 10 times higher than that in the fetus (38) and thus the placental enzymatic barrier helps to protect the fetus from excess cortisol exposure in the maternal circulation. Furthermore, under normal conditions throughout gestation, there is an upregulation of both maternal and fetal adrenal production of cortisol. This increase in levels of glucocorticoids are required by the fetus for lung maturation and surfactant production in preparation for birth (39-41) with a concurrent increase in 11 $\beta$ -HSD2 activity in the placenta (42) to prevent excessive overexposure to cortisol. However, the 11 $\beta$ -HSD2 barrier is not complete and due to this high proportional difference in maternal and fetal cortisol levels, a relatively small

increase in maternal cortisol concentrations can still result in a large increase in fetal concentrations (38), indicating that minor stressors for the mother may still cause significant changes in the developing fetus. Maternal stress and anxiety have been shown previously to down regulate 11 $\beta$ -HSD2 expression in the placenta in both human (43) and animal models (44), suggesting a feed-forward mechanism whereby maternal stress-induced increases in circulating cortisol concentrations may in turn inhibit the protective barrier. This would allow more cortisol to reach the developing fetus, dramatically increasing fetal cortisol concentrations. Pharmacological inhibition of 11 $\beta$ -HSD2 activity has been shown to produce anxiety-like behaviour in offspring (45), mirroring outcomes commonly associated with prenatal stress exposure, which indirectly implicates increased glucocorticoid exposure with altered behavioural phenotypes in the offspring.

The fetal adrenal glands also play an important role in regulating glucocorticoid exposure, beginning production of steroids from as early as 6-8 weeks of gestation in humans, predominantly producing Dehydroepiandrosterone (DHEA) and Dehydroepiandrosterone sulphate (DHEAS) (46). By the 10<sup>th</sup> week of gestation the fetal adrenal glands are capable of producing glucocorticoid, by the 20<sup>th</sup> week the fetus is capable of mounting a stress response (47, 48) and by late gestation these steroids are produced in large amounts with concentrations peaking at term (49). Therefore, the majority of fetal adrenal development occurs early in gestation, however remodelling and secretion of steroids peaks late in gestation, with birth causing the adrenal glands to undergo extensive structural changes to prepare for adult maturation of the glands postnatally (50). Prenatal stress has been linked with increased apoptosis in the adrenal gland of offspring (51), suggesting that stress in utero may alter the normal development of the fetal adrenal gland, potentially predisposing offspring to further stress-related disorders later in life. In combination with increases in maternal cortisol

concentrations and a potential inhibition of 11 $\beta$ -HSD2 expression in the placenta, perturbations in fetal adrenal development as a result of prenatal stress may have dramatic and long-lasting effects on the HPA functioning of offspring (24).

### **1.2.3 Inappropriate levels of glucocorticoids**

As previously mentioned in section 1.2.2, the increase in endogenous glucocorticoid production in late pregnancy is essential for the early life health of the neonate.

Administration of exogenous glucocorticoids just prior to birth is now common clinical practice in pregnancies at risk of delivering prematurely in order to aid in neonatal survival and reduce respiratory distress for those fetuses that may miss the rise in endogenous glucocorticoids due to premature birth. Indeed, for the past 40 years, the use of exogenous glucocorticoids has been an undisputable method of treatment to improve neonatal outcomes following preterm birth (52, 53). Glucocorticoids are also used for treatment of maternal asthma (54), congenital adrenal hyperplasia (55) and autoimmune disease during pregnancy (56) and are critical for the correct development of several fetal organs during gestation including the thyroid and kidney (57). Therefore maintenance of appropriate levels of glucocorticoids is essential for normal development.

In a 2006 Cochrane review assessing over 4000 infants, one course of exogenous glucocorticoid treatment (betamethasone, dexamethasone or hydrocortisone) was associated with a reduced risk for neonatal death, respiratory distress syndrome, cerebroventricular haemorrhage and necrotising enterocolitis (58). The need for respiratory support and intensive care admissions were reduced leading to recommendations for routine use of glucocorticoid treatment for preterm pregnancies (58). In extremely preterm infants (born <25 weeks gestation), neonatal mortality was dramatically reduced with one course of antenatal glucocorticoid treatment

(betamethasone or dexamethasone) as was the risk for severe intraventricular haemorrhage and neonatal mortality, however the rates of premature rupture of membranes and chorioamnionitis were increased (59). In a more recent Cochrane review in 2011, immediate benefits of repeated glucocorticoid doses (betamethasone or dexamethasone) after an initial course were similar to that previously reported (58) however in early childhood, no benefits of prior treatment were seen, raising questions regarding the long-term outcomes of offspring exposed to multiple courses of synthetic glucocorticoid treatment (60). Even since the first controlled trial of betamethasone treatment to pregnant mothers in 1972 (61), the use of repeated courses of glucocorticoids has been questioned, with associations observed in increased rates of growth restriction and behavioural abnormalities in rats (62, 63). Concerns regarding the long-term outcomes of offspring have been increasingly weighted against the immediate benefits seen in neonatal outcomes in both human and animal studies. Indeed, prolonged or repeated administration of exogenous glucocorticoids to expectant mothers is now suggested to be used with caution (64). In this regard, the parallels to prenatal stress exposure and rises in endogenous glucocorticoid levels are increasingly relevant. Studies assessing the outcomes of offspring after repeated maternal exogenous glucocorticoid treatment (more than one course) are not only more common than studies on prenatal stress in human populations, but they also do not require subjective means of identifying the maternal psychological state and therefore are useful in assessing the direct effects of overexposure to glucocorticoids during pregnancy.

In 1999, French et al, found in a Western Australian cohort that birth weight and head circumference (birth weight ratio) both significantly decreased with every increasing number of betamethasone courses (65), indicating that fetal growth and glucocorticoid exposure may share a direct relationship. Another study concurrently found in a North

American population that repeated courses of glucocorticoids (>3) were associated with decreased fetal growth, increased neonatal mortality, and alterations in adrenal function of offspring (66). Recently, the use of repeated courses of betamethasone was reported to have no adverse risk for cardiometabolic disease in early childhood (67) which is promising data for this necessary treatment however highlights the diversity in findings to date which may result from differences in the dose, timing, number and type of glucocorticoid treatments as well as the age at which outcomes are measured in offspring between studies. It is important to note however, that antenatal glucocorticoid treatment is indicated for pregnancies at risk of delivering preterm and therefore the potential causative factors that underpin preterm birth may not be relevant to extrapolation to prenatal stress. Outcome measures in these populations must also be interpreted with caution as offspring born preterm are known to have different health outcomes to term babies (68).

Animal studies provide detailed information on the effects of glucocorticoid programming on fetal growth. Studies in primates report no change in birth weight following dexamethasone treatment however high doses do show attenuated growth postnatally coupled with impaired glucose tolerance, increased blood pressure and altered HPA axis functioning (69). Repeated hydrocortisone administration prenatally in rats has been shown to negatively affect placental size, acting in both a concentration and time dependent manner (70). In sheep, administration of betamethasone to pregnant ewes was associated with decreased brain size and weight contributing to overall brain retardation at birth, effects that were also dose dependent (71). These investigators later found that repeated betamethasone administration in late gestation delayed myelination in the fetal sheep brain at term (72) and our group has also shown that repeated betamethasone treatment in late gestation negatively affected reactive astrocyte expression in the hippocampus, a result that was only seen in male guinea pig

fetuses (73). Interestingly, it has also been shown that males fail to mount an appropriate upregulation of 11 $\beta$ -HSD2 following glucocorticoid exposure in utero (41), a response which may help to explain the poor outcomes males appear to display following prenatal stress exposure. These findings have important implications for the potential development of neuropathologies later in life, particularly for male offspring. As mentioned previously, pharmacological inhibition of 11 $\beta$ -HSD2 in rats has been shown to result in reduced birth weight, which persists into adulthood, and is associated with increased baseline corticosterone and CRH levels in offspring (45). Interestingly, this study also reported decreased expression of GR in the hypothalamic paraventricular nucleus and the amygdala following inhibition of 11 $\beta$ -HSD2 which was further associated with poorer outcomes in both an open field and forced swim test in adulthood (45). Together, these observations have shown the effects of a disruption in the fetal endocrine environment not only on short term indicators of altered growth such as birth weight, but also on long term disturbances in the HPA axis and limbic system of adult offspring and the behavioural pathologies that these changes may predispose one to later in life. These data further highlight the striking relationship between the importance of appropriate levels of glucocorticoid exposure during development and the potentially altered neuroendocrine and behavioural profiles of offspring later in life.

#### **1.2.4 Pregnancy outcomes with prenatal stress**

Human studies to date have shown the effects of prenatal maternal stress on altering fetal growth and pregnancy outcomes. In 2011, Class et al assessed over 2 million pregnancies and found that exposure to maternal stress (death of the father or a first-degree relative) in the 5<sup>th</sup> or 6<sup>th</sup> month of pregnancy was associated with an increased risk for shortened gestational length, preterm birth, low birth weight and small for

gestational age offspring (74). In 2013, the investigators found that the risk of infant mortality was increased if stress was experienced in the preconception period (75). Others have also confirmed that prenatal stress exposure in the second trimester of pregnancy is associated with an increased risk for producing a small-for-gestational-age baby (76). Perhaps as expected, maternal stress in late pregnancy has been shown to be predictive of preterm delivery where this association was not present in early gestation (77). In a prospective study of women in their third trimester of pregnancy, each unit increase in self-reported prenatal life stress and pregnancy anxiety was associated with a 55 gram decrease in birth weight and a 3 day decrease in gestational length respectively (78). Furthermore, women who experienced a social disaster (9/11 World Trade Centre disaster) in their first trimester of pregnancy gave birth to offspring with significantly smaller head circumferences (a surrogate marker for compromised fetal growth), irrespective of proximity to the disaster (79, 80). A gestation specific alteration in the secondary sex ratio has also been noted after natural disasters, where male fetuses show increased vulnerability compared to females (80-82).

These findings from studies in human populations show diverse and often conflicting effects on outcomes following exposure to prenatal stress. There is a need for uniform reporting of pregnancy outcomes relative to the gestational age of onset and also for consistent and reliable measurements of maternal stress as discussed previously in section 1.2.1. However, the majority of pregnancy outcomes associated with prenatal stress exposure include an increased risk for preterm birth and low birthweight (reviewed in 83), both of which are contributors to neonatal death (responsible for 70%) and illness (responsible for 75%; 84), highlighting the importance of prenatal stress exposure in adverse outcomes. Taken altogether, these studies have shown that the effects of prenatal stress on pregnancy outcomes can be profound but that further research is needed to understand the mechanisms involved and which stage of

pregnancy is the most susceptible to stress in order to ultimately be able to prevent and/or offer treatment to those affected pregnancies and improve outcomes.

#### **1.2.5 Prenatal Stress and Programming of the HPA Axis**

The programming effects of prenatal stress on HPA axis functioning in offspring have been extensively investigated. Indeed, as the HPA axis is critical in mediating many cardiovascular, metabolic, reproductive and neurodevelopmental functions, potential alterations in such a central pathway during development can be profound (85). With this in mind, the outcomes associated with altered HPA development are seen to be variable due to the time at which development was affected, the species used, the method of prenatal stress exposure or the method of measuring maternal stress. The sex of the offspring and also the age at which outcomes are assessed, are also among the factors influencing the final outcome that is measured. Despite some variation in study designs, the current literature suggests that prenatal stress exposure (and synthetic glucocorticoid treatment as a surrogate) has major and potentially long lasting effects on the HPA functioning of offspring.

The first study to show this effect in the 1950s demonstrated that adult rats exposed to stress in early life had altered HPA axis responsiveness (86), indicating the potent programming effects of early life experiences on adult functioning. Since this time, others have found that prenatal stress exposure is associated with an increase in stress responsiveness in offspring, whereby there is a reduced sensitivity of negative feedback within the HPA axis (87). Specifically, children of a prenatally stressed pregnancy have shown increased cortisol responsiveness in adolescence (16 years of age), which is exacerbated if they were exposed to adversity between 6 and 11 years of age (88). This indicates a potential disadvantageous adaptation to stress in offspring which may have been programmed in utero. Maternal cortisol concentrations throughout pregnancy

have been shown to be predictive of infant cortisol concentrations in response to stress whereas self-reported maternal stress in pregnancy was associated with behavioural changes to a stressful stimuli and not cortisol itself in offspring (89). In animal models, male rats born to prenatally stressed mothers also demonstrate increased HPA responsiveness postnatally (90), as do guinea pig offspring exposed to prenatal stress in late pregnancy (91). In rats however, the development of the HPA axis is different from that of humans, sheep and guinea pigs whose cortisol levels rise throughout gestation to peak at term (92) and therefore must be considered carefully when interpreting studies into perinatal adversity.

Similar effects have been observed with the administration of synthetic glucocorticoids during pregnancy on outcomes of HPA axis functioning in offspring. In guinea pigs, a single course of synthetic glucocorticoids is associated with a suppressed cortisol response to stress at 18 days of age in both sexes (93) but multiple doses was associated with suppressed cortisol in male offspring only at 10 days of age (94). Offspring of non-primate mothers given a high dose of dexamethasone repeatedly from mid pregnancy also show increased cortisol responses to stress, which are coupled with increased blood pressure and impaired glucose tolerance at 12 months of age, highlighting the potentially broad effects that perturbations in HPA axis development and prenatal stress exposure may produce (69). In contrast, in human preterm infants, a reduction in HPA responsiveness was noted after a single course of betamethasone and persisted until 4-6 weeks after birth (95, 96). By 19 years of age, young adults did not show any effect of prior glucocorticoid treatment on basal cortisol levels (97) however this study was again conducted in a cohort of premature offspring born at <32 weeks gestation which may have different outcomes to term babies administered synthetic glucocorticoids. The only long-term human study assessing the effect of antenatal steroids on HPA axis functioning in 30-year-old adults has found an increase in morning cortisol

concentrations (98). This study however did not assess changes in stress responses in adulthood and also failed to show any significant changes in HPA activity when accounting for other factors such as sex, birthweight, body mass index and use of oral contraceptives (98).

Programming of the HPA axis of offspring is a complex interaction of maternal and fetal endocrine and neuronal processes which are also markedly influenced by the age of insult and assessment as well as the sex of the offspring. There remains a need for further studies which track the development of babies administered synthetic glucocorticoids during development. Also more extensive examination of pregnancies that are affected by significant stress should be assessed to determine if these populations do indeed have clear alterations in their HPA stress responsiveness and thus are susceptible to stress-related conditions later in life.

### **1.3 Structural and functional outcomes of prenatal stress**

#### **1.3.1 Normal neurodevelopment and the intrauterine environment**

Stress exposure during pregnancy is known to impact on the developing fetus according to the time at which the stressor is experienced and thus the ontogeny of developing fetal systems and organs. Many of the adverse outcomes commonly associated with prenatal stress exposure relate to the behaviour of the offspring as they mature. Thus, understanding the normal processes of maturation is critical in elucidating mechanisms by which stress-induced perturbations in fetal development increase the incidence of behavioural pathologies after birth (Figure 1.2).

Human neurodevelopment is a complex series of events which begin from as early as 2 weeks of gestation as the neural plate begins to form and is fully formed by 4 weeks of gestation (99). Neurodevelopment occurs in such a manner that essential structures and

processes are developed earlier in gestation and more complex connections developed towards birth and even postnatally (100). Indeed, neural differentiation and migration occurs during the first 6 weeks of gestation when the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon are formed. Further, more specific neuronal migration and differentiation occur to develop structures such as the brainstem early in gestation and therefore perturbations in development at this early stage can result in severe alterations in brain structure. Examples of improper early neural development include spina bifida and other neural tube defects.

In any given brain region during fetal development, neurons arise first, followed by astrocytes and finally by oligodendrocytes (101). Once generated, neurons migrate to their final destination either by passive displacement by new cell creation or by active migration along glial fibres (102). This neuronal migration begins early in development and peaks between the third and fifth months in the cerebral cortex (103). Axonal and dendritic growth is also active from early in gestation, however the largest (six-fold) increase in synaptic density seen at 28 weeks of gestation until a peak level is reached, the age of which is region specific (104). Myelination is one of the last processes to occur in brain development. Although oligodendrocyte precursors have been reported in the spinal cord as early as 12 weeks of gestation (105), precursors are apparent in the periventricular white matter later in pregnancy at around 27 weeks with mature myelin producing oligodendrocytes present thereafter with activity continuing well after birth (106).

Towards the end of pregnancy, the majority of the brain has formed and now selective synapse elimination and apoptosis (pruning) occurs to create complex interactions between different brain structures. In the third trimester, at around 80% of gestation, a large amount of cholesterol accumulation and myelination begins to occur in brain regions such as the hippocampus and the cerebellum (100). Late pregnancy is also a

time of considerable glial cell proliferation as well as increased synaptogenesis, neuronal and axonal migration and proliferation and various receptor maturational processes, all of which place high energy demands on the fetal brain (107, 108).

Two of the major brain regions undergoing extensive development both pre and postnatally that have been shown to be affected by stress exposure are the hippocampus and the cerebellum. The hippocampus is formed from the cortical plate from as early as 7 weeks of gestation and continues to develop and produce granule cells until term and into postnatal life (99). The majority of granule cells are produced within the dentate gyrus by birth in primates and the neurons of the CA1-CA3 regions of the hippocampus are also fully present by birth (109). The cerebellum also begins development from early in gestation however neurogenesis in this region is relatively late, occurring in the last trimester and into early postnatal life (102). In the cerebellum, granule cells migrate inward postnatally to form a functional inner granule cell layer by adulthood (110). Interestingly, the different lobes of the cerebellum develop at precise times during gestation with for example, lobe VIII developing relatively early in gestation and lobe X developing shortly before birth and into postnatal life (111).

These two brain regions represent important structures in perinatal brain development and demonstrate the vast differences that can exist between different brain structures and even cell types, which are important considerations when assessing the outcomes of a prenatal insult on fetal brain development.

The guinea pig and the human have remarkably similar brain development trajectories compared to other laboratory rodent species such as rats and mice (112). The onset of neurogenesis in the CA1 region of the hippocampus occurs in the first trimester of pregnancy at approximately 23 days of gestation in the guinea pig and 40 days of gestation in the human (113). Likewise, deep cerebellar nuclei are seen in both species during the first trimester (approximately 23 days of gestation in guinea pig and 42 days

of gestation in human; 113) and continue to develop throughout the rest of pregnancy and into early life (102). GABA positive neurons are first seen in the human and the guinea pig in the second trimester with myelinating occurring near term in the guinea pig and shortly after in the human (113).

