EFFECT OF PRE AND POSTNATAL NEUROSTEROID THERAPY ON NEURODEVELOPMENT AND BEHAVIOUR

Julia Catherine Shaw

Bachelor of Biomedical Science (Honours Class I)

School of Biomedical Sciences and Pharmacy

Faculty of Health and Medicine

University of Newcastle

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DECLARATION

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

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This thesis is dedicated to mama bear and papa bear,

Jacqueline and Steven Shaw.

Thank you for doing all that you do for me.

This would not have been possible without you.

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CONTENTS

ABSTRACT

Children that are born preterm are at an increased risk of developing late onset cognitive problems and behavioural disorders, such as attention deficit hyperactivity disorder (ADHD) and anxiety, with males being particularly vulnerable. The mechanisms by which this happens are poorly understood; however, actions and modulation of $GABA_A$ receptor signalling by the neurosteroid allopregnanolone has a major role in late gestation neurodevelopment, and we believe that the early loss of placentally-derived allopregnanolone following preterm birth is pivotal to the development of these disorders. There is increasing interest in the development of the hippocampus and cerebellum following preterm birth and the potential involvement of GABAergic pathways in neurodevelopmental disorders. In these studies, we propose that the early loss of the intrauterine trophic environment as a result of preterm birth alters the development of the hippocampus and cerebellum, contributing to ongoing neurobehavioral disorders. We anticipate that neurosteroid-replacement therapy with ganaxolone (GNX) following preterm birth may prevent the deficits in neonatal development that contribute to these disorders. We further propose that maternal administration of progesterone, which is commonly administered prophylactically to women at risk of preterm labour, may have unforeseen effects on fetal neurodevelopment due to the ability of progesterone to be metabolized to a number of steroids with varying effects on development including allopregnanolone and cortisol.

We found that there is an adaptive increase in the mRNA levels of $GABA_A$ receptors involved in neurosteroid action after term birth in the guinea pig neonate,

but not after preterm birth. The increased levels in the term neonate may compensate for the dramatic decline in allopregnanolone levels following separation from the placenta, and this lack of an adaptive increase in the preterm neonate may heighten the adverse effect of the premature decline in neurosteroid exposure. Preterm neonates also had deficits in myelination of the hippocampus, subcortical white matter, and cerebellum. At juvenile age these deficits remained in the hippocampus, subcortical white matter, and female cerebellum. Interestingly, increased myelination of the male cerebellum at juvenility, suggesting a deficit in axonal pruning, was observed in conjunction with a dysregulation of the cerebellar GABAergic system. In addition to the white matter alterations at juvenility, male guinea pigs that were born preterm exhibited a hyperactive, ADHD-type phenotype, whilst females had anxious behaviour. Unexpectedly, maternal progesterone therapy did not affect fetal allopregnanolone or cortisol steroid levels, nor did it have an effect on myelination of the hippocampus. Circulating maternal cortisol was increased, but fortunately the placental enzymatic barrier protected the fetus from this potentially damaging rise in cortisol. Novel neurosteroid-therapy replacement therapy using ganaxolone during the immediate neonatal period following preterm birth improved the neurobehavioural outcomes of the male juvenile offspring by ameliorating myelination deficits and returning behavioural phenotypes to term-born levels.

Therefore, through utilizing our guinea pig model of preterm birth, we were able to conclude that the GABAergic system and its effects on myelination are disrupted following preterm birth and that this occurs in parallel with a hyperactive phenotype in males, and conversely an anxious phenotype in females. Furthermore, whilst prenatal progesterone therapy does not influence fetal allopregnanolone levels,

restoring the *in utero* neurosteroid environment for preterm neonates following preterm birth may be a viable therapy to prevent the onset of behavioural and learning disorders.

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

11βHSD2	11β-hydroxysteroid dehydrogenase type 2
5α-DHP	5α-dihydroprogesterone
17-OHPC	17-hydroxyprogesterone caproate
5αR1	5α-reductase type 1
5αR2	5α-reductase type 2
ADHD	Attention deficit hyperactivity disorder
AKR	Aldo-keto reductase
ANOVA	Analysis of variance
ART	Assisted reproductive technologies
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLR	Brain-to-liver ratio
BSA	Bovine serum albumin
CA	*Cornu ammonis*
cDNA	Complimentary DNA
CNS	Central nervous system
CPAP	Continuous positive airway pressure
CPNA	Corrected postnatal age
CReDITTS	Critical research design, information technology, and statistical support
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DAB	3, 3'-diaminobenzidine
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DWM	Deep white matter
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GA	Gestational age
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid A
GAD65	Glutamic acid decarboxylase isoform 65
GAD67	Glutamic acid decarboxylase isoform 67
GAT1	GABA transporter type 1
GAT3	GABA transporter type 3
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
GNX	Ganaxolone
HMRI	Hunter Medical Research Institute
HPA	Hypothalamic pituitary axis
HRP	Horseradish peroxidase
IGL	Internal granule layer
IQ	Intelligence quotient
IUGR	Intrauterine growth restriction
KCC2	Potassium-chloride transporter member 5
LCMS	Liquid chromatography mass spectrophotometry
MAG	Myelin associated glycoprotein
MAP2	Microtubule associated protein 2
MBP	Myelin basic protein

mIPSCs	Miniature inhibitory postsynaptic currents
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NeuN	Neuronal nuclei
NG2	Neural antigen 2
NHMRC	National Health and Medical Research Council
NMDA	N-methyl-D-aspartate
NTC	No-template control
OLIG2	Oligodendrocyte transcription factor
$P450_{SCC}$	P450 enzyme complex, side-chain cleavage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFRα	Platelet-derived growth factor receptor α
PIP	Peak inspiratory pressure
PLP	Myelin proteolipid protein
PM	Postmortem
PND	Postnatal day
PEEP	Positive end expiratory pressure
PTSD	Post-traumatic stress disorder
PVDF	Polyvinylidene fluoride
PVL	Periventricular leukomalacia
qPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription

RT+	Reverse transcriptase positive
RT-	Reverse transcriptase negative
RT-PCR	Reverse transcription polymerase chain reaction
SCWM	Subcortical white matter
SEM	Standard error of the mean
TBI	Traumatic brain injury
TBS-T	Tris-buffered saline with Tween
TEA	Term equivalence age
THDOC	Tetrahydrodeoxycorticosterone
TMB	Tetramethylbenzidine
USA	United states of America

PUBLICATIONS FOR INCLUSION

Shaw JC, Palliser HK, Walker DW, and Hirst JJ. Preterm birth affects $GABA_A$ receptor subunit mRNA levels during the foetal-to-neonatal transition in guinea pigs. *The Journal of Developmental Origins of Health and Disease.* 2015; 6(3): 250-260. DOI: 10.1017/S2040174415000069
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Shaw JC, Palliser HK, Dyson RM, Hirst JJ, and Berry MJ. Long-term effects of preterm birth on behavior and neurosteroid sensitivity in the guinea pig. *Pediatric Research.* 2016; 80(2): 275-83. DOI: 10.1038/pr.2016.63
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Shaw JC, Palliser HK, Palazzi K, and Hirst JJ. Administration of progesterone throughout pregnancy increases maternal steroids without adverse effects on mature oligodendrocyte immunostaining in the guinea pig. *Reproductive Science.* 2017. DOI: 10.1177/1933719117715125
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Shaw JC, Palliser HK, Dyson RM, Berry MJ, and Hirst JJ. Dysregulation of the cerebellar GABAergic system in juvenile guinea pigs following preterm birth. Accepted by *International Journal of Developmental Neuroscience.* 2017.

Shaw JC, Palliser HK, Dyson RM, Berry MJ, and Hirst JJ. Neurosteroid replacement therapy using the allopregnanolone-analogue ganaxolone following preterm birth in the guinea pig. Prepared for submission to *Pediatric Research.*

ADDITIONAL PUBLICATIONS

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Hirst JJ, Cumberland AL, **Shaw JC**, Bennett GA, Kelleher MA, Walker DW, and Palliser HK. Loss of neurosteroid-mediated protection following stress during fetal life. *Journal of Steroid Biochemistry and Molecular Biology.* 2016; 160: 181-8. DOI: 0.1016/j.jsbmb.2015.09.012.

Bennett GA, Palliser HK, **Shaw JC**, Palazzi KL, Walker DW, and Hirst JJ. Maternal stress in pregnancy affects myelination and neurosteroid regulatory pathways in the guinea pig cerebellum. *Stress*. 2017. DOI: 10.1080/10253890.2017.1378637.

1.0 INTRODUCTION

Preterm birth is the leading cause of death and neurodevelopmental related disability in neonates[1]. In developing nations such as Australia, the incidence of moderate-late preterm birth specifically now accounts for ~80% of all preterm births[2, 3]. In the absence of overt white matter damage and neurodevelopmental delay at the time of birth, these offspring are at an increased risk of developing behavioural disorders such as anxiety and ADHD, in addition to learning delays at school age[2, 4-10]. Development of the brain continues throughout late gestation and following birth in areas such as the hippocampus and cerebellum, and reductions in brain volumes and functionality of these regions are evident in children that were born preterm[11-14]. In particular the process of myelination by mature oligodendrocytes is ongoing throughout this late gestation stage and is vulnerable to insults and excitotoxic damage associated with early exposure to the *ex utero* environment[15-17]. *In utero*, the fetal neurosteroid allopregnanolone is responsible for protection from insults by reducing seizure activity, encouraging the fetal 'sleep state', and for promoting myelination through its action on the inhibitory $GABA_A$ receptors of the central nervous system (CNS)[18-20]. Preterm birth is associated with an inappropriate and early separation from the placenta and thus, placental supply of allopregnanolone is prematurely lost in these neonates during a period when it is critical for proper neurodevelopment[21]. Whilst neurosteroid therapy is utilized for traumatic brain injury (TBI) and epilepsy[22-25], their therapeutic use following preterm birth has not been investigated and forms the basis of this thesis.

1.1 PRETERM BIRTH

1.1.1 OCCURRENCE AND CAUSES

Worldwide approximately 10% of babies are born preterm each year (i.e. <37 completed weeks of gestation)[26, 27]. Despite a large percentage of preterm births worldwide occurring in Africa, Asia, and Latin America, over the past two decades this figure has been increasing in developed nations, including the United Kingdom, Scandinavia, and the United States of America (USA) where the percentage of preterm births each year ranges from peaking in 2006 at 12.8% to 11.4% in 2013[3, 4, 26, 28]. In Australia, the rate of preterm birth was 8.6% in 2013[29, 30] and for twins this figure rose to 53.6%[31]. The increased use of assisted reproductive technologies (ART) has contributed to the rise in preterm births. Singleton pregnancies following ART that result in preterm birth are only slightly higher than the national average at 9.3%[32]; however, the figure for twin pregnancies is drastically higher at 64.4% in Australia and New Zealand[33]. Preterm birth can be separated into four categories with different rates of occurrence; very preterm (<28 weeks of gestation) account for only 6.4%, early preterm (28-31 weeks) account for 10.5%, with moderately and late preterm (32-36 weeks) accounting for a staggering 83.1% of all preterm births[3]. In Australia, there are approximately 16,000 late preterm (34-36 weeks) births each year, and 11% of these can be contributed to elective caesarean deliveries[2, 3].

The causes of preterm birth are in many cases idiopathic, recently however, the Global Alliance to Prevent Prematurity and Stillbirth in combination with the International Fetal and Newborn Growth Consortium published the first proposed phenotype classification system for causes of preterm birth[3]. The classification system followed a large, international, multicentre, prospective cohort study of 5828

preterm births occurring between 16 and 36 completed weeks gestation. The causal phenotype clusters and associated prevalence were: spontaneous labour (30%), infection (15.3%), pre-eclampsia/eclampsia (11.8%), bleeding (11%), multiple births (10.4%), suspected fetal growth restriction (5.8%), perinatal sepsis/congenital abnormalities (5.5%) antepartum stillbirth (3.7%), fetal distress (3.4%), and severe maternal conditions (3.1%)[34]. Interestingly, the cluster analysis of spontaneous preterm birth could then be separated into 5 groups: maternal stress (43.3%), premature membrane rupture (28.6%), familial factors (11.7%), maternal comorbidities (6.1%), and infection/decidual haemorrhage/placental dysfunction (10.3%)[35].

Risk factors for preterm birth are extensive and can be separated into three categories; maternal characteristics, reproductive history, and current pregnancy characteristics. The maternal risk factors for preterm birth include lifestyle factors such as tobacco use, drug use, low body mass index, low socio-economic status and educational attainment, maternal age (low or high), and race (African-American at highest risk). Diseases or disorders such as infections, uterine anomalies, family history of preterm birth, periodontal disease, depression and stress are also maternal risk factors for preterm birth. Factors associated with reproductive history include prior preterm birth or stillbirth, as well as induced abortion. Finally, the risk factors that fall under the current pregnancy characteristics category include vaginal bleeding, multiple gestation, polyhydramnios (excess amniotic fluid), the use of ART, and short cervical length[3].

1.1.2 Prevention of preterm labour

The mechanism of preterm birth is still largely unknown and thus therapies to prevent preterm labour are very limited. One of the most commonly used therapeutics to prevent preterm birth is progesterone. Progesterone is one of the major steroid hormones in the body, is produced by the corpus luteum during early pregnancy and then the placenta, and is essential in maintaining pregnancy. For this reason, oral and vaginal progesterone or intramuscular injection of the synthetic progestin 17-hydroxyprogesterone caproate (17-OHPC) is used to treat women who have a history of preterm labour, or have a short cervix, in an attempt to delay their delivery[36, 37]. The use of progesterone-based therapies has increased dramatically over the last decade[38, 39]; however, the effectiveness is heavily debated. Multiple small sample size studies utilizing progesterone therapy at doses ranging from 100 – 400mg/day administered from mid-gestation onwards report reductions in preterm birth[40-45]. However, therapy was also found to be ineffective at delaying birth once arrested preterm labour began[38, 39, 46]. Recently the largest multicentre double blind, placebo-controlled trial (OPPTIMUM) to date found no significant effect of prophylactic vaginal progesterone on gestational age, morbidity, or mortality at the time of delivery in singleton pregnancies when administered from mid-gestation onwards[47]. Similarly, another multicentre, double blind, placebo-controlled trial (PREDICT) observed the same in twin pregnancies[48].

The calcium-channel blocker nifedipine is a tocolytic agent used for maintenance therapy following threatened preterm labour to prevent the onset of further contractions and subsequent delivery. A Cochrance systematic review of six trials comparing nifedipine use to placebo/no treatment following threatened preterm

labour found that there was no difference in the incidence of preterm birth between those receiving the therapy and those without treatment, and there was also no difference in the number of women delivering preterm within 48 hours of receiving treatment compared to placebo[49]. Magnesium is another tocolytic agent that is used to prevent labour from continuing; however, trials using this therapy have also found no significant reduction in the occurrence of preterm birth compared to control[50]. Magnesium treatment continues to be used as a neuroprotective therapy despite this.

In women with a short cervix, one of the known risk factors of preterm birth, cervical cerclage is physically performed in an effort to reduce the chances of preterm birth occurring. Unlike some of the other therapies, this intervention occurs prior to the onset of contractions. A recent multicentre, randomized trial of women with a history of spontaneous preterm birth, and with a short cervix, found that cerclage reduced the rate of perinatal mortality and previable birth, but did not prevent birth at less than 35 weeks' gestation[51].

Another preventative therapy that has been investigated in clinical trials is the use of prophylactic antibiotics to reduce the rate of preterm birth as either maternal or chorioamnionitis infections are often present in women who experience preterm labour. However, there was no benefit identified in the use of oral or vaginal antibiotics in either low-risk or high-risk pregnancies[52]. Finally, although bed rest is widely used as the first step in treatment of women with threatened preterm labour, there is no quantitative evidence to suggest it reduces the rate of preterm birth[53].

1.1.3 Outcomes following preterm birth

Despite only comprising 10% of births, preterm birth is the leading cause of death and neurodevelopmental related disability in neonates, accounting for up to 50% of neonatal deaths when preterm-related consequences such as neonatal sepsis and birth asphyxia are included[27]. Furthermore, approximately 50% of survivors of preterm birth are then at an increased risk of developing a long-term neurodevelopmental disability[4, 54]. Large physical, psychological, and economic costs are associated with these disorders and thus have a major impact on the individual, their family, and the broader society. In fact, the USA estimated that their medical, educational, and loss of productivity costs associated with preterm birth exceeded $26.2 billion in 2005 alone and this is likely to be higher now[26].

During the neonatal period, premature infants suffer from greater incidences of temperature instability, respiratory distress, infections, apnea, hypoglycaemia, seizures, jaundice, feeding difficulties, necrotizing enterocolitis, periventricular leukomalacia, and subsequent rehospitalisation[55]. Long-term consequences of preterm birth include cerebral palsy, high blood pressure, bronchopulmonary dysplasia and high rates of respiratory illnesses, insulin resistance, hearing impairments, and visual impairments[55]. In addition to these established preterm birth related disorders, there is now a growing body of evidence suggesting that preterm infants from moderate-late preterm pregnancies are much more likely to develop neurodevelopmental morbidities and learning disorders that become apparent at school age[2, 5-10].

Anxiety and ADHD are the most commonly diagnosed of these disorders in school-aged ex-premature children[56, 57]. ADHD is characterized by a deficit in

behavioural inhibition, inattention, impulsivity and social difficulties, and in a Norwegian cohort of preterm/low birth weight children at 5 years old was more commonly diagnosed in males[58]. In the same cohort, the females were more likely to be diagnosed with anxiety[58] highlighting that the behavioural outcomes of preterm birth occur in a sex-dependent manner. In a large Danish cohort children born at 34-36 weeks' gestation (i.e. moderate-late preterm) had an 80% increased risk of being diagnosed with ADHD compared to children born after 37 weeks' gestation, a larger percentage of these were also male[57]. Furthermore, in a Swedish cohort, the amount of ADHD medication purchased for ex-premature school-aged males was more than three times as much than for females, and the amount purchased increased by degree of immaturity at birth[56]. In addition to anxiety and ADHD, incidences of autism and depression are also increased following premature birth. Children in the USA that were born moderate-late preterm have been reported to have twice the incidence of autism at 10 years of age[59]. Parent-reported mental health rates in the USA are also higher for ex-premature children than the general population for children and adolescents, with a prevalence of 22.9% compared to 15.5% in the general child population[60]. This study also revealed that ex-premature children have 61% higher risk of having serious emotional/behavioural problems, which broke down to a 33% higher chance of developing depression, and a 58% higher chance of developing anxiety in childhood and adolescence[60].

School problems also arise in children following preterm birth, with ex-premature children having an increased need for special education, increased risk of repeating a grade, and lower reading and mathematics scores compared to full term born children[5]. These findings appear to be universal with numerous cohort studies

observing that moderate-late ex-premature children have a 1.3 – 2.8 fold increased risk for requiring special education, and a 1.3 – 2.2 fold increased risk of repeating grades at ages 5-10[61-64]. Furthermore, another study identified reading, writing, and spelling difficulties in 9-11 year old ex-premature children compared to those born full term[65]. Even at just 3-4 years of age impairments to visuospatial processing, spatial working memory, and sustained attention have been documented following preterm birth where major neurological deficits were not present at the time of birth[66].

Cognitive outcome following preterm birth may also be impaired in some cases although findings appear to be variable. Studies comparing cognitive delays in toddlers at two years of age do not find any significant difference between preterm and term when corrected for prematurity[67-70]. Alternatively, studies based in Swedish, American, and French cohorts found that 5-10 year old ex-premature children are twice as likely to score <85 on an IQ (intelligence quotient) test than term born children and that this is correlated with gestational age at birth, with those being born more preterm at the highest risk of severe cognitive impairments[71-73]. However, a much larger and comprehensive longitudinal American study in late-preterm ex-premature 4-15 year olds found no significant differences in IQ based on 11 different cognitive tests[64]. These results suggest that intellectual disability may not be as prevalent following late-preterm birth as other outcomes such as behavioural disorders and poor school performance, suggesting that poor school performance may be a reflection of behavioural disruptions that impact ability to pay attention and learn during class, rather than a result of reduced cognitive capacity.

1.2 DEVELOPMENT OF THE BRAIN

In utero, the fetal brain begins to develop at 3 weeks of gestation, and continues to develop throughout gestation and following birth[74]. The first and most simple stages of neurodevelopment are neuronal induction, neuroblast proliferation, neuronal migration, and neuronal selective aggregation. The more complex stages of neuronal differentiation and formation of specific patterns of connection and myelination occur last[75]. The sequence of these stages means that the brain develops in an inside-out manner, with the formation of smaller and more specific connections and structures occurring last[76]. Gene expression along the neural tube initiates differentiation into the five brain vesicles (telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) between 3-6 weeks of gestation[77]. These five vesicles then go on to mature and further differentiate into the specific regions of the adult brain. The telencephalon gives rise to the cerebral cortex and the basal nuclei, whilst the diencephalon becomes the thalamus and hypothalamus. The midbrain, superior colliculi, and inferior colliculi are formed from the mesencephalon. Finally, the metencephalon differentiates into the pons and cerebellum, and the myelencephalon into the medulla. Thus, essential brain regions such as the brain stem are developed at 3-7.5 weeks of gestation, and are followed by development of higher order executive functioning brain regions, such as the hippocampus and cerebellum, from mid-gestation until after birth (refer to Figure 1.1) [76, 78]. Overall brain volume is reduced following premature birth[79-81] and two regions of the brain that are particularly vulnerable to damage and developmental delay following preterm birth are the hippocampus and the cerebellum[11-14] as both of these regions continue to mature throughout late gestation.

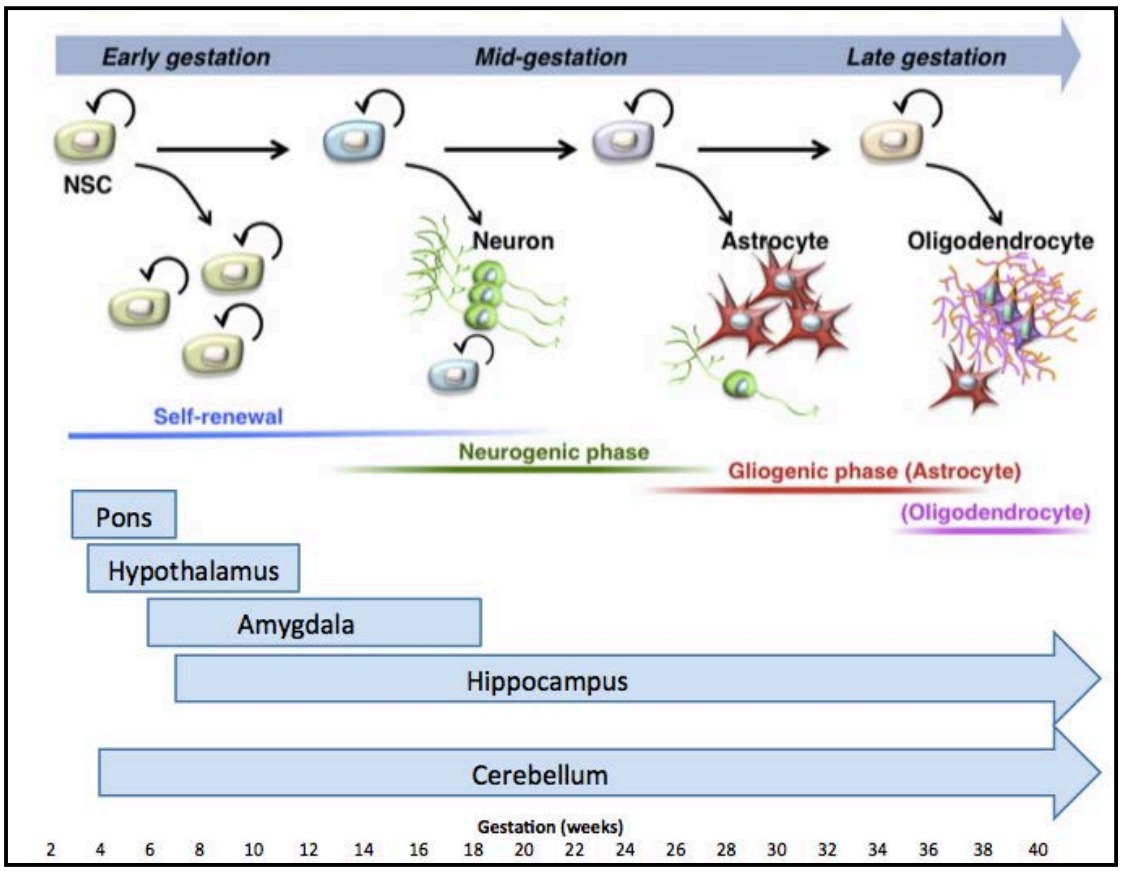

Figure 1.1 Development of the brain *in utero*. Development of cell types and regions of the brain follow a trajectory from most simple cells and regions involved in basic, essential functions (i.e. breathing and heart rate), up until higher-order executive functioning regions and cell types responsible for cognition and learning. In early gestation, neural stem cells replicate and then differentiate into neurons by mid-gestation. From mid-gestation onwards differentiation of astrocytes and oligodendrocytes occurs. Meanwhile, the development of brain regions such as the pons, the hypothalamus, and even the amygdala takes place during early gestation. Whilst the hippocampus and cerebellum begin to develop in early gestation, they are still maturing throughout mid-late gestation and after birth. Adapted from Bayer, *Neurotoxicology,* 1993[82], Howdeshell, *Environ Health Perspect,* 2002[83], and Kneusel, *Nat Rev Neurol*, 2014[84].

1.2.1.1 HIPPOCAMPUS

The hippocampus is located in the medial temporal lobe of the brain and spans across both hemispheres. Development begins in early gestation (approximately week 6) and continues throughout late gestation and after birth. The hippocampus features the *cornu ammonis* (CA) regions (CA1-3) as well as the dentate gyrus. Projections into and out of the hippocampus are via the entorhinal cortex, which is strongly connected with many cortical and subcortical structures within the brain, and information flowing out from the hippocampus generally outweighs the input entering the region. To process information axons from pyramidal cells in the entorhinal cortex project to the dentate gyrus, and from here the mossy fibres of the dentate gyrus pass on information to the CA3 pyramidal cells. Axons from these CA3 cells then extend up to dendrites within the CA1 and finally project back to the entorhinal cortex and thus the rest of the brain. In humans, the volume of the hippocampus doubles from mid-gestation until late-gestation, representing a period of rapid growth[85]. Cytoacrchitecture of the CA1-3 regions is complete by mid gestation[86], and cell proliferation in these areas is minimal during late gestation, therefore suggesting that large increases in volume are due to maturation of pre-existing neurons. By 32-34 weeks' gestation (mid-late gestation) the CA2 and CA3 regions have enlarged and matured, whilst in the CA1 region some neurons remain immature[15]. The dentate gyrus is the last area to develop and cell proliferation occurs throughout the second half of gestation as well as postnatally[86]. Myelination of the hippocampus does not become strongly evident until late gestation, and continues throughout postnatal life[15].

Interestingly different regions of the hippocampus are responsible for the various functions of the hippocampus. Cells of the dorsal hippocampus are demonstrated to perform the learning, memory, and spatial recognition roles and project mainly to the prefrontal cortex. Conversely, the ventral hippocampus is responsible for emotional processing and fear conditioning, as it largely projects to the amygdala.

Functional studies observing the effects of damage to the hippocampus on subsequent behaviour have demonstrated the differential functions of the hippocampus along its dorsoventral axis. The involvement of the ventral hippocampus with the fear response has been demonstrated in a study where rats with lesions to the ventral hippocampus exhibit reduced fear conditioning as evidenced by a failure to avoid the open-arms on the elevated plus maze, whilst those with lesions to the dorsal half of the hippocampus did not[87]. These rats also had decreased neuroendocrine stress responses when confined to a brightly lit chamber, further highlighting their altered response to fear. As the dorsal hippocampus supports spatial memory formation lesions to the dorsal hippocampus in rats resulted in a learning deficit during the water maze[88], and impaired performance in working memory tasks such as rewarded alternation in the T-maze[89]. Interestingly ablation of inhibitory interneurons in the dorsal hippocampus of adult mice resulted in a hyperactive phenotype (larger distances travelled in the open field), in addition to deficits in spatial learning ability during the water cross maze[90]. The dorsal hippocampus is also highly vulnerable to stress, with restraint and social stress resulting in selective death of synapses in the CA1 of the dorsal hippocampus, whereas the ventral hippocampus is unaffected[91].

1.2.1.2 CEREBELLUM

The cerebellum is located at the base of the brain and is connected to the rest of the brain by the cerebellar deep white matter. The development of the cerebellum begins early in gestation at weeks 4-5 and peaks in developmental activity at weeks 24-33, continuing to mature until after birth. Between weeks 20-40 the rapid growth of the cerebellum far outweighs that of any other brain region. The cerebellum has 3 lobes, which are then separated into 10 lobules (lobules I – X), and each of these lobes is responsible for different functions. The anterior lobe (lobules I – V) is active during motor performance, the posterior lobe (lobules VI – IX) interconnects with the prefrontal cortex and is engaged during cognitive tasks, and the flocculonodular lobe (lobule X) receives input from the vestibular nuclei and therefore has roles in balance[92]. Similarly to the cerebral cortex, the cerebellum has a developmental trajectory beginning with the earliest lobules responsible for basic function, up until the last lobules, which are involved with cognition[93].