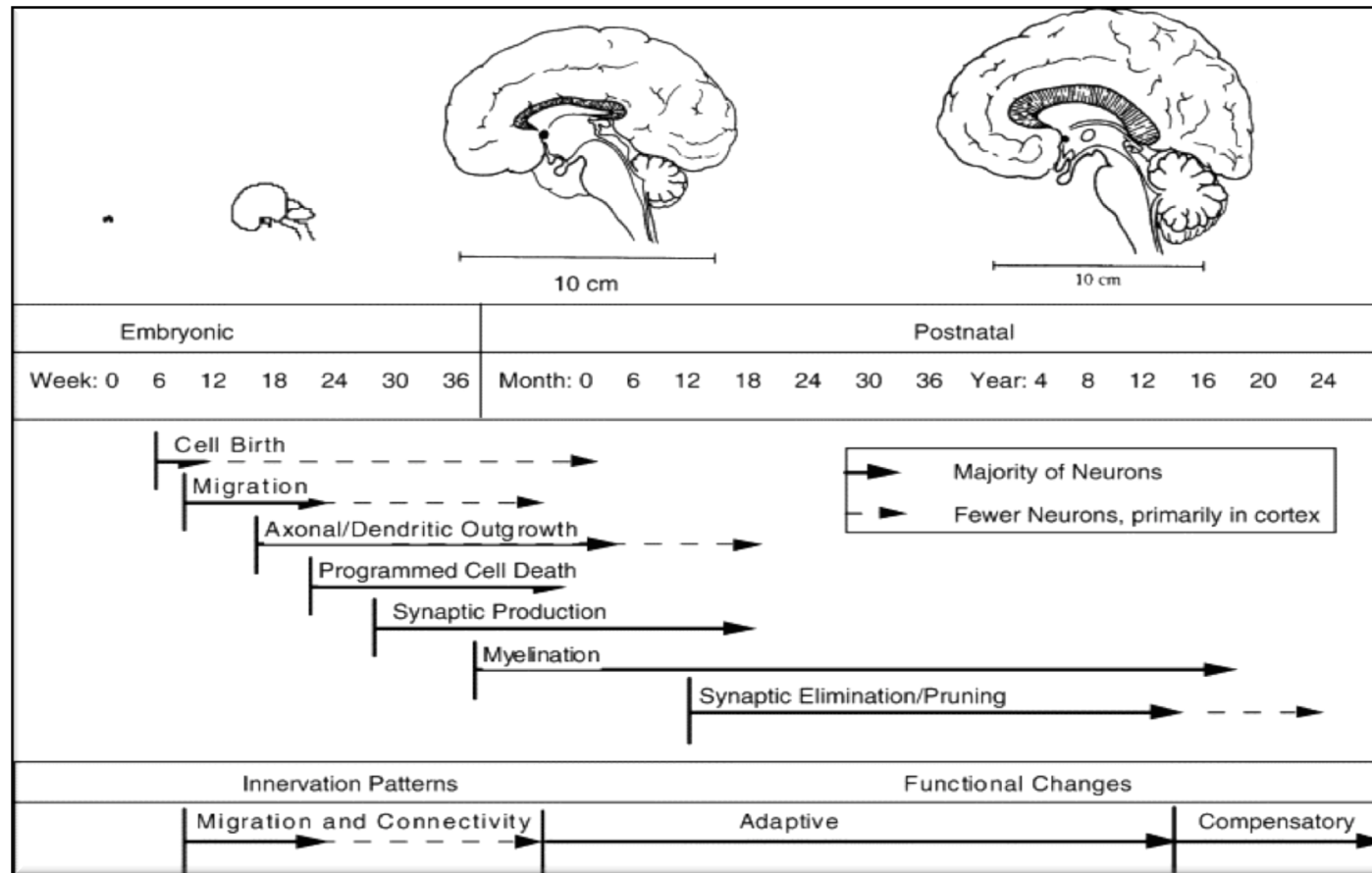


FIGURE 1.2 TIMELINE OF HUMAN BRAIN DEVELOPMENT. NEURODEVELOPMENT OCCURS IN PHASES, WITH MORE COMPLEX STRUCTURES AND CONNECTIONS OCCURRING IN LATER GESTATION AND EARLY LIFE. ADAPTED FROM ANDERSEN, 2003, WITH PERMISSION (100).

### **1.3.2 Prenatal stress and fetal neurodevelopment.**

Several studies have demonstrated adverse effects of prenatal stress and high levels of glucocorticoids on fetal brain development. The majority of studies to date have focused on brain regions which have likely contributory effects on functional outcomes observed following maternal stress. In this regard, the hippocampus has been the most widely studied brain regions as it has been implicated in learning and memory functions (114) which have also been shown to be affected by prenatal stress exposure (115). Uno et al, showed in a number of studies that the hippocampus is one of the primary brain regions influenced by the effects of prenatal stress and exogenous glucocorticoid treatment, finding a loss in hippocampal volume and neuron number (116-118). In 2003, Coe et al showed in rhesus monkeys that high fetal levels of cortisol (induced by chronic prenatal stress for 25% of gestation) both in early and late gestation were associated with reduced hippocampal sizes and disturbed development of neurons in the hippocampus of offspring, effects that persisted for up to 3 years after birth (119). The CA1 region of the hippocampus contains a high concentration of important efferent projections and pyramidal cells involved in cognitive functioning (114, 116, 120) and also has been shown to be selectively vulnerable to excitotoxic insults (121-123). This may be attributable in part, to the high levels of expression of GR (123) and MR in this region (124). Indeed, expression of GR has been reported in the guinea pig brain in the CA1 and CA3 regions of the hippocampus, the dentate gyrus, thalamus and amygdala with levels rising with increasing gestation (125).

Interestingly, in a study where neurogenesis was experimentally decreased in the rat hippocampus, stress responsiveness was shown to be altered, and this in turn resulted in an increased vulnerability to psychiatric disorders in adulthood (126) indicating that neurogenesis may be a critical factor in normal hippocampal functioning in adult life and therefore prenatal insults to neurogenesis may cause even more detrimental outcomes

for hippocampal functioning. Restraint stress in the last week of gestation in rats has shown a dramatic (50%) reduction in synaptic density in the CA1 region of the hippocampus at 90 days post birth (120) which indicates that connectivity may be another way in which hippocampal functioning is altered following stress exposure. Interestingly, one study has found a beneficial effect of mild acute prenatal stress in rats whereby a very short duration (30 minutes) of stress exposure is associated with increased neurogenesis and differentiation in the hippocampus whereas a longer lasting duration of stress exposure was detrimental to the development of the hippocampus (127). This study attributed the beneficial effects of stress exposure to MR activation under mild stress conditions and the detrimental effects to activation of GR under high stress conditions due to higher glucocorticoid affinity to MR in the hippocampus and thus preferential activation of MR when glucocorticoid concentrations are lower in mild stress conditions (127). In support of studies implicating excitotoxicity as a mediator in prenatal stress-induced developmental damage, this study also found increases in glutamate in the hippocampus following severe prenatal stress which was coupled with increased circulating corticosterone concentrations in offspring (127). This group further found similar results in the paraventricular nucleus of offspring rats where mild stress enhanced neurogenesis but severe stress resulted in poorer performance in learning tasks (128). Myelin sheaths and synaptogenesis were also found to be altered in the hippocampus of male rats after prenatal stress exposure where the largest alterations were seen when the stress was experienced from mid-late pregnancy more than just late pregnancy (129). This study suggests that neurodevelopmental outcomes resulting from prenatal stress exposure may also be timing and sex-dependent.

White matter tracts within the cerebral cortex are linked with neurodevelopmental damage in other pregnancy compromises such as preterm birth and intrauterine growth restriction (130). Cerebral cortical white matter damage is strongly associated (>90%

prevalence) with cognitive, behavioural and attention deficits in children born preterm (130-133), effects that are also seen after exposure to prenatal stress. Oligodendrocyte maturation within the cerebral cortical white matter in late gestation has also been shown to be a particularly vulnerable window during development to the effects of hypoxic-ischemic injury, often resulting in cerebral palsy and brain injury in premature infants (106, 134). There has been little research on the specific effect of prenatal stress on the development of the cortex and white matter. One study investigating the effect of repeated betamethasone administration (4 doses) found that it delayed myelination in the corpus callosum and disrupted fetal brain growth by reducing whole brain and cerebral weight in term lambs (71, 72). In rats, late gestation prenatal stress is also associated with altered reactive astrocyte expression and reduced synaptic density in the frontal cortex, striatum, and hippocampus of adult offspring (120), highlighting that multiple brain regions may be affected by the same stressor.

The amygdala is another brain region which is involved in learning and memory functions and is linked to the function of the hippocampus and limbic system (135). Studies in rats have shown that the volumes, neuron number and glial cell expression are all reduced in the amygdala of male offspring shortly after birth to mothers who were chronically stressed from mid gestation (136). This study and others have shown however that deficits seen in the development of the amygdala in early life after prenatal stress exposure are resolved by adulthood in rats (136, 137), suggesting that the amygdala may be able to resolve prenatal insults and repair itself after birth. The amygdala has also been implicated in regulating the HPA stress response due to its high concentrations of CRH (138). Following late gestational social stress prenatal stress, both male and female rat offspring showed increased concentrations of CRH mRNA in the amygdala however only male offspring showed increased anxiety (139). These data suggest that the amygdala may have a role in the regulation of and adaptation to

prenatal stress but does not appear to be the major brain region contributing to long-term altered neurodevelopmental outcomes.

The hypothalamus and prefrontal cortex (PFC) have also been shown to be affected by prenatal stress exposure. Prenatally stressed rat dams who were exposed to variable stressors throughout the last trimester of pregnancy delivered male offspring with a hypothalamus almost double the size of controls (140). Later in life however, these males went on to development hypothalamic sizes that were half the size of their control counterparts (140), indicating an important period of postnatal development of the hypothalamus. Hypothalamic development was unaffected in females in this study (140). These findings were confirmed by Kerchner et al, who found that late gestational prenatal stress caused a reduction in male hypothalamic size compared to controls with no effect on females (141). Additionally, Rees et al noted that the status of sexual activity was also important in determining hypothalamic size in male offspring exposed to prenatal stress (142). In the prefrontal cortex, prenatal stress in the third trimester in rats reduces the organisation of cells in this region in adult offspring of both sexes (143) however the power of this study was limited by low animal numbers and therefore a lack of sex differences is expected. Chronic restraint stress in adult male rats has shown dramatic changes in dendritic morphology in the prefrontal cortex with reductions up to 35% apparent in dendritic length (144), suggesting that this brain region may have particular sensitivity to the effects of stress. The prefrontal cortex is important for executive and cognitive functions (145) and therefore its role in mediating the stress response (146) may be critical for the development of cognitive related deficits that result from prenatal stress in utero.

In the last decade, the cerebellum has become increasingly recognised for its role in emotional regulation and cognitions, as well as its key function in motor control (147). The cerebellum also contains the highest number of glucocorticoid receptors of any

brain region (148) and has important links with the limbic system and HPA axis (149). Alterations in the structure and function of the cerebellum have been associated with major neuropathologies including autism (150), schizophrenia (151) and attention deficit disorder (152) and interestingly, these are also outcomes associated with prenatal stress exposure (section 1.3.4). Despite this link, very few studies have assessed the role of prenatal stress on the developing cerebellum. Granule and purkinje cells as well as interneuronal connectivity have been shown to be adversely affected following acute bouts of restraint stress in the first and second trimester in rat dams (153, 154). Late gestational restraint stress in mice has been shown to alter Purkinje cell morphology in adolescence and dendritic atrophy in adulthood in the cerebellum of offspring. Interestingly, these neural changes were not associated with anxious behaviour in a plus maze test (155), indicating that the cerebellum may not play a key role in the development of anxiety disorders.

These data suggests that different cell populations within the fetal brain contribute and respond differently to prenatal stress and further, that they confer different developmental qualities during gestation, perhaps relating to their functional relevance or stage of development at the time of stress.

### **1.3.3 Sexually dimorphic brain development and outcomes**

Males and females have been shown to respond differently to in utero insults including prenatal stress, showing sex-specific effects on endocrine, neurological and epigenetic development that may predispose to further complications during the lifespan (156). Epidemiological studies show that pregnancy complications, including fetal morbidity and mortality, and particularly disorders of neurodevelopment, have higher incidences in males than in female fetuses (157-159). In animal models assessing brain development, adult male rat offspring show reduced neurogenesis in the hippocampus

following exposure to prenatal restraint stress (160). Male adult mice who were exposed to chronic variable prenatal stress in early gestation showed altered behaviour in a tail suspension and forced swim test. In contrast, prenatally stressed females did not exhibit any changes in behaviour compared to controls (161). This is in agreement with clinical findings which suggest that maternal stress early in pregnancy is associated with an increased risk for development of schizophrenia later in life, particularly for males (162). Szuran et al reported that prenatal restraint stress in late pregnancy results in decreased hippocampal wet weight by approximately 15% in male rat offspring and approximately 8% in female offspring however only males demonstrate altered behaviour in water maze performance (163). These observations again highlight the vulnerability of males to prenatal stress-induced programming of brain development and behaviour. Using the same model, Szuran et al later found that although males showed poorer performance in spatial learning tasks, it was female offspring who showed increased basal corticosterone levels and decreased GR expression in the hippocampus (164), indicating that females may be more susceptible to HPA axis programming than males. In contrast, Schmitz et al found that just one acute episode of restraint stress in late pregnancy reduced hippocampal volume and granule cell number in the hippocampus by almost a quarter in female adult rat offspring with no effect on males (165). This result suggests that neurodevelopmental outcomes may be as highly dependent on the type and timing of the prenatal stressor as they are on the sex of the offspring. Many studies however have investigated the outcomes of prenatal stress exposure on the brain development of male offspring only (115, 120), or have not specified the sex of the offspring (127, 166), which potentially skews the results of this work.

Sexual differentiation of the brain has been shown to occur during critical periods of fetal neurodevelopment in both animal models and human studies. In fact, under

normal circumstances males and females show different structural features in the brain including brain volumes, neuron numbers and synaptic connections (167, 168) and therefore sexually dimorphic outcomes after prenatal stress are not unexpected. The first study to characterise the effects of steroid hormone levels during pregnancy on masculinization and defeminisation of the offspring's brain was performed in 1959 in guinea pigs and began the investigation into the organisational/activational hypothesis (169). This study found that sex hormones such as testosterone could program the development of the fetal brain (organisation), producing females with altered sexual phenotypes postnatally (activation) (169). Interestingly, a recent study in rats found that when testosterone conversion to estradiol is experimentally blocked during gestation, male offspring show altered sexual behaviour. This behaviour was similar in male offspring whose mother received the mild stress of vehicle injections (170), indicating that prenatal stress may have similar effects on sexual differentiation of the brain. In another study, the precise sexual behaviours observed in male rat offspring after prenatal stress were highly dependent on the type of stressor experienced by the mother (171). These studies showed that maternal immobilisation stress during pregnancy caused more feminine sexual behaviours postnatally and maternal water immersion stress facilitated masculine behaviours in offspring (171). Thus, this data suggests that male and female offspring may show different responses to prenatal stress exposure and as a result, fetal sex is a critical factor to consider when interpreting outcomes.

#### **1.3.4 Functional outcomes with prenatal stress**

There is now a substantial body of evidence highlighting the association between maternal stress during pregnancy and the cognitive, behavioural and emotional development of the offspring (10, 12, 172). As with other perinatal outcomes, the timing

of maternal stress during pregnancy has been found to be a major regulator of behavioural development. In non-human primates, early gestation prenatal stress has been associated with poorer scores relating to attention and neuromotor maturity, compared to mid and late gestation (173). Maternal bereavement stress in the first trimester and also in the first 2 months after birth has been associated with an increased risk for development of psychiatric disorders in offspring, including poorer temperament, slow adaptability, negative mood and easy distractibility (174). In a Danish study assessing maternal bereavement, over 29,000 women who experienced the unexpected loss of a child or the father during pregnancy had male offspring with a substantial 72% increased risk for development of Attention Deficit Hyperactivity Disorder (ADHD) with no effect on females (175). Interestingly, this association was also apparent if the loss occurred in the 6 months preceding conception, however to a lesser extent (175). The strongest association was also seen if the loss occurred during the third trimester of pregnancy (175), indicating that late pregnancy may be a vulnerable timepoint for the risk of developing ADHD. This finding is further supported by studies that show male offspring exhibit higher rates of learning and memory deficits and hyperactivity disorders following exposure to prenatal maternal stress (175, 176), particularly when the stress was experienced in late gestation (177, 178). Prenatal stress has also been associated with increased incidences of neuropathologies such as depression and schizophrenia which manifest later in life (179-181). Associations have been made between prenatal stress exposure from a natural disaster and poor neonatal outcomes, with negative effects seen on normal playing behaviours, cognition, language and even physical development (10, 12, 172). In one observational study which used the experience of war whilst pregnant as a measure of maternal psychosocial stress, fetuses affected in the first trimester, particularly during the third month of pregnancy, showed a 2-5 fold increase in the risk of developing mood disorders and bipolar disorder later in

life (182). Both objective and subjective reporting of first trimester prenatal stress has also been shown to predict higher scores for autism spectrum disorder at 6<sup>1/2</sup> years of age in offspring (183) however in a different cohort, self-reported prenatal stress exposure in the second trimester of pregnancy was associated with a 2 fold increased risk for development of ADHD symptoms at 4-5 years of age, particularly in male offspring (184). In the precocious guinea pig, prenatal stress in mid gestation has also been shown to be associated with anxious behaviour in male offspring (91) with the same results noted in adult male rats who were exposed to prenatal stress in late pregnancy (139). In pigs however, maternal stress in mid gestation resulted in anxious behaviour in adult female offspring that was also associated with abnormal maternal care in early life (139). This interaction between prenatal adversities and early life care and attachment to the mother are likely important interlinking factors given the ongoing nature of brain development after birth in many brain regions that may contribute to the overall behavioural development of offspring. Indeed, early life care has now been implicated as an important determinant for normal neurodevelopment, stress responsiveness and behavioural functioning (185). The effects of maternal care and prenatal stress are also known to produce changes in maternal behaviour transgenerationally, leading to a vicious cycle of stress and impaired care of young offspring, particularly for females (186, 187).

These studies show the persistent effects that prenatal stress can have on behavioural functioning of offspring both in early life and well into adulthood. The potential alterations in behaviour that result from prenatal stress exposure appear to be related to the type and timing of stress-related insult, the sex of the offspring and to the level of care received shortly after birth.

#### 1.4 Neurosteroids and neuroprotection

Neurosteroids are steroid hormones that are synthesised in the CNS and/or peripheral systems and are able to regulate CNS excitability (188). The most potent neurosteroid, allopregnanolone, is synthesised from progesterone, which is primarily released from the placenta during pregnancy with levels rising steadily throughout gestation (189).

Allopregnanolone acts to positively modulate  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptors to increase inhibition and thereby to decrease neural excitability. This action is particularly important during mid-late gestation at which time this action performs a critical neuroprotective role (190, 191).

The GABA<sub>A</sub> receptors are ligand-gated chloride channels which are organised in a heteropentameric structure consisting of a combination of 5 subunits out of a possible 19 subunits identified to date (192). The composition of these subunits determines their binding affinity and regional expression within the brain (192). The most common structure of GABA<sub>A</sub> receptor subunits in the brain contains 2  $\alpha$  units, 2  $\beta$  units and either a  $\delta$  or a  $\gamma$  unit. Receptors containing a  $\delta$  unit have a higher sensitivity for allosteric activation by allopregnanolone which acts through increasing the duration of the chloride channel opening, causing cell hyperpolarisation and thus inhibition of action potentials (193, 194). Extrasynaptic GABA<sub>A</sub> receptors, which are primarily responsible for tonic inhibition, are commonly comprised of the  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 6 and  $\delta$  subunits (195). Interestingly, the hippocampus predominantly expresses the  $\alpha$ 4,  $\alpha$ 5 and  $\delta$  subunits and the cerebellum predominantly expresses the  $\alpha$ 6 and  $\delta$  subunits (195) suggesting that tonic neural inhibition mediated by GABA<sub>A</sub> neurotransmission may be particularly important in these brain regions.

The synthesis of neurosteroids is interlinked, with many metabolites requiring the same synthesising enzymes (Figure 1.3). Notably, 5 $\alpha$ -Reductase is the rate-limiting enzyme in synthesis of all GABA<sub>A</sub> receptor modulatory neuroactive steroids and therefore is often

the subject of investigation in studies assessing neurosteroidogenesis. Indeed, our group has previously observed high levels of fetal arousal-like behavioural activity and neural excitability when neurosteroid synthesis is blocked with a 5 $\alpha$ -Reductase inhibitor, finasteride (196). Reduced neurosteroid levels are associated with reduced levels of in utero REM sleep (196) which in turn, may result in developmental delay (197). Enzymes 5 $\alpha$ -reductase and 3 $\alpha$ & $\beta$ -hydroxysteroid dehydrogenase are necessary for the synthesis of neurosteroids and these enzymes are capable of being synthesised in astrocytes, oligodendrocytes and neurons (198-200). In addition, there is evidence supporting region-specific generation of these enzymes in the hippocampus, dentate gyrus, cerebellar granule layer and purkinje cells (198).

Neurosteroids, in particular allopregnanolone, have been implicated in the stress response with acute stress shown to increase concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC) in the circulation and also locally in the cerebral cortex and hypothalamus (201). Presumably by activating inhibitory GABA<sub>A</sub> receptors in areas of high CRH activity during stress, these neurosteroids are then able to attenuate the stress response thereby preventing excitotoxic neural damage (202). Indeed, exogenous administration of allopregnanolone in rats has been shown to attenuate CRH induced anxiety (202), supporting an important role for these neurosteroids in regulating the stress response. This action may be particularly important during fetal life when relatively low neural activity is required for normal development and therefore neurosteroids may be of critical importance for maintaining normal brain development (203). Acute stress has been found to be associated with an acute increase in neurosteroid levels which restore normal GABAergic tone and alleviate anxiety (204). Chronic stress however, has been shown to be associated with increased allostatic load in the neurosteroid system which produces chronic deficits in expression (205).

This greater allostatic load may be maladaptive, and particularly in early life, when persistent alterations in such stress-responsive systems can predispose to other stress-related conditions such as depression later in life (206).

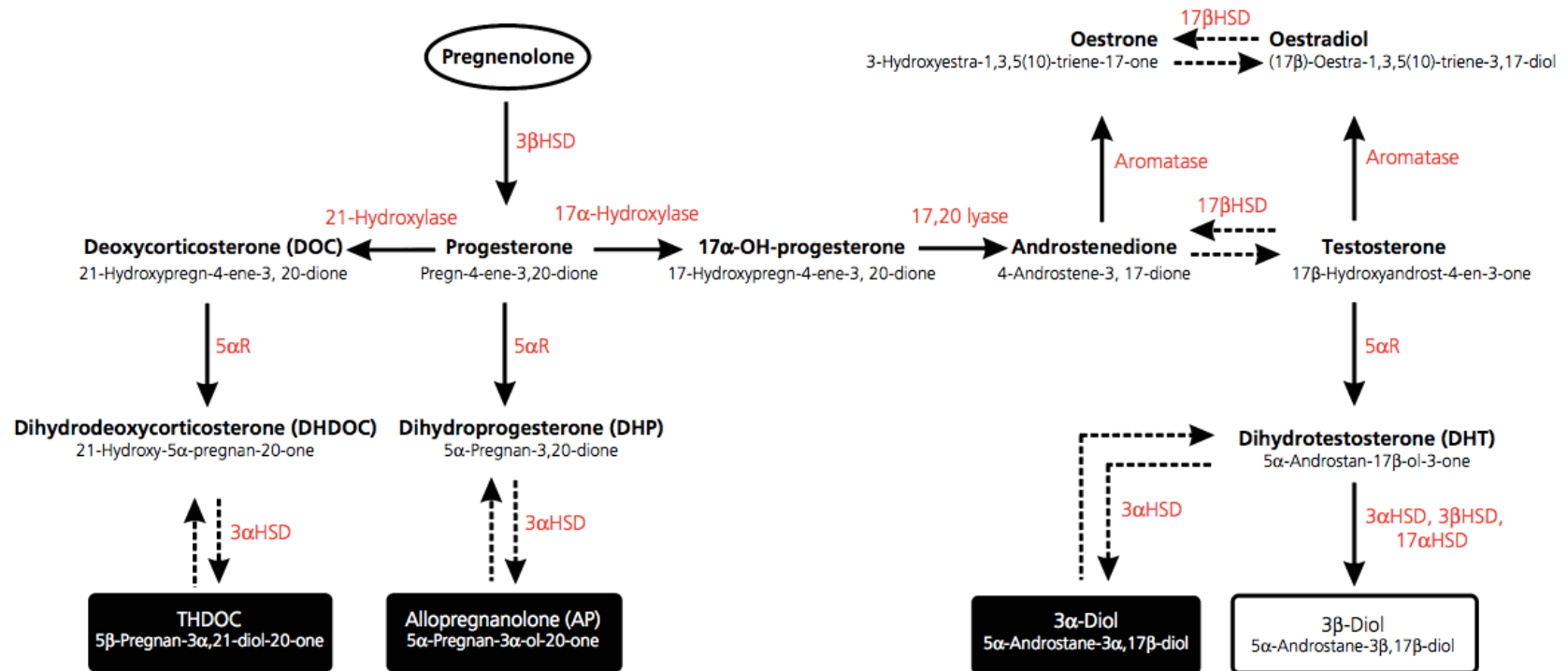


FIGURE 1.3 PATHWAYS OF NEUROSTEROID SYNTHESIS WITH POSITIVE MODULATORS OF GABA<sub>A</sub> RECEPTORS SHOWN IN BLACK BOXES. DASHED ARROWS INDICATE A REVERSIBLE REACTION. R=REDUCTASE; HSD=HYDROXYSTEROID DEHYDROGENASE. ADAPTED FROM BRUNTON ET AL, 2015, WITH PERMISSION (192).

In contrast, chronic stress during pregnancy has been associated with reduced levels of neurosteroids in the fetus and offspring. In adult rats, chronic isolation stress has been shown to dramatically reduce circulating and hippocampal concentrations of allopregnanolone which was also associated with reduced hippocampal neurogenesis and a more depressive/anxious phenotype (207). Interestingly, these effects were either prevented or restored to normal levels when rats were treated with allopregnanolone (207), which suggests that neurosteroids may indeed contribute to stress-induced outcomes. A number of psychiatric disorders including anxiety, depression and schizophrenia are also linked with reduced plasma/serum or cerebrospinal fluid (CSF) levels of neurosteroids in human populations (208-211). Recently, functional magnetic resonance imaging in human participants showed that exogenous allopregnanolone treatment increased connectivity between the amygdala and prefrontal cortex, which was associated with reduced anxiety (212). In fact, the neuroprotective properties of progesterone have also been trialled for the treatment of traumatic brain injury, with beneficial results (213) leading to current clinical trials of allopregnanolone for the treatment of traumatic brain injury and Alzheimer's disease (214, 215).

#### **1.4.1 Neurosteroids in pregnancy**

During pregnancy, allopregnanolone levels rise due to increasing placental progesterone production (189). After birth and the removal of the placenta, progesterone and allopregnanolone levels drop off substantially and this has been implicated in postpartum depression in women (216). In sheep, the expression of GABA<sub>A</sub> receptors has been shown to reach adult levels by the end of gestation however interestingly, allopregnanolone showed a higher affinity for these fetal receptors than adult receptors (217), indicating a potential selective expression of fetal GABA<sub>A</sub> receptor subunits that promotes allopregnanolone activation prenatally. During first half of human pregnancy,

these GABA<sub>A</sub> receptors are immature and promote excitatory neurotransmission until a 'development switch' occurs and these receptors cause inhibition. In the rat, however, due to the altricial nature of the late gestation fetus this switch occurs postnatally (218). In humans and guinea pigs, this occurs in mid-late pregnancy (219) and this is important for the action of endogenous neurosteroids exerting their inhibitory neuroprotective actions at this time.

Acute levels of stress in pregnancy are shown to increase allopregnanolone and other neurosteroid levels. Acute asphyxia of fetal sheep in utero has been shown to result in marked increases in allopregnanolone concentrations in the CSF (with more sustained increases in allopregnanolone in the fetal brain) which was paralleled by increases in expression of 5 $\alpha$ -Reductases and other enzymes required for neurosteroid synthesis (220). Previous studies have further shown that local production of allopregnanolone in the fetal brain is an important endogenous neuroprotection mechanism (191). This concept is supported by studies showing that neurosteroid levels as well as neurosteroidogenic enzymes are present in high concentrations in the fetal sheep brain (203). Furthermore these levels are sustained following hypophysectomy or adrenalectomy and thus, the fetal brain is capable of maintaining its own levels of neurosteroids, independent of hypothalamic-pituitary factors (221).

In contrast, chronic stress during pregnancy has been associated with reduced levels of neurosteroids in the fetus and offspring. In rats that were exposed to chronic late gestational prenatal stress, both male and female offspring showed reduced circulating allopregnanolone levels at birth and also in the brains of offspring when they displayed poorer cognitive abilities in juvenility (222). This same group found that when 5 $\alpha$ -Reductase activity is blocked during late gestation, offspring show similar impairments in cognitive abilities to that seen with stress exposure (223). Unlike males, female offspring in this cohort also showed increased basal corticosterone levels (223),

consistent with previous studies noting a female sensitivity to HPA axis programming after prenatal stress (224). Betamethasone administration in late gestation in guinea pigs lead to alterations in endogenous neurosteroidogenesis and reduced allopregnanolone levels in both the placenta and hippocampus, particularly in male fetuses (73). Testosterone is thought to impact on the expression on 5 $\alpha$ -Reductase in males (225) and recently, prenatal stress has also been shown to increase circulating testosterone in offspring in both rodent and human studies (226, 227). Given the intricate nature of steroid hormone synthesis (Figure 1.3), an interaction between neurosteroids and sex hormones seems likely, particularly considering the broad action of many of the synthesising enzymes involved, including 5 $\alpha$ -Reductase. Administration of allopregnanolone to mice prenatally has been shown to enhance memory and learning skills postnatally, effects that were attributed to increases in neurogenesis and myelination (228, 229). Conversely, when allopregnanolone synthesis was inhibited in sheep fetuses, an increase in apoptosis was observed (230), indicating the important functions of allopregnanolone in regulating both cell proliferation and cell death. Neurosteroids are clearly involved in a variety of processes, from normal brain development and neuroprotection to programming of altered functioning later in life. The role of neurosteroids in modulating GABA<sub>A</sub> receptor activity, enhancement of neurogenesis and neural signalling as well as the attenuation of HPA axis activation indicates that alterations in these important neuroendocrine factors may underlie some of the adverse effects associated with prenatal stress.

## **1.5 Summary, Rationale and Hypotheses**

### **1.5.1 Summary**

There is now a growing body of evidence supporting the association between maternal stress during pregnancy and a number of adverse perinatal outcomes. Some of the strongest associations in epidemiological studies include those relating to the cognitive and behavioural development of offspring. Learning difficulties, anxious behaviour and memory impairments are all outcomes that have been associated with stress in utero. Adverse behavioural outcomes following prenatal stress are supported by data in animal studies which link prenatal stress with perturbations in fetal brain development at particular vulnerable windows of fetal brain growth and thus these changes in neurodevelopment may underpin many of the altered functional outcomes reported. The neuroendocrine systems of affected offspring have also been associated with stress exposure, often noted to be more responsive to HPA axis activation after birth. Neurosteroids and in particular, allopregnanolone, are endogenous neuroprotectants that are produced in high amounts during pregnancy due to the abundance of placental precursors and can also be synthesised de novo in both the fetal and adult brain. Allopregnanolone has been linked with stress exposure, either increasing or decreasing depending on the nature of the insult. Allopregnanolone increases inhibitory neurotransmission through activation of GABA<sub>A</sub> receptors and is also capable of attenuating the HPA stress response, restoring homeostasis in the fetal brain. Alterations in allopregnanolone concentrations or activity may mediate the effects of prenatal stress by providing endogenous protection to the fetal brain.

### **1.5.2 Model design**

Multiple factors need to be considered when choosing an appropriate model to investigate the effects of prenatal stress. As discussed in section 1.2.1, there key difficulties in studying stress and ever more so, stress in pregnancy. Briefly, a lack of reliable and objective means for reporting and measuring prenatal stress is one major hurdle. Another is that of the numerous confounding factors that affect a pregnancy such as maternal nutrition, social support networks and parity. For these reasons, an animal model of pregnancy is a valuable means of assessing prenatal stress as primarily, stress can be induced. The choice of an animal model however is also complex. This thesis utilises a guinea pig model of pregnancy for a number of reasons. Firstly, guinea pigs are a precocious species with a relatively long pregnancy (~69 days). These rodents, unlike other rodents such as rats or mice, produce young that are mature at birth, fully covered in fur and eyes open (231).

Neurodevelopmentally, this species also produces mature offspring at birth, which is similar to the human, non-human primates and sheep (112). Unlike sheep however, guinea pigs do not exhibit a fall in progesterone concentrations just prior to birth and instead, a functional progesterone withdrawal has been proposed to initiate labour in both guinea pigs and humans (232). The guinea pig also has a haemochorial placenta like humans where the placenta secretes large amount of progesterone throughout gestation (233). This method of placentation as well as the relatively long gestation period in the guinea pig is important in maintaining sustained release of neurosteroids throughout gestation and thus provides a relevant model for studying developmental aspects of the neurosteroid system. The long gestation period also enables various experimental manipulations to be performed prenatally, for example as in the present work, the ability to induce maternal stress at different times in gestation.

The effects of glucocorticoids on HPA axis development in guinea pig offspring have been extensively studied (91, 234-236), making this animal model particularly useful in expanding the knowledge base to encompass specific neurobehavioural and neurosteroid influences on development after stress exposure in utero.

### **1.5.3 Research aims**

Despite a vast amount of research into the effect of maternal stress on perinatal outcomes for centuries, many of the precise mechanisms which alter neurodevelopment and behaviour are largely unknown. Therefore, the major aim of this thesis is to explore how prenatal stress exposure may alter key aspects of perinatal brain development, behaviour and neurosteroid expression in male and female offspring using a developmentally relevant model to human pregnancy, the guinea pig. Specifically, this thesis aims to investigate how prenatal stress induced at different times in gestation affects:

- 1) Perinatal brain development in key areas of interest i.e. the hippocampus, the cerebral cortex/subcortical white matter and the cerebellum;
- 2) The behaviour of offspring at a vulnerable time in development: childhood/juvenility; and
- 3) The neurosteroid system with and without maternal exogenous allopregnanolone treatment.

#### **1.5.4 Research design**

*Experiment 1: Effects of Prenatal Stress on Fetal Neurodevelopment and Responses to Maternal Neurosteroid Treatment in Guinea Pigs.*

Initial experiments had two main aims. The first was to determine the effect of maternal stress in mid-late gestation (an established model; 91, 234, 235) on key markers of fetal brain development in each sex cohort. The second aim was to investigate if maternal treatment with exogenous allopregnanolone could protect any adverse effect prenatal stress might have on the fetal brain. It was hypothesised that:

1. Prenatal stress in mid-late gestation will cause significant changes in markers of fetal brain development in the hippocampus and cerebral cortical white matter;
2. Fetuses will show altered endocrine parameters if subjected to prenatal stress;  
and
3. Maternal neurosteroid treatment will help to protect affected fetal brains from the effects of prenatal stress with no adverse effects on control fetuses.

*Experiment 2: Prenatal stress alters hippocampal neuroglia and increases anxiety in childhood.*

Following the investigation into fetal brain development in experiment 1 (Chapter 3), the second study aimed to characterise similar markers of brain development in the brains of offspring in childhood/juvenility who were exposed to the same maternal stressor and determine the effect of the sex of the offspring on outcome measures. This study also aimed to determine if behavioural functioning was altered in juvenile offspring and if their basal neurosteroid and endocrine levels were programmed prenatally by stress.

The hypotheses of this study were:

1. Prenatal stress in mid-late gestation will be associated with sustained changes in markers of brain development in the hippocampus and subcortical white matter of juvenile offspring;
2. Behaviour will be different in juvenile offspring exposed to stress in utero;
3. Prenatal stress exposure will alter endocrine concentrations in juvenile offspring; and
4. Prenatal stress exposure will change the expression of key GABA<sub>A</sub> receptor subunits in the hippocampus in parallel with neurosteroidogenic enzyme and local allopregnanolone expression.

*Experiment 3: Prenatal stress has persistent effects on the development of the cerebellum and GABA<sub>A</sub> receptor expression.*

The third study aimed to delineate the effect of prenatal stress exposure on perinatal brain development by assessing the cerebellum in male and female offspring. Therefore, this study had two primary hypotheses:

1. Prenatal stress in mid-late gestation will alter the development of the cerebellum both pre and postnatally; and
2. GABA<sub>A</sub> receptor subunit expression and circulating allopregnanolone concentrations will be altered in offspring exposed to prenatal stress.

*Experiment 4: The effect of early, late and acute prenatal stress on neurodevelopment, behavioural and neurosteroid responses in offspring.*

Finally, the last study in the thesis investigated how, using the same prenatal stressor at different times in gestation, could alter the brain development and behaviour of male and female offspring. The hypotheses of this study were:

1. Prenatal stress beginning early in gestation will have a greater impact on perinatal brain development than stress beginning towards the end of pregnancy due to increased exposure;
2. Prenatal stress beginning early in gestation will also result in more pronounced behavioural alterations in juvenile offspring than other stress exposure timepoints;
3. Circulating allopregnanolone levels will be higher in fetal populations than in juvenile offspring, in all stress timing groups; and
4. Prenatal stress beginning early in gestation will produce the most severe reductions in circulating allopregnanolone concentrations and levels will be higher in the group with the least stress exposure in utero.

Therefore the specific aims of this study were

1. To determine the effect of prenatal stress on markers of brain development in the hippocampus and subcortical white matter (cerebral cortical white matter) in fetuses and juvenile offspring when the maternal stress was induced from 0.5, 0.7 and 0.8 of gestation respectively; and
2. To determine if the timing of prenatal stress affected juvenile behaviour postnatally and finally if neurosteroid levels were changed as a result of the timing of prenatal insult.

## **Chapter Two: Extended Methods**

*This Chapter explains the methods for the studies in extended detail to that provided in individual data Chapters due to the complexities in the animal and behavioural testing protocols in particular as well as the word restrictions in published manuscripts. It is to be noted that as part of this thesis, initial experiments included one group of maternal stress induction (mid) with later experiments assessing the effect of different stress timing protocols on outcomes. As such, Chapters in this thesis are presented in this order. The methods detailed in this Chapter however have been presented as a cohesive protocol including all experimental groups.*

### **2.1 Animal Stress Protocol**

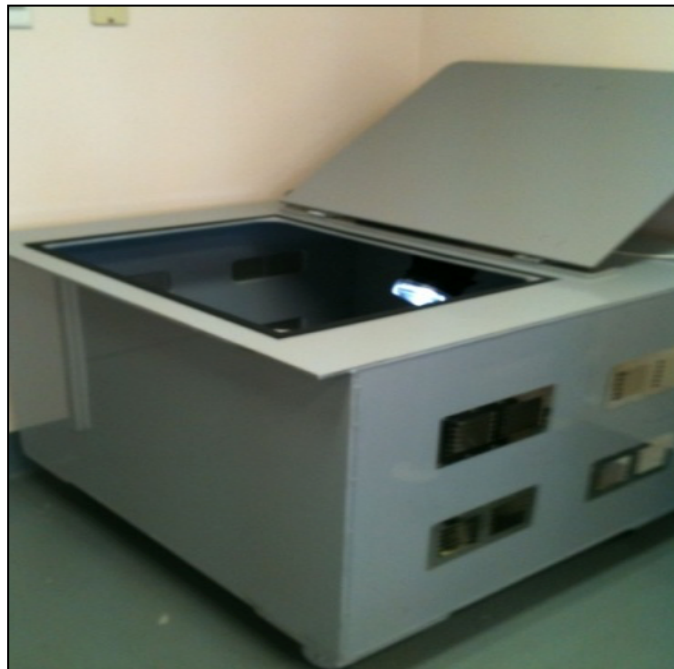
All procedures were approved by the University of Newcastle Animal Care and Ethics Committee under consecutive ethics protocols A-2009-109, A-2010-151 and A-2013-300 and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Research Support Unit of Newcastle University at the John Hunter Hospital campus provided time mated out bred tricolour pregnant guinea pigs. The dams were pregnancy tested by Research Support Unit staff using manual palpation of the abdomen and uterine horns at approximately gestational age (GA) 21 and upon confirmation of pregnancy, dams were allocated to the respective protocols. The pregnant guinea pigs were housed indoors in individual cages within visual and auditory contact of other animals in the study. Commercial guinea pig pellets, hay and water fortified with ascorbic acid (237) were provided *ad libitum* and as such, food (commercial guinea pig pellets and hay) and/or nutrient intake were not measured in this study. The guinea pigs were also subject to a 12 hour light/dark cycle daily to replicate natural environmental cycles. From the time of allocation, pregnant guinea

pigs were handled and monitored at least twice weekly to acclimatise the animals to contact with research personnel and the saliva sampling protocol. Monitoring included keeping written records detailing key indicators of health such as weight gain, abnormal vocalisations, quiet or overactive behaviour and reduced appetite. Cage changing occurred twice weekly with clean cages provided with soft paper-based bedding and loose hay to ensure comfort and cleanliness of the dams.

Upon entering the research protocol, dams were randomly allocated into either a stress (strobe light) exposure or control (handling but no strobe light exposure) group. A ventilated wooden box (dimensions: 1m x 1m x 0.7m; Figure 2.1) constructed by the University of Newcastle, Faculty of Health Workshop was fitted with a 20 Watt LED Strobe Light (Audio Visual Engineering, Dandenong, Victoria, Australia). Guinea pig home cages (maximum capacity 2 cages and hence 2 guinea pigs) were placed in this box and subjected to 2 hours (9am-11am) of strobe light exposure on designated days (every 5 days beginning on GA35 ('early' stress group), 50 ('mid' stress group) or 60 ('late' stress group); Figure 2.2). It is to be noted that dams were placed in the strobe light box within their normal home cages with all food and water provisions remaining unchanged and *ad libitum*. This stress container was appropriately ventilated and maintained at similar temperature to the surrounding housing areas (adjacent to the housing room). Both control and stress dams had been extensively handled prior to entering the experiment protocol and so were familiar with handling and with the area where the procedures were performed. Control groups of guinea pigs remained in their home cages in the normal housing room during the allocated 'no strobe light exposure' times. During this time, conditions of the housing and experimental area were monitored to ensure a consistent environment for the control animals.