The cerebellum is highly interconnected with various regions of the cerebral cortex, and is now known to have roles in cognition, procedural learning, skill acquisition, and affective regulation, in addition to its well understood role in movement. Based on the regions of the brain that different parts of the cerebellum are connected to, it is suggested that disrupted cerebellar development can have major impacts on both the structure and the function of the cortical regions to which it projects by a process known as 'developmental diaschisis'[94]. Following preterm birth the cerebellar volume is positively correlated to cognition, and specific reductions in the lateral portion of the cerebellum (lobules IV – X) of 7-year-old ex-preterm children are associated with decreased verbal comprehension, visual perception and perceptual

reasoning. Preterm survivors of cerebellar parenchymal injury (haemorrhage within the parenchyma) have impaired growth of the uninjured contralateral cerebral hemisphere in areas such as the dorsolateral prefrontal cortex and mid-temporal regions[95]. Furthermore, in a small study of age-matched 2-4 year olds 66% of survivors displayed social-behavioural deficits such as inattention and internalizing behaviour, as well as cognitive and language deficits[96]. The role of the cerebellum in learning is particularly associated with the acquisition of new skills, as adults with cerebellar lesions are shown to have deficits in new skill acquisition[97]. Furthermore, it is also suggested that cerebellar injury in early life has more dramatic and long-lasting effects than damage late in life, presumably due to this role in skill acquisition and lesser role in retention of learned behaviours[92, 93].

Finally, reductions in various regions of the cerebellum are common in neurodevelopmental disorders such as autism, ADHD, and dyslexia. Interestingly the regions of the cerebellum involved vary depending on the disorder, again highlighting the regional specificity of the cerebellum. A meta-analysis of 37 studies revealed that patients with autism had reduced gray matter in cerebellar regions that projected to the frontoparietal, somatomotor, and limbic regions, whereas reductions in gray matter of ADHD patients were functionally connected to the dorsal and ventral attention networks[98]. Furthermore, worsened ADHD symptoms are correlated with large reductions in gray matter of the posterior lobe, and one of the primary treatments for ADHD (methylphenidate) increases cerebellar activation in this region[99].

1.2.2 Cell types in the brain

Once neural stem cells reach their destined location they begin to differentiate into neurons and glial cells (see Figure 1.1). Neuronal cells become mature neurons, whilst glial cells can differentiate into a number of different cell types. Glial cells of the CNS are broadly categorized into microglia and macroglia. Microglia are essentially the macrophages of the CNS and protect neurons by phagocytosing foreign particles. Their activation is an integral factor in the defence of the nervous system against infectious diseases, inflammation, trauma, ischemia, and neurodegeneration. Macroglia are able to differentiate into four types of cell; the ependymal cells, which are responsible for the creation and secretion of cerebrospinal fluid, and radial cells, which are involved in the development of the neuroepithelia, in addition to astrocytes and oligodendrocytes.

Astrocytes are involved in a number of processes, including enveloping synapses (where they modulate the transmission of impulses), regulation of the ion concentration in the extracellular space, repair or replacement of damaged neurons, and neurotransmitter [including gamma-aminobutyric acid (GABA), glutamate, and adenosine triphosphate (ATP)] release and uptake[100]. Astrocytes are therefore seen as the support cells of the CNS and one of their roles is the promotion of myelinating activity by oligodendrocytes. The ATP released by astrocytes induces the secretion of a regulatory protein, which then acts directly on oligodendrocytes to stimulate myelin formation[101]. Astrocytes have been shown to promote myelination *in vitro*[102] and also to protect the central nervous system from trauma by preserving myelin and reducing white matter damage in a mouse model of spinal cord injury[103], in addition to demonstrating myelin repair properties in a mouse model knockout of

astrocytes[104]. Astrocyte expression is analysed immunohistochemically by glial fibrillary acidic protein (GFAP), which is an intermediate filament protein in the cytoskeleton of astrocytes[105].

Oligodendrocytes exist in a lineage, which spans from an early pre-progenitor cell up to a mature myelinating oligodendrocyte. Their main function is to provide support and insulation to axons by forming the myelin sheath, which is composed mostly of lipids. Important to note however, is that only mature myelinating oligodendrocytes are capable of producing myelin[106]. Myelin production by mature oligodendrocytes includes expression of three main proteins including myelin basic protein (MBP), myelin proteolipid protein (PLP), and myelin associated glycoprotein (MAG)[107, 108]. Cells throughout the entire lineage can also be analysed by oligodendrocyte transcription factor (OLIG2) expression, and those early in the lineage by neural glia antigen 2 (NG2) and platelet derived growth factor alpha (PDGFRα)[106]. Myelination by mature oligodendrocytes largely occurs from late gestation onwards and thus is highly vulnerable to late gestation pregnancy compromises. Mature oligodendrocytes strongly express glucocorticoid receptors due to the essential role of corticosteroids in proliferation and differentiation of oligodendrocytes[16]; however, this also makes them an especially vulnerable target for increased levels of glucocorticoids. Furthermore, mature oligodendrocytes are also known to be particularly vulnerable to hypoxia[17], which is a common consequence of preterm birth.

1.2.3 Importance of the placenta for neurodevelopment *in utero*

The mammalian fetus develops in the uterus of the mother in a system known as the feto-placental-unit. The placenta plays an essential role in ensuring fetal neurodevelopment occurs correctly by secreting growth regulating factors including steroid hormones throughout pregnancy. Insufficient development reduces the supply of steroids, nutrients, and oxygen to the fetus, resulting in reduced growth leading to intrauterine growth restriction (IUGR) and associated impaired neurodevelopment[109-112]. In addition to supplying hormones to the fetus, the placenta also protects the fetus from damaging hormones derived from the maternal circulation. Maternal stress is a risk factor for preterm delivery as maternal cortisol may pass from the maternal circulation to the fetal circulation via the placenta. Low levels of glucocorticoids, such as cortisol, are important for normal maturation and growth of the developing fetal brain; however, raised levels of metabolised maternal cortisol or synthetic glucocorticoids (such as betamethasone) have the potential to delay or disrupt myelination and glial cell proliferation, resulting in poor neurodevelopmental outcomes for the fetus[113]. When there is a risk of premature birth, betamethasone is administered to quickly encourage the growth of the lungs to ensure that the neonate is not born with ineffective lungs that cannot produce surfactant[114]; however, as mentioned excessive exposure can be detrimental. Animal studies in sheep and guinea pigs have confirmed this negative effect of glucocorticoids where repeated administration was shown to delay the maturation of neurons, glia, vasculature, and myelination, resulting in an overall decrease in brain volume, neuronal structures, and synapse formation[115-117]. In our guinea pig

model, repeated betamethasone administration also negated neurosteroid effects by reducing the amount of progesterone metabolized into allopregnanolone[118]. Prolonged stress in the primate is also associated with neurodegenerative effects[119].

The fetus is at least partially protected from damaging levels of cortisol by the placental expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) which rapidly catalyses cortisol into less active cortisone[120, 121]. This expression forms an enzymatic barrier that can prevent 80-90% of maternal cortisol reaching the fetus, but if maternal cortisol levels are raised the fetus is exposed to increased levels of cortisol[122]. Maternal stress can also affect the expression of the enzymatic barrier in the placenta as a rat model of maternal stress has demonstrated that placental expression and activity of 11βHSD2 is strongly reduced during chronic maternal stress[123]. We have previously shown that the combined effect of IUGR and maternal stress reduce circulating neurosteroid allopregnanolone concentrations as well as myelination of the fetal brain, particularly in the male[110]. Whilst factors that influence placental development and function can significantly alter the development of the fetus, the most damaging insult to the fetus is complete removal of the placenta before fetal development is complete by preterm birth.

1.2.4 *Ex utero* neurodevelopmental consequences following preterm birth

Following preterm birth, the newborn is exposed to the *ex utero* environment much earlier than if it had remained *in utero* until term (37-40 weeks' gestation). This premature exposure means that organs such as the lungs are expected to adapt earlier than usual, often resulting in periods of apnea and respiratory distress. The brain is

another organ that is also highly vulnerable following preterm birth as it is not only removed from placental precursor supply of fetal neurosteroids and other placentally supplied nutrients, but it is also prematurely exposed to a stimulating environment and excitotoxic damage. Damage to astrocytes and especially oligodendrocytes of the developing nervous system can occur in the vulnerable developmental window prior to term gestation. Astrocyte activation is involved in nervous system repair, and increased proliferation of astrocytes is demonstrated following activation of inflammatory pathways, such is the case following preterm birth and accompanying cerebral white matter damage[124, 125]. Astrocyte dysfunction has also been suggested to play a role in the development of psychiatric disorders, including ADHD and autism, where marked increases of astrogliosis and altered astrocyte signalling are present[126, 127].

Oligodendrocytes are highly sensitive to the external environment and are frequently injured by chemical and mechanical damage, which can happen following preterm birth and early exposure to the *ex utero* environment[128, 129]. Early demyelination and damage is apparent in Alzheimer's disease and multiple sclerosis, which are diseases that are also characterized by memory loss, impaired learning ability, and impaired motor coordination[130, 131]. Similarly, ex-premature children are also known to experience impaired learning ability and motor coordination, and a loss of myelination or loss of 'white matter' is evident following preterm birth suggesting a similar causal pathway[11]. Furthermore, reductions in myelination are apparent in a rat model of ADHD[132], and decreases in the white matter volumes of vulnerable regions such as the hippocampus and cerebellum are evident on magnetic resonance imaging (MRI) comparing term and preterm neonates[133].

Cerebral white matter injury in the preterm infant varies based on gestational age at the time of birth. Injury following early premature birth is characterised by multiple lesions, intraventricular hemorrhage, and periventricular leukomalacia (PVL)[124, 134]. In survivors of early preterm birth weighing <1500g approximately 10% develop cerebral palsy as a result of these gross insults and necrosis[135]. Immature oligodendrocytes are the major cellular target of PVL, with evidence suggesting that these cells are particularly vulnerable to the free radicals formed and increased excitatory glutamate released during ischemia-reperfusion, and thus PVL often occurs in combination with intraventricular hemorrhage and results in necrotic lesions[124]. The severity of PVL at the time of birth affects the extent of poor developmental outcomes, which has been demonstrated by a study following 100 ex-preterm children in Korea[136]. At the time of birth the severity of PVL was rated mild, moderate, or severe in infants born between 27 and 37 weeks' gestation and followed up with cognitive and behavioural testing between 3-9 years of age. Intellectual disability increased with severity of PVL, 27.8% had intellectual disability in the mild PVL group, 53.2% in the moderate group, and a staggering 77.1% in the severe PVL group had an intellectual disability during childhood. Behavioural problems, including anxiety and attention problems, were present in a third of ex-premature children but was not related to severity of PVL, suggesting a different underlying cause. Cognitive deficits following preterm birth have been demonstrated to be long lasting following white matter damage. In infants that were born <32 weeks' gestation, it has recently been shown that reductions in white matter volume in areas such as the fornix and the cingulum observed by MRI at the time of birth remained present until 19 years of age and were associated with impairments in memory functions[137]. The Stockholm

Neonatal Project has also recently published the results of a longitudinal trial following infants born <36 weeks' gestation up until 18 years of age when they undertook psychological assessment including general intelligence and executive functioning measures. Significantly poorer outcomes were observed for preterm offspring in areas such as IQ, attention, working memory, and cognitive flexibility[138]. Most importantly however, is that the executive functioning deficits did not correlate with reductions in white matter or gray matter volumes evident by MRI following birth, but the microstructure of white matter tracts was altered at adolescence. Thus, this study found that following preterm birth, and in the absence of obvious perinatal brain injury, the alterations observed in white matter microstructure during adolescence correlate with executive function and general cognitive abilities. Furthermore, suggesting that disruption to neural pathways, as opposed to reductions in brain volume, is involved in the impairment of neurodevelopment following preterm birth.

1.2.4.1 TREATMENT OPTIONS FOR NEUROLOGIC OUTCOMES

In an effort to prevent neurological deficits from occurring following very premature birth a number of therapies have been adopted; however, often these are not used following late preterm birth, as brain injury is not obvious at the time of birth. Maternal magnesium sulphate is frequently administered in anticipation and preparation of early preterm birth. A Cochrane systematic review of 5 large trials comprising 6145 babies found that the incidence of cerebral palsy in preterm neonates dropped from 5% to 3.4% following antenatal magnesium sulphate therapy[139]. Unfortunately, there was no benefit in terms of prevention of other neurological conditions such as learning disorders. A limitation of this therapy is the need for

antenatal rather than postnatal treatment, especially considering that up to 50% of preterm births are spontaneous and thus antenatal therapy is often not an option. Human and animal studies have also demonstrated a lack of neurological improvement following magnesium sulphate therapy in the context of chorioamnionitis induced preterm birth and asphyxia associated with preterm labour[140, 141], again limiting the effectiveness of this therapy. The antioxidant melatonin has also been investigated for its neuroprotective benefits in animal models due to its roles in modulating neuroinflammation and reducing reactive oxygen species[142]. In neonatal stroke and hemorrhage rat models, pre-treatment with melatonin reduced the neuroinflammation and damage associated with stroke, whilst post-treatment reduced the amount of tissue death and improved cognitive and sensorimotor outcomes[143, 144]. However, despite entering clinical trials there are few conclusive results available, in fact a Cochrane systematic review identified zero randomised trials published in the literature[145].

Hypothermia in late preterm and term infants exposed to hypoxia and stroke has demonstrated well-established benefits, such as reduced mortality and decreased long-term neurodevelopmental disability, if implemented within 6 hours of the insult occurring[146, 147]. Despite the obvious benefits however, this therapy is implemented with caution as side effects of body cooling can include an increase in sinus bradycardia and a significant increase in thrombocytopenia (low levels of platelets in the blood)[146]. The feasibility of this therapy for improving neurodevelopmental outcomes following preterm birth therefore is limited due to the immature thermoregulatory system in the preterm neonate. The risk of body temperature dropping below 32°C and resulting in impaired oxygenation, risk of

infection, and development of intracranial hemorrhage warrants considerable caution when treating a preterm neonate[147]. A small pilot trial investigating the feasibility of extending hypothermia treatment to preterm neonates at 34-35 weeks' gestation exposed to hypoxic ischemia observed hypothermia-associated complications in 90% of the preterm group versus 81.3% in the term cohort[148]. Injury to white matter was also increased in the preterm group following hypothermia therapy at 66.7% compared to just 25% in the term neonates with hypoxic ischemia injury. Furthermore, 100% of the deaths following the hypothermia therapy were in the preterm neonatal group. Currently the American Academy of Paediatrics committee advises that hypothermia should not be undertaken on preterm neonates due to the extensive risk associated, unless it is performed in a research setting[149].

1.3 NEUROSTEROIDS

1.3.1 THE *IN UTERO* NEUROSTEROID ENVIRONMENT AND FETAL NEURODEVELOPMENT

Neurosteroids are endogenous steroids that rapidly alter neuronal excitability through interaction with ligand-gated ion channels and other cell surface receptors. These steroid hormones can cross the blood-brain barrier and produce changes in mood and behaviour. Changes in neurosteroid levels are associated with various conditions including stress, pregnancy, and also neural development[150]. Neurosteroids can be classified as excitatory or inhibitory. Excitatory neurosteroids, such as dehydroepiandrosterone (DHEA), are potent negative allosteric modulators of the $GABA_A$ receptor and have excitatory effects on neurotransmission. In contrast,

inhibitory neurosteroids are potent positive allosteric modulators of the $GABA_A$ receptor and result in the inhibition of neurotransmission. One of the inhibitory neurosteroids, allopregnanolone, is found in very high concentrations in both the fetal plasma and fetal brain, compared to concentrations following birth, and is the main focus of this thesis[21, 151, 152]. Tetrahydrodeoxycorticosterone (THDOC) has similar actions to allopregnanolone as an inhibitory neurosteroid but it is produced from the mineralocorticoid deoxycorticosterone, as opposed to progesterone. There is very little literature regarding the role of THDOC during fetal and early neonatal life. The adrenal gland is responsible for production of deoxycorticosterone and in the fetus this gland may not be sufficiently developed to produce ample levels that would be required if THDOC were to have the same effects as allopregnanolone on the brain during development.

In utero, the elevated concentration of allopregnanolone provides neuroprotection for the developing fetal brain. In late gestation, the fetus is maintained in a sleep-like state characterized by low levels of arousal-like activity. This ensures that excitation of the brain is limited, ultimately providing sufficient energy for processes such as myelination to occur[18, 19]. This fetal 'sleep' state is maintained by an elevated level of allopregnanolone in the brain compared to the adult, and a decreased supply of this neurosteroid is shown to increase the excitation of the brain, potentially disrupting or delaying brain developmental processes[109, 153]. A reduction in the normal fetal neurosteroid environment is thus associated with adverse outcomes, such as an increase in potentially damaging seizures which can lead to permanent disruptions to neurodevelopment[154]. Following preterm birth there is a premature reduction in the supply of progesterone and allopregnanolone (see Figure

1.2, resulting in an already vulnerable premature neonate being exposed to the *ex utero* environment without neuroprotection. The excessive excitation induced by the *ex utero* environment, and associated lack of neurosteroids, can therefore inflict additional damage to the developing brain.

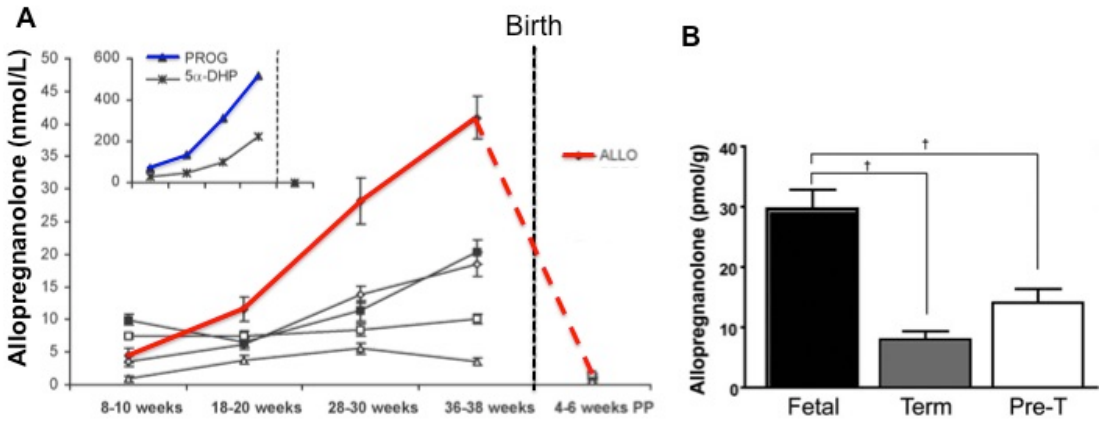

Figure 1.2 Allopregnanolone concentrations during gestation. In a) circulating allopregnanolone concentrations in the human increase from 8-10 weeks of gestation, up until term. Following birth there is a rapid decrease and levels are minimal by 4-6 weeks after birth. Progesterone (inset) follows a similar profile. In b) we have previously shown in the guinea pig that term and preterm (Pre-T) concentrations of allopregnanolone in the neonatal brain are less than the fetal. Adapted from Evans, *Gynecol Endocrinol*, 2005[155] and Kelleher, *Reproductive Sciences*, 2013[21].

Allopregnanolone is metabolized, by the rate limiting enzymes 5α-Reductases type 1 and 2 (5αR1 and 2), from progesterone[156, 157]. During early gestation the corpus luteum is responsible for producing progesterone; however, by mid-gestation the placenta is producing very large amounts of progesterone and metabolizing it into

allopregnanolone and its immediate precursors (see Figure 1.3 for synthesis pathway)[158, 159].

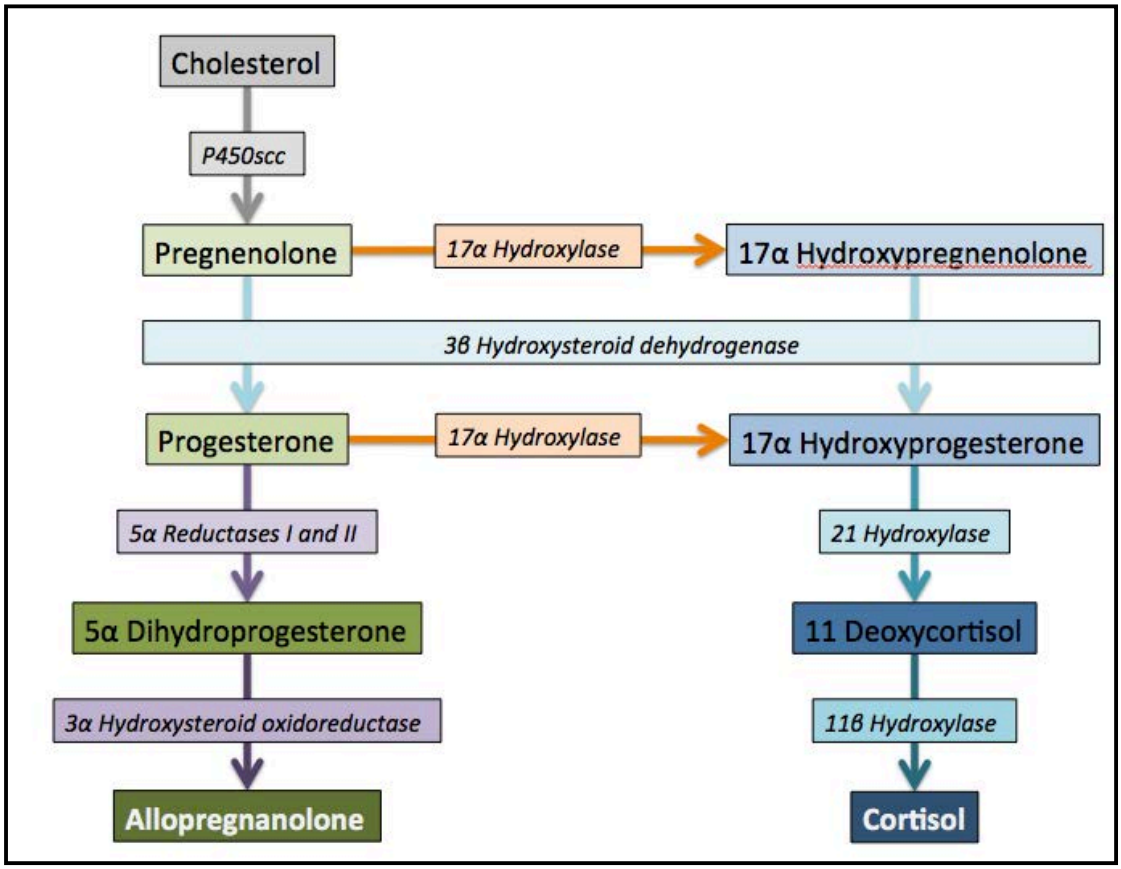

Figure 1.3 Steroidogenesis of allopregnanolone and cortisol from cholesterol. Circulating cholesterol is metabolised into pregnenolone and then progesterone in the maternal circulation and placenta. Placental supply of progesterone to the fetus can then be metabolised into the neurosteroid allopregnanolone, via the intermediate 5α dihydroprogesterone. Progesterone can also be metabolised into cortisol in the maternal, placental, or fetal circulation from the intermediates 17α hydroxyprogesterone and 11 deoxycortisol.

In addition to the allopregnanolone supplied by the placenta to the fetal circulation, the fetal brain is also capable of metabolizing allopregnanolone from placentally derived precursors and thus there is also a high level of allopregnanolone

produced and maintained within the fetal brain[152, 160]. We have previously shown in the guinea pig that following birth, and the loss of the placenta, progesterone and allopregnanolone levels decline rapidly within 24 hours, highlighting the necessity of the placenta for the supply of steroidogenic precursors[21]. The rate-limiting enzymes 5αR1 and 2 are strongly expressed in the placenta, and sheep and rat studies have confirmed that the 5αR2 isoform is expressed on neurons and glia within the developing fetal brain in late gestation[18, 156]. In a sheep model, administration of finasteride (inhibitor of the 5αR1 and 2 enzymes) to the fetus preventing the metabolism of progesterone to allopregnanolone within the fetal brain results in an increase of damaging excitation in the brain as a result of reduced suppression by allopregnanolone[161]. As a result of this excitation, there is also an increase in the numbers of dead cell in areas such as the hippocampus, cerebellum, and white matter tracts. In another study, when the synthesis of allopregnanolone was reduced as a result of the prevention of progesterone production by trilostane (a 3β-hydroxysteroid dehydrogenase inhibitor) there was a decrease in the normal incidence of sleep-like behaviour and an increase in arousal-like activity[162], which is associated with an increase in excitability of the brain and damaging seizures[163, 164]. Furthermore, when progesterone was replaced by exogenous supplementation, the occurrence of sleep-like behaviour returned to normal[162].

Exposure to finasteride has also been shown to increase apoptotic cells in the CA1 and CA3 regions of the hippocampus, and the cerebellar molecular and granular layers in fetal sheep, as well as increasing the number of dead Purkinje cells (GABAergic neurons) in the cerebellum[165]. Interestingly, when a double infusion of finasteride and alfaxolone (an allopregnanolone analogue) is administered this

deleterious effect is not seen. Similarly allopregnanolone itself has also been shown to protect the fetal brain when insults occur, in a sheep model the introduction of brief asphyxia in the presence of finasteride induced cell death in the hippocampus; however, when allopregnanolone was present in normal concentrations this asphyxia-induced damage did not occur[161]. *In utero* administration of finasteride to guinea pigs has also highlighted the role of allopregnanolone in myelination, as a reduction in myelination in the subcortical white matter was present following inhibition of allopregnanolone synthesis[109]. Interestingly, administration of the allopregnanolone precursor, progesterone, to *in vitro* rat cerebellum slices increased both the proliferation of myelinating oligodendrocytes and the rate of myelination[166]. Follow up studies then revealed that this effect was achieved by neurosteroids acting on the $GABA_A$ receptors[167]. Studies such as these emphasize how important the role of allopregnanolone is not just for development of the brain, but also for protection from insults, and highlights the damage that preterm brains are exposed to following periods of brief asphyxia, which are more common following premature birth.

A reduction in normal allopregnanolone concentrations during pregnancy can have long-lasting effects on the offspring as has been demonstrated in guinea pigs, where administration of finasteride during late gestation resulted in an anxiety-like phenotype in female offspring, along with reductions in components of the GABAergic pathway within the amygdala[168]. Furthermore, there was also decreased expression of neurosteroid-sensitive $GABA_A$ receptors and increased astrocyte activation within the cerebellum of these animals[169]. In a similar study, finasteride exposure during late gestation increased corticosterone levels, decreased hippocampal allopregnanolone levels and impaired performance in memory tasks of juvenile rat

offspring[170]. Studies inhibiting the production of allopregnanolone in adult rats highlight the importance of allopregnanolone for the prevention of neurodevelopmental disorders throughout life as reductions in the concentration of allopregnanolone within the hippocampus[171] or the amygdala[172] increased anxiety-like behaviours in these animals. Furthermore, multiple neurological conditions are characterised by a reduced level of allopregnanolone in adults, including post-traumatic stress disorder[173], major depressive disorder[174], and premenstrual dysphoric disorder[175, 176].

1.3.2 Allopregnanolone action on $GABA_A$ receptors

GABA receptors are separated into two broad categories, the ionotropic ligand-gated receptors, and the g-protein coupled metabotropic receptors. The bicuculline sensitive $GABA_A$ receptors and the bicuculline insensitive $GABA_C$ receptors are the ionotropic ligand-gated receptors, whilst $GABA_B$ are the g-protein coupled[177-179]. $GABA_A$ receptors specifically feature a gated chloride ionophore channel and specific binding sites for benzodiazepines, barbiturates, and anaesthetics; however neurosteroids are thought to bind to a separate allosteric steroid-binding site[177, 178]. $GABA_A$ receptors normally exhibit inhibitory effects in response to neurosteroid stimulation from mid gestation onwards; however they are capable of exhibiting excitatory actions under the right circumstances, and in early gestation when excitatory action is required for neuronal outgrowth[180, 181]. Whether the effect is inhibitory or excitatory is determined by the chloride gradient of the receptors, which is primarily regulated by the K+/Cl- co-transporter, known as KCC2[182, 183]. The expression and activity of this integral co-transporter is regulated by the

phosphorylation of its Ser940 residue, with dephosphorylation resulting in downregulation of the co-transporter, increasing the intracellular chloride concentration, and switching to excitatory GABA actions[184, 185].