Saliva samples were collected from all of the dams by mastication on a cotton bud for approximately 1 minute both immediately (within 2 minutes) before and after each of

the strobe light exposure or the control events. These samples were then centrifuged at 12000g at 4°C for 5 minutes to remove the saliva from the cotton bud and stored at -20°C until analysis. Changes in maternal diurnal cortisol concentrations were not assessed as a part of this study however the times of the stress protocol was controlled as previously described.



*FIGURE 2.1 STROBE LIGHT EXPOSURE APPARATUS. A WOODEN BOX MEASURING 1M X 1M X 0.7M FITTED WITH A STROBE LIGHT FOR INDUCING STRESS. CAPACITY OF THE APPARATUS IS 2 GUINEA PIG CAGES SIMULTANEOUSLY.*

## **2.2 Allopregnanolone Treatment**

As a part of experiment 1 in this thesis (Chapter 2), a subset of pregnant dams were allocated to receive either allopregnanolone (exogenous neurosteroid) or vehicle (45% cyclodextrin) beginning on GA60 and administered subcutaneously each day until term. Allopregnanolone (Dr R. H. Purdy, Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA) was suspended in 45% 2-hydroxypropyl- $\beta$ -cyclodextrin (Sigma Aldrich, Castle Hill, NSW, Australia) and sonicated in a warm water bath for approximately 24 hours, or until the allopregnanolone was completely dissolved. The

45% 2-hydroxypropyl- $\beta$ -cyclodextrin was also used as the vehicle treatment. The dams were administered either allopregnanolone (10mg/kg/day; 238) or vehicle (1ml/day) at approximately 9am and 5pm (half dose administered twice daily) from gestational day 60 to gestational day 68 (except on stress/non stress days when the first injection was given after the stress or control event at 11am). Drugs were administered by subcutaneous injection on the scruff of the neck with control (vehicle) dams being handled and injected in the same manner. Animals were monitored for approximately 30 minutes following each injection within their normal housing conditions.

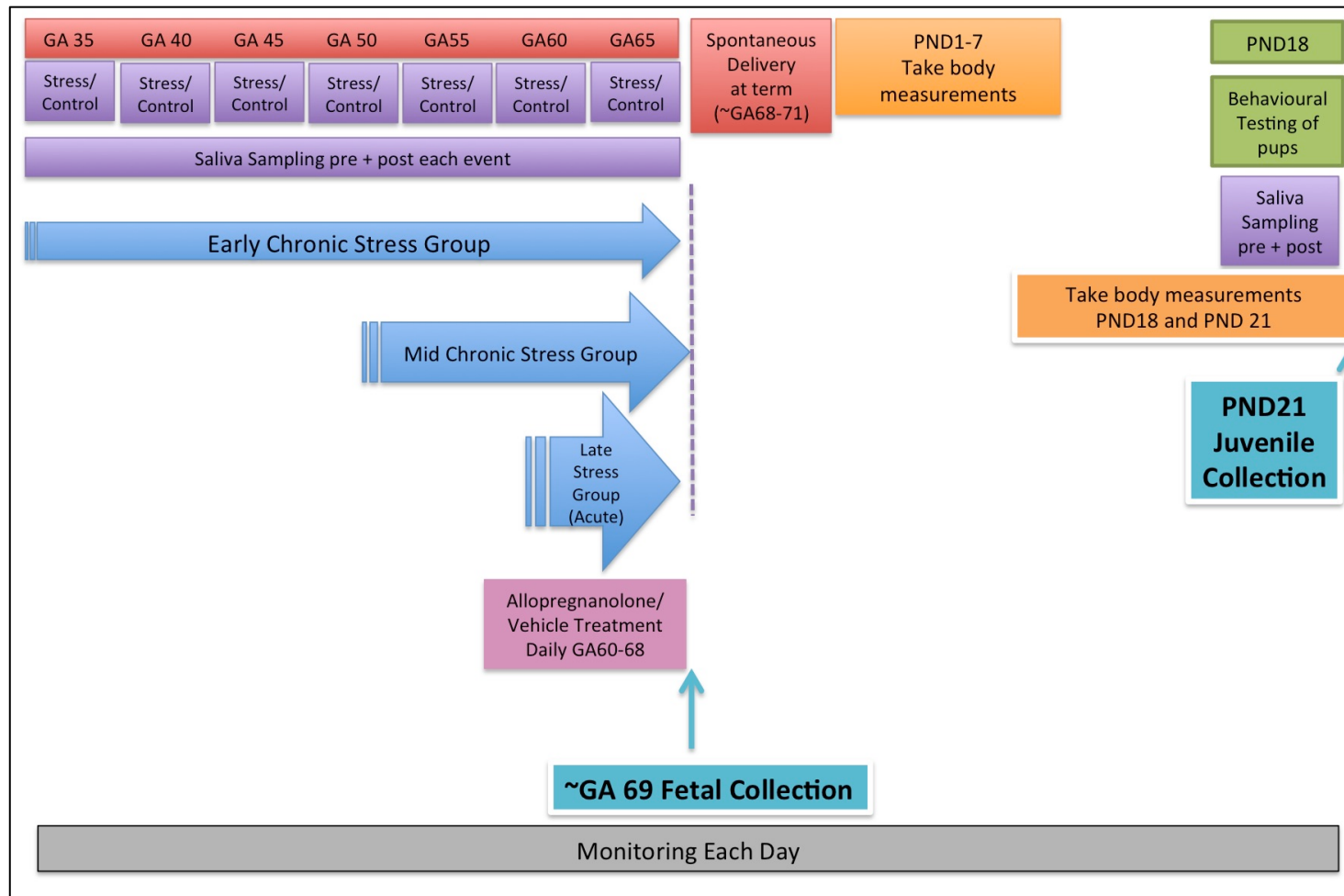


FIGURE 2.2 ANIMAL EXPERIMENTAL PROTOCOL. DARK BLUE ARROWS REPRESENT THE TIME AT WHICH PRENATAL STRESS WAS INDUCED IN PREGNANT DAMS (BEGINNING FROM GESTATIONAL AGE 35, 50 OR 60; EVERY 5 DAYS). RED BOXES INDICATE GESTATIONAL DAYS WITH PAIRED STRESS TIMEPOINTS AND SALIVA COLLECTIONS TIMEPOINTS IN PURPLE BOXES BELOW. ALLOPREGNANOLONE/VEHICLE TREATMENT IS SHOWN IN THE PINK BOX AND REPRESENTS A SUBSET OF DAMS WHO WERE TREATED FROM GESTATIONAL DAY 60-68. LIGHT BLUE ARROWS INDICATE THE TISSUE COLLECTION TIMEPOINTS FOR OFFSPRING (~GESTATIONAL AGE 69-FETAL OR POSTNATAL DAY 21- JUVENILITY). POSTNATAL DAYS AND TIMES FOR BODY MEASUREMENTS ARE SHOWN IN ORANGE BOXES. BEHAVIOURAL TESTING CONDUCTED ON POSTNATAL DAY 18 IS SHOWN IN THE GREEN BOX WITH PAIRED SALIVA SAMPLING IN THE PURPLE BOX BELOW IT. DAILY WELLBEING MONITORING OCCURRED FOR EVERY ANIMAL AND IS INDICATED BY THE GREY BAR.

### **2.3 Tissue collection**

Dams were further allocated randomly to a fetal tissue collection (GA69) or a postnatal day (PND) 21 tissue collection group. For those dams allocated to the postnatal arm of the protocol, dams were allowed to spontaneously deliver at term and using webcam monitoring (Logitech Webcam C170, Logitech Pty Ltd, Strathfield South, NSW, Australia), researchers were capable of identifying labour and confirming birth. Within 4 hours of delivery, pups were identified, weighed, measured for nose-rump-length (NRL), head length (HL), head circumference (HC) and abdominal circumference (AC). These measurements were recorded again at the time of post mortem on PND 21.

All pups and dams were monitored daily, as previously described, with all procedures carried out within visual and auditory contact of both the dam and remainder of the litter to minimise any stress to the neonate during handling. Dams were kept with their litters throughout the experiment.

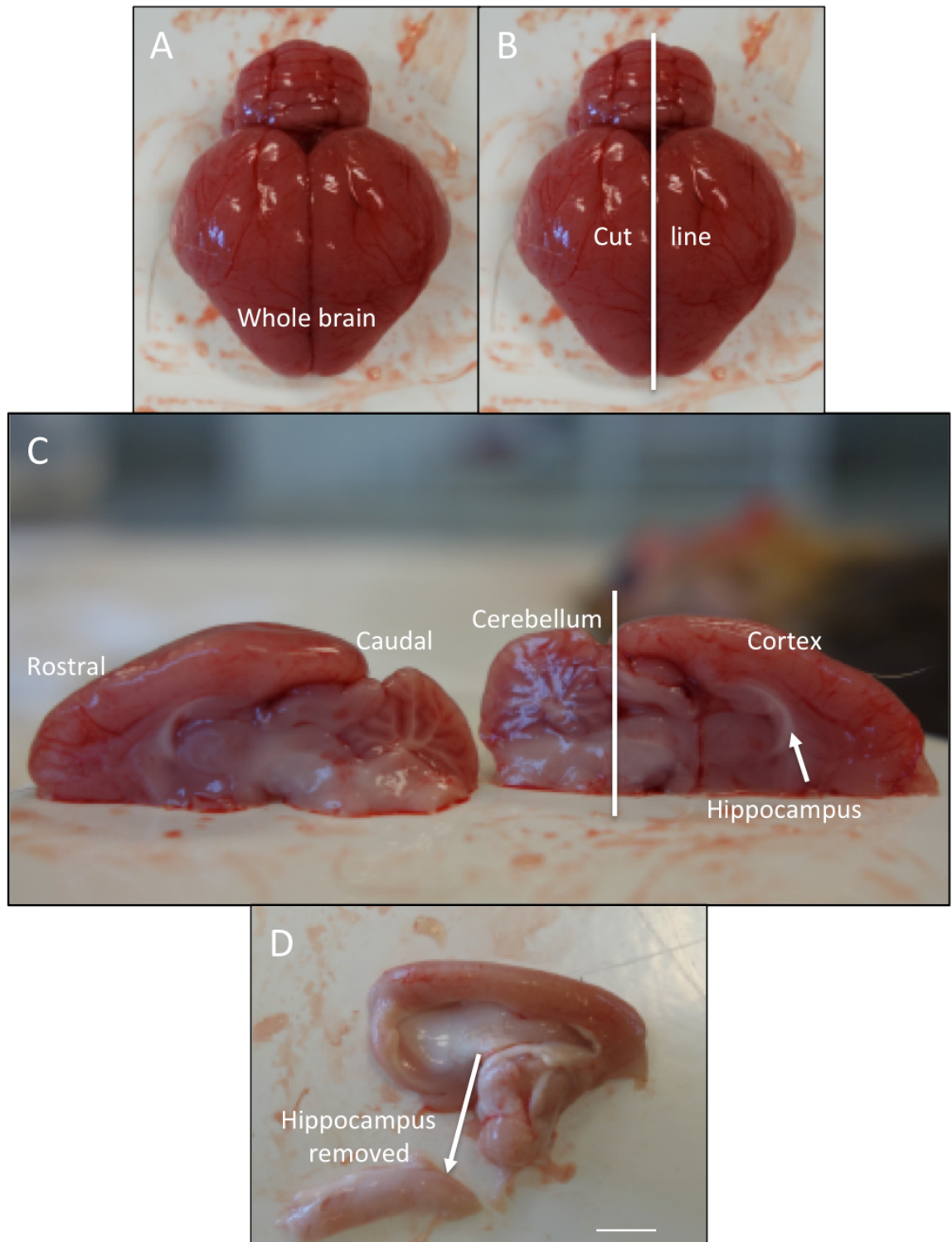
Post mortems were performed at PND21 to investigate the effects of prenatal stress on juvenile pups prior to weaning and the onset of sexual maturity.

For those tissues collected at term, pregnant dams were euthanased at approximately GA69 or on the second consecutive day of full pubic symphysis opening (>2cm diameter), which is a key indicator of imminent labour in the guinea pig (239).

All animals were sacrificed using 100% CO<sub>2</sub> inhalation for 8 minutes or until complete loss of heartbeat, pedal reflex and palpebral/corneal reflex (240). Once euthanased, an incision was immediately made along the abdomen. The diaphragm was incised as a secondary death measure and following this, in pregnant dams for the fetal collection, the uterus exposed allowing fetuses to be removed. Maternal, fetal and pups bloods were immediately collected in K2 Ethylenediaminetetraacetic acid (EDTA) vacuette tubes (Greiner Bio-one, Kremsmünster, Austria) to bind calcium ions and delay coagulation before centrifugation at 12000g for 10 minutes at 4°C to obtain plasma

samples. Plasma samples were then snap frozen and stored at  $-80^{\circ}\text{C}$  until analysis. Body weight, sex, nose-rump length as well as weights of organs were recorded, including the whole brain, hippocampus, placenta, heart, liver, kidney, and adrenal glands. Amniotic fluid was also collected from the amniotic sac in fetal collections. Brains, once removed from the skull and weighed (Figure 2.3A), were dissected in a sagittal plane (Figure 2.3B) and each hemisphere either sectioned coronally to isolate the hemisphere from the cerebellum; or dissected to isolate the hippocampus separately (Figure 2.3C,D). All brain tissues were then either snap frozen in liquid nitrogen at  $-80^{\circ}\text{C}$  or fixed via immersion in a 10% neutral buffered formalin solution (HT501128; Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia) for 24 hours at  $4^{\circ}\text{C}$  to create crosslinking of covalent bonds within the tissue, preserving the cytoskeleton and inhibiting any unwanted damage or contamination of the sample. Following fixation of the tissues, they were then placed in 0.1M Phosphate Buffer ( $\text{Na}_2\text{PO}_4$ ;  $\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$ ) until embedding in paraffin wax (Debbie Pepperall at Newcastle TAFE).

It is to be noted that in collection of brain tissues, it was only possible to collect samples for two separate types of analyses (out of three possibilities: one hemisphere frozen or fixed, and the hippocampus frozen separately) from each animal. As a result, due to the number of analyses performed and the method of collection of brain tissues, animal numbers differ between Figures presenting the results of different analyses. Animal numbers used are listed in parentheses where applicable.



**FIGURE 2.3 GUINEA PIG BRAIN DISSECTION.** THE WHOLE BRAIN IS SHOWN, REMOVED FROM THE SKULL (WITH OLFACTORY BULBS AND SPINAL CORD REMOVED; A). IT IS THEN DISSECTED SAGITTALLY TO SEPARATE THE TWO HEMISPHERES (B). ONCE SEPARATED, THE MIDLINE OF THE CEREBELLUM, HIPPOCAMPUS AND CORTEX CAN BE VIEWED (C). THE ROSTRAL AND CAUDAL ORIENTATION IS SHOWN FOR REFERENCE (C). THE HIPPOCAMPUS IS REMOVED SEPARATELY (D). SCALE BAR = 10MM

## **2.4 Behavioural Testing**

On PND18, the behaviour of offspring was assessed using an open field and object exploration test. Testing was performed on PND18 to allow for return to basal hormone levels (if a stress response was to be triggered) before post mortem tissue collection at PND21. Testing was performed by placing pups in a plastic, sterile open field arena (40 x 40 x 30cm) for 10 minutes with their behaviour tracked and recorded using the Stoelting ANY-Maze video tracking and analysis software (Stoelting Co., Wood Dale, IL, USA). Immediately following this open field test (which also served as an acclimatisation period to the testing arena), neonates were subjected to an object exploration test for a further 10 minutes. This test involved placing two identical objects (plastic building blocks and cups) at fixed locations in the outer quadrants of the arena and allowing the animal to explore these objects for the full duration of the test. Each object was secured to the floor of the arena using cloth tape to ensure that they did not move during the test i.e. if the animals bumped the objects. The objects and arena were cleaned after every use with 70% ethanol to remove any residual smells from previous testing.

Using the ANY-Maze tracking software, (Stoelting Co) the position of the animals' head and also the whole body were used to determine the distance travelled and time spent in different zones within the arena. The outcome measures from the open field test include total distance travelled (locomotor activity), and time spent and crossings into the inner zone of the arena (inner 9 squares of the field divided into 49 equal squares; anxious behaviour). The object exploration test was used to determine the time spent exploring both objects (object + 10% of object diameter as a border for total zone) to further determine anxiety and neophobic behaviours as a function of the motivation to explore novelty in the arena or avoid the perceived danger of unknown objects (241, 242). Animals were always placed into the arena facing away from the centre of the

arena (open field) and the objects (object exploration) to ensure no bias was introduced by placing the animals nearer a particular field or object.

Behavioural testing was performed in a well-lit (normal fluorescent lighting), temperate controlled room adjacent to the normal housing room within the animal facility. Dams and the remainder of the litter (where applicable) were maintained in their home cages within auditory contact of the behavioural testing area to minimise undue isolation stress to the pups undergoing testing. Saliva was collected by methods previously described immediately before and after the complete testing period (i.e. immediately before open field testing and immediately after object exploration) before centrifugation at 12000g for 5 minutes at 4°C to remove the saliva from the cotton bud and stored at -20°C until analysis. The arena was thoroughly cleaned between each test to ensure the removal of residual olfactory stimuli within the arena.

## **2.5 Immunohistochemistry**

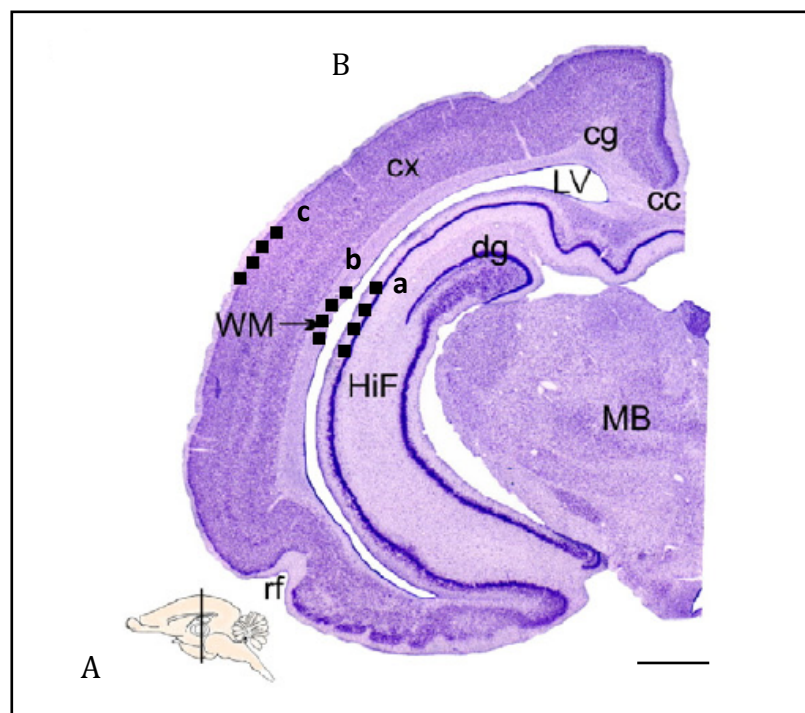
The fixed brain tissues were embedded in paraffin wax and processed for immunohistochemical staining and analysis using methods that have been previously described briefly (243). Paraffin embedded brain blocks were cooled to approximately 0°C and dampened with a cloth prior to cutting to ensure high quality sections. Sectioning was undertaken on Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde NSW, Australia) to produce 8µm brain sections. Three serial sections per slide were placed on SuperFrost Plus Slides (Menzel-Glaser, Braunschweig, Germany) and air dried for approximately 24 hours at room temperature.

Once the sections had dried, they were then rehydrated and dewaxed via immersion in serial chemical baths containing xylene for dewaxing, 100% ethanol (EtOH) and 70% EtOH for rehydrating the sections and 100% methanol with 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to inhibit endogenous peroxidase activity. Antigen recovery was carried out with

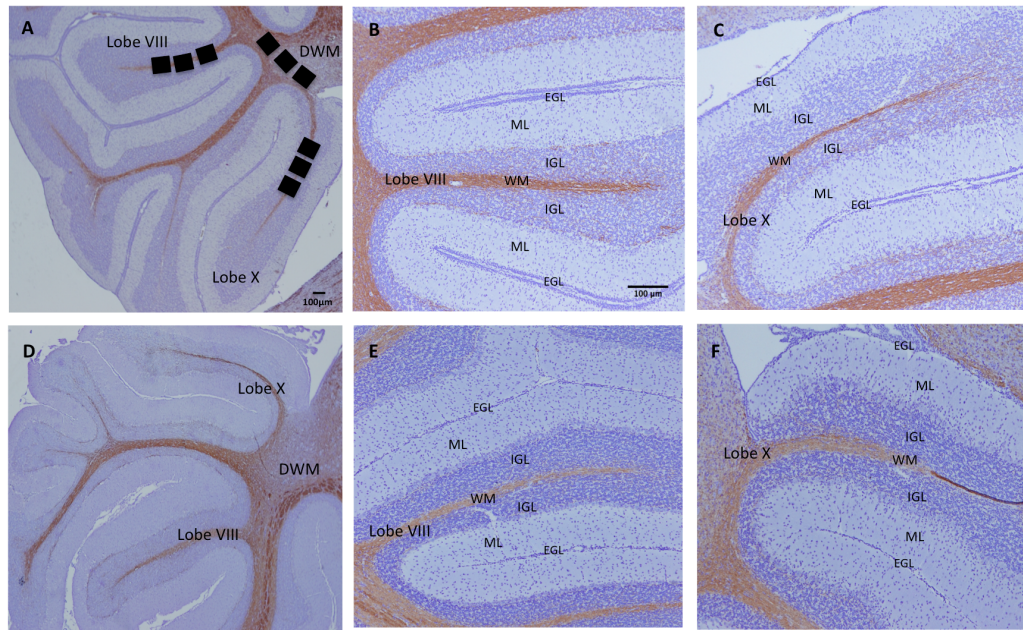
Reveal It Solution (ImmunoSolutions Pty Ltd, NSW, Australia) at 80-90°C to unmask epitopes. Following blocking with bovine serum albumin in phosphate buffered saline (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide) for 1 hour, sections were incubated in primary antibody solutions for myelin basic protein (MBP; Sigma Aldrich), glial fibrillary acidic protein (GFAP; Sigma Aldrich) and microtubule associated protein 2 (MAP2; Sigma Aldrich) overnight at concentrations 1:4000, 1:4000 and 1:30000 respectively. This was followed by incubation with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP and MAP2, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK). Subsequently slides were incubated in Streptavidin-Biotinylated HRP complex (RPN1051, Amersham) or VECTASTAIN® Elite ABC System (Vector Laboratories Inc., Burlingame, CA, USA; cerebellum slides) for immunodetection at room temperature for 1 hour. Finally, the slides were then incubated in 3,3'-diaminobenzidine (DAB) concentrate (Sigma Aldrich) with H<sub>2</sub>O<sub>2</sub> (Australian Scientific Pty Ltd, Kotara, NSW, Australia). One section on the slide was stained with 1% cresyl violet counter stain to allow for easy orientation under the microscope (and layer width measurements in the cerebellum). Slides were fixed with coverslips (ProSciTech Pty Ltd, Kirwan, QLD, Australia) using Microscopy DPX (Merck Australia, Kilsyth, VIC) and viewed with bright field microscopy on a Nikon Eclipse 90i microscope (Nikon Australia Pty Ltd, Lidcombe, NSW, Australia) with images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia).

Under the microscope, once general histology and orientation was achieved via examination of the cresyl violet stained section, the remaining two sections were imaged according to specific brain regions. In this thesis, the CA1 region of the hippocampus and the adjacent subcortical white matter were predominantly analysed, however the pial surface of the cortex and lobes VIII and X as well as the deep white

matter of the cerebellum were also included. To do this, four images were captured within the CA1 region of the hippocampus as well as four corresponding images in the inferior subcortical white matter cortex (MBP and GFAP) and pial surface of the cortex (MAP2 only; Figure 2.4. In the cerebellum, due to the length of the white matter tracts, three images were captured in each of the lobes and the deep white matter (Figure 2.5). All immunoreactivities were analysed by densitometry using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA), made binary by adjusting the threshold manually, with the percentage area of coverage recorded for each of the images captured, in two sections per animal yielding eight measurements per region analysed in the hippocampus and cortex, and six measurements for the lobes and white matter of the cerebellum. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.



**FIGURE 2.4** EXAMPLE OF GUINEA PIG BRAIN HISTOLOGY. THE BRAIN IS SECTIONED CORONALLY (A), MOUNTED ONTO A SLIDE AND THIONIN STAINED FOR GENERAL HISTOLOGY ANALYSIS (B). THE BLACK BOXES INDICATE WHERE THE IMAGES WERE TAKEN AND ANALYSED UNDER A MICROSCOPE IN THIS STUDY; WITHIN THE CA1 REGION OF THE HIPPOCAMPUS (A), THE CEREBRAL CORTEX (B) AND THE PIAL SURFACE OF THE CORTEX (C). WM = WHITE MATTER, CX = CORTEX, CG = CINGULUM, CC = CORPUS CALLOSUM, LV = LATERAL VENTRICLE, DG = DENTATE GYRUS, HiF = HIPPOCAMPAL FORMATION, MB= MIDBRAIN. SCALE BAR = 1MM. ADAPTED FROM TOLCOS ET AL, 2011, WITH PERMISSION (244)



**FIGURE 2.5 CEREBELLUM HISTOLOGY.** 8MM THICK CORONAL CEREBELLUM SLICES STAINED FOR MYELIN BASIC PROTEIN AND COUNTERSTAINED WITH CRESYL VIOLET SHOWING LOBES VIII (B,E) AND X (C,F) OF FETAL (A-C) AND 21 DAY OLD OFFSPRING (D-F). THE BLACK BOXES INDICATE WHERE THE IMAGES WERE TAKEN AND ANALYSED UNDER A MICROSCOPE IN THIS STUDY; LOBE VIII, LOBE X AND THE DEEP WHITE MATTER. THE EXTERNAL GRANULE CELL LAYER IS SHOWN AS EGL, MOLECULAR LAYER SHOWN AS ML, INTERNAL GRANULE CELL LAYER SHOWN AS IGL AND WHITE MATTER AS WM. SCALE BARS =100μm.

## 2.6 Western Blotting

Hippocampal tissues were crushed using dry ice and liquid nitrogen in a mortar and pestle and weighed into sterile aliquots of 20mg. These samples were then homogenised using Precellys soft tissue lysing kits (Bertin Technologies, France) in a Precellys 24 automated homogeniser (Bertin Technologies), according to manufacturer's instructions. Radioimmunoprecipitation assay (RIPA buffer) (50mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, Roche Complete Protease Inhibitor Cocktail and Roche PhosSTOP Phosphatase Inhibitor Cocktail (Roche Pty Ltd, Dee Why, NSW, Australia) was added to the crushed hippocampal samples immediately before homogenisation to initiate cell lysis and protein solubilisation. The supernatant, containing the crude protein extract was removed, transferred to a new tube and stored at -80°C.

Crude hippocampal protein samples extracted with RIPA buffer were quantified using a bicinchoninic acid (BCA) assay (BCA<sup>TM</sup> Protein Assay Kit, Pierce, Rockford, IL, USA). This assay uses the reduction of Cu<sup>+2</sup> to Cu<sup>+1</sup> by protein to form a detectable molecule with one cuprous cation. Using bicinchoninic acid as the reagent, this molecule forms a water-soluble complex which when exposed to spectrophotometry at 570nm shows clear absorbance. In this way, Bovine Serum Albumin (BSA) diluted in RIPA buffer was used to demonstrate known protein concentrations and create a standard curve for comparison to unknown samples (as per manufacturer's instructions). A 96 well plate was used for loading diluted BSA standards and extracted protein-of-interest samples in duplicate. The BCA reagent was then added to each well and incubated for 30 minutes at 37°C to enable colour development. Optical densities were then read at 570nm using the Fluostar Optima plate-reader (BMG Labtech, Ortenberg, Germany) and protein concentrations determined.

Hippocampal homogenates were quantified for 5 $\alpha$ -Reductase type 1 &2 enzymes and brain derived neurotrophic factor by western blot. Following homogenisation and total protein quantification, samples were prepared for gel electrophoresis with 70 $\mu$ g of protein. Protein samples were prepared with NuPAGE LDS sample buffer (4X) (Invitrogen, Mulgrave, Vic, Australia) at a volume equalling 25% of the total sample volume to initiate denaturing of the proteins and reduce disulphide bonds allowing for gel electrophoresis separation; reducing agent at a volume equal to 10% of total sample volume to initiate protein reduction and to ensure proteins are maintained in the reduced state and milliQ H<sub>2</sub>O (Millipore) as required for dilution. Samples were heated at 70°C for 10 minutes to activate the LDS, quenched on ice and then quickly vortexed and centrifuged for approximately 10 seconds at 12000g at room temperature.

NuPAGE Novex 4-12% Bis/Tris 1.5mm 10 well precast gels were washed with 1xNuPAGE MOPS SDS Running Buffer (50mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH

7.7) (Invitrogen, Mulgrave, Vic, Australia), placed in the XCell SureLock™ Mini-Cell system and excess Running Buffer was then added to fill the system. Protein samples were pipetted into the wells in duplicate as well as protein standards of known internal positive controls (pooled guinea pig adrenal sample) and Magic Mark XP Protein Standard. Electrophoresis was carried out at 200V, 120mA and 17W for 75 minutes or until the protein samples had visibly migrated to the bottom of the gel.

PVDF membranes were activated prior to western transfer via immersion in 5mL methanol for 1 minute followed by immersion in cold 1X Transfer Buffer (20X NuPAGE Transfer Buffer solution, 20% methanol and MilliQ H<sub>2</sub>O) for 30 minutes. Pre-cut filter paper and blotting pads were also immersed in cold 1X Transfer Buffer prior to the transfer process. Following electrophoresis, gels were removed from their castings and placed in the XCell II™ Blot Module (Invitrogen, Mulgrave, Vic, Australia) along with the PVDF membranes, filter paper and blotting pads to form the transfer sandwich. Small rollers were used to ensure that no air bubbles formed in the transfer sandwich. The inner XCell II™ Blot Module was filled with cold 1X Transfer Buffer and the outer chamber of the module filled with cold MilliQ H<sub>2</sub>O (Millipore, Billerica, USA). This transfer module was run at 30V, 160mA and 25W for 46 minutes. On completion of the transfer process, the transfer sandwich was removed and the PVDF membranes allowed to air dry for approximately 1 hour.

Once dried, the PVDF membranes were reactivated via immersion in methanol and placed in a blocking solution (5% BSA and 5% skim milk (1% w/v skim milk powder, Home-Brand, Woolworths, Bella Vista, NSW, Australia) solution in 1X TBS-T; 25mM Tris-HCL, 15mM NaCl, 0.1% Tween-20) at room temperature for 1 hour to reduce any non-specific antibody binding. Following blocking, membranes were incubated in a primary antibody solution for either 5 $\alpha$ -Reductase type 1 (goat anti-SRD5A1, NB100-1491, Novus Biologicals, Littleton, CO, USA), 5 $\alpha$ -Reductase type 2 (goat anti-SRD5A2, Ab27469,

Abcam, Cambridge, UK) or BDNF (Goat polyclonal antibody to BDNF (P-14) sc-33905-Santa Cruz Biotechnology Inc., Texas, USA). Membranes were incubated in 5% BSA, 5% skim in Tris-Buffered Saline and Tween 20 (TBST) with these primary antibody solutions at a final concentration of 1:1000 overnight at 4°C on a gentle rocker.

Following primary antibody incubation, membranes were washed with TBS-T to ensure excess amounts of primary antibody were removed so as to not interfere with secondary antibody incubation. The same secondary antibody (rabbit anti goat-Horseradish Peroxidase conjugate, PO449, DakoCytomation, Glostrup, Denmark) was used on 5 $\alpha$ -Reductase type 1 and type 2 and BDNF quantification in a 3% skim in TBS-T solution to a final concentration of 1:5000. Membranes were again washed in TBS-T and then 1X TBS (25mM Tris-HCL, 15mM NaCl) before immersion in Amersham ECL Plus™ Western Blotting Detection Reagent (Amersham, GE Healthcare, Buckinghamshire, England) for 1 minute to enable the HRP induced oxidation of luminol, which when exposed to UV light via in the LAS-3000 Imager (Fuji Photo Film, Japan) produced a detectable chemiluminescent signal. Quantification of the specific bands for 5 $\alpha$  Reductase type 1 and 2, as well as BDNF (5 $\alpha$ R1 ~26kDa; 5 $\alpha$ R2 ~29kDa and BDNF ~32kDa) were analysed using the Mutiguage software (Fuji Photo Film, Japan).

Following detection of 5 $\alpha$  reductase type 1 and 2 and BDNF, membranes were stripped and re-probed for  $\beta$ -actin in order to control for differences in total protein concentration per well due to inaccuracies in loading or protein quantification. Briefly, the membranes were washed in tap water for 5 minutes to remove the Amersham ECL detection reagent. NaOH (0.1M) was subsequently added to the membranes and incubated for 10 minutes on a rocker to effectively strip the membranes of both primary and secondary antibodies. Following this, membranes were again incubated in a blocking solution made up of 3% skim milk powder in 1X TBST-T for 1 hour at room temperature. A loading control polyclonal antibody against  $\beta$ -actin (Rabbit anti- $\beta$ -actin,

ab8227, Abcam) was diluted in a 3% skim milk powder solution in TBS-T to a final concentration of 1:8000 and added to the membrane for 1 hour at room temperature. Secondary antibody HRP-conjugate (Goat anti-rabbit IgG HRP conjugate 12-348; Upstate Cell Signalling Solutions, Millipore) was added to the membrane and incubated for 1 hour at room temperature. Detection was performed in the same manner as described previously. Blocking peptides specific to each primary antibody were used as control membranes to confirm specificity of each respective antibody (5 $\alpha$ R1 NB100-1491PEP, Novus Biologicals; 5 $\alpha$ R2 ab45681, Abcam; BDNF sc-33905 P, Santa Cruz). Using the Mutiguage software (Fuji), desired bands of protein of interest were isolated and quantified via calculation of the area under the curve minus the background signal, relative to  $\beta$ -actin loading control (ab8227-50 Abcam) and an internal loading control (guinea pig adrenal protein) on each gel.

## **2.7 Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)**

Hippocampal and cerebellar tissues were crushed manually using dry ice and liquid nitrogen in a mortar and pestle with 20mg subsequently homogenized in RLT Plus Buffer (Qiagen RNeasy Plus Mini Kit, Qiagen Pty Ltd, VIC, Australia) containing  $\beta$ -mercaptoethanol ( $\beta$ -ME) using Precellys soft tissue lysing kits and the Precellys 24 dual-tissue homogenizer (Bertin Technologies, France), according to manufacturers instructions. Briefly, tissue samples were homogenized in tubes containing 1.4mm ceramic (zirconium oxide) beads for 3 rounds of 30 seconds at 5000rpm with 20 seconds break in between each round to completely lyse the samples. Homogenised samples were then quenched on ice and centrifuged at 13000rpm for 10 minutes at 4°C to obtain the supernatant. Homogenate supernatant was then extracted for RNA using an RNeasy miniprep kit (Qiagen, Clifton Hill, VIC, Australia) using methods previously described (73)

and according to manufacturer's instructions. This extraction method involved adding 70% ethanol to the supernatants and performing serial elutions through the RNeasy spin columns to remove genomic DNA and purify the RNA. By carefully discarding the supernatants after each elution, RNA was obtained by finally washing the spin columns with RNase free water (Qiagen) into sterile collection tubes. The quantity and quality of the RNA was determined using the ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and also by agarose gel electrophoresis. A260-A280 ratios of 2.1-2.9 were obtained for all samples and bands at 18s and 28s at a ratio of 1:2 were confirmed using a UVP benchtop UV transilluminator chamber (BioDoc-It Imaging System, Upland, CA, USA) to confirm sample integrity after extraction. A total amount of RNA (1µg) was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to manufacturers instructions. Substrates from this system including oligo(dt) (deoxy-thymine nucleotides), random and hexamers and dNTP (Deoxynucleotide Triphosphate) were added to the samples at 65°C for 5 minutes allowed for binding to the RNA. After quenching samples on ice, cDNA synthesis mix (Invitrogen) was added to the samples containing reverse transcriptase buffer, 25mM MgCl<sub>2</sub>, 0.1M DTT, RNase out and SuperScript II Reverse Transcriptase. A negative Reverse Transcriptase (RT) mixture was also made with RNase free water (Qiagen) added instead of RT as negative control samples. Both positive and negative RT samples were then denatured at 25°C for 10 minutes, cDNA synthesized at 50°C for 50 minutes, reaction terminated at 85°C for 5 minutes and finally samples chilled on ice at approximately 4°C. Samples were then vortexed briefly and spun at 1000rpm for 10 seconds at room temperature before addition of RNase H (Invitrogen) to each tube and incubation at 37°C for 20 minutes to complete to reverse transcription. Sample cDNA was diluted with RNase free water (Qiagen) to a final working concentration of 10ng/µL.

Primers were designed in house based on predicted guinea pig sequences available ([http://www.ensembl.org/Cavia\\_porcellus/Info/Index](http://www.ensembl.org/Cavia_porcellus/Info/Index)) and sourced from Life Technologies (Life Technologies, Mulgrave, VIC, Australia; Table 2.1). Conditions including primer and cDNA concentrations were optimised in house prior to running of samples to ensure optimal amplification. Each 10 $\mu$ L reaction contained SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia), 400nM forward and reverse primer ( $\beta$ -actin, 5 $\alpha$ R1 and GABA $_A$  receptor subunit  $\delta$ ), 600nM forward and reverse primer (GABA $_A$  receptor subunit  $\alpha$ ) or 800nM forward and reverse primer (5 $\alpha$ R2) and 10ng sample cDNA. RT-PCR was performed using the 7500 ABI real-time machine (Applied Biosystems), with results analysed by Sequence Detection Software v2.01 (Applied Biosystems). Default parameters of thermal cycling were used with target cDNA amplified in triplicate with negative reverse transcriptase and no-template controls included to ensure no fluorescent signal was generated by residual genomic DNA or primer interactions, respectively. The comparative Ct method ( $2^{-\Delta\Delta Ct}$ ) was used to calculate fold changes in the mRNA levels of each target gene relative to a house-keeping gene ( $\beta$ -actin) and a calibrator sample of pooled guinea pig hippocampal and cerebellar brain samples, as appropriate. Consistent Ct values were obtained for  $\beta$ -actin across all stress and control fetal and juvenile samples.

*Table 2.1: Complete guinea pig specific primer sequences for quantitative real-time reverse transcriptase-polymerase chain reaction*

| Gene of interest                       | Forward primer sequence        | Reverse primer sequence     | Concentration |
|----------------------------------------|--------------------------------|-----------------------------|---------------|
| 5 $\alpha$ -Reductase type 1           | CGA GGA GGG AAG CCA ACA        | TAA CCA CAA GGC ACA ACC AGC | 400nM         |
| 5 $\alpha$ -Reductase type 2           | TCA GAA AGC CTG GAG AAG TCA TC | CCG AGG AAA CAA AGC GTG AA  | 800nM         |
| GABA <sub>A</sub> R subunit $\alpha$ 5 | CAC GGG CGA ATA CAC GAT TA     | CAA TCA GAG CAG AGA ACA CGA | 400nM         |
| GABA <sub>A</sub> R subunit $\alpha$ 6 | ATA AGG AGT CAG TCC CAG CA     | ACG AAA GCA AAG CAT ACA GC  | 600nM         |
| GABA <sub>A</sub> R subunit $\delta$   | GCG TCT ACA TCA TCC AGT CC     | AAT GGG CAA AGG CAT ACT CC  | 400nM         |
| $\beta$ -actin                         | TGC GTT ACA CCC TTT CTT GAC A  | ACA AAG CCA TGC CAA TCT CAT | 400nM         |

*PRIMER*

*SEQUENCES DESIGNED FOR GUINEA PIG 5 $\alpha$ -REDUCTASE TYPES 1 AND 2, GABA<sub>A</sub> RECEPTOR  $\alpha$ 5,  $\alpha$ 6 AND  $\delta$  SUBUNITS AND B-ACTIN GENE EXPRESSION. PRIMER SEQUENCES ARE DISPLAYED FROM 5'–3' FOR FORWARD AND REVERSE PRIMER.*

## 2.8 Allopregnanolone radioimmunoassay

Plasma samples were thawed on ice, vortexed and centrifuged at 13,000g for 30 seconds at 4°C to remove any clots in the samples. Brain homogenates were crushed using dry ice and liquid nitrogen in a mortar and pestle. 200mg of tissue was then weighed into a plastic tube and homogenised in 1mL ice-cold 50% acidified methanol (50% v/v methanol containing 1% acetic acid) using an Ultra-Turrax T-25 homogeniser (IKA Labortechnik, Staufen, Germany). The homogenate was then centrifuged in a J-6 M/E centrifuge (Beckman Coulter, Gladesville, NSW, Australia) at 2500rpm for 25 minutes at 4°C, the supernatant decanted and homogenised again using another 1mL ice-cold 50% acidified methanol (50% v/v methanol containing 1% acetic acid). The supernatants were then pooled in glass tubes (12 x 75mm, Kimble Chase, Vineland, NJ, USA) ready for the allopregnanolone assay.