Inhibitory neurosteroids including allopregnanolone exert an inhibitory effect over the brain and suppress excessive excitation. This suppression is achieved by increasing GABAergic inhibition[20]. Allopregnanolone is an allosteric agonist of the extrasynaptic $GABA_A$ receptors and specifically enhances $GABA_A$ receptor mediated inhibition, which results in anxiolytic, anti-convulsant, anaesthetic, analgesic, and sedative effects (Figure 1.4)[186-192].

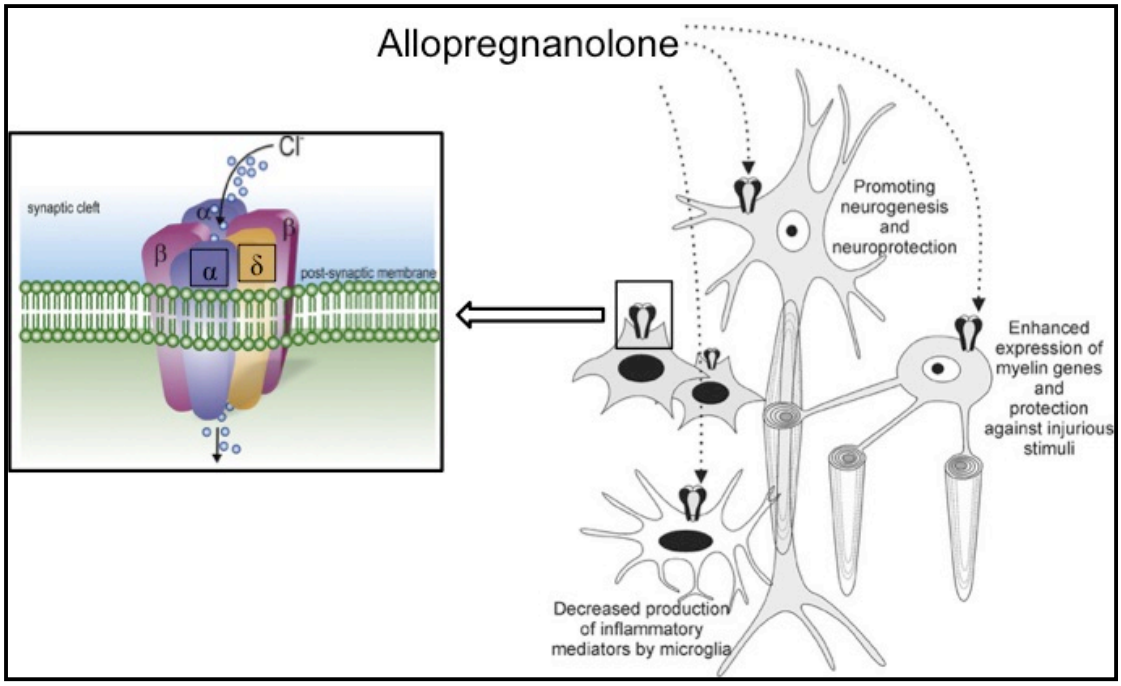

Figure 1.4 The action of allopregnanolone on $GABA_A$ receptors. The neurosteroid allopregnanolone acts directly on α and δ subunit containing $GABA_A$ receptors (see inset picture) of the central nervous system. These receptors are located extrasynaptically on a number of different cell types including oligodendrocytes. Allopregnanolone increases the inhibitory action of GABA on $GABA_A$ receptors and has roles such as promoting neurogenesis and neuroprotection, enhancing expression of myelin genes and protection against injurious stimuli, and decreasing production of inflammatory mediators by microglia. Adapted from Noorbakhsh, *Front Cell Neurosci,* 2014[193] and Brunton, *Prog Neurobiol*, 2013[194].

Thus, $GABA_A$ receptors are involved in a broad range of functions including controlling the excitability of the brain, modulation of anxiety, as well as cognition, memory, and learning[195]. Extra-synaptic neurosteroid sensitive receptors are also highly expressed on glial cells including oligodendrocytes throughout the fetal brain from mid-gestation onwards[158, 196, 197]. The expression of $GABA_A$ receptors in the fetal brain increases as gestation advances, reaching their highest levels of expression by full term gestation in most areas, such as the cerebral cortex and hypothalamus[18, 196, 198]. $GABA_A$ receptors exist in a pentameric formation of 5 subunits with a central selective chloride anion channel. The 5 subunits come from a pool of 19 different subunits, α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 and subunit composition varies greatly depending on the function of the receptor[199, 200]. Synaptic receptors, which are responsible for fast, synaptic transmission, usually contain the α1-3, β1-3, and γ2 subunits[201], whilst the extra-synaptic receptors (responsible for tonic inhibition)[200] are composed of the α4-6 and δ subunits[202]. Rather than produce an increase in amplitude of miniature inhibitory postsynaptic currents (mIPSCs), neurosteroids have been shown to increase the duration of the amplification by altering the kinetics of the $GABA_A$-gated ion channels[203]. This increase in duration is neuron specific, with different brain regions requiring different concentrations of neurosteroids to induce the same effect. Specifically, the CA1 neurons of the hippocampus, cerebellar granule cells, and Purkinje cells appear to be more sensitive to neurosteroids, only requiring low nanomolar concentrations to increase duration of amplification[204, 205], and this is primarily due to subunit composition.

Receptor subunit composition plays an important role in determining receptor affinity for various ligands. Benzodiazepines for example are known to be attracted to

receptors containing a γ subunit, whilst those featuring α6 are unresponsive to benzodiazepines[178]. Whilst there is a specific binding site for neurosteroids such as allopregnanolone, the composition of subunits affects the sensitivity of the receptor to stimulation[206, 207]. Regional specificity also exists for these receptors, for example in a knockout of the δ subunit in a mouse model tonic conductance was significantly reduced in the cerebellum; however in the CA1 region of the hippocampus there was no effect on conductance[208]. This regional specificity is due to differences in expression of various subunits throughout the brain and whilst the α6 and δ subunits (which are co-expressed in receptors) are highly expressed in the cerebellum, tonic conductance in the hippocampus is controlled by receptors containing the α4 and α5 subunits in addition to those containing the δ subunit.

The role of specific neurosteroid-sensitive subunits in behaviour has been revealed in knockout animal models. For example, in a mouse model the global deletion of the δ subunit significantly reduced the anxiolytic and anti-convulsant effects induced by the allopregnanolone analogue ganaxolone, confirming that neurosteroids bind to the δ subunit of $GABA_A$ receptors to exert their functions[209]. Increased anxiety-like behaviour was also present in a mouse model knockout of the α4 subunit as demonstrated by an increased preference for dark enclosed spaces in a T-maze[210], and seizure susceptibility has also been shown as increased following this knockout[211]. Similarly, it has been demonstrated that proepileptic behaviour is increased in mice lacking the δ subunit[209, 212, 213]. Together these data implicate the necessity of appropriate configurations of the $GABA_A$ receptors for neurosteroid binding and downstream effects on behaviour.

1.3.2.1 NEUROSTEROID MODULATION OF $GABA_A$ RECEPTORS

Extensive investigation has also demonstrated that sensitivity to neurosteroids can change over time. When mIPSCs are recorded from the cerebellar purkinje cells of 10 day old rats they show a greater sensitivity to neurosteroids compared to mIPSCs recorded in cerebellar purkinje cells of 20 day old rats[205]. The plasticity of the dentate gyrus cells of the hippocampus is also capable of changing. Allopregnanolone levels are raised after a seizure and exhibit anticonvulsant effects[214], but following an induced epileptic seizure it has been demonstrated that synaptic $GABA_A$ receptors of dentate gyrus cells become insensitive to neuroactive steroids[215]. Another example of neurosteroid induced $GABA_A$ receptor plasticity is demonstrated by the magnocellular oxytocin neurons of the hypothalamus. The release of oxytocin from these neurons occurs during parturition and is mediated by neuronal inhibition. One day prior to parturition, the circulating levels of oxytocin are relatively high as allopregnanolone acts to prolong the spontaneous inhibitory postsynaptic currents from the oxytocin neurons. When the levels of allopregnanolone drop after birth the synaptic $GABA_A$ receptors become insensitive to neurosteroids, changing the inhibitory tone and the release of oxytocin[216, 217]. The rising endogenous neurosteroid levels throughout pregnancy in the fetus depend on availability of progesterone precursors, which drop after birth. Interestingly when the supply of progesterone and allopregnanolone to the rat fetus is withdrawn, the expression of the α4 subunit of $GABA_A$ receptors in the hippocampus increases[218]. These examples highlight the modulatory role that neurosteroids may have on $GABA_A$ receptor subunit expression in the CNS and thus on their own action.

1.3.2.2 $GABA_A$ RECEPTORS AND GLUCOCORTICOIDS

$GABA_A$ receptor subunit expression can be influenced by glucocorticoids including cortisol. Specifically, acute stress has been linked with an increase in expression of the δ subunit and raising of baseline tonic conductance[219]. Additionally, administration of THDOC, which is also released endogenously in conditions of high stress, is capable of rapidly increasing the expression of the δ subunit[220]. A further effect of THDOC is the depolarizing of the GABA equilibrium potential, causing a shift from inhibitory to excitatory GABAergic transmission in corticotropin releasing hormone (CRH) neurons[221]. In this manner, THDOC acts on δ subunit containing $GABA_A$ receptors to initiate positive feedback to the hypothalamic-pituitary-adrenal (HPA) axis and respond to increased levels of stress[221]. Alternatively, as it has previously been stated, chronic stress has the opposite effect. In cases of chronic stress or chronically raised concentrations of glucocorticoids, levels of neurosteroids are decreased and the overall stimulation of the $GABA_A$ receptors is decreased[222]. It is unknown at this stage whether this is due to an alteration of the $GABA_A$ receptor subunit expression profile. Interestingly, one study in rats observed a sex-dependent influence of stress in early life on $GABA_A$ receptor subunit expression in later life. The exposure of a restraint and a social stress to the male rats in their juvenile age period was found to significantly increase the expression of their α2, α3, and α5 subunits in adulthood; however, the same response was not seen in the female rats[223]. This response could be an adaptation to early life stressors as the behaviour of the males in adulthood was less anxious compared to the females who displayed more anxious behaviour.

Interestingly, it has been suggested that the output of CRH neurons is regulated by GABAergic action[224]. It is known that following acute stress there is a collapse of the chloride gradient in CRH neurons[225]. The proposed explanation for this collapse is that acute stress results in dephosphorylation of the co-transporter responsible for controlling inhibitory and excitatory actions of GABA, KCC2[221]. The dephosphorylation results in a reduced surface expression of KCC2, as opposed to total levels of the co-transporter. This decrease in surface expression results in a switch to excitatory action of neurosteroids on CRH neurons[221]. Based on these findings, it has been proposed that the action of neurosteroids on $GABA_A$ receptors initiates a positive feedback mechanism onto the CRH neurons of the HPA axis, therefore regulating the physiological response to stress[221].

1.3.2.3 THE GABAERGIC SYSTEM

The GABAergic system is pivotal to proper functioning of the nervous system as it plays a large role in essential processes including the regulation of neuronal activity levels, control of size and propagation of neuronal assemblies, neuronal plasticity, and synapse formation[226]. GABA, the main neurotransmitter of the GABAergic system, is responsible for inhibition throughout the brain by hyperpolarizing the membrane and shunting excitatory inputs[180]. GABA is synthesized by the glutamate decarboxylase (GAD) enzyme, which exists in two isoforms (GAD65 and GAD67). Transporters for GABA are expressed throughout the nervous system, including on neurons [GABA transporter type 1 (GAT1)] and on astrocytes [GABA transporter type 3 (GAT3)].

Dysfunction of the GABAergic system has been implicated in a number of psychiatric disorders including ADHD, anxiety, epilepsy, and depression, which

premature offspring are at an increased risk of developing, as well as myelin degenerative disorders such as Parkinson's disease[227]. MRI in children aged between 8-12 with ADHD identified reduced concentrations of GABA in the brain of children with ADHD compared to normal controls[228]. Additionally, reduced extracellular GABA concentrations in the hippocampus were identified in a rat model of ADHD compared to controls[229]. Deficits in the expression of $GABA_A$ receptors in the hippocampus, frontal lobe, and temporal lobe are observed in patients with major depressive disorder, as is reduced GABA concentrations in the plasma, cerebrospinal fluid, and occipital cortical brain[230-232]. Additionally, this decrease in GABA coincides with an increase of the excitatory neurotransmitter glutamate[233]. $GABA_A$ receptor deficits are also present in patients with post-traumatic stress disorder and anxiety[234, 235]. These deficits in human patients have also been confirmed in various animal studies, with a number of studies demonstrating an increase in anxiety-like behaviour following a global deletion of the δ subunit of the $GABA_A$ receptor as mentioned previously[209, 212, 222, 236]. These findings suggest that impairment of the GABAergic system and an imbalance between inhibitory and excitatory arms may be involved in the development of neuropsychiatric disorders.

1.3.3 Allopregnanolone following preterm birth

Birth-associated loss of gestational allopregnanolone concentrations occurs earlier than normal in neonates that are born prematurely and is damaging, as evidenced by the deleterious effects of finasteride exposure *in utero*. Recent studies have shown there is a dramatic drop in brain allopregnanolone concentrations following birth at term (and preterm) compared to fetal levels[21, 237]. Furthermore,

preterm delivered animals also had significantly decreased myelination (evidenced by reduced MBP expression) in the CA1 region of the hippocampus and adjacent subcortical white matter 24 hours after delivery compared to animals delivered at term[21]. Additional studies performed in our laboratory with preterm guinea pig neonates at term equivalence have shown a continued lack of myelination in the CA1 region of the hippocampus and subcortical white matter in addition to the cerebellum[238].

1.3.4 Neurosteroids as a therapeutic intervention

Steroid hormones, such as progesterone and its metabolite allopregnanolone, can exert neuroprotective effects following damage to neurons and glia by preventing necrosis, apoptosis, and inflammation, and also by increasing remyelination and regenerative mechanisms[239]. Studies in adult rats have demonstrated the therapeutic effect of progesterone injections on TBI where progesterone administration reduced brain edema, neuronal loss, and lipid peroxidation[240-242]. Similarly, allopregnanolone administration was shown to reduce memory deficits and loss of neurons in the frontal cortex of these rats following bilateral injury[240]. In rat astrocytes and oligodendroglial progenitor primary cell cultures, *in vitro* progesterone exposure upregulated expression of the promyelinating factor insulin-like growth factor 1[243] and in organotypic slice cultures of rat cerebellum, myelination was stimulated by progesterone following its metabolism into allopregnanolone and action on $GABA_A$ receptors[166]. Both progesterone and allopregnanolone have been shown to be effective at reducing the pro-apoptotic activity of caspase-3, reducing astrogliosis as evidenced by GFAP staining, and improving performance in both the spatial learning

task and memory function in the Morris water maze in adult male rats[244]. Furthermore, rat studies have identified reductions in inflammatory cytokines TNF-α and IL-1β following TBI and subsequent progesterone or allopregnanolone administration[245]. Following the obvious benefits of progesterone therapy observed in animal studies, a randomized clinical trial of progesterone (ProTECT) for treatment of acute TBI in adults has proceeded into Phase III following findings that progesterone administration resulted in a lower 30-day mortality risk and that patients were more likely to have a moderate to good outcome than those receiving placebo[246]. Likewise, a large clinical trial in China is showing similar therapeutic benefits following progesterone therapy[23].

As progesterone is the precursor of the neurosteroid allopregnanolone, and there have been a number of positive studies relating to the use of progesterone, studies in our laboratory have previously trialed this hormone in preterm neonates as a replacement therapy. Unexpectedly, a detrimental effect of progesterone administration on neurodevelopment in the neonatal guinea pig was identified, particularly in the male offspring, which is in contrast to earlier studies on TBI in rats. From this preliminary study, it appears that progesterone is metabolized differently by the male neonates and instead of producing allopregnanolone, it is converted to cortisol (as shown in Figure 1.3). These males, with high plasma and salivary cortisol concentrations, also had reductions in myelination of the cerebellum and subcortical white matter, highlighting the vulnerability of these male neonates to increased cortisol as a result of increased postnatal progesterone[238].

Previous studies have also investigated the potential use of allopregnanolone to restore neurosteroid deficits. Preliminary findings however, suggested that

allopregnanolone was not effective, potentially due to the very short half-life of allopregnanolone, or other possible metabolic conversion in the placenta (data unpublished) resulting in the lack of a rise in allopregnanolone levels. To avoid both of these issues with allopregnanolone therapy, and the metabolism of progesterone into cortisol mentioned above, a possible postnatal therapy that is explored in this thesis is ganaxolone.

1.4 GANAXOLONE

Ganaxolone is a 3β-methylated synthetic analogue of allopregnanolone initially developed by Edward Monaghan at CoSensys in 1998, and in 2004 Marinus Pharmaceuticals Inc., acquired the development and commercialisation rights[247]. Marinus Pharmaceuticals then carried out a number of clinical trials using ganaxolone (see 1.4.1 section), some of which are still ongoing. Ganaxolone features a methyl group that prevents metabolism into other active steroids[248], and a half-life of 12-20 hours in humans[249]. This methyl group markedly improves therapeutic value as ganaxolone acts in a very similar manner to allopregnanolone and binds to the neurosteroid-binding site of $GABA_A$ receptors, producing similar anxiolytic and anti-seizure effects, but with the added benefit that it cannot be metabolized to other steroids that may bind elsewhere and produce unwanted effects[248].

Animal pharmacokinetic studies demonstrate that ganaxolone has a large volume of distribution as administration of radioactively labelled ganaxolone has shown wide distribution of the drug to all tissues and that it becomes highly concentrated in the brain with a brain-to-plasma concentration of between 5 and

10[24, 247]. In addition to pharmacokinetic studies there have been a number of animal studies relating to the use of ganaxolone and behavioural disorders. In an adult mouse model of Angelman syndrome (which is characterized by severe developmental delay, motor impairments, and epilepsy) treatment with ganaxolone over a period of 4 weeks was shown to ameliorate behavioural abnormalities associated with the syndrome[250]. Other mouse models of neurodevelopmental disorders have highlighted the therapeutic benefits of ganaxolone, such as an adult mouse model of autism where ganaxolone reversed the autistic phenotype[251], and an adult post-traumatic stress mouse model where again ganaxolone therapy improved behavioural deficits such as aggression towards other animals and anxiety in the elevated plus maze[252]. Despite numerous animal models of behavioural disorders demonstrating the therapeutic potential of ganaxolone in ameliorating disease states, there has not been any data regarding neurodevelopment or myelination in these models. There has been one model where administration of ganaxolone to Niemann-Pick Type C diseased adult mice identified protection against Purkinje cell death, which is similar to the previously reported protective mechanisms of allopregnanolone[253]. Furthermore, there has only been one neonatal animal model using ganaxolone therapy, a rat model of infantile spasms where the onset, number, and duration of spasms were reduced by ganaxolone therapy[254]. Doses used in these animal models have ranged from 3.75mg/kg to 50mg/kg, and due to the limited number of studies that have taken place in a neonate, as well the added vulnerability of the preterm neonate, we decided to use a dose of 2.5mg/kg twice daily (see Chapter 7).

1.4.1 Ganaxolone Clinical Trials

A number of Phase 1 and 2 clinical trials have examined the use of ganaxolone for epilepsy and infantile spasms, as well as for posttraumatic stress disorder, migraine, and Fragile X syndrome, and in some instances these trials are still ongoing[24, 25]. Daily drug doses of up to 1875mg in adults and 54mg/kg in children have been trialed, and it has been shown that a single oral dose of 1600mg can result in peak plasma concentrations of up to 460ng/mL. Recently a randomized, double blind, placebo-controlled phase 2 trial for ganaxolone as an add-on therapy for seizures took place in 147 adults[255]. The subjects received 1500mg/day spread over 3 doses for 8 weeks. The treatment resulted in an 18% decrease in mean weekly seizure frequency, compared to a 2% increase in the placebo group. The treatment was generally safe and well tolerated with similar rates of discontinuation due to adverse effects in each group (ganaxolone 7.1% versus 6.1% for placebo). The most common side effects were classified as mild to moderate and included dizziness (16.3% versus 8.2% in placebo), fatigue (16.3% versus 8.2%), and somnolence/constant sleepiness (13.3% versus 2.0%).

1.5 Preterm Model, Hypothesis, and Aims

1.5.1 Guinea Pig Preterm Model

Studies in this thesis utilises the guinea pig model of preterm birth that was first developed by Jonathan Hirst, Hannah Palliser, and Ian Wright at the University of Newcastle[21]. Mary Berry and Rebecca Dyson at the University of Otago then adapted this model[256]. The original model featured preterm neonates delivered by caesarean

section, in which the dams were not recovered, at gestational age 62 (0.8 of full gestation), resulting in preterm neonates that are functionally equivalent to a 29-week human infant in terms of microvasculature and respiratory systems, and a 35-week human infant when looking at neurodevelopment[21, 257]. To enable long-term studies to take place, the model was adapted to use pharmacological premature induction of labour to ensure survival of the dam[256].

There are a number of benefits to using the guinea pig for pregnancy and neurodevelopment studies over other small laboratory animals. This includes the relatively long gestation of the guinea pig, and more importantly the similar endocrine profile of the placenta, fetus, and neonate compared to that of humans. In a number of species, including the human and the guinea pig, the placenta becomes responsible for the production of progesterone by taking over from the corpus luteum during pregnancy[158]. As with humans, guinea pig pregnancy also ends in a functional withdrawal of progesterone, as opposed to a decline in progesterone in other laboratory species[258]. Furthermore, the guinea pig is a precocial species meaning that it has a highly developed CNS at the time of birth and again is therefore more comparable to humans where the majority of brain development happens *in utero* (as opposed to *ex utero* in species such as the mouse and rat where myelination primarily takes place on postnatal days 12-30[259]). Whilst the guinea pig neonate has a reasonably well-developed CNS at the time of birth, the preterm neonates require intensive respiratory, thermal, and nutritional support during the initial neonatal period, much like a human premature infant thus making this an appropriate model to investigate the effects of preterm birth on neurodevelopment. Additionally, long gestation species such as the human, guinea pig, and sheep undergo an excitatory to

inhibitory switch in GABAergic activity during gestation due to increases in maturation of chloride channels and changes in ion levels inside the cell[181, 182, 260], which leads to $GABA_A$ receptor stimulation becoming inhibitory[196, 261-263]. As emphasized previously, this is advantageous for fetal neurodevelopment as excessive excitation is damaging in the second half of gestation. In contrast to long gestation species, rodents such as rats and mice do not undergo the excitatory to inhibitory switch in GABAergic activity until after birth[226, 264] meaning they are less clinically relevant models for fetal and neonatal regulation of excitability and brain development[182].

1.5.2 Hypotheses

1. a) We hypothesize that preterm birth will disrupt the $GABA_A$ receptor subunit expression in the fetus and neonate (Chapter 3).

   b) We suggest that neurosteroidogenesis in the newborn will be disrupted and that this will in turn alter the neurodevelopment and behaviour of ex-premature juveniles in a sex dependent manner (Chapter 4 and 5).
2. We hypothesize that progesterone administered to prevent preterm birth disrupts the normal neurosteroidogenesis in the placenta and fetal brain, and that this may occur in a sex dependent manner, and have overall adverse effects on fetal neurodevelopment (Chapter 6).
3. We propose that Ganaxolone therapy in the immediate neonatal period will ameliorate the adverse effects of preterm birth on neurodevelopment and behaviour (Chapter 7).

1.5.3 AIMS

1. a) Ascertain the impact that preterm delivery has on neurosteroid action, with particular focus on $GABA_A$ receptor neurosteroid sensitivity.

   b) Examine the effect of preterm birth on behaviour at juvenility, with particular focus on hyperactive and anxious behaviour.

   c) Determine the effect of preterm birth on key markers of brain development to identify potential detrimental effects on the juvenile.

2. a) Examine the effect of prenatal exposure to progesterone therapy on neurosteroid profiles in the plasma and placenta of preterm and term fetuses.

   b) Determine the effect of prenatal exposure to progesterone therapy on key markers of brain development to identify potential detrimental effects on the preterm and term fetus.

3. Investigate if neurosteroid replacement therapy (ganaxolone) after preterm birth can restore normal brain structure, brain function, and behaviour in later life.

2.0 METHODS

Unless otherwise specified, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, NSW, Australia). Animals used in these studies were housed in two separate locations; Newcastle (Australia) and Wellington (New Zealand) as a collaboration with the University of Otago. All tissue analyses took place in Newcastle.

2.1 ANIMAL ETHICS

Approval for all of the animal experiments and procedures carried out throughout the studies in both Newcastle and Wellington was obtained from the University of Newcastle Animal Care and Ethics Committee. The animal experiments carried out in Wellington were also approved by the University of Otago, Wellington, Animal Ethics Committee prior to commencement of the study. In addition, all experiments and procedures were carried out in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care and Use of Animals for Scientific Purposes, and the New Zealand Animal Welfare Act.

2.2 ANIMAL MODELS

2.2.1 PRETERM FETUS AND NEONATE

Four guinea pig groups were used in chapter 3 and are detailed below. Meredith Kelleher, Rebecca Dyson, and Hannah Palliser performed the delivery of

guinea pigs and tissue collection as part of ongoing studies within the Newcastle laboratory.

a) Term fetus: Pregnant dams allocated to the term fetus group were euthanized (see section 2.5.1) just prior to term at gestational age 69 (GA69) and fetal tissues and fluids were rapidly collected.
b) Preterm fetus: Pregnant dams allocated to the preterm fetus group were euthanized at GA62 (equivalent to 0.87 of average gestation length) and fetal tissues and fluids were rapidly collected.
c) Term neonate: Guinea pig pups allocated to the term neonate group were delivered by caesarean section (see section 2.3.3) at either GA69 or following two days of pubic symphysis gap of 1.5cm which is a known precursor to natural delivery. Term neonates were then euthanized 24 hours after delivery (postnatal day 1, PND1) and their tissues and fluids collected.
d) Preterm neonate: Guinea pig pups allocated to the preterm neonate group were delivered by caesarean section at GA62. As with the term neonates, the preterm neonates were euthanized 24 hours after delivery and their tissues and fluids collected.

2.2.2 PRETERM JUVENILE

The guinea pigs used in chapter 4 and 5 were based in Wellington, New Zealand and were performed as part of collaboration with Max Berry and Rebecca Dyson at the University of Otago. Two guinea pig groups were used in this study and are detailed below.

a) Term juvenile: Guinea pig pups were delivered by spontaneous labour at term (GA70), behavioural testing was undertaken at PND25 (see section 2.4), and tissues were collected at PND28.

b) Preterm juvenile: Guinea pig pups were delivered by preterm induction of labour at GA62 (see section 2.3.4), behavioural testing was undertaken at corrected PND25 and tissues were collected at corrected PND28.

2.2.3 PRENATAL PROGESTERONE THERAPY

Chapter 6 used four guinea pig groups, these animals were based in Newcastle and groups are detailed below.

a) Preterm vehicle therapy fetus: Pregnant dams received oral administration of vehicle (45% β-cyclodextrin, 500µL/kg) once daily from GA29 – GA61. Fetal tissue collection took place on GA62.

b) Preterm progesterone therapy fetus: Pregnant dams received oral administration of progesterone (5mg/kg in 45% β-cyclodextrin, 500µL/kg) once daily from GA29 – GA61. Fetal tissue collection then occurred on GA62.

c) Term vehicle therapy fetus: Pregnant dams received vehicle therapy as detailed previously, however fetal tissue collection occurred on GA69 (one week after therapy was ceased).

d) Term progesterone therapy fetus: Pregnant dams received progesterone therapy as detailed previously, however fetal tissue collection occurred on GA69.

2.2.4 PRETERM NEUROSTEROID-REPLACEMENT THERAPY

Chapter 7 involved three guinea pig groups, which were also based in Wellington and are detailed below.

a) Term control juvenile: This group is identical to the "term juvenile" group in chapter 4 and 5 where pups were allowed to be delivered spontaneously at term. Behavioural testing was undertaken at PND25, and tissues were collected at PND28.

b) Preterm vehicle therapy juvenile: Guinea pig pups were delivered by preterm induction of labour at GA62 and received subcutaneous injection of vehicle (45% β-cyclodextrin in sterile water 1.25µL/g) twice daily until term equivalence (PND7). Behavioural testing was undertaken at correct PND25, and tissues were collected at corrected PND28.

c) Preterm ganaxolone (neurosteroid-replacement) therapy juvenile: Guinea pig pups were delivered by preterm induction of labour at GA62 and received subcutaneous injection of ganaxolone (2.5mg/kg in 45% β-cyclodextrin, 1.25µL/g) (Steraloids, Newport, RI, USA) twice daily until term equivalence. Behavioural testing was undertaken at corrected PND25, and tissues were collected at corrected PND28.