Radioimmunoassay was performed according to a previously published protocol (245). Plasma and brain homogenate was combined with allopregnanolone tracer (1000-1500CPM, 5 $\alpha$ -[9,11,12,3H(N)]; PerkinElmer Life and Analytical Sciences, Boston, USA) in assay buffer (0.05M PBS, 0.025M EDTA, 0.1% BSA, 0.1% NaN<sub>3</sub>) and 50% methanol containing 1% Acetic Acid to acidify the samples. Plasma steroids were then extracted using a C<sub>18</sub> Sep-Pak cartridge (Waters Associates, Milford, MA, USA), which was pre-primed with consecutive washes of 100% methanol, 50% methanol and 50% methanol with 1% acetic acid. Samples were eluted in 100% methanol and the eluent dried in a heating block at 50°C under a stream of nitrogen gas (5L/minute). Samples were then resuspended in MilliQ H<sub>2</sub>O (Millipore) and vortexed well. For fetal and maternal plasma, potassium permanganate solution (0.5%) was then added and vortexed well and incubated for 30 minutes at room temperature in order to oxidise saturated steroids, particularly progesterone, which is present in high concentrations during pregnancy and

can cross-react with the allopregnanolone antibody (245). Cross-reactivities of the antisera have previously been described (73). In order to re-extract the plasma steroids from the aqueous potassium permanganate solution, n-hexane and diethyl ether (1:1 v/v) was added, each sample was vortexed and the samples frozen on dry ice. The n-hexane/diethyl ether was then decanted into a fresh tube and the solvent extraction repeated twice more. Samples were again dried under nitrogen gas before being resuspended in assay buffer. Extraction recovery was then calculated for each sample by counting tritium activity using a liquid scintillation  $\beta$ -counter (Beckman Coulter, Gladesville, NSW, Australia) with extraction recoveries then used in the calculation of unknown allopregnanolone concentrations following radioimmunoassay. Average recovery of allopregnanolone was  $72.3 \pm 1.3\%$  from brain homogenate and  $78.6 \pm 0.6\%$  from plasma with recoveries values used in the final calculation of allopregnanolone tissue concentration to adjust for extraction losses.

For the radioimmunoassay, anti-allopregnanolone sheep IgG antisera (Dr R.H. Purdy) at a final concentration of 1:1000 and tritiated allopregnanolone tracer ( $\sim 7800$ - $8000$ cpm) was added to each sample (plasma and brain homogenate) and incubated overnight at  $4^{\circ}\text{C}$ . The following day, a charcoal suspension was added and each sample was centrifuged at  $10000g$  for 10 minutes at  $4^{\circ}\text{C}$  to remove any unbound tracer. The sample supernatants were added, in duplicate, to scintillation vials (Beckman Coulter) along with 5mL scintillation fluid (BSC Scintillation Cocktail, GE Healthcare) and tritium activity determined using a liquid scintillation  $\beta$ -counter. Unknown allopregnanolone concentrations were calculated relative to a standard curve and quality control (QC) samples were run in every assay. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. The limit of detection was  $25\text{pg/mL}$  and the inter- and intra- assay coefficients of variation were 6.4% and 6.7% respectively.

## 2.9 Cortisol and Progesterone Immunoassay

Cortisol concentrations were determined in maternal and neonatal saliva using a salivary assay kit (Salimetrics Inc., State College, PA, USA), as per manufacturers instructions. This kit provided a 96 well plate coated with monoclonal antibodies against cortisol and has previously been used to quantify cortisol in guinea pig saliva (235, 246-249). Cortisol standards (at concentrations of 3.0, 1.0, 0.333, 0.111, 0.037, 0.012  $\mu\text{g}/\text{dL}$  respectively), cortisol controls (accurate to approximately 0.2  $\mu\text{g}/\text{dL}$  for high control and 0.02  $\mu\text{g}/\text{dL}$  for low control) and unknown samples were added to the plates in duplicate. A cortisol enzyme conjugate (containing cortisol linked with horseradish peroxidase) was then added to the plate, which competes with cortisol in the controls, standards and samples for the available antibody binding sites. Following a 1 hour incubation, unbound cortisol conjugate was washed away using the manufacturers wash buffer solution and subsequently, substrate solution (Tetramethylbenzidine; TMB) was added to visualise the bound cortisol peroxidase. This substrate was incubated for 30 minutes in the dark before the reaction was stopped via addition of a 3M sulphuric acid stop solution to the plate. The plate was then read on a Fluostar Optima plate-reader (BMG Labtech, Ortenberg, Germany) at 450nm (corrected at 490nm) with the resulting optical density being the reading of bound cortisol peroxidase, which is inversely proportional to the amount of cortisol present in each samples. Using a 4-parameter fit sigmoid curve calculation, the unknown concentration of samples was calculated. Sensitivity of the assay was 0.012  $\mu\text{g}/\text{dL}$  to 3.0  $\mu\text{g}/\text{dL}$  and inter- and intra- assay coefficients of variance were 9.39% and 5.52% respectively.

Cortisol and progesterone concentrations were quantified in fetal and maternal plasma by immunoassay by Hunter Area Pathology Service (HAPS). The assays were conducted on the UniCel Dxl800 Access Immunoassay System (Beckman Coulter Inc., Gladesville, NSW, Australia), as per manufacturers instructions. Briefly, samples, controls and

standards were incubated simultaneously with anti-cortisol IgG and cortisol conjugated to horseradish peroxidase. During incubation, the samples, controls or standards competed with enzyme-labelled cortisol to bind anti-cortisol antibody. Unbound reagents were removed by washing. Upon addition of chromogen substrate, colour development occurred which is inversely proportional to the amount of cortisol in the sample. The intensity of the colour was measured and the concentrations of samples were obtained by reference to the standards. The same protocol was followed using standards and antibodies relevant to progesterone quantification. It is to be noted that progesterone in amniotic fluid was not detectable therefore no data is presented in this regard. The inter- and intra- assay coefficients of variance for cortisol were 5.17% and 4.3% respectively and for progesterone 8.2% and 7.9% respectively.

## **2.10 Statistical Analysis**

These studies used the Hunter Medical Research Institute's Clinical Research Design, Information Technology and Statistical Support (CReDITSS) service to assist in statistical design.

For fetal data collected in experiment 1 (Chapter 3), a linear mixed model was used to compare the differences between main independent variables as fixed factors: group (stress or control), drug treatment (vehicle and allopregnanolone) and sex (male or female). A two-way Multiple Analyses of Variance test (ANOVA) was used to further characterise specific relationships within each sex cohort. In this cohort, in order to prevent pregnancy bias, only one male and one female fetus was used from each pregnancy for analysis. In a number of pregnancies, there was only male or female fetus and therefore only one fetus was used for analysis. A two-way Multiple Analyses of Variance test (ANOVA) was also used to assess differences between maternal plasma allopregnanolone data with a repeated measures Multiple Analyses of Variance test

(RM-ANOVA) test used to assess maternal repeated salivary cortisol sampling. For this fetal study (Chapter 3), unless otherwise stated, all data is presented as mean  $\pm$  standard error of the mean (SEM) with F statistic and degrees of freedom listed. Significance considered as  $p < 0.05$ .

Similarly, for all other perinatal outcome measures (Chapters 4-6), a linear mixed model was used to characterise differences between prenatally stressed and control pups using prenatal stress exposure as a fixed factor in the model as well as the sex of the pups. However, a more sophisticated statistical model was created for this data set to allow for multiple pups from the same pregnancy to be included. To do this, a random factor was created within the linear mixed model to account for any possible familial correlation. In this way, any correlation between pups from the same dam was appropriately accounted for ensuring that each pregnancy was equally represented in the model. Furthermore, a Bonferroni correction was used to determine multiple comparisons between the groups within each sex and perinatal age cohort. All data in Chapters 3-5 is expressed as  $\beta$ -coefficient values, 95% confidence intervals and  $p < 0.05$  considered significant. Unless otherwise specified, all data shown graphically is expressed as mean  $\pm$  SEM.

All statistical analysis was performed using SPSS software (version 21, SPSS Inc. IBM, Chicago Ill., USA) with graphs made using Graphpad Prism Software (version 6, Graphpad Software Inc., La Jolla, CA, USA).

### **Chapter Three: Effects of Prenatal Stress on Fetal Neurodevelopment and Responses to Maternal Neurosteroid Treatment in Guinea Pigs**

**Greer A. Bennett**, Hannah K. Palliser, Britt Saxby, David Walker, Jonathan Hirst.

This Chapter contains a manuscript published in the *Journal of Developmental Neuroscience* (2013; 35:416–426) and is a detailed assessment of the neurodevelopment of fetuses affected by prenatal stress in mid-late gestation. This manuscript also assesses the effect of exogenous maternal neurosteroid treatment as a potential compensatory mechanism for stress affected fetal brains. The format of the manuscript has been altered for the purposes of this thesis. The original manuscript is included in appendix A.

## Statement of Author Contributions

| Author          | Contribution                                                                                                                                                  | Signature |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Greer Bennett   | Experimental design, animal stress protocols and tissue collection, laboratory procedures, data analysis and manuscript preparation, revision and submission. |           |
| Hannah Palliser | Experimental design, animal stress protocols and tissue collection, laboratory procedures, data analysis and manuscript corrections.                          |           |
| Britt Saxby     | Laboratory procedures.                                                                                                                                        |           |
| David Walker    | Experimental design.                                                                                                                                          |           |
| Jon Hirst       | Experimental design and manuscript corrections.                                                                                                               |           |

Digitally signed by Robert Callister  
 DN: cn=Robert Callister, o=University of  
 Newcastle, ou=Faculty of Health and  
 Medicine,  
 email=robert.callister@newcastle.edu.au  
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 Date: 2015.07.31 10:25:38 +10'00'

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 Signed: Prof Robert Callister  
 Faculty Assistant Dean (Research Training)

31/7/15

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 Date:

### **3.1 Abstract**

Maternal psychosocial stress during pregnancy is associated with adverse neonatal outcomes. These outcomes result from changes in fetal brain development and lead to disrupted cognitive, behavioural and emotional development. The neurosteroid, allopregnanolone, has been shown to reduce neural excitability and aid in protecting the fetal brain from excitotoxic insults. The objectives of this study were to assess the effect of prenatal maternal stress on fetal brain development with and without maternal allopregnanolone treatment.

Pregnant guinea pigs were subjected to stress induced by exposure to a strobe light at 50, 55, 60 and 65 days gestation. Salivary cortisol levels were measured before and after each exposure. Fetal brains were assessed for markers of brain development using immunohistochemistry and plasma allopregnanolone was measured by radioimmunoassay.

Female, but not male prenatal stress exposed fetuses demonstrated higher brain to liver ratios (BLR). Male fetuses showed significantly reduced expression of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and both males and females showed reduced expression of microtubule associated protein 2 (MAP2). These markers were not affected by maternal allopregnanolone treatment. However, maternal allopregnanolone treatment resulted in an increase in fetal plasma allopregnanolone concentrations in control pregnancies but concentrations were not raised after prenatal stress exposure.

These findings indicate that the effects of prenatal stress on fetal brain development are sexually dimorphic with more pronounced negative effects seen on male neurodevelopment. Allopregnanolone treatment was not effective in raising fetal plasma concentrations after prenatal stress suggesting a stress-induced dysregulation of neurosteroid pathways during gestation. Interestingly, this study directly implicates

prenatal stress in the disruption of fetal neurosteroid levels, such that it may mediate some of the deleterious effects on fetal neurodevelopment by facilitating a deficit in normal endogenous neuroprotective mechanisms.

### **3.2 Introduction**

There is now growing evidence supporting developmental origins of various diseases, including development of neuropathologies later in life, such that prenatal stress is now associated with many behavioural and cognitive problems postnatally. Prenatal stress may disrupt growth of the fetal brain resulting in increased susceptibility to neurodevelopmental disorders, however many of the precise mechanisms leading to this disruption are not known.

Stress can be thought of as the adaptive response of an organism to ready itself for a threat to survival. The stress response is often classified by the increased release of cortisol and the downstream effects this glucocorticoid produces. There is now a substantial body of evidence highlighting the association between maternal psychosocial stress during pregnancy and a number of adverse perinatal outcomes. Some of the strongest associations in epidemiological studies include those relating to cognitive, behavioural and emotional development of offspring (10, 12, 172). Maternal stress during pregnancy is associated with increased incidences of childhood behavioural problems in infancy and at school age (250-255) with male offspring showing higher rates of learning and memory deficits and hyperactivity disorders (175, 176), particularly when the stress was experienced in late gestation (177, 178). Prenatal stress has also been associated with disorders in offspring stretching beyond childhood including increased incidences of neuropathologies such as depression and schizophrenia later in life (179-181).

Adverse behavioural outcomes following prenatal stress are supported by data in animal studies which link prenatal stress with perturbations in fetal brain development at particular vulnerable windows of fetal brain growth (256). Late pregnancy is a time of considerable myelination and glial cell proliferation as well as increased synaptogenesis, neuronal and axonal migration/proliferation and various receptor maturational processes, all of which place high energy demands on the fetal brain (107, 108). Therefore this period has been identified as a vulnerable period for neurodevelopmental delay or damage (91). Animal studies have linked prenatal stress to alterations in the hippocampus that result in a higher susceptibility to neuropathologies later in life (115, 119, 191). Late gestational social stress has been shown to increase anxiety behaviours in male offspring (139). High levels of cortisol in fetal circulation following exposure to an acoustic stressor have also been shown to cause disturbed hippocampal development in rhesus monkeys (119). Prenatal restraint stress in late gestational rats leads to dendritic atrophy in the hippocampus of the offspring as a result of excitotoxicity (257). Therefore stress-induced increases in glucocorticoid levels and neural excitation may mediate some of these deleterious effects.

During gestation there are high levels of endogenous neurosteroids, which act at inhibitory GABA<sub>A</sub> receptors to reduce neural excitability. We have previously observed high levels of fetal arousal and neural excitability when neurosteroid synthesis is blocked with finasteride (196) and that a reduction in neurosteroid levels is also associated with reduced levels of REM sleep which in turn, may result in developmental delay (197). During pregnancy, the placenta has a key role maintaining the endogenously protective neurosteroid levels by providing considerable amounts of precursors for their synthesis. This accounts for the remarkably high levels of the potent neurosteroid allopregnanolone, which is synthesised from progesterone, in the fetus throughout late gestation (189, 196, 258-260). We have chosen to administer allopregnanolone in late

gestation (from gestational day 60) to mimic this high endogenous production and to model normal responsiveness to stress exposure at this time. Furthermore, we have previously reported that administration of exogenous glucocorticoids during late pregnancy alters levels of the endogenous allopregnanolone by suppressing synthesising enzymes in the placenta (73). Our studies have also shown a decrease in reactive astrocyte marker expression in the brains of these fetuses, and interestingly, the males and not the female fetuses demonstrated these adverse effects in response to glucocorticoid exposure (73). Our previous studies have shown that allopregnanolone has potent neuroprotective effects against acute excitotoxicity following hypoxia/ischemia and that reduced concentrations of allopregnanolone conferred increased vulnerability to brain injury in late gestation (230).

In the present study we examined the effect of prenatal maternal stress and the associated increase in glucocorticoid exposure on fetal brain development during key growth periods in gestation. We then investigated the effect of prenatal stress when allopregnanolone was administered during the last 8 days of gestation (0.8 of gestation).

### **3.3 Methods**

#### *Animal stress protocol*

Time mated, outbred pregnant guinea pigs were obtained from the University of Newcastle colony. All 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. The dams were randomly allocated into either a stress (light) exposure or control (the same handling but no light exposure) group. At 50 days of gestational age (GA), dams in the stress exposure group commenced a procedure developed by Matthews et al (91, 234, 235) in which stress was induced by exposure to strobe light. Briefly, the animals were placed in

a ventilated light proof container and exposed to a strobe light for 2hr (9-11am). The high frequency strobe light intensity was 75 joules per 10 seconds. This protocol was repeated on GA 55, 60 and 65 (term 69 days). Dams in the control groups were treated in the same way with handling performed but no exposure to the strobe light. Saliva samples were collected from all of the dams by mastication on a cotton bud for approximately 1 minute both immediately (within 30 seconds) before and after each of the strobe light exposure or the control events.

Food (commercial guinea pig pellets and hay) and/or nutrient intake were not measured in this study, as food and water were available to dams *ad libitum*.

#### *Allopregnanolone treatment*

Allopregnanolone was obtained from Dr. R. H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA) and administered subcutaneously in a 45% 2-hydroxypropyl- $\beta$ -cyclodextrin vehicle solution (Sigma Aldrich, Castle Hill, NSW, Australia). Stress and control dams were randomly allocated to either receive allopregnanolone (10mg/kg/day) or vehicle injections twice daily at 9am and 5pm from GA60 to GA68.

#### *Tissue collection*

Pregnant dams were euthanased at term (69 days GA) or on the second consecutive day of full pubic symphysis opening (>2cm diameter), which is an established indicator of imminent labour in the guinea pig (239). Dams were euthanased by inhalation of 100% CO<sub>2</sub>. Maternal and fetal blood was collected immediately. Fetal placement, body weight, sex, nose-rump length as well as weights of organs were recorded, including the whole brain, placenta, heart, adrenal glands and liver. Fetal brains were dissected in a sagittal plane with one half snap frozen at -80°C or and the other half fixed via immersion in a

formalin solution (4% w/v Paraformaldehyde in 0.1M Phosphate Buffer ( $\text{Na}_2\text{PO}_4$ ;  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ ) (Sigma Aldrich).

### *Immunohistochemistry*

The fixed brain tissues were embedded in paraffin wax and processed for immunohistochemical staining and analysis using methods we have previously described (243). Briefly, 8 $\mu\text{m}$  brain sections were processed by a method involving dewaxing in xylene, rehydration in a series of ethanol/water washes and finally incubation in a hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and methanol solution to inhibit endogenous peroxidase activity. Antigen retrieval was then performed by incubation in Reveallt Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia). Following blocking with bovine serum albumin in phosphate buffered saline (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide), sections were incubated with primary antibodies for myelin basic protein (MBP; Sigma Aldrich), glial fibrillary acidic protein (GFAP; Sigma Aldrich) and microtubule associated protein 2 (MAP2; Sigma Aldrich) overnight at concentrations 1:4000, 1:4000 and 1:30000 respectively. This was followed by incubation with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP and MAP2, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK). Subsequently slides were incubated in Streptavidin-Biotinylated HRP complex (RPN1051, Amersham). Finally, the slides were then incubated in 3,3'-diaminobenzidine (DAB) concentrate with  $\text{H}_2\text{O}_2$ . Slides were fixed with coverslips. Slides were viewed with bright field microscopy on a Nikon Eclipse 90i microscope and images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). All immunoreactivities were analysed by densitometry using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA), made binary by adjusting the threshold manually, with the percentage area of coverage recorded for four fields of

view per brain region on two sections per animal. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

*Allopregnanolone radioimmunoassay and cortisol enzyme immunoassay*

Allopregnanolone was extracted from fetal and maternal plasma as previously described (73). Briefly, plasma was treated with 50% methanol with 1% acetic acid in Sep-Pak C<sub>18</sub> cartridges (Waters, Milford, MA, USA), vacuum dried and then treated with potassium permanganate to reduce cross-reactivity of progesterone (261). The addition of tritium-labelled allopregnanolone (1000–1500 cpm., 5 $\alpha$ -[9, 11, 12, 3H(N)]); PerkinElmer Life and Analytical Sciences, Boston, MA, USA) allowed determination of sample recovery. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. 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 assay and cross-reactivities of the antisera have previously been described (73). The limit of detection for allopregnanolone was 35.0 + 2.5pg/tube. The inter and intra-assay coefficients of variation were 12.3% and 8.5% respectively.