2.3 ANIMAL CARE

2.3.1 HOUSING AND FEEDING

The animals housed in Newcastle were outbred, tricolour guinea pigs that were obtained from the University of Newcastle Research Support Unit. The guinea pigs were housed indoors with a 12 hour light/dark cycle and supplied with a diet consisting of commercial guinea pig pellets, lucerne hay, and water supplemented with ascorbic acid. Female guinea pigs were time-mated during post partum oestrous and then housed separately with their litters. Weaning occurred at 21 days and at this point pregnancy was confirmed by gentle palpitation of the abdomen. Confirmed pregnant females were then allocated to the study and housed in separate cages. Daily monitoring for signs and symptoms of illness or distress was carried out for each guinea pig in the study. Pregnant females were also weighed daily to monitor weight gain as an indicator of fetal growth, in addition to being closely monitored for signs of spontaneous or early labour indicated by pubic symphysis opening. Pregnant females allocated to vehicle or progesterone therapy received once daily oral doses of the appropriate therapy from GA29 – GA61. Saliva samples were also obtained daily from these animals prior to their daily dose, by gently encouraging chewing on a cotton bud.

The animals housed in Wellington were mature breeding Dunkin Hartley females and were obtained from the University of Otago Biomedical Research Unit. At oestrous they were placed with a male for 24 hours after which they were returned to the female group pen. The guinea pigs were housed indoors in floor pens and supplied with a diet consisting of standard commercial guinea pig pellets, lucerne hay, fresh fruit, and fresh vegetables. Their water was supplemented with vitamin C. Once

confirmed pregnant, dams were allocated to either the preterm delivery or term delivery group. Dams allocated to the term delivery group received no further interventions during pregnancy (besides daily monitoring and weighing) and their pups were allowed to deliver spontaneously in the floor pens and received no additional respiratory or nutritional support. Preterm pups were delivered by preterm induction of labour as described below. Preterm and term neonates were weighed daily and body measurements taken. The pups remained with their mothers in floor pens until weaning occurred at PND21, after which they were placed into floor pens with animals of the same sex.

2.3.2 CAESAREAN SECTION DELIVERY

In chapter 3, guinea pig pups from both neonatal groups (term and preterm) were delivered by caesarean section[21, 257]. At 24 hours and 12 hours prior to the surgery the pregnant females were given subcutaneous injections of Celestone Chronodose (1mg/kg betamethasone sodium phosphate/betamethasone acetate, Schering-Plough, North Ryde, NSW, Australia). In preparation for the delivery the pregnant females were placed into a chamber and anaesthetised by 4% isoflurane in medical grade oxygen. Throughout the procedure the anaesthesia was maintained by using a mask supplying 2% isoflurane. The pregnant dam was placed on a heat pad in the supine position with an elevated head for the duration of the surgery. An initial incision was made along the ventral abdomen, extending from above the umbilicus to the pubic symphysis, through the skin and muscle layers to expose the uterus. Once each fetus was located within the uterus an incision was made through the myometrium with extra care to avoid rupturing the amnion, and amniotic fluid was

then collected by insertion of a needle into the amniotic sac. Following quick removal of the amnion from the fetal nose and mouth, the umbilical cord was tied off with surgical silk and severed. Each neonate was delivered within 2 minutes of the previous neonate and continuously monitored for signs of distress. Once all neonates were delivered the maternal guinea pig was euthanized by a cardiac injection of lethabarb (344mg/mL Sodium pentobarbitone, Virbac, Regents Park, NSW, Australia).

2.3.3 PRETERM CARE FOLLOWING CAESAREAN SECTION

Respiration was stimulated by vigorously rubbing the neonates immediately after delivery. Excess fluid was removed from the pups lungs and respiratory tract by briskly inverting each pup. A 50µL dose of surfactant (Curosurf, 80mg/mL Poractant alfa, Douglas Pharmaceuticals, Baulkham Hills, NSW, Australia) was then delivered into the oropharynx of each pup by using an apparatus that consisted of a neonatal feeding tube attached to a luer lock syringe (Terumo Medical Corporations, Somerset, NJ, USA). A specialised small animal anaesthesia mask (Harvard Apparatus, Holliston, MA, USA) attached to a Neopuff infant T-piece resuscitator (Fisher and Paykel Healthcare, Melbourne, VIC, Australia) was used to administer a continuous positive airway pressure (CPAP) and create a seal over the nose and mouth of the guinea pig pup. Once the seal was made, a positive end expiratory pressure (PEEP) and peak inspiratory pressure (PIP) of 7mm H_2O and 20mm H_2O respectively at 50% medical oxygen and 50% medical air was applied at a flow rate of 8L/minute. Initially, neonates were given a sustained PIP of 20 seconds duration. If the neonate did not begin spontaneous respiration after this initial PIP, further PIP ventilation was provided until normal respiration was established. At 3 hours post-delivery all neonates were given

an additional 50µL dose of surfactant and CPAP. If neonates exhibited unstable respiration during the 24 hour experiment, CPAP was repeated until normal respiration was re-established. Neonates that were unable to maintain normal respiration for extended periods of time were humanely euthanized.

The resuscitation of each neonate was carried out under animal heat lamps and using animal heat pads in order to maintain the temperature of each neonate. Once respiration became stable, the neonates were dried off and placed into a humidified incubator (small animal intensive care incubator, Thermocare, Incline Village, NV, USA) with a constant temperature of ~30°C. Neonatal respiration, posture/muscle tone, activity, and urination was monitored closely and scored every 2 hours for the 24 hours (see Table 2.1). In addition, body weight, body temperature, incubator temperature, and humidity were also recorded.

Feeding of neonates was carried out every 2 hours with a commercial guinea pig milk replacement formula (Impact guinea pig replacement milk, Wombaroo Food Products, Adelaide, SA, Australia). For the first 4 hours, the milk replacement formula was provided at 100µL/g made up in 50% v/v water and glucose solution (5% glucose solution, Baxter Healthcare, Coorparoo, QLD, Australia). After the first 4 hours the milk replacement was given without the added glucose. The milk replacement was administered using a feeding tube composed of 20cm long polyvinyl tubing (internal diameter 0.8mm, external diameter 1.2mm, Microtube Extrusions, North Rocks, NSW, Australia) that was attached to a 1mL syringe (Terumo). Each feeding tube was blunted 1cm from the open end and markings measured at 6, 7, and 8cm from this blunted end. For neonates that did not have a strong swallowing reflex, the tube was carefully inserted into the oesophagus to ~7cm and the syringe slowly depressed to allow the

milk replacement to gently come out without excessive air bubbles. Neonates with stronger swallowing reflexes were fed slowly into their mouths with extra care taken to ensure that the pups did not aspirate the milk replacement into their lungs. At these 2 hour feeding time points the volume of milk replacement fed to each pup along with their wellbeing score (based on criteria in Table 2.1) was recorded. After feeding the pups urination and defecation was stimulated by using a moistened cotton tip. Throughout the monitoring process any neonate that showed signs of distress or symptoms of illness was humanely euthanized.

2.3.4 PRETERM INDUCTION OF LABOUR

Induction of preterm labour[256] was used to deliver the preterm neonates in Wellington to ensure survival of the dam (chapters 4, 5 and 7). At 48 and 24 hours before planned induction, a subcutaneous dose of Celestone Chronodose (Betamethasone 1mg/kg) was given to the pregnant dam to encourage fetal lung maturation. At 24 hours before delivery, and on the morning of delivery, a subcutaneous dose of a progesterone receptor antagonist (Aglepristone, 10mg/kg, Provet, Palmerston North, New Zealand) was given to pharmacologically inhibit progesterone-induced maintenance of pregnancy. At 1 hour after the second aglepristone dose, oxytocin was given by intramuscular injection (3 IU/kg, Provet) to stimulate uterine contractions. Oxytocin was given in repeated doses every 30 minutes until all pups and placentas were delivered. Most dams received 4 doses of oxytocin and delivered within 2 hours.

Table 2.1 Criteria for wellbeing score in neonatal guinea pigs

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

Scoring for each category were obtained every 2 hours during the first 24 hours of life for each neonate. Each category was assigned a score from 0-4 (with 0 being the poorest, and 4 being the optimal score). Scores were then added together to give a total out of 12 for each time point. Total scores between 0-3 indicated very poor wellbeing, and 4-6 indicated poor wellbeing, whereas 7-9 indicated good wellbeing, and 10-12 indicated very good wellbeing.

2.3.5 PRETERM CARE FOLLOWING INDUCTION OF LABOUR

Resuscitation, respiratory support, and nutritional support of preterm neonates following induced labour was similar to support provided following caesarean section but with some differences detailed below.

Pups were placed on a heated pad and respiration was encouraged by gently rubbing the chest wall. Respiratory support was provided for at least 5 minutes to all preterm pups by CPAP support at 5cm H_2O using the “Neopuff” t-piece infant

resuscitator (Fisher & Paykel, Auckland, New Zealand) with blended oxygen delivered at 5L/min. If spontaneous respiration was not achieved or sustained, positive pressure ventilation at 60 breaths/min with an inflation pressure of 12cm H_2O and expiratory pressure of 5cm H_2O was provided until spontaneous respiration was observed. All preterm pups were also given an initial fractional inspired oxygen concentration of 30% that was adjusted based on their colour, heart rate, and respiratory activity. Pups that were unable to achieve and maintain independent respiration, or showed other signs of distress or illness were humanely euthanized.

The preterm pups were housed with their mothers and littermates in a warm humidified infant incubator (Dräger 8000 IC, Drägerwerk AG and Co., Lübeck, Germany) at an ambient temperature of 33°C (titrated down to 28°C over the course of first 24 hours), and humidity at 60% (titrated down to 35% by 12 hours). Preterm pups were unable to sufficiently suckle from their mothers and therefore received nutritional support. Until 24 hours old the preterm pups received 0.3 – 0.5mL of replacement colostrum (Impact guinea pig colostrum replacement, Wombaroo Food Products) orally by a 1mL syringe within the first hour after birth and then every 3 hours. Between PND1-7 the pups were fed 0.5 – 2.0mL of replacement milk (Impact guinea pig replacement milk) every 3 hours or as needed to supplement independent suckling from their mothers. Pups that were allocated to the vehicle or ganaxolone therapy groups received subcutaneous injections of the appropriate therapy at 8am and 8pm daily until term equivalence.

On PND7 (one day before term equivalence) preterm pups were transferred with their mothers from the infant incubator into a standard cage at room temperature, and on PND8 (term equivalence) they were transferred into the nursery

floor pen with all other mothers and pups. As mentioned previously, pups remained with their mothers in the nursery until weaning occurred at correct PND21 at which point they were placed into floor pens with animals of the same sex.

2.4 Behavioural testing

2.4.1 Open field

The open-field arena was used to measure anxiety and locomotion of the juvenile (corrected PND25) guinea pigs[168, 265]. The arena was 40cm x 40cm with walls to prevent the guinea pigs jumping out. Before each guinea pig was placed into the arena and allowed to explore for 10 minutes, the arena was wiped clean with ethanol wipes. Before the open field test a pre-test saliva sample was obtained from each guinea pig by encouraging chewing on a cotton bud. Cotton buds were then cut in half and placed into eppendorf tubes (cut end down) and centrifuged to separate the saliva out, which was then stored at -40°C until required.

2.4.2 Environment exploration

The environment exploration test measured the guinea pigs exploratory behaviour and anxiety[168]. Following the open field test the guinea pig was placed back into a carry box whilst two identical objects (blue cubes) were positioned in the middle of the top two quadrants of the arena. The guinea pig was then placed back into the arena and allowed to explore the foreign objects for 10 minutes. On

completion of the environment exploration test a post-test saliva sample was obtained.

2.4.3 Social interaction

The social interaction test was used to measure behaviour towards a familiar guinea pig. The test guinea pig was placed into the arena (same arena as the open field/environment exploration) with a guinea pig of the same sex and age, from the same home pen. For 5 minutes the pair were allowed to interact. The animals were monitored throughout testing, and if excessive aggression occurred, the test was immediately brought to a cease.

2.4.4 Analysis

All behavioural tests were recorded from above using a GoPro Hero 4 (GoPro, San Mateo, CA, USA) attached to a stand. The videos were then exported and analysed using ANY-maze tracking software version 4.7 (Stoelting, Wood Dale, ILL, USA). When analysing the open field videos, an inner zone (measuring 20cm x 20cm) in the centre of the arena was designated on the program. Parameters measured included total distance travelled in the entire arena, time mobile in the entire arena, and also total distance and time mobile in the inner zone in addition to number of entries into the inner zone. For the environment exploration test the parameter measured were total object investigation time and number of interactions, which was signified by smelling or touching of the objects. When analysing the social interaction test the parameters measured included approaching or running away from the familiar animal, as well as

having an affectionate (grooming) or agonistic (aggressive) interaction towards the familiar animal.

2.5 TISSUE AND FLUID COLLECTION

2.5.1 EUTHANASIA

In Newcastle (chapters 3 and 6), euthanasia was performed by carbon dioxide inhalation for 10 minutes. Absence of pedal, palpebral, and ocular reflexes signified death however a secondary death measure of diaphragm puncture was performed.

In Wellington (chapters 4, 5, and 7), the guinea pigs were sedated by a combination of ketamine (40mg/kg) and domitor (0.5mg/kg) for cardiac puncture, and then euthanasia performed by lethabarb injection (3mL) directly into the heart.

2.5.2 PLASMA

Whole blood was collected by cardiac puncture immediately following euthanasia from the guinea pigs based in Newcastle (chapters 3 and 6). In Wellington (chapters 4, 5, and 7), whole blood was collected by cardiac puncture once the guinea pigs were sedated and pedal and palpebral reflexes were absent, but prior to administration of lethabarb.

Whole blood was collected in different sized syringes and using different sized luer lock needle gauges (Terumo) based on the size of the animal. For example, fetal and neonatal blood was collected using 23G needles and 1mL syringes, whereas juvenile blood was collected using 21G needles and 5mL syringes. Blood was then

centrifuged in ethylenediaminetetracetic acid (EDTA) blood collection Vacuette tubes (Greiner Bio-One, Monroe, NC, USA) for 15 minutes in a refrigerated centrifuge at 4°C and the plasma collected from the top layer transferred into sterile 1.5mL eppendorf tubes which were then snap frozen in liquid nitrogen.

2.5.3 BRAIN

A midline incision through the scalp exposed the skull of each guinea pig. The skull was then removed, with extra care to avoid damaging the underlying brain tissue. The cranial nerves and spinal cord were severed before gently easing the whole brain out. Each brain was then weighed and sliced down the midline in the sagittal plane to separate the two hemispheres. Each left hemisphere was collected and fixed for immunohistochemistry, whilst the right hemisphere was frozen in liquid nitrogen and used for further tissue processing such as western blot.

The left hemisphere was cut into three sections and fixed separately (see Figure 2.1). The ventral section contained mainly the frontal cortex (Region A), the middle section contained the cortex, along with the hippocampus and thalamus (Region B), and the dorsal section contained the cerebellum (Region C). The right hemisphere was cut into five sections and frozen separately (see Figure 2.1). The most ventral part, the frontal cortex, was collected first and then the hippocampus was isolated and weighed before being frozen, with the overlaying cortex being the third piece to be collected. The cerebellum was then weighed and frozen, before the remainder of the brain (mainly brain stem and thalamus) made up the fifth frozen section of the brain.

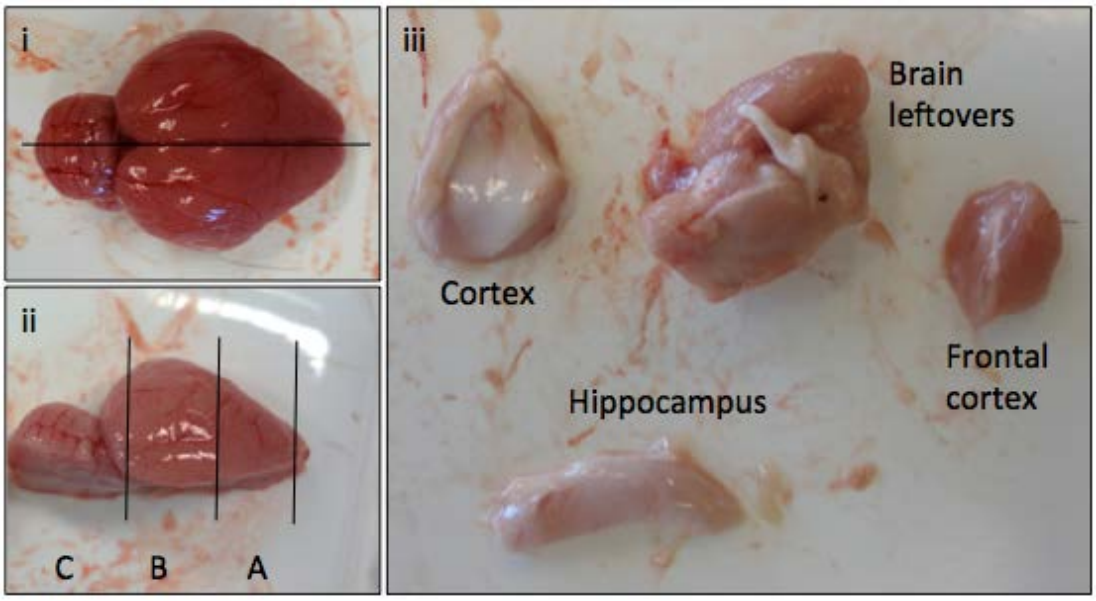

Figure 2.1. Brain dissection for fixed and frozen regions. Guinea pig brain hemispheres were separated down the midline (i), and then the left hemisphere was cut into three regions (A, B, and C) for fixation (ii), whilst the right hemisphere was separated into 5 regions [cortex, brain leftovers, hippocampus, frontal cortex, and cerebellum (not shown)] and snap frozen (iii).

2.1.1 ORGANS

In Newcastle, additional organs weighed and collected included the adrenals (frozen) and liver (fixed and frozen). The heart and kidneys were also dissected and weighed, but were not collected. Placentas and amniotic fluid were collected from fetal collections only.

In Wellington, as the guinea pigs were used by a number of different groups in collaboration, many additional organs were collected including the heart, lungs, kidney, subcutaneous and visceral fat, muscle, eye, and skin. The tissues collected for the purpose of our collaboration were the brain as detailed above, the adrenals, and the liver.

2.2 PLASMA STEROID ANALYSES

2.2.1 ALLOPREGNANOLONE PLASMA RADIOIMMUNOASSAY

Jonathan Hirst and Britt Saxby measured plasma allopregnanolone concentrations by radioimmunoassay. This first involved pipetting 60µL of plasma into glass tubes and adding 600µL of radioactive allopregnanolone tracer (approximately 2000cpm/600µL, 5α-pregnane-3α-ol-20-one, 5α-{9, 11, 12, ^{3}H[N]}, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). Solid phase extraction was then performed using Sep-Pak Classic C_{18} cartridges (360mg, 55-105mm, Waters Corporation, Milford, MA, USA) primed with 2.5mL of 100% methanol, 2.5mL of methanol, and 2.5mL of acidified methanol. The samples within the cartridges were then washed using 2.5mL 50% acetic acid and 50% acidified methanol before eluting the extracted steroids by adding 3mL of 100% methanol. The eluted steroids were then dried at 50°C under a nitrogen gas stream using a dry block heater (Ratek, Boronia, VIC, Australia).

The next step of the process involved oxidising progesterone in the samples. The dried steroids were resuspended in 450µL of distilled water and 50µL of 5% potassium permanganate solution was added to remove the non-saturated steroids (including progesterone). The solution was then incubated at room temperature for 25 minutes before extracting the remaining steroids by adding 2mL of 50% n-hexane and 50% diethyl-ether and mixing vigorously. The solution then separated into two layers, the aqueous (containing the steroids) and the organic (the n-hexane and diethyl-ether solution) layers. The aqueous layer was collected and frozen on dry ice for 1 minute whilst the organic layer was placed into a fresh tube. To improve the recovery of steroids from the organic layer, the addition of 50% n-hexane and 50% diethyl-ether

was repeated 3 times. Once completed, the samples were again dried under a nitrogen stream at 50°C for 30 minutes.

To calculate the percentage of allopregnanolone successfully recovered following the oxidation process, 600µL of assay buffer [0.05M phosphate-buffered saline (sodium chloride, disodium hydrogen phosphate anhydrous, sodium dihydrogen monohydrate) (PBS) and 0.025M EDTA], 0.1% bovine serum albumin (BSA), and 0.1% sodium azide was added to the dried samples. Of this solution, 50µL was pipetted into mini Poly-Q polyethylene scintillation vials (Beckman Coulter, Gladesville, NSW, Australia) in duplicate along with 5mL of BCS liquid scintillation cocktail (GE Healthcare, Little Chalfont, UK) for determination of recovery percentage. The samples were then placed into the LS3801 Liquid Scintillation Counter (Beckman Coulter) and the β-radiation emitted from the samples was measured. The average extraction recovery was 83.5% ± 1.4%.

Prior to running the radioimmunoassay to determine concentrations of allopregnanolone within each sample, the standards, tracer, and antisera were prepared. The standards were created from a primary stock (Steraloids) in 100% ethanol to a concentration of 200µg/mL. Serial dilutions in assay buffer from 25-8000pg/mL were used to prepare a standard curve. Quality controls of pre-prepared medium (400pg/mL) and high (2000pg/mL) concentrations were also run in each assay. A tritium-labelled allopregnanolone tracer (Perkin Elmer Life and Analytical Sciences) was diluted in 100% ethanol and assay buffer to a concentration of 7800cpm of allopregnanolone. The antisera anti-allopregnanolone polyclonal antibody (Agrisera, Sapphire Bioscience, Vannas, Sweden) was used at a dilution of 1:2000 for the assay.

To begin the assay, 250µL of standards, quality controls, and samples were added to glass tubes on ice. The antisera (250µL) was then added to the tubes and incubated for 30 minutes before addition of the tracer (250µL) and incubation at 4°C overnight. Following the overnight incubation, 200µL of charcoal-dextran mixture [0.5% w/v Norit-A activate charcoal, 0.1% w/v Dextran T70 (Pharmacosmos, Holbaek, Denmark), and 0.05% γ-globulin] was added to the tubes and the samples were then centrifuged at 1800xg for 15 minutes (j-6 M/E centrifuge, Beckman Coulter) to separate out the bound antisera from the unbound tracer. The bound fraction was collected and 500µL of this was added to the scintillation vials with 5mL of scintillation cocktail and counted by the β-counter. Standard curves were then generated from the counts produced from the standards, which then allowed for calculation of concentrations in the samples. Each assay also had additional tubes to allow for determination of total counts, non-specific binding, and tracer-antisera binding. The final allopregnanolone concentrations calculated also incorporated the recoveries following extraction as detailed previously. The limit of detection for this assay was 25pg/mL, whilst the inter- and intra-assay coefficients were 9.13% and 5.52% respectively.

2.2.2 CORTISOL PLASMA ENZYME IMMUNOASSAY

The concentration of cortisol in plasma was analysed by Pathology North (Newcastle, NSW, Australia) using the Architect Chemiluminescent Microparticle Immunoassay system (Abbott Laboratories, Abbott Park, IL, USA) for cortisol (B8D150). For each sample, 100µL of plasma was loaded onto the Architect *i* System in the provided sample cups, as were the calibrator and control samples. The system then

automatically aspirated the samples into reaction vessels, and added monoclonal anti-cortisol coated microparticles and cortisol labelled enzyme conjugate to be mixed and incubated together with the sample. The pre-trigger and trigger solutions were then added and allowed for measurement of chemiluminescent emissions to determine the quantity of cortisol in the sample. The inter- and intra-assay coefficients for the assay were 4.3% and 6.6% respectively, whilst the limit of detection was 0.8µg/dL.

2.2.3 PROGESTERONE PLASMA CHEMILUMINESCENT MICROPARTICLE IMMUNOASSAY

The concentration of progesterone in plasma was analysed by Pathology North using the Architect Chemiluminescent Microparticle Immunoassay system (Abbott Laboratories) for progesterone (B7K770). This system allows for the quantitative determination of progesterone in serum or plasma. For each sample, 150µL of plasma was loaded onto the Architect *i* System in the provided sample cups, as were the calibrator and control samples. As for the cortisol assay, the system then automatically aspirates the samples into reaction vessels, and adds monoclonal anti-fluorescein fluorescein progesterone complex coated microparticles and anti-progesterone enzyme conjugate to be mixed and incubated together with the sample. The pre-trigger and trigger solutions are then added and allow for measurement of chemiluminescent emissions to determine the quantity of progesterone in the sample. The inter- and intra-assay coefficients of variance for the assay were 7.9% and 6.1% respectively, whilst the limit of detection was 0.1ng/mL.

2.3 SALIVA STEROID ANALYSES

2.3.1 CORTISOL SALIVA ENZYME IMMUNOASSAY

Saliva samples obtained from neonatal, juvenile, and maternal guinea pigs were analysed using the Salimetrics Salivary Cortisol Assay kit (Salimetrics LLC, State College, PA, USA) to assess circulating cortisol concentrations. Studies consistently report high correlations between serum and salivary cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels[266, 267]. The Salimetrics kit is a competitive enzyme immunoassay that allows for quantitative measurement of cortisol and was performed by following the manufacturers instructions.

To begin 25µL of the provided standards (3.0, 1.0, 0.333, 0.111, 0.037, and 0.012µg/dL) and controls (high = 1.014µg/dL, and low = 0.095µg/dL), in addition to the samples, were pipetted into duplicate wells. Based on trial runs, the saliva samples were diluted out to a concentration of 1:10 in the supplied assay diluent. Once all samples were pipetted into appropriate wells of the plate, 200µL of the supplied enzyme conjugate was added to each well using a multi-channel pipette and the plate was incubated for 55 minutes. Following incubation, 3 x 200µL quantities of wash buffer were used to flush the wells before 200µL of 3,3',5,5'-Tetramethylbenzidine (TMB) solution was added and the plate incubated in the dark for a further 25 minutes. To complete the assay, 50µL of stop solution was pipetted into each well. The absorbance was then read at 450nm using a plate reader (SPECTROstar Nano Microplate Reader, BMG Labtech, Ortenberg, Germany). The limit of detection for this assay is 0.007µg/dL. The inter- and intra-assay coefficients were 7.1% and 4.8% respectively.

2.3.2 PROGESTERONE SALIVA ENZYME IMMUNOASSAY

Saliva samples obtained from maternal guinea pigs were analysed using the Salimetrics Salivary Progesterone Enzyme Immunoassay kit (Salimetrics). The kit is a competitive enzyme immunoassay specifically designed and validated for the quantitative measurement of progesterone in saliva, and was performed by following the manufacturers instructions.

To begin 50µL of standards (2430, 810, 270, 90, 30, and 10pg/mL), controls (high = 1079.59pg/mL, and low = 42.79pg/mL), and samples were pipetted into duplicate wells. Based on trial runs the samples were diluted out to 1:10 for the saliva obtained from pregnant dams receiving vehicle therapy, and 1:50 for the dams receiving progesterone therapy. Dilutions were made using the assay diluent. Once all samples were pipetted into appropriate wells, 150µL of the supplied enzyme conjugate was pipetted into all wells using a multi-channel pipette. The plate was then incubated for 1 hour at room temperature on a MX-M microplate shaker (Daigger Scientific, Vernon Hills, IL, USA) at 500rpm. Following the incubation the wells were then washed by 4 x 300µL of wash buffer. After flushing the wells a multi-channel pipette was used to add 200µL of TMB solution, before mixing on a plate shaker for 5 minutes and incubation in the dark for a further 25 minutes. To complete the assay, 50µL of stop solution was pipetted into the wells. The absorbance was then read at 450nm using a plate reader as above. The inter- and intra-assay coefficients were 9.76% and 16.5%, with a limit of detection of 5pg/mL.

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 TISSUE PREPARATION

The left half of each brain was fixed by immersion in neutral-buffered formalin for 24 hours on an orbital rocker at 4°C. Following this the tissue was stored in 0.1M PBS (pH 7.4) with 0.05% sodium azide until embedding was performed. The tissues were then dehydrated, paraffin-embedded, and mounted on cassettes by Debbie Pepperall (Newcastle TAFE) and the Core Histology Facility at the Hunter Medical Research Institute (HMRI). Once embedded and mounted, each block was cut into 8µm sections using a Leica RM2145 Microtome (Leica Microsystems Pty, Ltd, North Ryde, NSW, Australia). Cut sections were floated out into a water bath and then mounted onto SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany) in sets of three per slide and then air dried overnight before storage at 4°C.

Prior to immunostaining, each slide was allowed to warm to room temperature before dewaxing and rehydrating. This involved each slide being washed in xylene for 3 x 5 minutes, followed by 2 x 3 minute and 1 x 2 minute washes in 100% ethanol, and lastly a 1 x 2 minute wash in 70% ethanol and 1 x 1 minute wash in running distilled water.

2.4.2 STAINING PROCEDURES

Once dewaxed and rehydrated, slides were washed in 0.1M PBS for 3 x 5 minutes before antigen retrieval was performed by placing the slides into a beaker of citrate buffer (trisodium citrate pH 6.0) at 90-95°C on a hotplate for 25 minutes. The slides remained in the citrate buffer on the bench for a further 25 minutes before a 10

minute wash in running distilled water followed by 3 x 5 minute washes in PBS. Endogenous peroxidase blocking was carried out in PBS supplemented with 3% H_2O_2 for 20 minutes before 3 x 5 minute washes in PBS. Non-specific staining block (2% normal goat serum, 4% BSA, with 0.3% Triton-X in PBS) was then applied for 1 hour.

Following the non-specific staining block the slides were incubated overnight at room temperature in the appropriate primary antibody and required dilution. All primary and respective secondary antibodies used are detailed in Table 2.2. Primary and secondary antibodies were diluted in 4% BSA with 0.3% Triton-X in PBS. Before adding the secondary antibody, slides were washed 3 x 5 minutes in PBS. All secondary antibodies were incubated for 1 hour at room temperature at a dilution of 1:300 as detailed in Table 2.2. After thoroughly washing in PBS for 3 x 10 minutes slides were incubated in the tertiary reagent streptavidin-biotin-horseradish peroxidase (HRP) complex (RPN1051V; Amersham, GE Healthcare) diluted to 1:400 in PBS for 1 hour at room temperature. Following the tertiary reagent incubation slides were again washed in PBS for 3 x 5 minutes and then incubated in 3,3'-diaminobenzidine (DAB) tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce, Thermo Fisher Scientific) for 2-5 minutes to reveal the immunolabelling. Following the DAB reaction, all slides were rinsed in distilled water for 2 x 10 minutes and then allowed to air dry overnight before staining one of the three sections per slide with cresyl violet and then cover slipping using DPX mounting media.

Table 2.2 Primary and secondary antibodies for immunohistochemistry

Primary		Secondary	
Antibody	Dilution	Antibody	Dilution
Monoclonal anti-myelin basic protein (MBP) M9434 (Sigma)	1:1000	Biotin goat anti-rat B7139 (Sigma)	1:300
Monoclonal anti-microtubule associated protein (MAP2) M9942 (Sigma)	1:20,000	Biotin goat anti-mouse B6649 (Sigma)	1:300
Monoclonal anti-glial fibrillary acidic protein (GFAP) G3893 (Sigma)	1:1000	Biotin goat anti-mouse B6649 (Sigma)	1:300
Monoclonal anti-neuronal nuclei (NeuN) MAB377 (Millipore*)	1:1000	Biotin goat anti-mouse B6649 (Sigma)	1:300
Polyclonal anti-calbindin ab49899 (Abcam**)	1:1000	Biotin donkey anti-rabbit RPN1004 (Amersham)	1:300
Monoclonal anti-GAD67 ab26116 (Abcam**)	1:2000	Biotin goat anti-mouse B6649 (Sigma)	1:300

The type, name, product code, company and optimised dilution of primary and corresponding secondary antibodies used for Immunohistochemical analysis of proteins in the guinea pig brain. (* EMD Millipore Corporation, Billerica, MA, USA.) (** Abcam, Cambridge, UK.)

2.4.3 ANALYSIS

Stained slides were digitally scanned using the Aperio Digital Pathology Slide Scanner (Leica Biosystems, Mt Waverly, VIC, Australia). Imagescope (Leica Biosystems) was then used to locate the required part of each section at the appropriate magnification (Figure 2.2 and Table 2.3) and export each image for analysis. On each slide, images were captured for both stained sections and the cresyl violet section. Exported images were then analysed using Image J version 1.48 (National Institutes of Health, Bethesda, MD, USA).

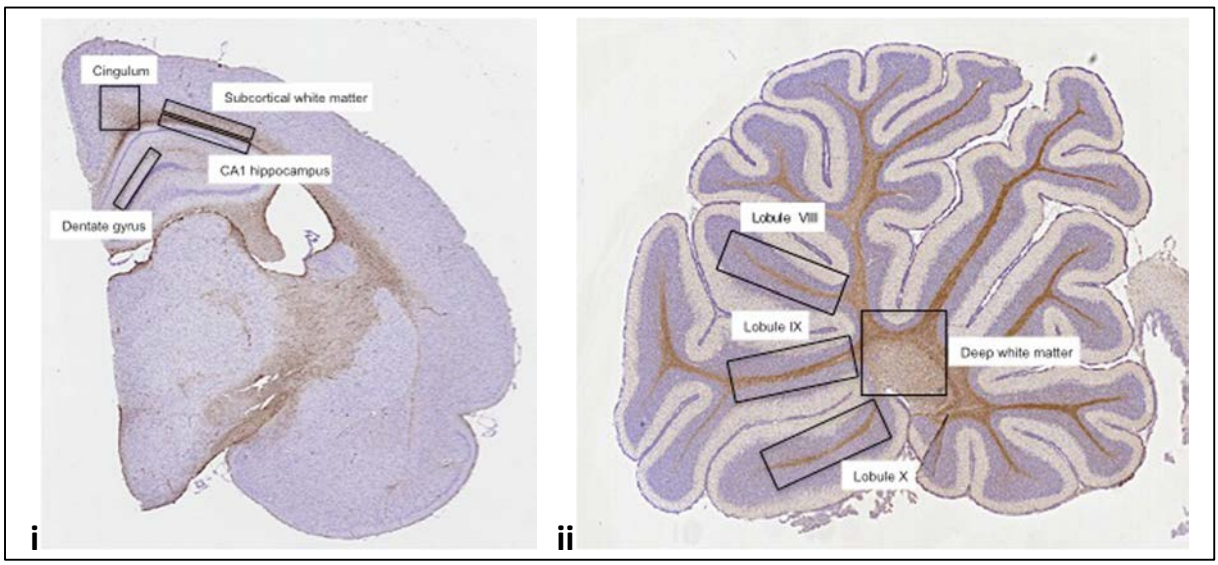

Figure 2.2. Areas of interest for guinea pig brain immunohistochemistry. Cresyl violet and myelin basic protein stained sections of the (i) Region B and (ii) Region C (cerebellum sectioned along the midline cerebellar vermis in the sagittal plane) parts of the guinea pig brain. Black boxes and labels signify areas that were analysed within each region.

Three methods of analysis were used in Image J, area coverage of staining as a percentage, relative width of cell layers, and cell count (Table 2.3). To perform the area coverage analysis the image was first converted to grayscale (8 bit) and then converted to a binary (black and white) image. The threshold was then manually adjusted so that all stained processes appeared black and all areas with no staining appeared white. Image J measured the percentage of area that was black and an average of the 8 consecutive images measured across the two stained sections was calculated to give an overall value of area stained for each animal in each region analysed.

Table 2.3 Methods of immunohistochemical analysis for brain regions

Brain Region	Magnification	Sections per animal	Images per section	Analysis
CA1 region of hippocampus	20x	2	4	Area coverage
Subcortical white matter	20x	2	4	Area coverage
Cingulum	20x	2	3	Area coverage
Cerebellum				
Deep white matter	20x	2	3	Area coverage
Lobule IX	20x	2	3	Area coverage
Lobule X	20x	2	3	Area coverage
Lobule X (molecular layer)	4x	1	1	Relative width
Lobule X (Purkinje cell layer)	4x	1	1	Relative width
Lobule X (Internal granule cell layer)	4x	1	1	Relative width
Lobule IX (Purkinje cells)	4x	2	1	Cell count
Lobule X (Purkinje cells)	4x	2	1	Cell count

Guinea pig brain regions of interest used for immunohistochemical analyses including the magnification at which images were obtained, the number of consecutive images obtained per section of tissue, as well as the analysis method that was used.

The relative width of cerebellar cell layers was measured on the cresyl violet stained sections by drawing a straight line from one side of the lobule to the other (at the end of the central myelin tract for lobule X, and the point of branching for lobule IX) and calculating the length, and then drawing straight lines between each of the cell layers and measuring their length. Each cell layer width was then expressed as a percentage of total lobule width. Finally, positively stained Purkinje cells were counted

using the cell counter function on Image J, again this was performed on both sections on each slide and therefore an average was calculated for each animal.

2.5 Real-time polymerase chain reaction

2.5.1 Tissue preparation

Frozen hippocampus and cerebellum of the right hemisphere were dry crushed into a fine powder using a mortar and pestle. Once crushed into a powder the tissue was stored in a sterile eppendorf tube. Throughout crushing of the tissues, the tissue and equipment was kept frozen using dry ice and liquid nitrogen.

For the purpose of ribonucleic acid (RNA) extraction and subsequent reverse transcription polymerase chain reaction (RT-PCR), 20-30mg of each tissue sample was weighed out into a sterile tube with ceramic beads (Qiagen, Chadstone Centre, VIC, Australia). Each tube then had 600µL of RLT Plus Buffer (obtained from the Qiagen RNeasy Plus Mini Kit, Qiagen) supplemented with 10µL/mL β-mercaptoethanol added before homogenisation was performed using a Precellys 24 dual tissue homogenizer (Bertin Technologies, Provence, France). Following the homogenisation, the samples were centrifuged for 3 minutes at 16,200xg at room temperature.

2.5.2 RNA extraction and gel

Extraction of RNA from the tissue homogenate was performed using the Qiagen RNeasy Plus Mini kit and by following the manufacturer's instructions. Provided in the kit were the necessary reagents and buffers including the RLT Plus

(used in the previous homogenisation step), RW1, and RPE, as well as the necessary columns and tubes. For the duration of the protocol the reagents were kept at room temperature and centrifugation performed at 9,600xg for 15 seconds at room temperature unless otherwise stated.

The supernatant obtained from the previous homogenisation step was transferred into a genomic deoxyribonucleic acid (gDNA) elimination column inside a 1.5mL collection tube and then centrifuged for 30 seconds at 16,200xg, after which the gDNA column was discarded. The flow through was thoroughly mixed with 600µL of 70% ethanol by gentle pipetting up and down. Half of the combined solution was then transferred into a new spin column inside a 1.5mL collection tube, centrifuged at 9,600xg for 30 seconds and the flow through discarded. This was repeated with the second half of the solution. The column was then washed by adding 700µL of RW1 buffer into the spin column, followed by centrifuging at 9,600xg for 30 seconds, and discarding of the flow through. Next, 500µL of RPE buffer was added to the spin column, centrifuged at 9,600xg for 30 seconds and flow through discarded, before repeating with a second 500µL of RPE buffer and centrifuging for 2 minutes. Following this 2 minute centrifuge, the spin column was placed into a new collection tube and centrifuged for a further 1 minute at 16,200xg to dry the membrane within the spin column. To elute the RNA the spin column was then placed into a new sterile 1.5mL eppendorf tube and 30µL of distilled RNase free MilliQ water (Millipore) was pipetted directly onto the membrane and centrifuged for 1 minute. Following this final centrifugation the spin column was discarded and 1µL of RNase inhibitor (RNase Out, Thermo Fisher Scientific, Scoresby, VIC, Australia) was pipetted into each sample.

The NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) connected to a laptop was used to quantify the RNA in each sample. To calibrate the NanoDrop, 2µL of distilled MilliQ water was pipetted onto the fibre optic point. Once calibrated, a second 2µL of distilled MilliQ water was used as a blank. Then, 2µL of each sample was pipetted onto the fibre optic point and the absorbance at 260nm, the 280/260 and 230/260 ratios, and the RNA concentration in ng/µL was recorded. Between measurements of each sample the fibre optic point was wiped clean using a lint-free cloth. The ratio of optical densities at the 260nm and 280nm ranges was used to assess the purity of each sample. A value of ~1.8-2.0 for the 260/280 ratio, and ~2.0-2.2 for the 260/230 ratio indicated pure RNA. A score of less than 1.6 for either ratio indicated possible contamination due to proteins or organic compounds. These samples were subsequently re-extracted.

Following extraction, RNA obtained from each sample was run on a 1% agarose gel to examine the quality and integrity of the RNA. The gel apparatus was initially soaked for 30 minutes in 0.1M sodium hydroxide to remove RNases. To make the gel, agarose powder was dissolved in TBE (89mM Tris, 89mM Boric acid, 2mM EDTA; pH 8.0) buffer by gently heating in a microwave. Once fully dissolved, 1µL of SYBR safe (Thermo Fisher Scientific) was added to the gel solution. The agarose solution was then poured into a gel cast and left to set for 30 minutes. While the gel was setting, 6x Blue/Orange loading dye (Promega, Alexandria, NSW, Australia) was added to each sample at a ratio of 1:6 (1µL loading dye to 5µL of sample). Once the gel had set the gel comb was removed and the gel placed into the RNase free tank. TBE buffer was then added to cover the wells before 5µL of sample was loaded into each well. A 1kb TrackIt DNA ladder (Invitrogen, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) was loaded

into the first well and used to measure the size of bands in the RNA samples. The gel was then run for 60 minutes at 100V.

To image the gels a UVP benchtop UV transilluminator chamber (BioDoc-It Imaging System, Upland, CA, USA) was used. The gel was examined for bands at the 18s and 28s positions at a ratio of 1:2. Smearing of bands indicated RNA degradation, whilst bands appearing in the wells indicated DNA contamination. Any samples that showed signs of degradation and/or contamination were re-extracted.

2.5.3 Reverse transcription

The Superscript III Reverse Transcriptase kit (Invitrogen) was used to perform reverse transcription using a GeneAmp 9700 PCR machine (Applied Biosystems, Foster City, CA, USA) to perform the thermocycling for cDNA synthesis.

The protocol supplied by the Superscript III Reverse Transcriptase kit was used to synthesise a final complementary DNA (cDNA) concentration of 33.3ng/µL for each sample. For the duration of the protocol all reagents and samples were kept on ice. For each RNA sample a reverse transcriptase positive (RT+) and a reverse transcriptase negative (RT-) sample was made. All reagents were added to both tubes (RT+ and RT-) in equal amounts for each sample, except one tube contained the reverse transcriptase required to form the cDNA (RT+) whilst the other tube contained MilliQ water and no reverse transcriptase (RT-). This second tube was used as the control for DNA contamination for each sample during the real time polymerase chain reaction (PCR).

To prepare each RNA sample for the reverse transcription 1µg of the sample diluted in MilliQ water was pipetted into two sterile 0.2mL PCR tubes (one labelled RT+ and the other RT-). Separately, $5x10^6$ copies of Alien RNA was combined with 50ng/µL of random hexamers, and 10nM deoxyribonucleotide triphosphate (dNTP). To each RT+ and RT- sample, 3µL of this mixture was added before incubation at 65°C for 5 minutes using the GeneAmp 9700 PCR machine.

During this incubation, the RT+ and RT- reaction mixes were made. To prepare the RT+ mixture, 10xRT buffer, 25mM magnesium chloride, 0.1M dithiothreitol (DTT), and 200U/µL of Superscript III reverse transcriptase were combined in a sterile eppendorf tube. The same reagents were used to prepare the RT- mixture however instead of the superscript, MilliQ water was added. Once the samples had completed the 5 minute 65°C incubation, 9µL of the appropriate reaction mix (RT+ or RT-) was added to the samples. The samples were then placed back into the GeneAmp 9700 PCR machine where they were denatured for 10 minutes at 25°C which was immediately followed by cDNA synthesis for 50 minutes at 50°C. The reaction was terminated by heating to 85°C for 5 minutes and then cooling back down to 4°C.

To complete the reverse transcription process 1µL of RNase H was added to each sample followed by incubation for 20 minutes at 37°C. All samples were then stored at -40°C until required for real time PCR.

2.5.4 PRIMER DESIGN

Forward and reverse primers were designed in our laboratory for the 5 primers used in this study, the α4, α5, α6, and δ $GABA_A$ receptor subunits, as well as the

housekeeping gene β-actin. The guinea pig specific primers for each transcript are detailed in Table 2.4. The known mRNA sequence for the guinea pig (GeneBank accession number AF508792) was used to design the β-actin primer, whilst the $GABA_A$ receptor subunit primers were designed from sequences obtained from the UCSD Genome Browser and the Broad Institute February 2008 CavPor3 draft assembly of the guinea pig. Each primer was designed to cover an exon boundary and had an amplicon size between 120-314 base pairs.

To ensure that the correct gene sequence was targeted and amplified, a qPCR plate was run featuring the primer pair at varying concentrations and the qPCR product was sequenced by the Australian Genome Research Facility (Westmead Millennium Institute, Westmead, NSW, Australia) and assessed against all known and predicted sequences using BLAST (National Centre for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA). To prepare the samples for sequencing the real time PCR products were run on a 2% agarose gel and the DNA bands were cut out of the gel. The DNA was then extracted from the gel using a Genelute Gel Extraction kit. The extraction involved pre-washing the supplied spin columns with MilliQ water, followed by centrifuging the cut-out DNA bands at 16,200xg for 10 minutes.

Once the sequencing results were obtained and a primer pair concentration chosen (200-600nM, see Table 2.4), the efficiency of the chosen primer pair concentration alongside β-actin was tested by performing real time PCR with 1, 2.5, 5, 10, 20, and 30ng/μL of sample cDNA. To determine the (delta cycle threshold) ΔCt for each cDNA concentration, the mean cycle threshold (Ct) value for the primer was subtracted from the mean Ct value of the β-actin primer. The ΔCt value was then

measured against the log concentration of the cDNA to form a slope, which would ideally be <0.1. A cDNA concentration of 10ng/µL was deemed to be appropriate for all primers.

Table 2.4 Guinea pig specific primer sequences for real time PCR

Gene of Interest	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (base pairs)	Primer Concentration (nM)
$GABA_AR$ α4 subunit	TGG GCA AAC AGT GTC AAG TG	GAC ACT TTG GGC AGA GAA TG	261	100
$GABA_AR$ α5 subunit	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA	314	400
$GABA_AR$ α6 subunit	ATA AGG AGT CAG TCC CAG CA	ACG AAA GCA AAG CAT ACA GC	148	600
$GABA_AR$ δ subunit	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC	255	400
β-actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT	72	400

Primer sequences designed for guinea pig Y-amino-butyric-acid A receptor ($GABA_AR$) α4, α5, α6, δ subunits, and β-actin gene expression. Primer sequences are displayed from 5'-3' for forward and reverse primers.

2.5.5 Real time polymerase chain reaction

Prior to beginning the real time PCR the cDNA samples were diluted out to a concentration of 10ng/µL. For the purpose of these studies, 5 primer pair sets were used and are detailed above. Before the real time PCR was carried out a primer master mix was made for each primer pair, which involved combining 50µL of the forward primer stock (100µM) with 50µL of the reverse primer stock (100µM) and adding 900µL of water. This ensured that a primer master mix concentration of 5pmole/µL was used.

The SYBR Green (Applied Biosystems) DNA binding dye method of real time PCR was used to detect the PCR products of each gene examined. Master mixes containing SYBR green, forward and reverse primers, and MilliQ water, were made up to final primer concentrations as detailed in Table 2.4. When preparing the real time PCR plate, all RT+ samples were run in duplicate wells with their corresponding RT- in a third well to ensure there was no DNA contamination. On each plate a calibrator sample was run in triplicate to enable normalisation of samples across plates. In addition, a no-template control (NTC) was run in triplicate on each plate to ensure that there was no contamination of the PCR plate or of the primer mixes, and to ensure that there was no formation of primer-dimers. For each well 2µL of sample (either RT+, RT-, calibrator, or NTC) were pipetted into the well followed by 8µL of the appropriate SYBR Green primer mix. The plate was then sealed with a PCR cover-shield and centrifuged for 1 minute at 466xg in a Sorvall refrigerated centrifuge (SH-3000 rotor, Thermo Fisher Scientific) to remove air bubbles and ensure mixing of the reagents. DNA/RNA/RNAse free PCR plates (Applied Biosystems) were used for each PCR run.

A 7500 ABI real-time machine (Applied Biosystems) performed the real time PCR, and the results were analysed by Sequence Detection Software version 2.0.6 (Applied Biosystems). Samples were detected at a Ct automatically set for a log scale of 0.2 fluorescence of cDNA expression above the threshold. Formation of a dissociation curve was also added to each reaction set-up to ensure there was no non-specific amplification, contamination, or primer-dimer formation.

2.5.6 COMPARATIVE CT METHOD OF ANALYSIS

To analyse the results of the real time PCR, the comparative Ct method was used as efficiencies had previously been determined to be similar. This method involved firstly calculating the ΔCt for the calibrator by subtracting the average calibrator value of the housekeeping gene β-actin from the average calibrator for each gene of interest. Secondly, the ΔCt for the sample was calculated by subtracting the average Ct value for β-actin from the average Ct value for each gene of interest. Thirdly, the ΔΔCt value was found by subtracting the ΔCt for the calibrator from the ΔCt for the sample. The final step was to then calculate the relative fold change difference by taking the –ΔΔCt value to the power of 2[268].

$$\Delta Ct_{calibrator} = \text{average calibrator } Ct_{gene} - \text{average calibrator } Ct_{\beta\text{-actin}}$$

$$\Delta Ct_{sample} = \text{average sample } Ct_{gene} - \text{average sample } Ct_{\beta\text{-actin}}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$$

$$\text{Relative fold change of gene expression} = 2^{-\Delta\Delta Ct}$$

2.6 WESTERN BLOT

2.6.1 RIPA EXTRACTION

To prepare for the western blot, protein was first extracted from frozen cerebellum and placenta by radioimmunoprecipitation assay (RIPA) extraction. Frozen cerebellum and placenta was dry crushed as described previously (section 2.9.1) and then 20-30mg (cerebellum) or 50mg (placenta) was weighed out into extraction tubes containing ceramic beads. RIPA buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% NP-40,

0.5% Na deoxycholate, 0.1% SDS) was prepared and 1 x Complete Protease Inhibitor Cocktail (Roche, North Ryde, NSW, Australia) and 1 x PhosSTOP Phosphatase Inhibitor Cocktail (Roche) tablets were dissolved per 10mL. For each sample, 250µL of the RIPA buffer containing tablets was added before homogenisation using the Precelleys machine (Bertin Technologies). The homogenates were then centrifuged at 4°C for 10 minutes at 16,200xg. Supernatants were then pipetted into fresh 1.5mL eppendorf tubes and stored at -80°C until required. Prior to freezing 6µL of each sample was pipetted into a separate tube for protein quantification by bicinchoninic acid (BCA) assay.

2.6.2 BCA ASSAY

The Pierce BCA Protein Assay kit (Thermo Fisher Scientific) is a two-component, high-precision, detergent-compatible assay that allows for total protein concentration measurement against a set of protein standards. Before plating the assay, the samples were diluted 1:10 in MilliQ water, and the protein standards were prepared by following the manufacturers instructions. This involved diluting the provided BCA stock standard (concentration 2mg/mL) with diluent (RIPA buffer diluted to 1:10) to achieve the required protein standard concentrations (2000, 1500, 1000, 750, 500, 250, 125, 25, and 0µg/mL). Once samples and standards were prepared, 25µL of each was pipetted into duplicate wells of the plate. The provided Reagent A and Reagent B were then combined in a ratio of 50:1, and of this solution 200µL was pipetted into all wells using a multi-channel pipette. After briefly mixing on a plate shaker, the plate was incubated at 37°C for 30 minutes. Following incubation and allowing the plate to cool back down to room temperature, the absorbance was read at 570nm using the plate

reader (SPECTROstar Nano Microplate Reader). Protein concentrations of the samples were then calculated based on the standard curve created by the supplied protein standards.

2.6.3 WESTERN BLOT

Protein was electrophoresed using the Bolt Mini Gel Western Blot system (Thermo Fisher Scientific). Samples were prepared by diluting to the total protein concentration required (see Table 2.5) in MilliQ water, and then supplementing with 25% Bolt LDS and 10% Bolt Reducing agent, before incubating at 70°C for 10 minutes. Each gel also included one sample that was the same across all gels, the internal calibrator, which was a pooled sample of protein obtained from guinea pig brain or placenta tissue. The samples were then run on a 4-12% Bis Tris 1.0mm Bolt gel at 165V for 45 minutes in a tank containing supplied Bolt MOPS Running Buffer and Bolt Antioxidant.

The proteins in the gel were then transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific) during the transfer, which occurred at 20V for 1 hour. The transfer was prepared by soaking the sponge pads and filter paper in cold Bolt transfer buffer. The PVDF membrane was activated by a 1 minute wash in methanol before also soaking in the transfer buffer. Once the transfer module was assembled it was placed into the tank, filled with transfer buffer, and surrounded by cold MilliQ water.

2.6.4 STAINING PROCEDURES

Once the transfer was complete, the membranes undergoing protein detection for GAD67 and GAT1 were blocked overnight in 5% BSA/5% Skim milk in 0.1% TBS-T (Tris HCl, NaCl, Tween-20) at 4°C. Membranes were then incubated for two hours at room temperature in the appropriate primary antibody (see Table 2.5) in 5% BSA in 0.1% TBS-T. Membranes undergoing protein detection for 11βHSD2 were blocked for 1 hour at room temperature in 5% BSA/5% Skim milk in 0.1% TBS-T, and then incubated overnight at 4°C in primary antibody (see Table 2.5) in 5% BSA/5% Skim milk in 0.1% TBS-T. Washes for 3 x 5 minutes in 0.1% TBS-T were performed before incubation in secondary antibody (see Table 2.5) for 1 hour at room temperature in 5% BSA (GAD67 and GAT1) or 5% Skim milk (11βHSD2). Following the final incubation, the membranes were again washed 3 x 5 minutes in 0.1% TBS-T before a final wash in TBS (Tris HCl, NaCl pH 7.4).

To detect the staining of protein bands, the enhanced chemiluminescence (ECL) (Amersham, GE Healthcare) detection method was used. This involved combining equal parts of Reagent A and Reagent B, and then incubating the membrane for 1 minute in the resulting solution before protein detection by the Amersham Imager 6000 (Amersham, GE Healthcare).

Following ECL detection of protein, staining for the loading control (β-actin) was performed. This first involved 1 x 5 minute wash in tap water before stripping of the membrane for 10 minutes in 0.2M NaOH. The membrane was then washed for 1 x 5 minutes in distilled water and blocked in 3% Skim milk in 0.1% TBS-T for 1 hour at room temperature. The primary incubation then followed again for 1 hour at room temperature in 3% Skim milk in 0.1% TBS-T. Before the secondary incubation, the

membrane was washed 3 x 5 minutes in 0.1% TBS-T followed by incubation under the same conditions. The remainder of the protocol was then performed the same as the antibodies of interest.

Table 2.5 Primary and secondary antibodies for western blot

Tissue Type	Protein Conc. (µL/µg)	Primary		Secondary	
		Antibody	Dilution	Antibody	Dilution
Cerebellum	10	Polyclonal anti-GAT1 ab426 (Abcam) 67kDa	1:2000	Goat anti-rabbit conjugated HRP 12-348 (Millipore)	1:2000
Cerebellum	50	Monoclonal anti-GAD67 ab2611 (Abcam) 67kDa	1:1000	Goat anti-mouse conjugated HRP 65-6420 (Invitrogen)	1:5000
Cerebellum and Placenta	N/A	Polyclonal anti-β-actin ab8227 (Abcam) 42kDa	1:8000	Goat anti-rabbit conjugated HRP 12-348 (Millipore)	1:10,000
Placenta	80	Polyclonal anti-11βHSD2 ab80317 (Abcam) 44kDa	1:1000	Goat anti-rabbit conjugated HRP 12-348 (Millipore)	1:3000

The type, brand, molecular weight, and optimised dilution for primary and corresponding secondary antibodies used for protein detection by western blot in guinea pig cerebellar and placental tissues.

2.6.5 Analysis

The analysis for the western blots was performed using the Amersham Imager 6000 software. The software calculated the area of positive staining for selected bands of interest. Each value was then divided by the value obtained for the loading control (β-actin) of that sample. As all samples were run in duplicate, the average of these two resulting numbers was taken. To obtain the final value used for statistical analysis each

average of the samples was then normalised to the internal control on each membrane.

2.7 STATISTICAL ANALYSIS

2.7.1 CHAPTER THREE

Data was analysed and graphs made using Graphpad Prism software (version 6.01, Graphpad Software Inc., La Jolla, CA, USA). Two-way analysis of variance (ANOVA) was used to identify any statistically significant differences within sex or delivery groups. When significant differences were identified, Tukey's post-hoc tests and corrections for multiple comparisons were performed. The data was expressed as mean ± SEM and significance considered $p<0.05$.

2.7.2 CHAPTER FOUR

As in paper one, data was analysed and graphs made using Graphpad Prism software version 6.01. Unpaired t-tests in same-sex groups were used to identify significant differences between deliveries. Two-way ANOVA and Tukey post-hoc tests were performed as above.

2.7.3 CHAPTER FIVE

Data was analysed and graphs made using Graphpad Prism Software (version 7, Graphpad Software Inc.). Unpaired t-tests in same-sex groups were used to identify

significant differences between delivery groups. The data was expressed as mean ± SEM and significance considered $p<0.05$.

2.7.4 CHAPTER SIX

Statistical analyses for paper three were performed in conjunction with the Clinical Research Design, Information Technology, and Statistics Support Unit (CReDITSS) at HMRI. Data was analysed using SPSS version 2.3 (IBM SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA) and graphs made using Graphpad Prism software version 7.