Cortisol concentrations were determined in maternal saliva obtained before and after each stress or control event using a salivary assay kit (Salimetrics Inc., State College, PA, USA), as per manufacturers instructions. Sensitivity of the assay was 0.012  $\mu$ g/dL to 3.0  $\mu$ g/dL and inter- and intra- assay coefficients of variance were 6.89% and 5.52% respectively.

Cortisol and progesterone were quantified in fetal and maternal plasma by immunoassay by Hunter Area Pathology Service (HAPS). The assays were conducted on the UniCel Dxl800 Access Immunoassay System (Beckman Coulter Inc., Gladesville, NSW, Australia), as per manufacturers instructions. The inter- and intra- assay coefficients of

variance were 5.17% and 4.3% respectively for cortisol and 8.2% and 7.9% respectively for progesterone.

### *Statistical Analysis*

For all fetal data, a linear mixed model was used to compare the differences between main independent variables as fixed factors: group (stress or control), drug treatment (vehicle and allopregnanolone) and sex (male or female). This statistical model accounted for familial variations as well as interactions between the main variables.

A two-way Multiple Analyses of Variance test (ANOVA) was used to further characterise specific relationships within each sex cohort. This same test was also used to assess differences between maternal plasma allopregnanolone data. A repeated measures Multiple Analyses of Variance test (RM-ANOVA) test was used to assess maternal repeated salivary cortisol sampling. All data analysis was performed using the SPSS statistical software package (version 19, SPSS Inc. IBM, Chicago, IL, USA). In order to prevent pregnancy within litter association and bias, only one male and one female fetus was used from each pregnancy in the analysis. In a number of pregnancies, there was only one male or female fetus and therefore only one fetus was available to be used for analysis. All data are presented as mean + SEM with  $P < 0.05$  considered significant.

## **3.4 Results**

### *Effect of prenatal stress on fetal physiological characteristics*

Female fetuses showed significantly (ANOVA  $p < 0.05$ ,  $F = 5.172$ ,  $df = 1$ ) larger brain-weight-to body-weight ratios than their male counterparts, irrespective of stress exposure or drug treatment (Table 3.1). Female fetuses exposed to prenatal stress were also the only group to show a significantly (ANOVA  $p < 0.05$ ,  $F = 3.393$ ,  $df = 1$ ) reduced liver-weight-to-body-weight ratio with no significant changes observed in the male cohort or in the

cohort of female fetuses exposed to allopregnanolone treatment. Brain to liver ratio (BLR) in the females, but not males, was significantly higher (ANOVA  $p < 0.05$ ,  $F = 6.472$ ,  $df = 1$ ) in fetuses exposed to stress compared to those in control groups, indicating asymmetric growth and brain sparing. No significant effect of stress, drug treatment or sex was found on body weight or nose-rump length. There were also no significant effects of stress, drug treatment or sex on placental weight, heart weight or adrenal weight when adjusted for individual differences in body weight. It should also be noted that pregnancies exposed to PS showed a modest reduction in GA at the time of post mortem, indicating a shorter gestational length (control  $68.43 \pm 0.47$  and stress  $67.7 \pm 0.35$ ; ANOVA  $p < 0.05$ ,  $F = 4.286$ ,  $df = 1$ ). There was no significant effect of stress exposure or drug treatment on litter size or average litter weight, nor were there any effects on maternal weight gain during pregnancy (data not presented). This suggests that maternal weight gain was not responsible for any difference in fetal growth data.

*Table 3.1: Fetal Physical Characteristics*

| Sex    | Treatment Group      | Body weight  | Brain to Body weight     | Nose-Rump Length | Placenta to Body weight | Heart to Body weight | Liver to Body weight       | Adrenal Gland to Body weight | BLR                      |
|--------|----------------------|--------------|--------------------------|------------------|-------------------------|----------------------|----------------------------|------------------------------|--------------------------|
| Male   | Cont + Veh (n=10)    | 98.45 ± 4.34 | 2.49 ± 0.07              | 14.50 ± 0.42     | 5.84 ± 0.30             | 0.50 ± 0.03          | 5.06 ± 0.14                | 0.03 ± 0.002                 | 0.49 ± 0.02              |
|        | Cont + Allo (n=10)   | 92.79 ± 4.39 | 2.66 ± 0.08              | 14.20 ± 0.22     | 5.55 ± 0.20             | 0.47 ± 0.04          | 4.49 ± 0.22                | 0.03 ± 0.003                 | 0.60 ± 0.06              |
|        | Stress + Veh (n=10)  | 86.09 ± 4.25 | 2.71 ± 0.12              | 14.61 ± 0.23     | 5.69 ± 0.23             | 0.59 ± 0.05          | 4.66 ± 0.22                | 0.03 ± 0.003                 | 0.56 ± 0.03              |
|        | Stress + Allo (n=13) | 89.61 ± 3.36 | 2.72 ± 0.12              | 14.25 ± 0.19     | 5.51 ± 0.22             | 0.53 ± 0.03          | 4.49 ± 0.22                | 0.04 ± 0.006                 | 0.61 ± 0.04              |
| Female | Cont + Veh (n=7)     | 89.39 ± 4.72 | 2.87 ± 0.11 <sup>b</sup> | 14.31 ± 0.33     | 5.30 ± 0.30             | 0.55 ± 0.02          | 5.15 ± 0.16                | 0.03 ± 0.002                 | 0.55 ± 0.03              |
|        | Cont + Allo (n=7)    | 85.07 ± 4.72 | 2.94 ± 0.41 <sup>b</sup> | 14.22 ± 0.40     | 5.75 ± .0.24            | 0.56 ± 0.04          | 5.02 ± 0.40                | 0.04 ± 0.002                 | 0.51 ± 0.06              |
|        | Stress + Veh (n=11)  | 88.75 ± 4.84 | 2.84 ± 0.18 <sup>b</sup> | 14.25 ± 0.38     | 5.28 ± 0.14             | 0.52 ± 0.03          | 3.88 ± 0.25 <sup>'a'</sup> | 0.07 ± 0.03                  | 0.67 ± 0.03 <sup>a</sup> |
|        | Stress + Allo (n=12) | 82.91 ± 4.85 | 2.90 ± 0.18 <sup>b</sup> | 13.89 ± 0.38     | 5.14 ± 0.29             | 0.48 ± 0.02          | 4.64 ± 0.31                | 0.05 ± 0.02                  | 0.64 ± 0.04 <sup>a</sup> |

ALL VALUES ARE REPRESENTED AS A PERCENTAGE OF BODY WEIGHT AT THE TIME OF POST MORTEM WITH THE EXCEPTION OF BRAIN TO LIVER WEIGHT RATIO (BLR), WHICH IS A RATIO VALUE OF BRAIN WEIGHT TO LIVER WEIGHT. THIS VALUE IS INDICATIVE OF GROWTH RESTRICTION AND BRAIN SPARING, WHEREBY A VALUE OF >0.9 IS USED TO CLASSIFY GROWTH RESTRICTED FETUSES. <sup>A</sup> INDICATES SIGNIFICANT (P<0.05) EFFECT OF STRESS, AND <sup>B</sup> INDICATES SIGNIFICANT (P<0.05) EFFECT OF SEX. NO EFFECT OF DRUG TREATMENT WAS FOUND. VALUES ARE EXPRESSED AS THE MEAN PERCENTAGE ± SEM AND ARE CALCULATED FOR ANIMAL NUMBERS SHOWN IN PARENTHESES. ALLO =ALLOPREGNANOLONE, BLR = BRAIN TO LIVER RATIO, CONT= CONTROL, VEH =VEHICLE.

### Maternal Salivary Cortisol Concentrations

Maternal salivary cortisol data (presented as the fold change in concentrations from immediately before to after each stress or control handling exposure) is shown in Figure 3.1. Maternal allopregnanolone treatment did not affect cortisol levels and therefore these data are combined with the vehicle treated animals. As expected, dams in stress exposed groups demonstrated significantly higher (RM-ANOVA  $p < 0.001$ ,  $F = 82.18$ ,  $df = 1$ ) salivary cortisol concentrations after each exposure compared to their control handled counterparts, who showed no change after each event. In addition, there was no difference in the fold change in salivary cortisol concentrations compared to controls, even when adjusting for advancing gestational age.

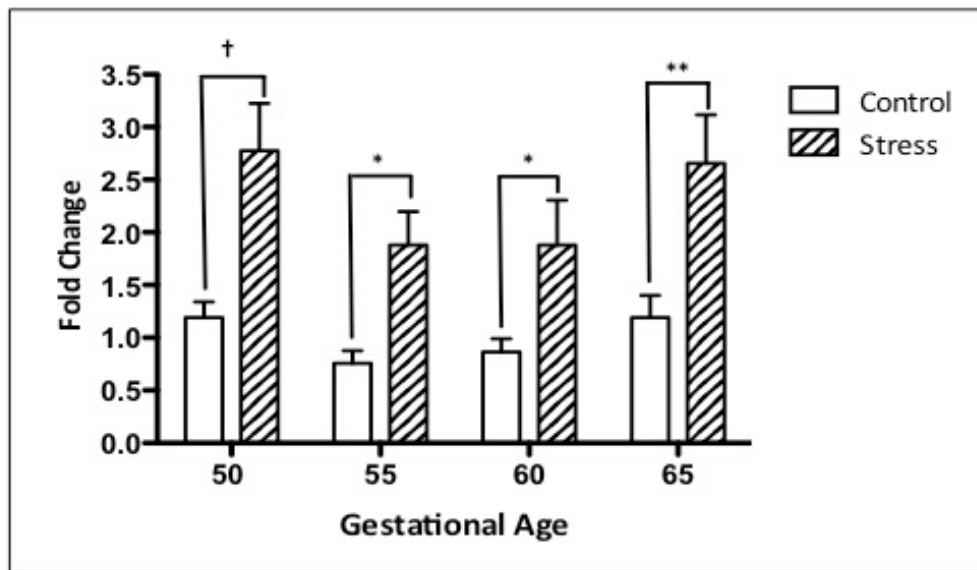


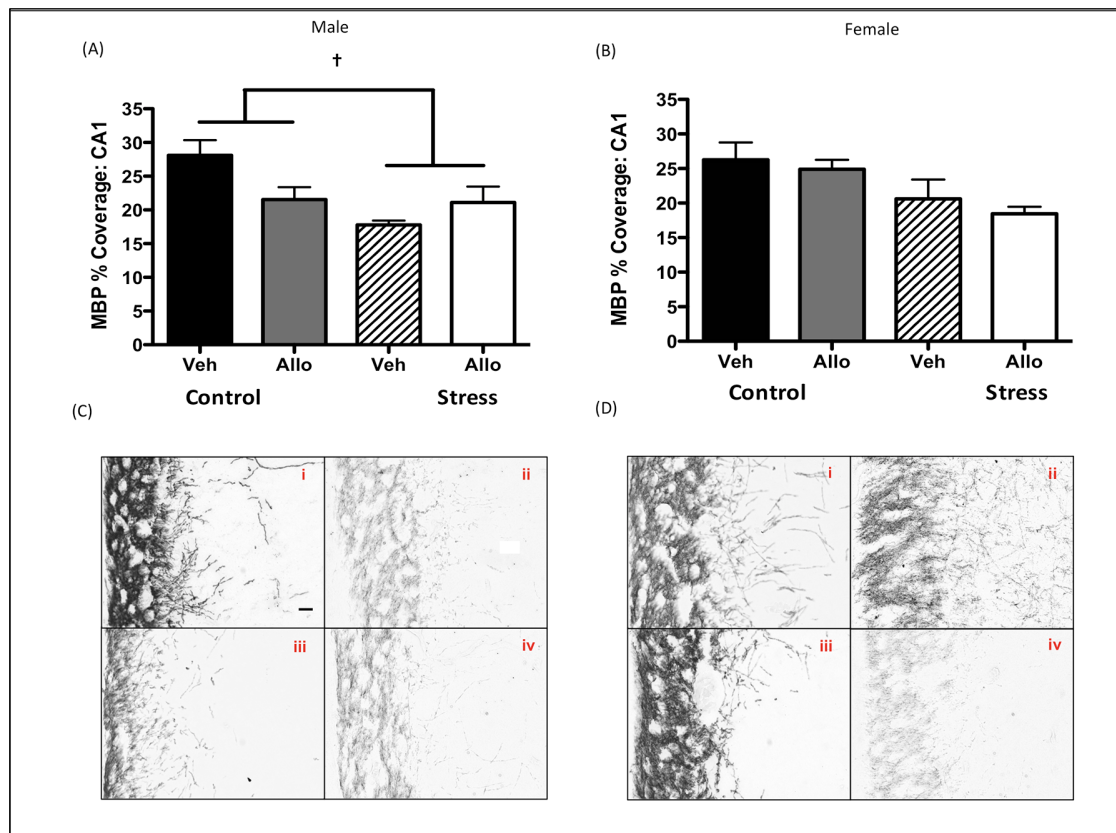
FIGURE 3.1 EFFECT OF STROBE LIGHT-INDUCED STRESS EPISODES ON MATERNAL SALIVARY CORTISOL CONCENTRATIONS. DATA ARE PRESENTED AS FOLD CHANGE OF SALIVARY CORTISOL BETWEEN SALIVARY SAMPLES TAKEN IMMEDIATELY BEFORE AND AFTER EACH STRESS (HATCHED BARS) OR CONTROL (HANDLING WITHOUT STRESS EXPOSURE, OPEN BARS) EPISODES THAT WERE PERFORMED AT 50, 55, 60 AND 65 DAYS GA. STRESS EXPOSED ( $N = 14$ ) AND CONTROL ( $N = 14$ ) GROUPS CONTAIN BOTH VEHICLE AND ALLOPREGNANOLONE TREATED GUINEA PIG DAMS. '\*'  $P = 0.05$ ; '\*\*'  $P < 0.01$  AND '+'  $P < 0.001$  INDICATES SIGNIFICANCE LEVEL BETWEEN CHANGES SEEN BETWEEN CONTROL AND STRESS GROUPS.

Maternal plasma cortisol concentrations taken at the time of post mortem were significantly correlated with cortisol levels in saliva collected at the time of post mortem ( $p < 0.001$ ; Spearman  $r = 0.75$ ; data not presented) supporting the use of salivary cortisol

as a measurement of circulating cortisol concentrations. Furthermore, maternal plasma cortisol negatively correlated with fetal BLR ( $p=0.03$ ; Spearman  $r=0.33$ ; data not presented), indicating that fetal brain growth may be negatively affected as maternal cortisol levels rise.

*Myelin basic protein (MBP) expression and allopregnanolone response*

Figure 3.2C and D show representative micrographs of MBP immunostaining in the CA1 region of the hippocampus of prenatally stressed, control, vehicle and allopregnanolone treated fetuses. There were significantly lower levels of MBP expression in male prenatally stressed fetuses in the CA1 region of the hippocampus (ANOVA  $p<0.001$ ,  $F=13.576$ ,  $df=1$ ; Figure 3.2A and C), and in the cerebral cortical white matter (ANOVA  $p<0.001$ ,  $F=14.840$ ,  $df=1$ ; data not shown), compared to controls. This result was not seen in the female cohort (Figure 3.2B and D). A significant negative correlation was also found between maternal salivary cortisol concentrations at the time of post mortem and MBP expression in the CA1 region of the hippocampus when male and female groups were combined ( $p=0.02$ ; Spearman  $r=-0.53$ ) and between maternal plasma cortisol at the time of post mortem and MBP expression in the cerebral cortical white matter when male and female groups were combined ( $p<0.001$ ; Spearman  $r=-0.62$ ), indicating the relationship between increased cortisol exposure and reduced fetal brain myelination.

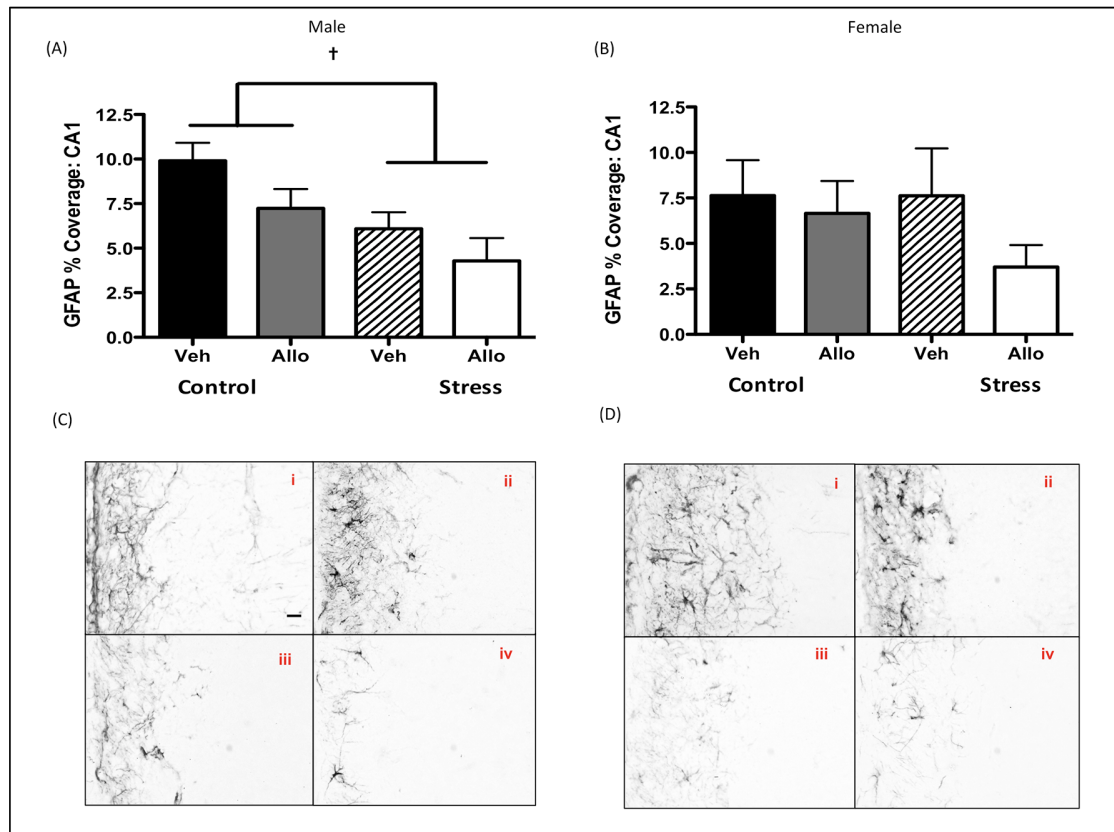


**FIGURE 3.2 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT (A). REPRESENTATIVE IMAGES OF MBP IMMUNOSTAINING FOR MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM SHOWING STAINING IN A CONTROL + VEHICLE TREATED (i), CONTROL + ALLOPREGNANOLONE TREATED (ii), STRESS + VEHICLE TREATED (iii) AND STRESS + ALLOPREGNANOLONE TREATED FETUS (iv). MBP EXPRESSION CALCULATED COVERAGE AREAS OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS) IN MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM. MBP STAINING COVERAGE IN: CONTROL + VEHICLE GROUPS IN BLACK BARS (MALE N=7; FEMALE N=5), CONTROL + ALLOPREGNANOLONE GROUPS IN GREY BARS (MALE N=9; FEMALE N=4), STRESS + VEHICLE GROUPS IN HATCHED BARS (MALE N=5; FEMALE N=4) AND STRESS + ALLOPREGNANOLONE GROUPS IN OPEN BARS (MALE N=5; FEMALE N=5). ‘†’  $p < 0.001$  INDICATES SIGNIFICANCE LEVEL BETWEEN CONTROL AND STRESS GROUPS OF MALES FETUSES. ALLO = ALLOPREGNANOLONE; VEH = VEHICLE. SCALE BAR = 50  $\mu$ M.**

#### *Glial Fibrillary Acidic Protein (GFAP) and allopregnanolone response*

Representative micrographs of GFAP immunostaining in the hippocampus are shown in Figure 3.3C and D. Analysis of immunostaining showed there was a marked effect of stress in the male cohort with reduced expression of GFAP in the CA1 region of the hippocampus (ANOVA,  $p < 0.001$ ,  $F = 9.347$ ,  $df = 1$ ; Figure 3.3A and C) and the cerebral cortical white matter (ANOVA  $p < 0.05$ ,  $F = 6.480$ ,  $df = 1$ ; data not shown), which was not