Maternal salivary progesterone data was measured over time [categorised into: pre- (before commencement of treatment), early- (GA35 – 44), mid- (GA45 – 54), late- (GA55 – 60), and post-treatment (after treatment has ceased)]. Differences between vehicle and progesterone groups (overall and for each time category) were examined using linear mixed modelling to account for correlation of repeated measures (random intercept for mother), and including an interaction term between treatment and time category. Treatment effect for maternal allopregnanolone and cortisol data was analysed using two-way ANOVA (treatment*delivery) and independent t-test respectively.

The association between treatment and each fetal outcome measure was examined by linear mixed modelling including a 3-way interaction term (delivery*treatment*sex), all 2-way interactions, and main effects. A mixed model was used to account for correlation of errors for multiple pups per mother (random intercept for mother) with Bonferroni correction to adjust for multiple comparisons.

2.7.5 CHAPTER SEVEN

Data was analysed and graphs made using Prism v7.0 software (Graphpad Software Inc.). All data were presented as mean ± SEM for each group and significance considered $p<0.05$. In order to identify differences between groups the data was first analyzed by one-way ANOVA. Post-hoc tests with Tukey corrections for multiple comparisons was performed when the ANOVA was $p<0.05$. When data were not normally distributed, non-parametric Kruskal-Wallis test with post-hoc tests with Dunn's correction for multiple comparisons were performed. The exception to this was the supplemental feeding data and fractional weight gain, which were analysed by repeated measures two-way ANOVA, with corrections for multiple comparisons by Bonferroni.

3.0 "PRETERM BIRTH AFFECTS $GABA_A$ RECEPTOR SUBUNIT MRNA LEVELS DURING THE FOETAL-TO-NEONATAL TRANSITION IN GUINEA PIGS"

This published manuscript examines the changes in $GABA_A$ receptor subunit composition before and after birth at preterm and term gestational ages in the vulnerable hippocampus and cerebellum in a guinea pig model of preterm birth.

Published manuscript

Author	Contribution	Signature
Julia C Shaw	Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K Palliser	Experimental design Animal protocols and tissue collection Manuscript corrections	
David W Walker	Experimental design	
Jonathan J Hirst	Experimental design Manuscript corrections	

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Assistant Dean Research Training

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ORIGINAL ARTICLE

Preterm birth affects $GABA_A$ receptor subunit mRNA levels during the foetal-to-neonatal transition in guinea pigs

J. C. Shaw[1,2]*, H. K. Palliser[1,2], D. W. Walker[3] and J. J. Hirst[1,2]

[1]*School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, Australia*
[2]*Hunter Medical Research Institute, Mother and Babies Research Centre*
[3]*Monash Institute of Medical Research, Ritchie Centre*

Modulation of gamma-aminobutyric acid A ($GABA_A$) receptor signalling by the neurosteroid allopregnanolone has a major role in late gestation neurodevelopment. The objective of this study was to characterize the mRNA levels of $GABA_A$ receptor subunits (α4, α5, α6 and δ) that are key to neurosteroid binding in the brain, following preterm birth. Myelination, measured by the myelin basic protein immunostaining, was used to assess maturity of the preterm brains. Foetal guinea pig brains were obtained at 62 days' gestational age (GA, preterm) or at term (69 days). Neonates were delivered by caesarean section, at 62 days GA and term, and maintained until tissue collection at 24 h of age. Subunit mRNA levels were quantified by RT-PCR in the hippocampus and cerebellum of foetal and neonatal brains. Levels of the α6 and δ subunits were markedly lower in the cerebellum of preterm guinea pigs compared with term animals. Importantly, there was an increase in mRNA levels of these subunits during the foetal-to-neonatal transition at term, which was not seen following preterm birth. Myelination was lower in preterm neonatal brains, consistent with marked immaturity. Salivary cortisol concentrations, measured by EIA, were also higher for the preterm neonates, suggesting greater stress. We conclude that there is an adaptive increase in the levels of mRNA of the key $GABA_A$ receptor subunits involved in neurosteroid action after term birth, which may compensate for declining allopregnanolone levels. The lower levels of these subunits in preterm neonates may heighten the adverse effect of the premature decline in neurosteroid exposure.

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Key words: $GABA_A$ receptors, neurosteroids, preterm birth

Introduction

Preterm birth is the leading cause of death and neurodevelopment-related disability in neonates, accounting for up to 70% of neonatal deaths, and with ∼50% of the survivors developing a long-term neurodevelopmental disability.[1–3] Of the 133 million births each year, roughly 10% are preterm.[4] There is a growing body of evidence suggesting that late preterm infants are more likely to develop neurodevelopmental morbidities and exhibit poor school performance compared with term infants.[5–7] Furthermore, mothers and teachers of children who are born moderately to late preterm indicate higher rates of anxious and depressed behaviour in conjunction with learning difficulties at primary school age.[8] Aiding postnatal development of vulnerable brain regions following preterm birth remains a therapeutic target for the prevention of these neurodevelopmental disorders. Two regions of the brain that are particularly vulnerable to damage and developmental delay following preterm birth are the hippocampus and the cerebellum.[9,10] Development and maturation of the cerebellum, which is responsible for the regulation and co-ordination of movement with additional roles in attention and language, continues throughout late gestation until after birth in the guinea pig and in humans.[11] The same is true for the hippocampus, which has a major role in learning, memory formation and spatial recognition.[12]

Myelination, which occurs during late gestation, is reduced in neonates born preterm and may be the result of a reduced number of mature oligodendrocytes at the time of birth.[13,14] Appropriate levels of excitability are essential for normal neurodevelopment, including myelination,[13,15–17] and during foetal life they are regulated by the suppressive action of the neurosteroid allopregnanolone in the foetal brain.[18,19] Throughout gestation, the precursors required for allopregnanolone synthesis are supplied in high concentrations by the placenta and are, therefore, lost at birth. Thus, allopregnanolone levels decline rapidly after removal of the placenta and reach levels observed in neonates within 24 h after birth.[20] The resultant decrease in neurosteroid exposure may contribute to the continued delay in myelination following preterm birth, in addition to reducing inhibitory tone and exposing these immature brains to damaging excitotoxicity. Neurosteroids, including allopregnanolone, exert their suppressive action by increasing GABAergic inhibition. These effects of allopregnanolone on central nervous system (CNS) activity are due to agonist actions at the gamma-aminobutyric acid A ($GABA_A$) receptors, specifically to enhance $GABA_A$ receptor-mediated inhibition.[19,21] Steroid-sensitive $GABA_A$ receptors are highly

*Address for correspondence: J. C. Shaw, Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2308, Australia.
(Email julia.shaw@uon.edu.au)

expressed throughout the foetal brain from mid-gestation, including on oligodendrocytes.[22] Compared with the receptors found in the adult brain, those found in the foetal brain are more sensitive to modulation by allopregnanolone and are strongly activated by the concentrations of allopregnanolone found in foetal brain extracts.[23] The premature loss of allopregnanolone supply following preterm birth, therefore, may have consequences on $GABA_A$ receptor expression and ultimately in the reduction of inhibitory tone in the developing brain. Excessive excitation is damaging during the second half of gestation, and in long-gestation species such as the guinea pig and the sheep the $GABA_A$-mediated inhibitory pathway is active in the foetal brain from mid-gestation onwards.[22,24,25] Animal studies have shown that reducing allopregnanolone levels in the foetus *in utero* leads to increased cell death within the brain and a reduction in myelination.[24,26,27] Interestingly, there is evidence demonstrating the plastic nature of $GABA_A$ receptors, as neurosteroid withdrawal increases the expression of the α4 subunit.[28] This highlights the adaptive nature of $GABA_A$ receptors and the potential of replacement therapies that increase neonatal allopregnanolone concentrations to prevent excitotoxicity and associated cell death in premature brains.

$GABA_A$ receptors exist in a pentameric form comprising five subunits. The subunit composition of receptors varies greatly. Extra-synaptic subunits, which have a major role in tonic inhibition, commonly comprise α4, α5, α6 and δ subunits, with the expressions of α4 and α5 subunits high in the hippocampus and that of α6 and δ subunits predominant in the cerebellum.[29] Receptors containing these four subunits are highly sensitive to modulation by neurosteroids with consequent suppression of foetal CNS excitation,[30] whereas reduced expression of these receptor subunit types may lead to excitotoxic brain injury and suboptimal development. Previous studies have shown that $GABA_A$ receptor subunit expression is influenced by changes in glucocorticoid exposure. Specifically, acute stress has been linked with the increased expression of α5 and δ subunits along with raising baseline tonic conductance, which may be a compensatory mechanism to cope with the stress and prime for re-exposure.[31,32] Alternatively, chronic stress has been found to have the opposite effect, lowering expressions and overall inhibitory functions of the $GABA_A$ receptors.[33] Early exposure of the foetus to the *ex utero* environment is a potentially highly stressful situation; however, it is unclear whether this exposure may have a further effect on the expressions of $GABA_A$ receptor subunits in the newborn brain. The potential alterations to the $GABA_A$ receptor subunit expression profile following preterm birth warrants investigation, as this appears to be one component involved in the susceptibility of preterm brains to *ex utero* vulnerability and subsequent development of long-term neurodevelopmental disorders, and will, therefore, aid in developing effective prevention strategies. The objective of the present study was to characterize the mRNA levels of key $GABA_A$ receptor subunits in the hippocampus and cerebellum of preterm and term guinea pig foetuses and 1-day-old neonates. In addition, myelination and cortisol concentrations were assessed in these neonates to ascertain neurodevelopmental immaturity and *ex utero* stress exposure. We hypothesize that preterm animals will have lower $GABA_A$ receptor subunit mRNA levels. Furthermore, we suggest that this immaturity will be associated with reduced myelination within the hippocampus and cerebellum, and with high salivary cortisol levels, once exposed to the *ex utero* environment.

Method

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, NSW, Australia).

Animals

Before beginning this work, approval for all the animal experiments and procedures carried out throughout this study was obtained from the University of Newcastle Animal Care and Ethics Committee. In addition, all experiments and procedures were carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Time-mated outbred tricolour guinea pigs were obtained from the University of Newcastle Research Support Unit. Guinea pigs were housed indoors with a 12-h light/dark cycle, and were supplied with a diet consisting of commercial guinea pig pellets, Lucerne hay and water supplemented with ascorbic acid. The following animal groups were studied: term and preterm foetuses, and term and preterm neonates at 24 h of age. One male and one female foetus or neonate was used from each dam to prevent pregnancy bias. Term (GA69) and preterm (GA62) neonates were delivered by caesarean section as previously described.[20] After vigorously rubbing and quickly inverting each pup to remove fluid from the airways and to stimulate respiration, a 50 µl dose of surfactant (Curosurf, 80 mg/ml Poractant alfa, Douglas Pharmaceuticals, Baulkham Hills, NSW, Australia) was administered into the oropharynx. To encourage respiration, continuous positive airway pressure (CPAP) was administered using a small animal anaesthesia mask (Harvard apparatus, Holliston, MA, USA) attached to a Neopuff infant T-piece resuscitator (Fisher and Paykel Healthcare, Melbourne, VIC, Australia). Positive end expiratory pressure of 7 mm H_2O and peak inspiratory pressure (PIP) of 20 mm H_2O were applied at a flow rate of 8 l/min. Medical oxygen and air were adjusted to give a fraction of inspired O_2 of 60% during CPAP. An initial sustained PIP of 20 s was administered. CPAP and PIP were performed again when respiration became unstable, and an additional dose of 50-µl surfactant was administered at 3 h. Neonates were then placed in a humidified incubator (small animal intensive care incubator, Thermocare, Incline Village, NV, USA) at 34°C.

Pups were monitored for well-being, fed using a gastric tube and their saliva was collected every 2 h, up until 24 h.[20] The monitoring for well-being involved scoring respiration, posture

and alertness out of a maximum of 12 points at every 2-h interval. Each category was assigned a score from 0 to 4 (with 0 being the poorest and 4 being the optimal score); scores were then added together to give a total out of 12. Total scores between 0 and 3 indicated very poor well-being, 4–6 indicated poor well-being, 7–9 indicated good well-being and 10–12 indicated very good well-being.

The feeding regime used was commercial guinea pig milk-replacement formula (Wombaroo Food Products, Adelaide, SA, Australia), at 100 μl/g/24 h, made up in 50% v/v water and glucose solution (5% glucose solution, Baxter Healthcare). Following the fourth feed, glucose solution was no longer added to the milk formula.

Immediately after the 24-h cortisol sample collection, the neonates were euthanized and tissues were collected. Term and preterm foetal tissues were also obtained from pups at the time of caesarean section. At the time of tissue collection, body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the two hemispheres. The left hemisphere was fixed for immunohistochemistry, whereas the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

Immunohistochemistry

Immunodectection of myelin basic protein (MBP), a protein that is present in mature myelinating oligodendrocytes, was used to assess myelination within the neonatal brains. Hippocampal CA1, sub-cortical white matter and cerebellum sections were immunostained for MBP as previously described.[27] In brief, for each region, 7-μm sections were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia). Before immunostaining, each slide was de-waxed and incubated in methanol containing 3% hydrogen peroxide. Antigen retrieval was performed using Reveal-It solution (ImmunoSolution Pty Ltd, QLD, Australia), according to the manufacturer's instructions. Blocking for 30 min occurred at room temperature in bovine serum albumin (BSA) Blocking Solution (0.5% w/v BSA, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1 M PBS). Primary antibody (rat monoclonal anti-MBP antibody, M9434; Sigma-Aldrich) diluted to 1:4000 in BSA Blocking Solution was incubated overnight at room temperature. The secondary antibody, biotinylated anti-rat IgG (B7139; Sigma-Aldrich) diluted to 1:300 in BSA Blocking Solution, was incubated for 2 h at room temperature. Finally, incubation in the tertiary reagent, streptavidin–biotin–horseradish peroxidase complex (RPN1051V; Amersham), diluted to 1:300 in blocking solution (instead of Sodium Azide, 0.05% v/v thimerasol was added), was incubated for 2 h at room temperature. To reveal the immunolabelling, incubation in 3,3′-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce) was carried out. The samples were stained with cresyl violet and coverslipped using DEPX (Merck) the following day.

Stained slides were imaged using a Nikon upright microscope eclipse Ni-U with a Nikon DS-Ri1 camera attached (Nikon Instruments Inc., New York, USA) and NIS-Elements Advanced Research software (Nikon Instruments Inc.). Four consecutive images of the CA1 region of the hippocampus, sub-cortical white matter, lobe VIII, lobe X and deep white matter of the cerebellum from two sections per region were used for each animal. To quantify MBP expression, ImageJ (version 1.47, National Institutes of Health, USA) was used to calculate the area coverage of MBP expression. This was carried out by, first, removing the background and converting the image to 8-bit greyscale. The threshold of the image was then adjusted until all processes were visible, enabling the programme to calculate the total area coverage percentage of the stained regions. An overall average of MBP area coverage was then calculated for each neonate by taking the average of the four images captured per section.

Real-time PCR

Real-time PCR was performed as previously described.[34] Frozen cerebellum and hippocampus samples were homogenized in RLT Plus Buffer (obtained from the Qiagen RNeasy Plus Mini Kit, Qiagen Pty Ltd, VIC, Australia) using Precellys 24 dual-tissue homogenizer (Bertin Technologies, France). Extraction of RNA from the homogenate was carried out using the Qiagen RNeasy Plus Mini Kit by following the manufacturer's instructions. The NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the RNA in each sample. RNA purity was also confirmed using the NanoDrop, with A260/A280 ratios of 2.1–2.19 obtained for each sample. The RNA was then run on 1% agarose gel to confirm quality and integrity. The gels were imaged using a UVP benchtop UV transilluminator chamber (BioDoc-It Imaging System, Upland, CA, USA), and were examined for bands at 18S and 28S at a ratio of 1:2.

cDNA was synthesized on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) using the Superscript III Reverse Transcription kit (Invitrogen), according to manufacturer's instructions. Five primer pairs (α4, α5, α6 and δ $GABA_A$ receptor subunits, as well as the housekeeping gene β-actin) were designed and optimized within our laboratory for detection in the guinea pig and are detailed in Table 1. For each primer, a primer master mix concentration of 5 pmole/μl was used. The SYBR Green (Applied Biosystems) DNA-binding dye method of RT-PCR was used to detect the PCR products of each gene examined. Master mixes containing SYBR green, forward and reverse primers and MilliQ water (Millipore system treatment includes RNase and DNase filtration) were made up to final primer concentrations of 100 nM for the $GABA_A$ receptor α4 subunit, 600 nM for the α6 subunit and 400 nM for the α5 subunit, δ subunit and for β-actin. RT-PCR was performed using a 7500 ABI real-time machine

Table 1. *Guinea pig-specific primer sequences*

Gene of interest	Forward primer sequence	Reverse primer sequence
$GABA_AR$ α4 subunit	TGG GCA AAC AGT GTC AAG TG	GAC ACT TTG GGC AGA GAA TG
$GABA_AR$ α5 subunit	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA
$GABA_AR$ α6 subunit	ATA AGG AGT CAG TCC CAG CA	ACG AAA GCA AAG CAT ACA GC
$GABA_AR$ δ subunit	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC
β-actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT

Primer sequences designed for Guinea pig $GABA_A$ receptor α4, α5, α6, δ subunits and β-actin gene expression. Primer sequences are displayed from 5′–3′ for forward and reverse primer.

(Applied Biosystems), and the results were analysed by Sequence Detection Software v2.01 (Applied Biosystems). The comparative C_t method ($2^{-\Delta\Delta C_t}$) was used to calculate relative fold changes in the mRNA levels of each gene. A house-keeping gene (β-actin) and a calibrator sample were used as controls in the comparative C_t method of analysis. Consistent C_t values were obtained for β-actin across the term/preterm, male/female and foetal/neonatal samples. The calibrator was a pooled sample of hippocampal and cerebellar brain samples, and was used as a control on every PCR plate.

Cortisol EIA

The Salimetrics Salivary Cortisol Assay competitive immunoassay kit (Salimetrics Inc., State College, PA, USA) was used to measure the concentration of cortisol in guinea pig saliva samples, and was performed by following the manufacturer's instructions. Saliva samples were obtained from term and preterm neonates by encouraging the neonates to chew on a cotton bud. For each animal, samples collected at 2 and 4 h were pooled together to create a 2–4-h sample, and the same was followed to obtain a 22–24-h sample. As previously described, the sensitivity of the assay was 0.012–3.0 μg/dl, with the inter- and intra-assay coefficients of variance being 6.89% and 5.52%, respectively.[35]

Statistical analysis

Data were analysed by two-way ANOVA using Graphpad Prism software (version 6.01, Graphpad Software Inc., La Jolla, CA, USA). When a significant difference was found, Tukey's *post-hoc* tests and corrections for multiple comparisons were performed. Unless otherwise stated, all data were expressed as mean ± S.E.M., and significance was considered at $P < 0.05$.

Results

Foetal and neonatal physical characteristics

Table 2 depicts the mean birth weights and organ-to-body weight ratios for foetuses collected at term and preterm. Within each sex, preterm foetuses had lower body weight ($P < 0.0001$) than their term counterparts. In addition, brain-to-body weight ratios (males $P = 0.0023$ and females $P = 0.0094$) and placenta-to-body weight ratios were higher for preterm foetuses (males $P = 0.015$ and females $P = 0.0029$), as well as heart-to-body weight ratios (males $P = 0.019$, and females $P = 0.04$). Brain-to-liver ratios were not significantly different, nor did they indicate growth restriction. Adrenal-to-body weight ratio was not significantly different between groups. No differences were found between the sexes in either the preterm or term foetal groups.

Body weights and organ-to-body weight ratios for term and preterm neonates at 24 h of age are shown in Table 3. Birth weights ($P = 0.011$) and postmortem weights ($P = 0.018$) of the preterm male neonates were significantly lower than the term male neonates. However, no differences were identified between preterm and term female neonates. Placenta-to-body weight ratios were significantly higher for the preterm neonates (males $P = 0.001$ and females $P = 0.039$). No other organ-to-body weight ratios were significantly different between sexes or gestational ages (GAs), nor was brain-to-liver ratio.

Neonates were also assessed for well-being during the 24-h period using a set of criteria that included resuscitation requirements, posture, alertness and movement. Average scores (average of the scores taken every 2 h over the 24-h period) were significantly lower for the preterm neonates compared with the term neonates ($P < 0.0001$), the same was found for final scores (the final score measured at 24 h; $P < 0.0001$; Table 3). The preterm neonates also had periods of apnea, forelimb spasticity and irregular respiration.[20] No differences were found between the sexes in either the preterm or term foetal groups.

Confirmation of neonatal prematurity

MBP immunostaining in the CA1 region of the hippocampus was significantly lower in the preterm female neonates compared with the term female neonates ($P = 0.014$, Fig. 1b). Area coverage in the hippocampus did not differ between preterm and term male neonates. In the sub-cortical white matter and lobe X of the cerebellum, MBP coverage was significantly reduced in preterm neonates compared with term neonates ($P = 0.048$, Fig. 1d, and $P = 0.043$, Fig. 1f, respectively). There were no differences in MBP immunostaining in lobe VIII and the deep white matter of the cerebellum observed

Table 2. *Foetal physical characteristics*

Sex	Group	*n*	Body weight (g)	Brain-to-body weight	Placenta-to-body weight	Heart-to-body weight	Liver-to-body weight	Adrenal gland-to-body weight	BLR
Male	Preterm	8	62.39 ± 2.94*	3.29 ± 0.13*	6.17 ± 0.23*	0.65 ± 0.03*	4.95 ± 0.2	0.04 ± 0.002	0.68 ± 0.04
	Term	11	93.44 ± 3.86	2.53 ± 0.11	4.95 ± 0.31	0.52 ± 0.03	4.81 ± 0.15	0.03 ± 0.002	0.53 ± 0.03
Female	Preterm	8	60.14 ± 3.35*	3.40 ± 0.20*	6.61 ± 0.24*	0.62 ± 0.04*	5.48 ± 0.45	0.04 ± 0.002	0.65 ± 0.06
	Term	11	89.57 ± 4.34	2.71 ± 0.13	5.10 ± 0.24	0.50 ± 0.02	5.11 ± 0.16	0.04 ± 0.003	0.54 ± 0.03

All values are represented as a percentage of body weight at the time of postmortem with the exception of BLR, which is a ratio value of brain weight to liver weight. The BLR value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted foetuses. Values are expressed as the mean percentage + S.E.M. and are calculated for animal numbers.

*Denotes a significant effect of age within a sex group ($P < 0.05$).

Table 3. *Neonatal physical characteristics*

Sex	Group	*n*	Birth Weight (g)	Postmortem weight (g)	Brain-to-body weight	Placenta-to-body weight	Heart-to-body weight	Liver-to-body weight	Adrenal gland-to-body weight	BLR	Average score	Final score
Male	Preterm	9	72.17 ± 1.83*	66.81 ± 1.57*	3.19 ± 0.08	6.37 ± 0.28*	0.63 ± 0.03	4.49 ± 0.22	0.05 ± 0.006	0.73 ± 0.04	8.05 ± 0.28*	8.31 ± 0.49*
	Term	9	89.12 ± 5.23	84.13 ± 5.36	2.94 ± 0.15	4.82 ± 0.30	0.63 ± 0.02	4.04 ± 0.17	0.04 ± 0.007	0.76 ± 0.04	11.63 ± 0.19	11.89 ± 0.11
Female	Preterm	11	70.26 ± 3.32	66.3 ± 3.14	3.20 ± 0.12	5.73 ± 0.27*	0.62 ± 0.03	4.64 ± 0.18	0.05 ± 0.004	0.70 ± 0.04	7.85 ± 0.46*	8.61 ± 0.63*
	Term	12	78.58 ± 3.80	73.83 ± 3.75	3.078 ± 0.22	4.79 ± 0.15	0.60 ± 0.03	4.08 ± 0.12	0.05 ± 0.01	0.76 ± 0.08	11.64 ± 0.11	11.96 ± 0.04

All values are represented as a percentage of body weight at the time of postmortem with the exception of BLR, which is a ratio value of brain weight to liver weight, and the average and last scores. The BLR value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted foetuses. Average and final scores are indicative of well-being and are scored out of a maximum 12, whereby 12 indicates good well-being. Values are expressed as the mean percentage + S.E.M. and are calculated for animal numbers.

*Denotes a significant effect of age within a sex group ($P < 0.05$).

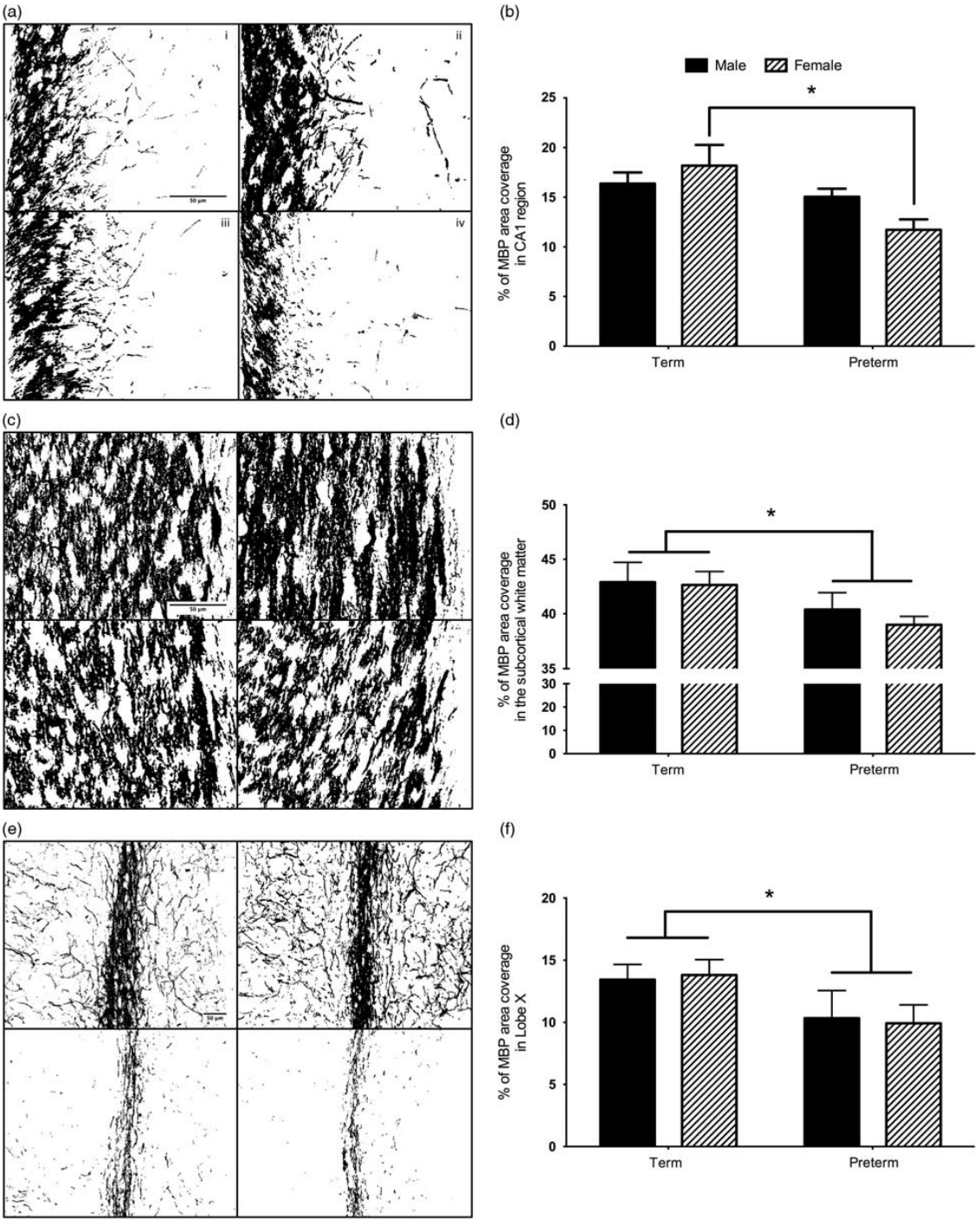

Fig. 1. Representative photomicrographs of myelin basic protein (MBP) immunolabelling and percent coverage in the neonatal Guinea pig external capsule adjacent to the CA1 region of the hippocampus (a and b), the sub-cortical white matter (c and d) and lobe X of the cerebellum (e and f). Scale bar = 50 μm; (i) male term, (ii) female term, (iii) male preterm and (iv) female preterm for all photomicrographs. Mean ± S.E.M. Males = black bars, females = hashed bars, *indicates $P < 0.05$, all groups are $n = 4–6$.

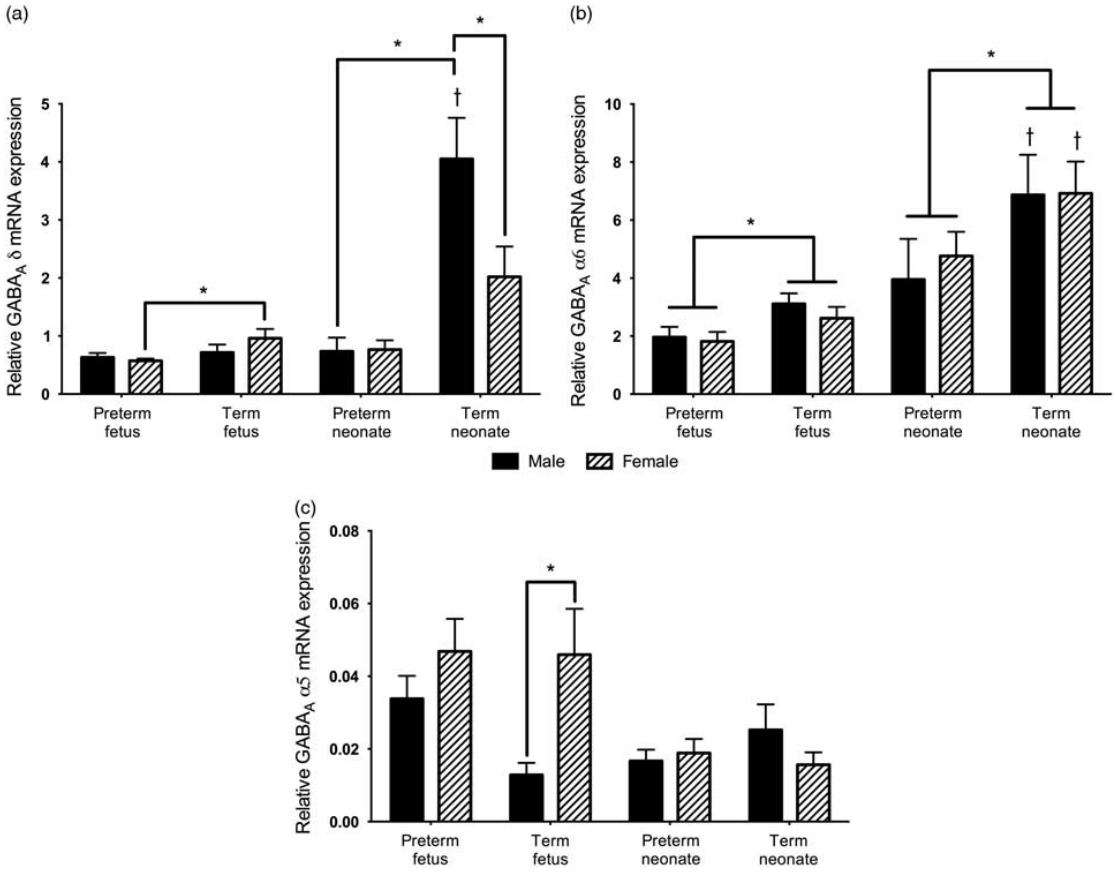

Fig. 2. Relative $GABA_A$ receptor (a) δ, (b) α6 and (c) α5 subunit mRNA levels in the cerebellum. Values are for preterm (62 day) and term (69 day) foetuses, and preterm and term neonates at 24 h of age for males (filled bars) and females (hashed bars). All groups are $n = 5–8$, * indicates $P < 0.05$ within gestational age or sex, † indicates $P < 0.05$ between foetal and neonatal groups within gestational age and sex groups.

between preterm and term neonates (data not shown), nor were any sex differences observed in any of the regions examined.

GABA$_A$ receptor subunit mRNA levels

In cerebellar tissues, foetal mRNA levels of the δ subunit were significantly higher in term females compared with the preterm females ($P = 0.047$; Fig. 2a), but did not reach significance in the neonates ($P = 0.28$). Conversely, term male neonates exhibited significantly higher δ subunit mRNA levels compared with preterm male neonates ($P = 0.0003$) and term female neonates ($P = 0.026$). In addition, cerebellar δ subunit mRNA levels in the term male neonates were significantly higher than in the term male foetuses ($P < 0.0001$), and approached significance for higher levels in the term female neonates compared with term female foetuses ($P = 0.065$). Cerebellar δ subunit mRNA levels between the preterm foetal and neonatal populations did not change. Preterm foetuses and neonates showed significantly lower cerebellar mRNA levels of the α6 subunit compared with the term foetuses ($P = 0.011$) and neonates ($P = 0.044$; Fig. 2b). In addition, the mRNA levels of both the male and female term neonates were significantly higher than levels of the male and female term foetuses ($P = 0.017$ and $P = 0.0073$, respectively). Cerebellar α5 subunit mRNA levels were significantly lower in term male foetuses compared with term female foetuses ($P = 0.047$; Fig. 2c) in the foetal cohort. By 24 h, however, levels of α5 mRNA were similar between term female and male neonates. No significant difference was identified in the levels of α4 subunit mRNA in either the foetal or neonatal cerebellum (data not shown).

In the hippocampal tissues, there were no significant differences in relative mRNA levels identified for the α4, α5, α6 and δ subunits within either the foetal cohort or the neonatal cohort, between sexes or between GAs (data not shown).

Neonatal salivary cortisol

Salivary cortisol levels were assessed in neonates at 2–4 and 22–24 h. Overall, preterm neonates were found to have

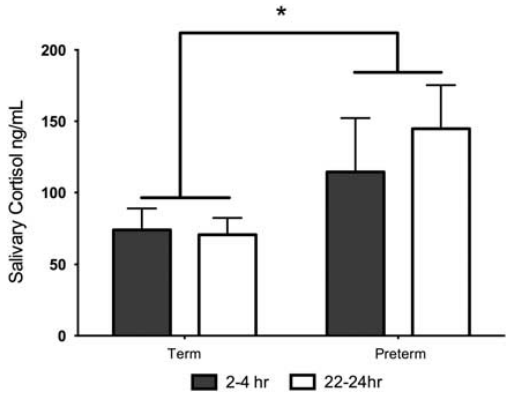

Fig. 3. Comparison of salivary cortisol between term and preterm neonates. Male and female neonates are combined to create overall term and preterm groups at 2–4 h (filled bars) and 22–24 h of life (open bars). *Indicates $P < 0.05$, all groups are $n = 10$.

significantly higher levels of salivary cortisol compared with the term neonates ($P = 0.035$; Fig. 3). Furthermore, the concentrations did not increase from birth to 24 h within the term or preterm neonatal group. No differences were observed between males and females; therefore, these data were combined.

Discussion

The major finding of this study shows that, for the first time using the established guinea pig model, neonates that are born preterm have disrupted $GABA_A$ receptor subunit mRNA levels. Interestingly, these changes were region specific, as levels in the cerebellum were markedly affected, whereas no changes in the subunits examined were found in the hippocampus. Specifically, preterm neonates have lower mRNA levels of the α6 and δ (male only) subunits in the cerebellum compared with neonates delivered at term. Interestingly, term neonates exhibited higher α6 (male and female) and δ (male only) subunit mRNA levels than term foetuses. As this increase was not present in the preterm neonatal cohort, these results indicate that birth at this earlier stage in gestation not only leaves these neonates with an immature mRNA profile for α6 and δ subunits, but also, unlike term neonates, no further increase occurs during the 24 h following birth. Although the regulatory mechanism remains to be determined, the increase in subunit mRNA levels in term males and females over this initial 24-h period may be an adaptation for the transition to the neonatal environment, which is not available to preterm neonates. Separation of the foetus from the placenta at the time of birth results in a loss of supply of progesterone required for allopregnanolone synthesis.[20] Therefore, after birth, allopregnanolone levels decline rapidly, such that markedly lower neonatal levels are observed by 24 h after delivery. This dramatic drop highlights the importance of the increased mRNA levels of the α6 and δ subunits following birth in the term neonates, which may compensate for the dramatic reduction in the supply of allopregnanolone, and therefore maximize allopregnanolone action at the $GABA_A$ receptors. The results of this study suggest that preterm neonates are incapable of increasing their α6 and δ mRNA levels, which would lead to further vulnerability to excitation-induced brain injury and suboptimal development following preterm birth.

Although term male foetuses had lower cerebellar α5 subunit mRNA levels compared with term female foetuses, a finding not present in the neonatal population, levels for this subunit and that for α4 were very low, suggesting that the role of these subunits in the cerebellum may not be important in determining receptor-binding affinities. The observation that there were no differences in the mRNA levels of $GABA_A$ receptor subunits in the hippocampus, in both the foetal and neonatal cohorts, suggests that this region has matured at least in terms of $GABA_A$ receptor development before the time of preterm delivery in our model (62 days GA). As mentioned, sex differences were identified in this study, with the expression of the α5 subunit in term foetuses and the δ subunit in term neonates differing between males and females. These differences between sexes were only identified in the term populations, suggesting that they are physiologically normal differences in neurodevelopment between males and females. Interestingly, the same differences were not observed in the preterm populations, providing further evidence for a deregulated transition to the *ex utero* environment. Specifically, the large sex difference present in the term neonate population, with the males expressing higher levels of the δ subunit, is not apparent in the preterm population. This may be an indicator of vulnerability in the immediate neonatal period for preterm males, but seemingly the levels for preterm females reached term equivalent.

These findings suggest that the action of neurosteroids in the preterm brain may differ due to the marked disparity in $GABA_A$ receptor subunit composition and mRNA levels compared with the term brain, therefore leading to major differences in neurosteroid sensitivity after birth. The implications of these alterations, however, requires further investigation, including long-term animal studies to determine whether these mRNA profiles 'catch up' to those present in term neonates or whether the differences are permanent and whether they are associated with changes in behaviour or susceptibility to hyperactivity states, as has been demonstrated in other rodent models of $GABA_A$ receptor subunit knockouts. Behaviour following a knockout of the δ subunit (which is known to commonly group with the α6 subunit) has been extensively studied and linked with development of multiple neurodevelopmental conditions, a cause for concern if the low mRNA levels in preterm neonates identified in this study are permanent. Various knockout studies in mice and rats have demonstrated an increase in behaviour common to preterm infants, such as anxiety-like and pro-epileptic behaviour in the absence of the δ subunit, which may be a result of reduced sensitivity to neurosteroids.[36–38] Studies in humans have

shown that preterm infants have smaller cerebellar volume and white matter, and at school age they are described as 'clumsier' and with worse fine motor control, such as hand dexterity, than those born at term age.[39–41] Preterm guinea pig neonates in this study also demonstrated seizure-like behaviour and ataxia in the initial 24-h period, suggesting that motor function of the cerebellum may be affected. Although there is limited information concerning $GABA_A$ receptors and motor function, one study has examined subunit mRNA levels in a mutant mouse strain that presented ataxia and head tossing. This study showed that mRNA levels of extrasynaptic receptors containing both the α6 and δ subunits were significantly decreased in the cerebellum compared with wild-type mice, providing evidence for the role of $GABA_A$ receptors in this region.[42] In the case of preterm birth, the initial effect of the deficit in mRNA levels would be compounded by the low allopregnanolone concentrations seen after delivery. As the δ subunit has a major role in controlling the steroid sensitivity of extrasynaptic $GABA_A$ receptors, and that neurosteroids have a role in regulating tonic levels of excitability, differences in the levels of this subunit could influence overall levels of excitability in a period when inhibitory action over the brain is crucial for neurodevelopment. Recent studies support previously unidentified roles of the cerebellum in cognitive functions. Functional imaging and clinical studies have shown that the cerebellum is involved in language processing and reading, working memory and associative learning.[43–45] It is also now well documented that children born preterm function more poorly in school, with learning difficulties and lower IQs compared with term children.[6,8] Future behavioural studies with a cohort of preterm neonates may further support the role of the $GABA_A$ receptor cerebellar deficits, observed here, in these poor neurodevelopmental and motor outcomes that are common in preterm children.

A key finding was that salivary cortisol concentrations of preterm neonates over the initial 24-h period were significantly higher than term neonates, suggesting increased stress exposure. In human premature newborns, studies looking at cortisol concentrations are contradictory due to interactions with prenatal glucocorticoid treatment. Despite this, however, one study found that as both GA and birth weight decreased, circulating cortisol concentrations increased.[46] Furthermore, cortisol levels have been shown to influence $GABA_A$ receptor subunit levels.[31–33] However, this does not appear to account for the differences found between the term and preterm neonates here, as similar $GABA_A$ receptor mRNA levels were evident in the foetal preterm population before the exposure to the *ex utero* environment, and therefore the associated stress. The lower mRNA levels of the α6 and δ subunits in the preterm neonates in this case instead appear to be due to immaturity at the time of birth and lack of a birth-associated rise as opposed to being induced by high cortisol exposure over the 24-h period.

Children and adolescents born preterm have higher incidences of a range of behavioural, cognitive and motor disorders including anxiety, depression, internalizing behaviour and hyperactivity disorders.[8,47,48] Reduced $GABA_A$ receptor-related inhibition might have a contributory role in these conditions. There have been suggestions that differing $GABA_A$ subunit expression and actions of neurosteroids may be involved in controlling cortisol release and the response to stress.[49,50] $GABA_A$ receptors possessing the δ subunit are highly expressed in the paraventricular nucleus, where the corticotropin-releasing hormone (CRH) neurons involved in the stress response are located.[49] Interestingly, knockout of the δ subunit reduces the sensitivity of these CRH neurons to the stress-induced neurosteroid tetrahydrodeoxycorticosterone, ultimately affecting the response to stress.[49] Further long-term studies in juvenility, adolescence and adulthood are required to determine the long-term consequences of the differences in subunit mRNA levels that we observed, particularly for the development of anxiety-like behaviour in the preterm neonates. This would determine whether preterm neonates permanently experience higher levels of cortisol and differences in mRNA levels of the $GABA_A$ receptor subunits implicated in the stress response, and whether this, in part, contributes to the higher incidences of neurobehavioural illnesses and delays that preterm neonates develop later in childhood and adolescence.

The development of neurobehavioural delays in children born preterm are suggested to be partly attributed to a decreased white matter volume at the time of birth.[13] A decrease in the area coverage of myelination in several regions of the hippocampus and cerebellum was identified in the preterm neonates in our study, confirming previous findings within our laboratory and the prematurity of these neonates.[20] The differences identified in the sub-cortical white matter and lobe X of the cerebellum appear to be quite small; however, imaging studies in preterm humans show that white matter injuries in the immediate postnatal period persist long-term and are correlated with poor cognitive outcome and motor impairment.[39,51] Furthermore, when the difference between total percentages covered (as opposed to area coverage) is compared between the term and preterm neonates in lobe X, for example, the reduction in total myelination is quite substantial. Future analysis of these regions may also focus on axon length and markers of oligodendrocyte progenitors and apoptosis to explore the differences in more detail, as the analysis used in this study focused on mature oligodendrocytes only. The most striking decrease in mature oligodendrocyte expression was observed in the CA1 region of the female preterm neonates. This is further evidence for the differing neurodevelopmental trajectories of males and females.

Previous studies within our laboratory have shown that allopregnanolone levels following preterm birth are significantly lower compared with foetal levels.[14] These lower concentrations may potentially disrupt the long-term development of myelin within these vulnerable brains. Other studies have identified the importance of allopregnanolone in the formation of myelin. This has been demonstrated in rat brain slice cultures, where administration of allopregnanolone enhanced

myelination.[52] Similarly, in a mouse model of neurodegeneration, allopregnanolone treatment delayed damage to myelin and increased survival.[53] Our studies have also shown that inhibition of allopregnanolone synthesis using finasteride reduces myelin maturation.[27] Furthermore, changes in receptor expression have been identified in human brain tissues in Alzheimer's and Parkinson's disease, but their role in disease progression is unknown.[54] Based on this knowledge that neurosteroids promote myelination, and that their primary site of action is $GABA_A$ receptors, it is feasible that catch-up myelination in preterm neonates may be further impaired due to the lack of the α6 and δ subunits. Depending on the results of future long-term studies, there is the potential of neurosteroid replacement therapy to act on the limited levels of neurosteroid-sensitive subunits in the preterm brain and mimic *in utero* conditions to encourage correct neurodevelopment of preterm brains, increasing receptor expression to term equivalent levels, and thus decreasing damaging excitation in this vulnerable window for neurodevelopment, ultimately improving outcomes for these neonates.

Overall, this study has highlighted key differences in $GABA_A$ receptor subunit mRNA levels in the cerebellum of neonates born prematurely and has identified an additional vulnerability that preterm neonates face. The lack of a birth-related adaptive increase in the mRNA levels of cerebellar α6 and δ $GABA_A$ receptor subunits after birth potentially reduces the effect of allopregnanolone postnatally and may contribute to neurodevelopmental disability in preterm neonates by exposing the immature brain to damaging excitotoxicity. This finding provides a basis for investigation in long-term studies of behavioural outcome.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes) and has been approved by the institutional committee (University of Newcastle Animal Care and Ethics Committee).

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4.0 "Long-term effects of preterm birth on behaviour and neurosteroid sensitivity in the guinea pig"

This published manuscript investigates the effects of preterm birth on neurodevelopment of the vulnerable hippocampus, in addition to behavioural phenotypes and hippocampal $GABA_A$ receptor subunit expression, of the juvenile guinea pig.

Published manuscript

Author	Contribution	Signature
Julia C Shaw	Experimental design Animal protocols and tissue collections Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K Palliser	Experimental design Animal protocols and tissue collections Manuscript corrections	
Rebecca M Dyson	Experimental design Animal protocols and tissue collections Manuscript corrections	
Jonathan J Hirst	Experimental design Manuscript corrections	
Mary J Berry	Experimental design Animal protocols and tissue collections Manuscript corrections	

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Long-term effects of preterm birth on behavior and neurosteroid sensitivity in the guinea pig

Julia C. Shaw[1,2], Hannah K. Palliser[1,2], Rebecca M. Dyson[3], Jonathan J. Hirst[1,2] and Mary J. Berry[4,5]

BACKGROUND: Ex-preterm children and adolescents are at risk of developing late-onset neurodevelopmental and behavioral disorders. The mechanisms by which this happens are poorly understood and relevant animal models are required.
METHODS: Ex-preterm (delivered at 62 d gestation) and term (spontaneously delivered) juvenile guinea pigs underwent behavioral testing at 25 d corrected postnatal age, with tissues collected at 28 d. Neurodevelopmental markers (myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP)) were analyzed in the hippocampus and subcortical white matter by immunohistochemistry. Gamma-aminobutyric acid A ($GABA_A$) receptor subunit mRNA levels were quantified by reverse transcription polymerase chain reaction (RT-PCR), and salivary cortisol measured by enzyme-linked immunosorbent assay.
RESULTS: Preterm males travelled greater distances, were mobile for longer, spent more time investigating objects, and approached or interacted with familiar animals more than controls. Myelination and reactive astrocyte coverage was lower in the hippocampus and the subcortical white matter in preterm males. Hippocampal levels of the α5 subunit were also lower in the preterm male brain. Baseline salivary cortisol was higher for preterm males compared to controls.
CONCLUSION: We conclude that juvenile ex-preterm male guinea pigs exhibit a hyperactive phenotype and feature impaired neurodevelopment, making this a suitable model for future therapeutic studies.

Children born preterm (birth at <37 wk gestation) have an increased risk of developing a long-term neurodevelopmental disability (1,2). Importantly, this risk exists even in those thought to be "well" at the time of discharge from neonatal care and in those with no evidence of the structural brain injuries known to be associated with adverse neurodevelopmental outcomes (e.g., intraventricular hemorrhage or periventricular leukomalacia) (3,4). Thus, although intraventricular hemorrhage and, or, periventricular leukomalacia explain neurodevelopmental problems in a subset of high-risk infants, they do not account for the overall burden of neurodisability seen in the ex-preterm population.

Although major neurodevelopmental problems are usually picked up early, subtle behavioral or psychiatric disorders may not become apparent until school age, at a time distant from the causative insult (5,6). Anxiety disorder and attention deficit hyperactivity disorder are the most commonly diagnosed disorders in school-aged ex-preterm children (7,8). Attention deficit hyperactivity disorder has a male preponderance and is characterized by a deficit in behavioral inhibition, inattention, impulsivity and social difficulties, whereas anxiety disorder is more commonly diagnosed in ex-preterm females (7,8). Thus, the behavioral outcomes of preterm birth occur in a sex-dependent manner. Other neuropathologies, including depression, and impaired cognitive performance are also increased in those born preterm compared to children and adolescents born at term (5,9–11). Hypo-myelination of white matter tracts with reduction in the white matter volume of brain regions such as the hippocampus and frontal cortex is also described in ex-preterm neonates, children, and adolescents (12–14). Despite the clear link between preterm birth and later neurological impairment, the causal mechanisms underpinning preterm-associated brain injury remain poorly understood.

One possible mechanism linking preterm birth with the development of these neurological morbidities and accompanying white matter reductions may be the early loss of exposure to neurosteroids. Neurosteroids, particularly allopregnanolone, are present in high levels during fetal life, especially the latter stages of gestation. Preterm birth leads to a premature loss of placental support of allopregnanolone synthesis and a decline in plasma and brain levels in the newborn compared to the fetus at equivalent postconceptual age (15). Allopregnanolone acts as an agonist at the extrasynaptic $GABA_A$ receptor and exerts an inhibitory effect, suppressing excessive neuronal excitation and maintaining tonic enhancement of GABA-mediated synaptic inhibition (15–17).

The $GABA_A$ receptor composition has a key role in regulating neurosteroid action. Receptors containing the δ and α4–6 subunits are highly sensitive to allopregnanolone concentrations

[1]School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, Australia; [2]Hunter Medical Research Institute, Mothers and Babies Research Centre, Newcastle, Australia; [3]Department of Paediatrics, Graduate School of Medicine and IHMRI, University of Wollongong, Wollongong, Australia; [4]Centre for Translational Physiology, University of Otago, Wellington, New Zealand; [5]Department of Paediatrics and Child Health, University of Otago, Wellington, New Zealand. Correspondence: Julia C. Shaw (Julia.shaw@uon.edu.au)

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observed in the fetal brain and this interaction has been suggested to be integral to proper neurodevelopment (16,18). The receptors are highly expressed throughout the brain, including on myelinating oligodendrocytes, however regional variation exists with subunits such as the δ and α4–5 subunits highly expressed in the hippocampus specifically (15,17). Mouse knockout studies involving these subunits result in increased anxiety-like behavior and seizure susceptibility (19,20). Interestingly, in humans markedly lower levels of the δ subunit are observed in the frontal cortex in patients with major depressive disorder (21,22). Additionally, lower levels of both the α5 and δ subunits are present following chronic stress and in patients suffering post-traumatic stress disorder and anxiety, implicating decreased expression of these subunits with major depressive disorders (23,24). Previously, we have reported that the expression of these subunits differed between the preterm and term brain at the time of delivery, or allopregnanolone withdrawal, in guinea pigs. Allopregnanolone withdrawal resulted in an adaptive increase in the expression of the δ and α6 subunits in the brain of term guinea pig neonates, and thus represents the feedback impact of allopregnanolone levels on $GABA_A$ receptor subunit expression (25). This increase was seemingly absent in the preterm cohort, with expression remaining unchanged between the preterm fetal and neonatal animals 24 h after birth. Currently, it is unknown whether these changes in expression persist into childhood, or are rectified in the initial neonatal period.

Our previous studies have demonstrated a dramatic drop in brain allopregnanolone concentrations following birth at term and preterm compared to fetal levels (26). This reduced allopregnanolone-mediated neuroprotection increases the vulnerability of the neonatal brain to insults such as hypoxia. Furthermore, these preterm animals also show decreased myelination in the CA1 region of the hippocampus and the adjacent subcortical white matter 24 h after delivery, and in the cerebellum at term equivalence (25–27).

In this study, we examined the effect of preterm birth on brain development, focusing on the mature myelinating oligodendrocytes and reactive astrocytes that are essential for proper neuronal circuitry and synaptic transmission, to identify effects at adolescence in guinea pigs. We also aimed to ascertain the impact that preterm delivery has on the expression of neurosteroid-sensitive $GABA_A$ receptor subunits, in addition to behavior at juvenility, with a particular focus on the hyperactive and anxious behaviors that are common to ex-preterm males and females respectively. We hypothesize that early loss of neurosteroid supply alters $GABA_A$ receptor subunit expression, which then contributes to immature and delayed neurodevelopment following preterm delivery. We further hypothesize that these changes in neurosteroid sensitivity and neurodevelopment, specifically reduced oligodendrocyte and astrocyte expression, will contribute to the development of a hyperactive phenotype and an anxious phenotype, in male and female ex-preterm juveniles respectively, in a sex-dependent manner.

RESULTS

Physical Characteristics

The average litter size across the term and preterm deliveries was three, and the number of pups that were either stillborn or were euthanized in the early neonatal period was approximately 10% for term pups and 30% for preterm pups.

Mean gestational age at delivery and birth, postmortem, and organ weights for juveniles collected at corrected postnatal day 28 are depicted in **Table 1**. Within each sex, birth weights of the preterm neonates were lower (males $P = 0.0135$, females $P = 0.002$). No difference in postmortem weight was observed, however the interaction between gestational age and sex was significant ($P = 0.0213$). Percentage of weight gain from birth until postmortem was higher for the preterm males and females compared to their term counterparts (males $P < 0.0001$, females $P = 0.0013$). The preterm males also had a higher percentage of weight gain than the preterm females ($P = 0.0027$). Liver weight was significantly higher for the preterm males compared to the term males ($P = 0.035$), and was also higher than the preterm females ($P = 0.0029$). The interaction between gestational age and sex for liver weight was also significant ($P = 0.0151$).

Behavior

Open field and environment exploration. Distance travelled in the open-field arena (**Figure 1a**) and inner zone (**Figure 1b**) was higher for preterm males at corrected postnatal day 25 compared to term males ($P = 0.026$ and $P = 0.046$ respectively). The time mobile in both the open-field arena (**Figure 1c**) and inner zone (**Figure 1d**) was higher for preterm males ($P = 0.036$ and $P = 0.021$ respectively). Term females exhibited higher distances travelled in both the open field and inner zone, and more time spent mobile in the open field compared to term males ($P = 0.0065$, $P = 0.026$, and $P = 0.049$ respectively). Preterm males spent more time

Table 1. Juvenile physical characteristics

	Pups (*n*)	Dams (*n*)	Gestational age delivery	Birth weight (g)	Postmortem weight (g)	Brain weight (g)	Hippocampus weight (g)	Half cerebellum weight (g)	Liver weight (g)
Term male	10	8	68.87 ± 0.29	94.2 ± 4.22	270.1 ± 10.79	3.36 ± 0.042	0.12 ± 0.0041	0.53 ± 0.055	12.12 ± 0.57
Term female	10	9	68.88 ± 0.51	99.86 ± 5.47	286.74 ± 16.54	3.35 ± 0.048	0.12 ± 0.0075	0.48 ± 0.047	11.61 ± 0.90
Preterm male	10	8	62 ± 0†	76.35 ± 1.88†	322 ± 7.28	3.32 ± 0.047	0.12 ± 0.0075	0.45 ± 0.031	15.26 ± 0.79†,‡
Preterm female	8	8	62 ± 0†	74.51 ± 5.16†	265.75 ± 21.7	3.18 ± 0.082	0.12 ± 0.0048	0.42 ± 0.026	10.88 ± 0.66

Values are expressed as mean ± SEM and are calculated for animal numbers.
† Denotes a significant difference between preterm and term within a sex, whereas ‡ denotes a significant difference between sexes within a gestational age group ($P < 0.05$).

investigating the foreign objects placed into the open-field arena compared to term males (Figure 1e, $P = 0.036$).

Social interaction test. The number (Figure 2a), and frequency of approaches (Figure 2b), toward a familiar animal in the arena was higher for preterm males compared to term counterparts ($P = 0.0072$, and $P = 0.0062$ respectively). Similarly, the number (Figure 2c) and frequency (Figure 2d) of affectionate interactions toward the familiar animal was also higher for preterm males compared to terms ($P = 0.033$, and $P = 0.034$ respectively). There were no differences identified for number or frequency of agonistic interactions (data not shown).