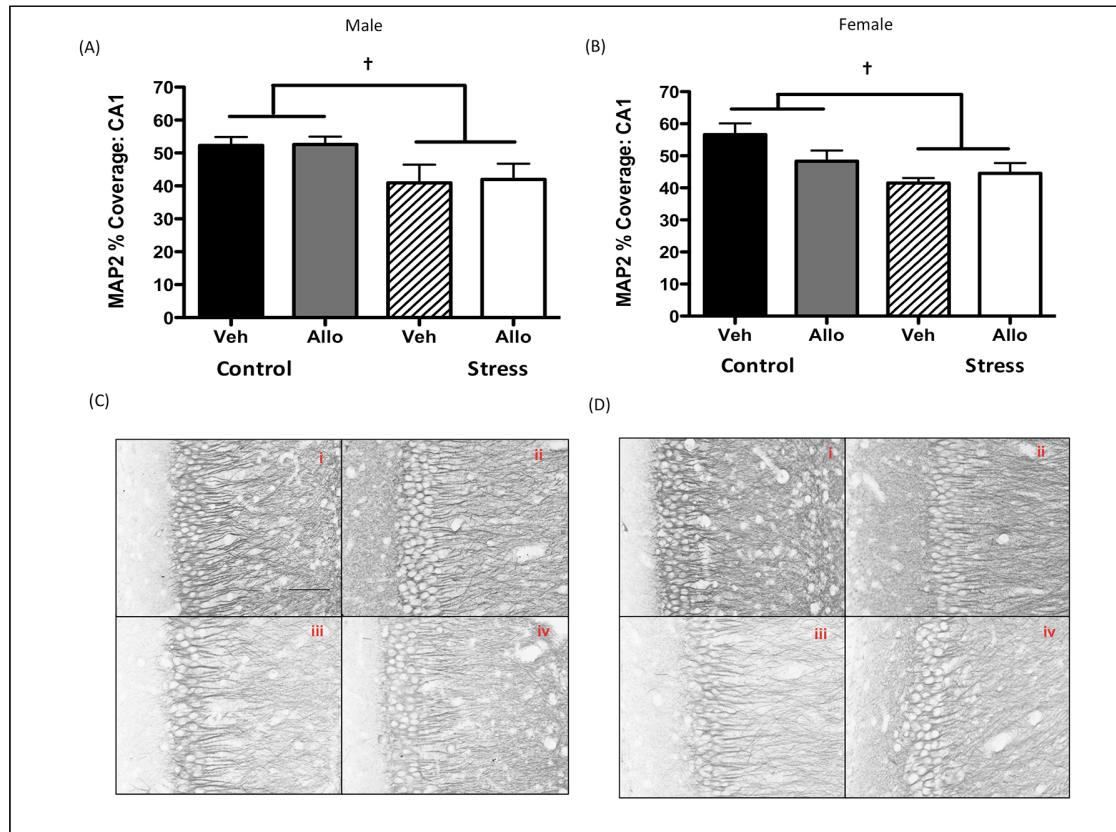
seen in the female cohort (Figure 3.3B and D). Also within the male cohort, there was an interaction between stress and allopregnanolone treatment (ANOVA  $p < 0.05$ ,  $F = 4.541$ ,  $df = 1$ ) indicating that the combination of stress and allopregnanolone treatment caused a differential effect on GFAP expression in the CA1 region, which was not seen in any of the other experimental groups.



**FIGURE 3.3 EFFECTS OF PRENATAL STRESS ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT (A). REPRESENTATIVE IMAGES OF MBP IMMUNOSTAINING FOR MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM SHOWING STAINING IN A CONTROL + VEHICLE TREATED (i), CONTROL + ALLOPREGNANOLONE TREATED (ii), STRESS + VEHICLE TREATED (iii) AND STRESS + ALLOPREGNANOLONE TREATED FETUS (iv). GFAP EXPRESSION CALCULATED COVERAGE AREAS OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS) IN MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM. GFAP STAINING COVERAGE IN: CONTROL + VEHICLE GROUPS IN BLACK BARS (MALE  $N = 7$ ; FEMALE  $N = 5$ ), CONTROL + ALLOPREGNANOLONE GROUPS IN GREY BARS (MALE  $N = 9$ ; FEMALE  $N = 4$ ), STRESS + VEHICLE GROUPS IN HATCHED BARS (MALE  $N = 5$ ; FEMALE  $N = 4$ ) AND STRESS + ALLOPREGNANOLONE GROUPS IN OPEN BARS (MALE  $N = 5$ ; FEMALE  $N = 5$ ). ‘†’  $p < 0.001$  BETWEEN CONTROL AND STRESS GROUPS OF MALES FETUSES. ALLO = ALLOPREGNANOLONE; VEH = VEHICLE. SCALE BAR =  $50\mu\text{m}$ .**

*Microtubule Associated Protein 2 (MAP2) and allopregnanolone response*

Figure 3.4 C and D show representative micrographs showing MAP-2 immunostaining in the hippocampus of prenatally stressed, control, vehicle and allopregnanolone treated fetuses. Analysis showed the significance of stress on MAP2 expression in the CA1 region of the hippocampus revealing stress reduced MAP2 expression in both male and female cohorts (Males ANOVA  $p < 0.01$ ,  $F = 6.443$ ,  $df = 1$ ; Females ANOVA  $p < 0.001$ ,  $F = 11.743$ ,  $df = 1$ ; Figure 3.4A and B respectively). There was however, no effect of allopregnanolone treatment on either males or females. There was no effect of stress exposure or allopregnanolone treatment on MAP2 expression within the cerebral cortical white matter (data not shown).



**FIGURE 3.4 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN-2 (MAP-2) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS WITH AND WITHOUT ALLOPREGNANOLONE TREATMENT (A). REPRESENTATIVE IMAGES OF MBP IMMUNOSTAINING FOR MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM SHOWING STAINING IN A CONTROL + VEHICLE TREATED (i), CONTROL + ALLOPREGNANOLONE TREATED (ii), STRESS + VEHICLE TREATED (iii) AND STRESS + ALLOPREGNANOLONE TREATED FETUS (iv). MAP2 EXPRESSION CALCULATED COVERAGE AREAS OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS) IN MALE (A) AND FEMALE (B) GUINEA PIG FETUSES AT TERM. MAP-2 STAINING COVERAGE IN: CONTROL + VEHICLE GROUPS IN BLACK BARS (MALE N=7; FEMALE N=5), CONTROL + ALLOPREGNANOLONE GROUPS IN GREY BARS (MALE N=9; FEMALE N=4), STRESS + VEHICLE GROUPS IN HATCHED BARS (MALE N=5; FEMALE N=4) AND STRESS + ALLOPREGNANOLONE GROUPS IN OPEN BARS (MALE N=5; FEMALE N=5). '†'  $P < 0.001$  BETWEEN CONTROL AND STRESS GROUPS IN MALE AND FEMALE FETUSES. ALLO = ALLOPREGNANOLONE; VEH = VEHICLE. SCALE BAR = 100  $\mu$ m.**

### *Allopregnanolone treatment and fetal plasma concentrations*

Maternal plasma allopregnanolone concentrations remained elevated 12 hours after the last maternal allopregnanolone administration in both control and stressed pregnancies compared to vehicle treated controls (RM-ANOVA  $p < 0.05$ ,  $F = 4.859$ ,  $df = 1$ ; Figure 3.5).

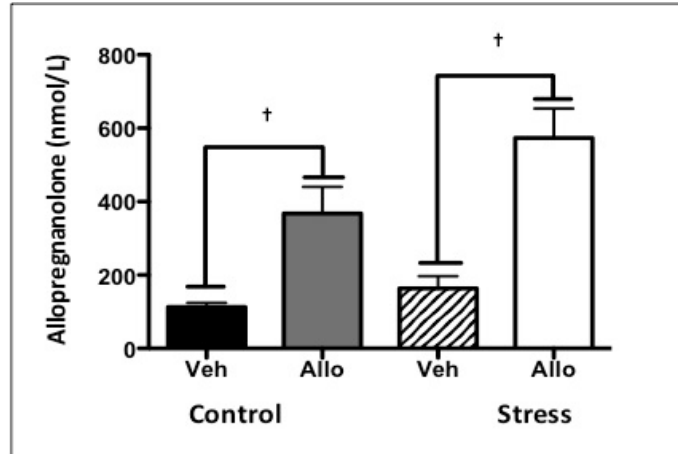


FIGURE 3.5 MATERNAL PLASMA ALLOPREGNANOLONE CONCENTRATIONS IN STRESS AND CONTROL DAMS 12 HOURS AFTER LAST VEHICLE (CONTROL + VEHICLE GROUP, BLACK BARS,  $N = 3$ ; STRESS + VEHICLE, HATCHED BARS,  $N = 4$ ) OR ALLOPREGNANOLONE ADMINISTRATION (CONTROL + ALLOPREGNANOLONE, GREY BARS,  $N = 4$ ; STRESS + ALLOPREGNANOLONE, OPEN BARS,  $N = 4$ ). ‘\*’  $p < 0.05$ , ‘†’  $p < 0.001$  BETWEEN VEHICLE AND ALLOPREGNANOLONE TREATMENT. . ALLO= ALLOPREGNANOLONE; VEH = VEHICLE.

In control pregnancies, maternal allopregnanolone treatment resulted in marked fetal plasma allopregnanolone concentrations in both male and female fetuses (ANOVA  $p < 0.001$ ,  $F = 14.598$ ,  $df = 1$ ; Figure 3.6A and B respectively). In contrast, neither male nor female fetal allopregnanolone concentrations were elevated in pregnancies exposed to stress and allopregnanolone administration (open bars, Figure 3.6). This observation is consistent with the finding of a significant (ANOVA  $p < 0.05$ ,  $F = 4.090$ ,  $df = 1$ ) interaction between stress and allopregnanolone treatment in fetal plasma indicating that the fetal allopregnanolone levels in response to maternal treatment was altered by stress exposure.

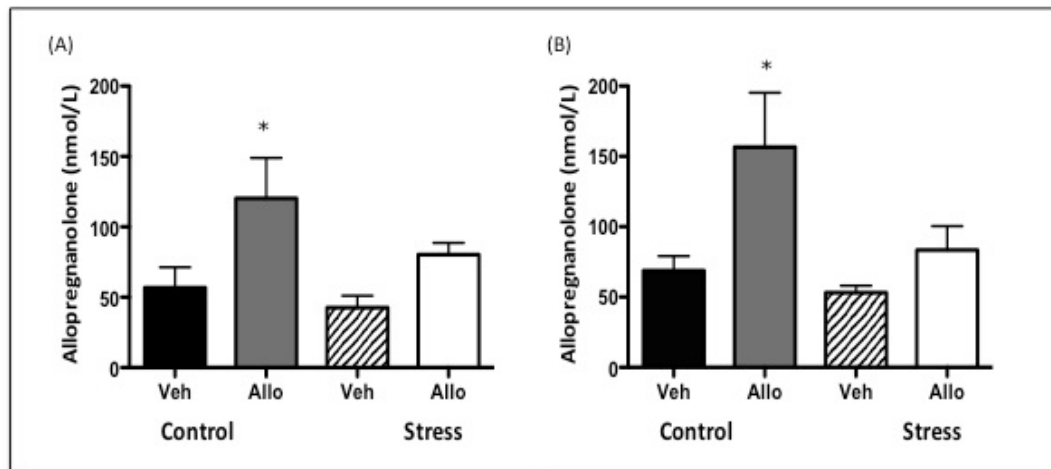


FIGURE 3.6 FETAL PLASMA ALLOPREGNANOLONE CONCENTRATIONS IN STRESS AND CONTROL DAMS 12 HOURS AFTER LAST VEHICLE OR ALLOPREGNANOLONE ADMINISTRATION IN MALE (A) AND FEMALE (B) FETUSES. DATA SHOWN ARE FOR: CONTROL + VEHICLE (BLACK BARS; MALES N=5, FEMALES N=5), CONTROL + ALLOPREGNANOLONE (GREY BARS; MALES N=6, FEMALES N=5), STRESS + VEHICLE (HATCHED BARS; MALES N=5, FEMALES N=5) AND STRESS + ALLOPREGNANOLONE TREATMENT (OPEN BARS; MALES N=6, FEMALES N=6). ‘\*’  $P < 0.05$  DIFFERENCE BETWEEN LEVELS IN THE CONTROL + ALLOPREGNANOLONE GROUP AND ALL OTHER GROUPS.. ALLO= ALLOPREGNANOLONE; VEH = VEHICLE. SCALE BAR =  $10\mu\text{M}$ .

There was no difference in plasma progesterone concentrations between any of the experimental groups in fetal plasma at the time of post mortem (average fetal levels were  $2845.82\text{nmol/L} \pm 308.45\text{nmol/L}$ ). There was also no significant effect of stress exposure or allopregnanolone treatment on maternal plasma progesterone levels at the time of post mortem (average maternal levels were  $10442.83\text{nmol/L} \pm 1936.95\text{nmol/L}$ ).

### 3.5 Discussion

The major finding of this study was that prenatal maternal stress had profound, sexually dimorphic effects on the guinea pig fetus. In this study, female brain sparing is seemingly a neuroprotective growth adaptation which may have partially preserved brain growth and development. In contrast, male fetuses demonstrated reduced expression of markers for three major brain cell types (myelinating oligodendrocytes, reactive astrocytes and mature neurons) in both brain regions assessed (CA1 region of

the hippocampus and cerebral cortical white matter). These observations suggest an increased vulnerability of males to the effects of prenatal stress on fetal brain growth and development. This study also highlights the differing effects of prenatal stress on each cell type and brain region during fetal neurodevelopment. These findings are consistent with clinical and experimental studies showing an inherent disadvantage of males to prenatal insults such as prenatal stress as well as the vulnerability of the hippocampus to damage (81, 161, 252, 262).

There is now increasing evidence for the developmental origins of neuropathologies, such that disturbances in processes such as myelination and neural migration during critical windows of brain development can predispose offspring to abnormalities in efficient synaptic transmission and neural connectivity at birth or later in life. Thus, decreases in MBP, GFAP and MAP2 expression in the male brain indicate decreased myelination, neurogenesis and stability of axons where a lack of sufficient repair processes may confer vulnerability and susceptibility to injury at birth or later in life. The present findings indicate oligodendrocyte maturation and myelination is reduced by late gestation stress. Reactive astrocytes have been shown to have important neuroprotective qualities (263) and are key to supporting development of the CNS (264), such that a reduction in these cells may have a role in the development of detrimental outcomes. The results of this study are consistent with previous studies, which have shown reductions in fetal myelination following brain sparing (265) in both the CA1 region of the hippocampus and the cerebral cortical white matter that are associated with increased incidences of postnatal behavioural pathologies (266). We assessed white matter tracts within the cerebral cortex, where disturbances during development have been linked to neurodevelopmental damage in other forms of pregnancy compromise such as preterm birth and intrauterine growth restriction (130). This cerebral cortical white matter deficiency is strongly associated (>90% prevalence) with

cognitive, behavioural and attention deficits in children born preterm (130-133).

Previous studies have also found site-specific (CA1 region) disturbances in hippocampal development following exposure to prenatal stressors (267-269). The CA1 region of the hippocampus contains a high concentration of important efferent projections and pyramidal cells involved in processes such as memory and learning, which are known to be affected by exposure to prenatal stress (114, 116, 120). Furthermore, this region of the hippocampus has also been shown to be selectively vulnerable to the deleterious effects of glucocorticoid exposure (122, 123). This may be attributable in part, to the high levels of expression of glucocorticoid receptors in the CA1 region of the hippocampus (123). In addition, neurons and glial cells within the CA1 region of the hippocampus express high concentration of GABA<sub>A</sub> receptors potentially leading to sensitivity to endogenous neurosteroid-dependent neuroprotection. The observation that allopregnanolone metabolism pathways were disrupted by prenatal stress may therefore further contribute to vulnerability.

Astrocytes, mature oligodendrocytes and neurons all express steroidogenic enzymes required for neurosteroid synthesis and stress-induced perturbations in the number of these cells could alter endogenous neurosteroid production and therefore contribute to disruption of CNS developmental processes (270). Allopregnanolone has been shown to have potent inhibitory effects, modulating the GABA<sub>A</sub> receptor in the late gestation fetus supporting proper neurodevelopment. Thus, fetuses affected by prenatal stress may also be susceptible to damage due to perturbations in neurosteroidogenesis creating an additive environment for damage. These observations have implications for perinatal brain development and psychopathology later in life, particularly for affected male fetuses.

We have found a negative effect of stress on the expression of the mature neuronal marker, MAP-2, in both males and females suggesting that females may not be totally

protected from maternal stress. This may in part, be attributable to the vulnerability of the hippocampus to prenatal insults including prenatal stress (115) and that some of the first stress episodes, conducted at GA50 and/or 55 (0.7 of gestation) may have damaged these neurons during their peak growth period, before an effective growth adaptation (brain sparing) was employed. Studies have shown the vulnerabilities of the hippocampus to stress-induced reductions in neurogenesis and resultant learning and memory, supporting the idea of a selective effect of stress on certain cell populations and regions within the fetal brain (115, 119, 271-273). Male offspring have also shown reduced neurogenesis in the hippocampus following exposure to prenatal restraint stress (160) and experimentally induced hippocampal damage confers increased vulnerability to psychiatric disorders in adulthood (126). These data suggests that different cell populations within the fetal brain respond differently to prenatal stress.

The model of transient stress used in this study was previously developed by Matthews and colleagues, and was used to evaluate the effect of prenatal stress on fetal hypothalamo-pituitary adrenal axis development (91, 235). The maternal salivary cortisol levels observed in this study following prenatal stress exposure were similar to those previously reported (235). Maternal plasma cortisol concentrations at the time of post mortem were positively correlated to maternal salivary cortisol concentrations at this time, suggesting the reliability of this measurement of cortisol whilst minimising the stress of venepuncture which would be difficult to quantify (274, 275). Furthermore, we have found a significant correlation between maternal cortisol levels and fetal BLR indicating that the higher the maternal cortisol level, the greater the effect on the fetus. The findings of the present study are consistent with the programming effect proposed by Glover et al (276). These investigators suggested that in compromised pregnancies, it may be more advantageous for female fetuses to alter their physical growth in order to

maintain optimal brain development whereas males may grow to larger in size to the detriment of their neurodevelopment (276).

This study is the first to demonstrate potential disruption of placental transfer or maternal or fetal metabolism of neurosteroids in pregnancies complicated by prenatal stress. The present observations show whilst maternal administration of allopregnanolone raised fetal circulating allopregnanolone concentrations in normal pregnancies, this was not achieved in both male and female fetuses of stressed pregnancies. This indicates the marked effects of prenatal stress on the neurosteroid environment. The mechanisms leading to the stress-induced suppression of allopregnanolone levels are unclear. Stress may affect maternal metabolism thereby reducing the effectiveness of exogenous allopregnanolone administration. Alternatively, placental metabolism of allopregnanolone may be increased to result in diminished levels present in the fetal circulation. It is also possible that prenatal stress reduced placental efficiency and therefore reduced the capacity for allopregnanolone to cross the placenta. Investigation of placental function is warranted to further address the mechanisms influencing neurosteroid production and metabolism pathways in the placenta. Regardless of the mechanism involved, the absence of a fetal response to exogenous allopregnanolone treatment suggests that the adverse effects of prenatal stress on the fetal brain may be caused by a loss of neurosteroid responses that are normally both trophic and neuroprotective (220). A stress-induced reduction in allopregnanolone levels could result in exacerbation of brain injury that exceeds normal regenerative processes in the fetal brain, thus resulting in psychopathologies postnatally. A chronic loss of allopregnanolone induced by stress could also directly result in disorders involving excess neural excitation such as increased vulnerability to seizures.

In conclusion, this study has shown the pronounced effects of prenatal stress on both fetal brain development and whole body growth adaptations, effects that were sexually dimorphic. The effect on the male brain is consistent with observational studies in humans, which indicate there is an inherent vulnerability of males to prenatal insults and subsequent behavioural abnormalities later in life. Further studies investigating neonatal behaviour following prenatal stress in a guinea pig cohort would be valuable in elucidating the ongoing effects of these changes, the extent to which the placenta impacts fetal adaptation to stress, and the development of treatments and compensatory approaches.

### **3.6 Acknowledgements**

Hunter Area Pathology Service (HAPS) were responsible for the measurement of cortisol and progesterone in plasma and amniotic fluid.

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