Immunohistochemistry

Oligodendrocytes. Mature oligodendrocyte cell areas were compared by immunostaining for MBP. MBP area coverage

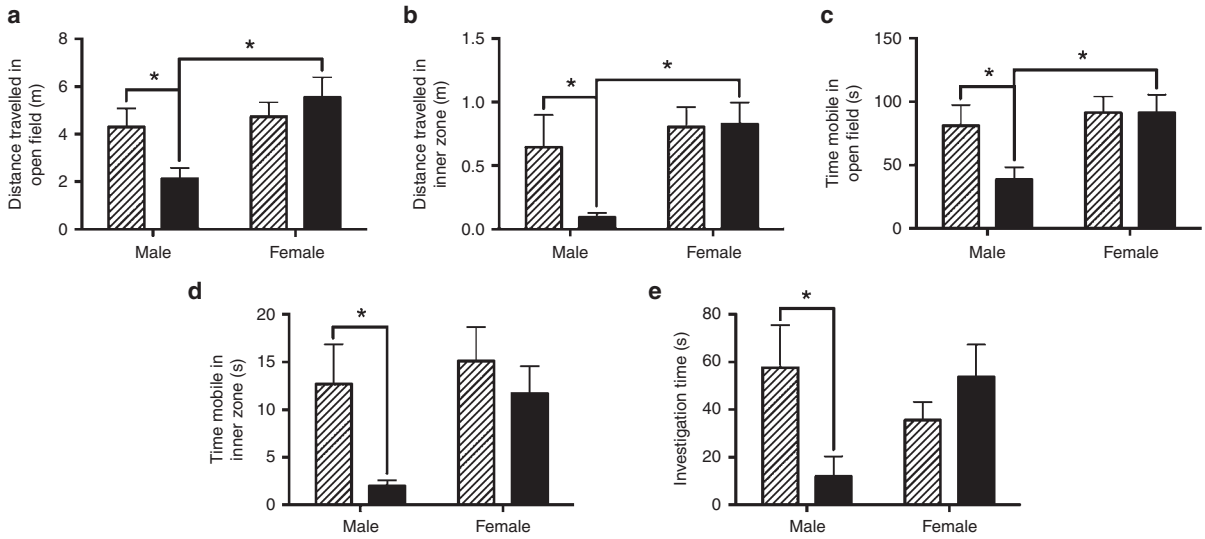

Figure 1. Open field and environment exploration behavioral measurements. (**a**) Distances travelled by preterm and term juvenile guinea pigs at corrected postnatal day 25 in the open-field arena and (**b**) the inner zone. (**c**) Time spent mobile in the open-field arena and (**d**) the inner zone. (**e**) Total time spent investigating foreign objects in the open-field arena. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal numbers are preterm males = 10, term males = 10, preterm females = 8, and term females = 10 for **a–d**. Animal numbers are preterm males = 8, term males = 6, preterm females = 6, and term females = 10 for **e**).

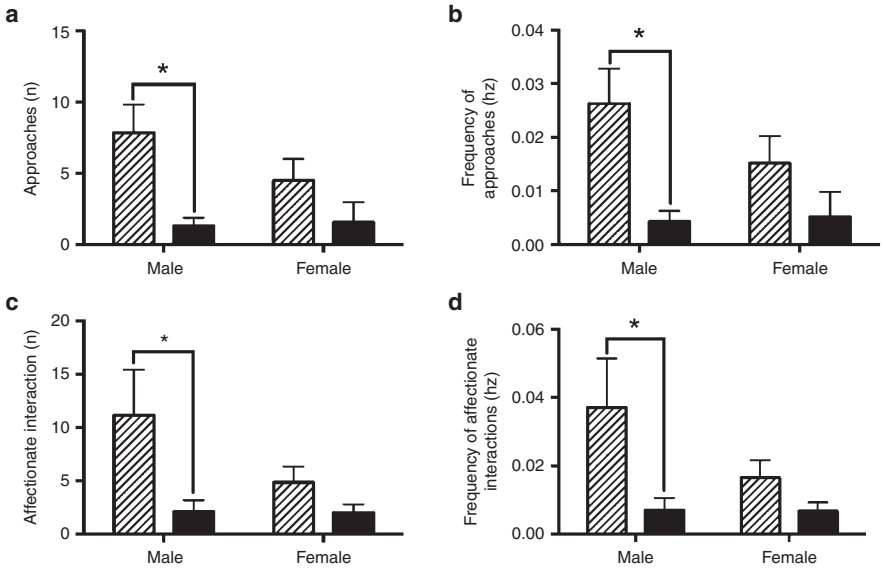

Figure 2. Social interaction behavioral measurements. (**a**) Total number and (**b**) frequency of approaches, and affectionate interactions (**c** and **d** respectively) toward a familiar animal by preterm and term juvenile guinea pigs at corrected postnatal day 25. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal numbers are preterm males = 7, term males = 10, preterm females = 6, and term females = 7.

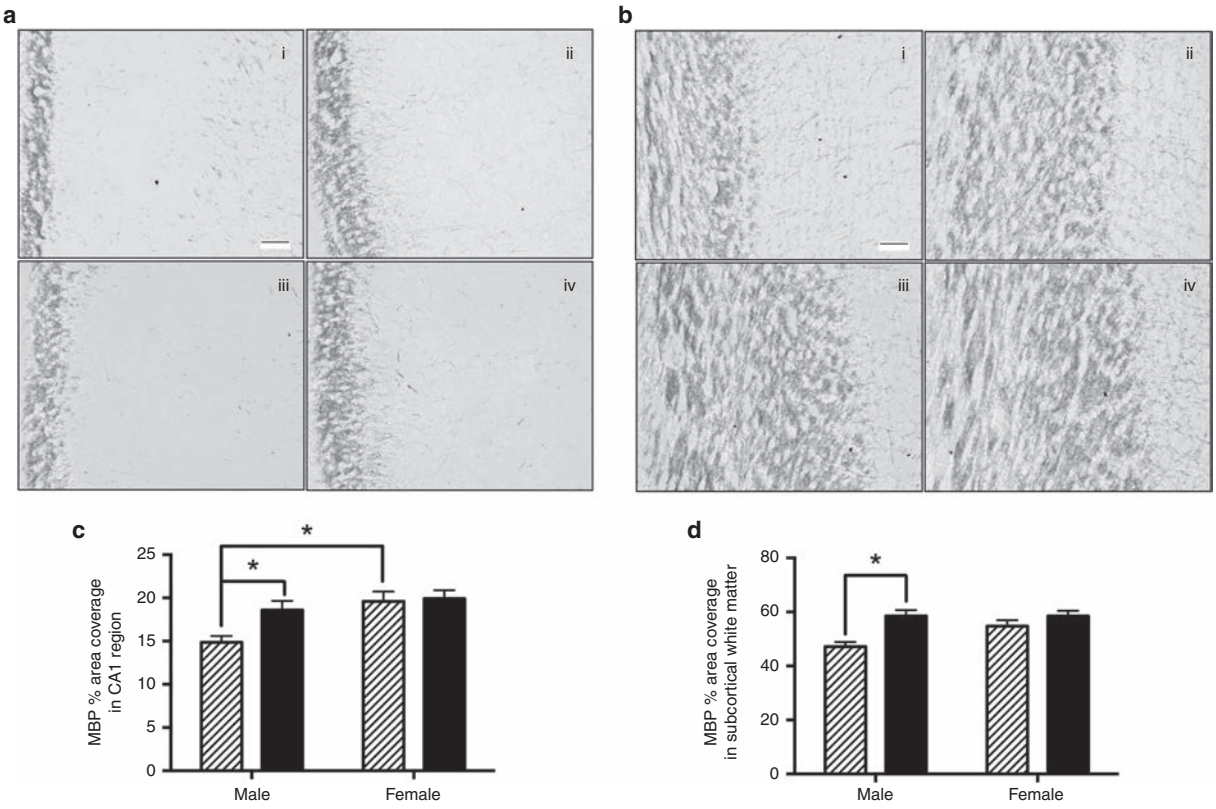

Figure 3. Myelin basic protein immunolabeling of juvenile guinea pig brain regions. Representative photomicrographs at 20× magnification of myelin basic protein immunolabeling and percent coverage in the juvenile guinea pig external capsule adjacent to the CA1 region of the dorsal hippocampus (**a** and **c**) and the adjacent subcortical white matter (**b** and **d**). Scale bar = 50 μm; (i) male preterm, (ii) male term, (iii) female preterm, and (iv) female term for all photomicrographs. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal groups are preterm males = 8, term males = 6, preterm females = 6, and term females = 7.

in the CA1 region of the dorsal hippocampus was lower for preterm males compared to both their term counterparts (Figure 3c, $P = 0.05$), and to preterm females ($P = 0.0098$). In the immediately adjacent subcortical white matter, preterm males exhibited decreased area coverage of MBP compared to term males (Figure 3d, $P = 0.0019$).

Astrocytes. The areas occupied by reactive astrocytes were compared by immunostaining for GFAP. GFAP area coverage in the CA1 region of the dorsal hippocampus was lower in the preterm males compared to both their term counterparts (Figure 4c, $P = 0.032$), and to preterm females ($P = 0.012$). Interaction between gestational age and sex was also significant in the CA1 region ($P = 0.010$). In the adjacent dentate gyrus, males exhibited decreased area coverage of GFAP compared to the females (Figure 4d, $P = 0.023$). Subcortical white matter expression was also analyzed, however, there were no significant differences identified between sexes or gestational ages (data not shown).

$GABA_A$ Receptor Subunit Expression

In hippocampal tissues, males had higher relative expression of the δ subunit compared to females (Figure 5a, $P < 0.0001$). Relative expression of the α5 subunit was reduced in the preterm males compared to their term counterparts (Figure 5b, $P = 0.0432$). Relative expression of the α6 subunit also appeared to be somewhat reduced in both the male and female preterm cohorts compared to their term counterparts, however, these differences in mean values did not reach significance (Figure 5c, $P = 0.0883$).

Salivary Cortisol Concentration

Salivary cortisol was assessed in juveniles before (baseline) and after (response) behavioral testing. Preterm males had higher cortisol concentrations prior to behavioral testing when compared to both term males and preterm females (Figure 6a, $P = 0.0147$, and $P = 0.0273$ respectively). Following behavioral testing, cortisol concentration was markedly higher in preterm females when compared to the term females (Figure 6b, $P = 0.0483$).

DISCUSSION

The key findings of this study were the marked behavioral differences of the preterm and term animals at an age equivalent of juvenility in the guinea pig and the associated impact

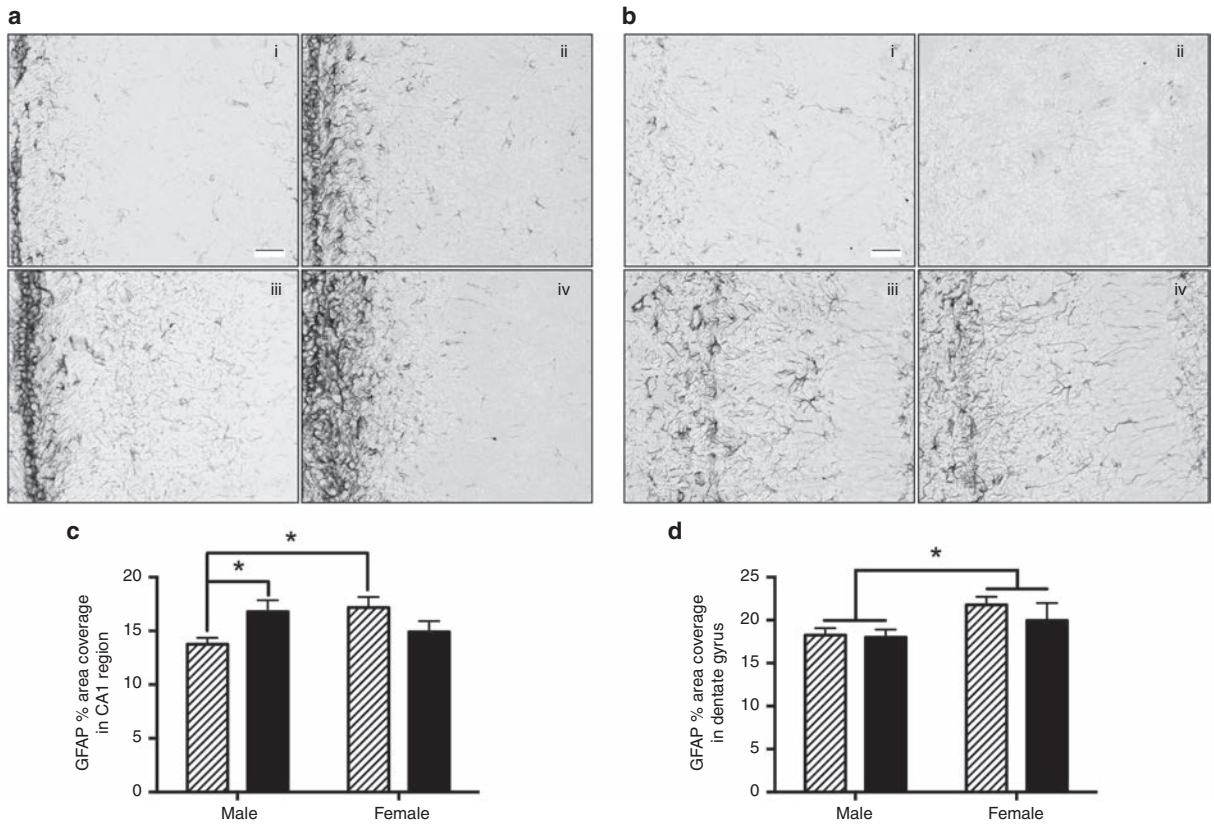

Figure 4. Glial fibrillary acidic protein immunolabeling of juvenile guinea pig brain regions. Representative photomicrographs at 20× magnification of glial fibrillary acidic protein immunolabeling and percent coverage in the juvenile guinea pig external capsule adjacent to the CA1 region of the dorsal hippocampus (**a** and **c**), and the dentate gyrus of the dorsal hippocampus (**b** and **d**). Scale bar = 50 μm; (i) male preterm, (ii) male term, (iii) female preterm, and (iv) female term for all photomicrographs. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal groups are preterm males = 7, term males = 8, preterm females = 7, and term females = 6.

of preterm delivery on the long-term expression of $GABA_A$ receptor subunits that influence neurosteroid sensitivity. Additionally, the results indicate that there are sex differences in the influences of prematurity on these parameters, with males seemingly more affected by early delivery. Furthermore, one of the most important findings was that the deficiencies in neurodevelopment of ex-preterm males seen previously in neonates remained until juvenility, whereas females were able to restore their initial reductions (25,26).

Our previous studies in guinea pigs showed markers of mature oligodendrocytes were reduced in preterm compared to term gestational age fetuses, and were markedly affected by preterm delivery in the neonatal hippocampus and subcortical white matter in both sexes (25,26). The use of an ex-preterm juvenile model in the present study allowed us to ascertain whether oligodendrocyte expression remains affected until childhood, when many of the school-related disabilities become apparent clinically. The study demonstrated the lasting impact of preterm delivery on males, with reductions of oligodendrocyte immunostaining in the late developing CA1 region of the dorsal hippocampus and adjacent subcortical white matter. In this study, mature myelinating oligodendrocyte expression was reduced in the CA1 region of the hippocampus in conjunction with reactive astrocyte expression. Oligodendrocytes are the cells responsible for myelination, whereas astrocytes are seen as the support cells of the central nervous system (CNS) and one of their roles is the promotion of myelinating activity by oligodendrocytes. ATP released by astrocytes induces secretion of a regulatory protein, which then acts directly on oligodendrocytes to stimulate myelin formation (28). Astrocytes have been shown to promote myelination *in vitro* and also to protect the CNS from trauma by preserving myelination and reducing white matter damage in a mouse model of spinal cord injury, in addition to demonstrating myelin repair properties in a mouse knockout of astrocytes (29–31). Therefore, a reduction in myelination in the hippocampus may be a result of the reduction in reactive astrocytes, however further investigation such as determining expression of early markers of immature and preoligodendrocyte cells may aid in teasing out this relationship. Reductions in reactive astrocyte expression were also apparent in males compared to females in the dentate gyrus, which may be due to general sex-specific differences in neurodevelopment rather than an effect of preterm delivery.

Quantitation of $GABA_A$ receptor δ, α5, and α6 subunit expression revealed that overall males exhibited increased

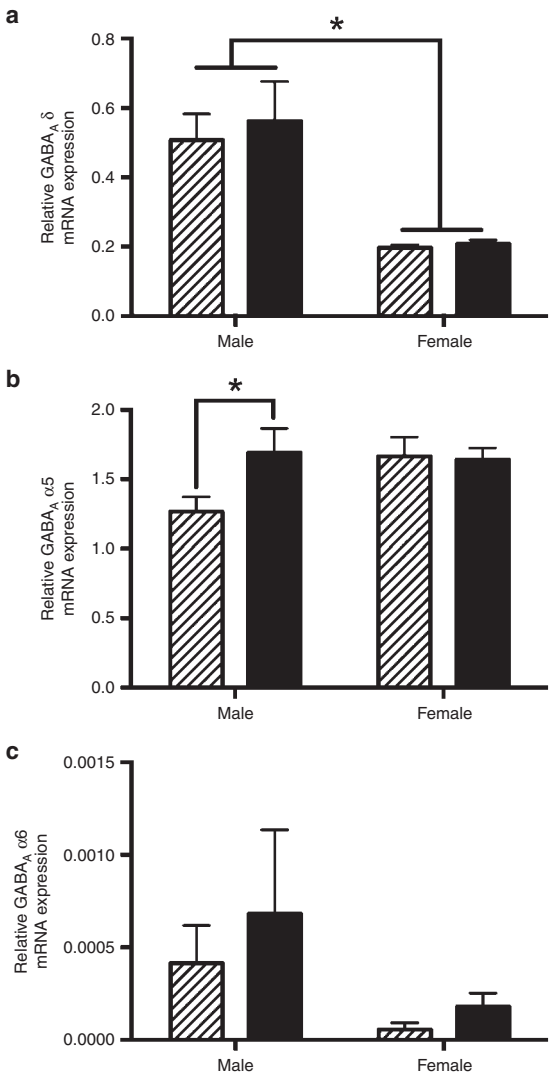

Figure 5. mRNA expression of GABA$_A$ receptor subunits in juvenile guinea pig hippocampus. Relative GABA$_A$ receptor (**a**) δ, (**b**) α5, and (**c**) α6 subunit mRNA expression in the hippocampus. Values are for preterm (62 d) and term (69 d) 28-d juvenile guinea pig hippocampal tissue. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal groups are preterm males = 10, term males = 8 (term males = 7 for **b**), preterm females = 7, and term females = 10 (term females = 9 for **b**).

expression of the δ subunit compared to females. This finding was unexpected as this subunit has a major role in allopregnanolone-receptor binding and increased levels would tend to increase allopregnanolone-mediated suppression of excitation. One possibility may be a dysregulation of neurosteroid action in response to changes in the balance of inhibitory and excitatory activity, however such possibilities require further examination. Interestingly, the expression of the α5 subunit was reduced in preterm males compared to the controls, with females seemingly unaffected. Animal studies have demonstrated that this subunit mediates tonic inhibition in the CA1 and CA3 of the hippocampus and is required for associative learning and memory (32,33). Therefore, the reduced expression of this subunit may have implications for learning and memory in school aged ex-preterm male children. A reduction in this subunit may be affecting overall tonic inhibition and suppression of excitation in the hippocampus, and thus contributing to the hyperactivity often exhibited by ex-preterm males. Targeting of the α5 subunit is a potential avenue for treating cognitive deficits, as this has become an option for schizophrenia patients where alterations in the functionality of the GABAergic system are suggested to contribute to impaired cognition (34).

The deficiencies in the markers of neurodevelopment and neurosteroid-sensitive receptors were associated with increased activity in the open field and environment exploration tests, with the preterm males more active than the control term males. Similar results are seen in mice expressing an attention deficit hyperactivity disorder-like phenotype, where the distance travelled and time spent travelling is markedly higher for the affected mice compared to the controls (35). This hyperactive type behavior has parallels with clinical studies on ex-premature male children that show an increased incidence of disorders that to some extent involve hyperactivity (7,8). This similarity in behavioral outcome supports the potential suitability of the guinea pig model for investigating key mechanistic links between prematurity related changes and the causation of these disorders and as a platform to investigate therapeutic treatment to target the development of these disorders. Additionally, preterm male juveniles exhibited reduced social inhibition in the familiar social testing. These findings could potentially be explained by the raised cortisol concentrations of the preterm males prior to the behavioral testing compared to the other cohorts, which suggests that they have a higher baseline level of stress than control animals. This higher cortisol baseline may be contributing to their hyperactive-type phenotype, however this relationship requires further investigation as other steroid hormones such as testosterone may be playing a role in the development of this phenotype. For all tests, the preterm females did not behave differently to the control term females, indicating that they are more resistant to effects on behavior, or are better able to adapt, despite their initial vulnerability in early life. This also parallels clinical findings that show females born preterm are more resistant to adverse outcomes compared to males, however are at higher risk of developing anxiety. Cortisol levels, and hence stress associated with the tests, suggested that the preterm females experience anxiety when introduced to the foreign behavioral testing experience as salivary cortisol was increased following the testing.

Some of the limitations that may be addressed in future studies include the preterm delivery dams receiving injections, which the term delivery dams did not, which may have introduced a degree of maternal stress due to the associated minimal pain. Prior to delivery, however, the dams were treated equally and it is unlikely this short-term minimal exposure to pain would affect the fetus shortly before delivery. Some of the

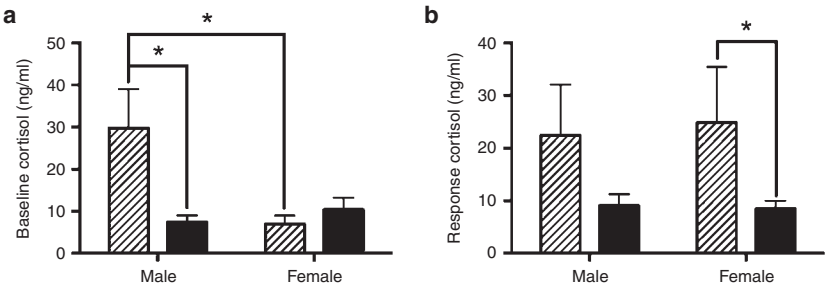

Figure 6. Salivary cortisol concentrations at baseline and in response to behavioural testing. Baseline (**a**) and response (**b**) salivary cortisol concentrations. Values are for saliva samples taken from preterm (62 d) and term (69 d) 25-d juvenile guinea pigs before (baseline) and after (response) behavioral testing. Mean ± SEM. Preterm = hashed bars, term = black bars; * indicates $P < 0.05$ between bars linked by black lines. Animal numbers are preterm males = 8 (preterm males = 9 for **b**), term males = 9 (term males = 10 for **b**), preterm females = 6, and term females = 10.

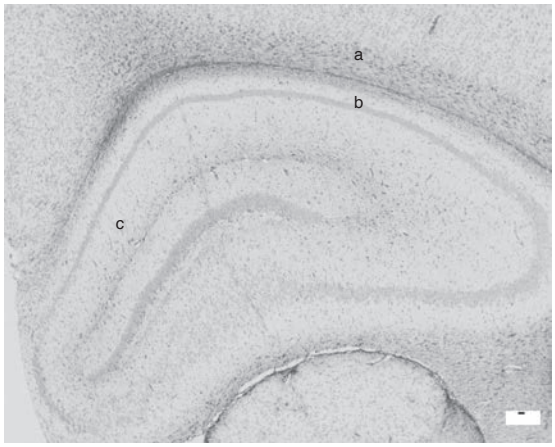

Figure 7. Representation of areas imaged for immunohistochemical analysis at 2× magnification. The above cresyl violet and glial fibrillary acidic protein-stained section highlights the specific areas of the (**a**) subcortical white matter, (**b**) CA1 region of the dorsal hippocampus, and (**c**) dentate gyrus.

injections that were only given to mothers delivering preterm were the two injections of betamethasone, which were given at a timing and dose that reflects current clinical practices. This minimal exposure to betamethasone is necessary for the preterm pup to survive and we suggest that any associated impact on neurosteroid synthesizing enzymes is minimal, as previous studies showing an effect have used much higher doses that were given repeatedly (36). Another essential limitation of the study was the altered treatment that the preterm pups required, including the resuscitation, nutritional supplementation, in addition to their lack of maternal grooming, which again the term delivered pups were not exposed to. These experiences are likely to have introduced an element of stress to the pups however are clinically relevant experiences for a preterm newborn to have, and thus means that our model mimics the treatment of a preterm newborn in the neonatal intensive care unit. A further unavoidable limitation was the absence of protein data for the $GABA_A$ receptor subunits examined, unfortunately due to a lack of guinea pig-specific antibodies, we were unable to obtain protein data and thus have presented the mRNA data.

Overall, this study has highlighted key late childhood differences in $GABA_A$ receptor subunit expression, myelination, and reactive astrocyte expression in the dorsal hippocampus of ex-preterm male juvenile guinea pigs, which coincided with increased salivary cortisol and a hyperactive-type phenotype. Alternatively, the females were found to be robust and seemingly unaffected by preterm delivery except for having a rise in cortisol associated with foreign situations, which parallels clinically with an increased risk of anxiety in ex-preterm females. These findings provide a basis for integral investigation of therapeutic options to protect the vulnerable ex-preterm male.

METHODS

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, Australia).

Animals

Approval for all animal experiments and procedures carried out throughout the study was obtained from both the University of Otago and the University of Newcastle Animal Care and Ethics Committees.

Mature breeding Dunkin Hartley female guinea pigs were obtained from the University of Otago Biomedical Research Unit. Guinea pigs were housed indoors and supplied with a diet consisting of standard commercial guinea pig pellets, lucerne hay, fresh fruit, and vegetables. Pregnant dams were randomly allocated to either preterm delivery or term delivery. Dams allocated to term delivery received no further interventions during pregnancy, with pups delivered spontaneously and receiving no respiratory or nutritional support. Preterm (GA62) pups were born following preterm induction of labor as previously described (37). Briefly, dams received Betamethasone 1 mg/kg subcutaneously (Celestone chronodose; Merck, Sharp & Dohme, Auckland, New Zealand) 48 and 24 h prior to delivery to accelerate fetal lung maturation. Aglepristone 10 mg/kg subcutaneously (Provet, Auckland, New Zealand), was administered 24 h prior to, and on the morning of delivery to inhibit progesterone-based continuance of pregnancy. Oxytocin 3 IU/kg intramuscularly (Provet) was administered to stimulate uterine contractions, beginning 1 h after the second Aglepristone dose and repeated in 30-min intervals until all pups and placentas were delivered.

Resuscitation and respiratory support of pups occurred as previously described (37). Briefly, pups were placed on a heated pad and respiration encouraged. Further respiratory support was given using "Neopuff" t-piece infant resuscitator (Fisher & Paykel, Auckland, New Zealand) with blended oxygen delivered at 5 l/min. If spontaneous respiration was not achieved or sustained, positive pressure ventilation at 60/min with an inflation pressure of 12 cm H_2O and expiratory pressure of 5 cm H_2O was provided until spontaneous respiration was observed. Additionally, continuous positive airway pressure support at 5 cm H_2O was given when pups exhibited adequate respiratory

drive but increased respiratory effort. All preterm pups were also given an initial fractional inspired oxygen concentration of 30% that was adjusted based on their colour, heart rate, and respiratory activity. Pups were housed with their mothers and littermates in a warm humidified human infant incubator (Dräger 8000 IC; Drägerwerk AG & Co., Lübeck, Germany); ambient temperature 30 °C, 70% humidity. Pups that were unable to achieve and maintain independent respiration, or showed other signs of distress (such as lack of weight gain) were excluded from the study and humanely euthanized.

Preterm pups received 0.3–0.5 ml of replacement colostrum (Impact guinea pig colostrum replacement; Wombaroo Food Products, Adelaide, Australia) orally by a 1 ml insulin syringe within the first hour after birth and then every 3 h until 24 h old. Between postnatal days 1–7, pups were fed 0.5–2.0 ml of replacement milk (Impact guinea pig milk replacement; Wombaroo Food Products) every 3 h or as needed to supplement independent suckling from the mother.

On postnatal day 6 (1 d prior to term equivalence), pups were transferred with their mothers from the infant incubator into a standard single cage at room temperature, and on postnatal day 7 into the nursery pen with other mothers and pups. Preterm and term neonates remained with their mothers in the nursery pen until weaning occurred at corrected PND21, after which they were placed into floor pens with animals of the same sex.

Juveniles were euthanized at corrected PND28 by sodium pentobarbitone (Lethabarb; Virbac, Milperra, Australia) 0.3 ml. At this time, body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the two hemispheres. Each left hemisphere was fixed for immunohistochemistry, whilst the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

Behavioral Testing

Juveniles underwent behavioral testing at corrected PND25. A pretest saliva sample was obtained from each animal immediately prior to the first test by allowing the pup to chew a cotton bud. A post-test sample was taken no more than 30 min later following the completion of the environment exploration testing. ANY-maze tracking software version 4.7 (Stoelting, Wood Dale, Illinois) was used to analyze the videos. All salivary samples and behavioral testing was performed by staff familiar to the animals, in a designated space free from the sight, sound, or scent of other animals.

Open Field and Environment Exploration Test

The open-field test measured anxiety and locomotion and was performed as previously described (38). Guinea pig pups were allowed to explore the arena (40 × 40 cm) for 10 min, with entries into the inner zone (an area 20 × 20 cm in the centre of the arena) recorded.

The environment exploration test measured the animals' exploratory behavior and anxiety (38). Following the open-field test, the animal was removed from the arena and two identical objects were placed in the center of the top two quadrants of the arena. The animal was then placed back into the arena and allowed to investigate the objects for 10 min.

Parameters measured included distance travelled in the entire arena and total time mobile, in addition to distance travelled and time spent mobile within the inner zone, and time spent investigating the foreign objects.

Familiar Social Test

The familiar social test measured social interactions towards a familiar animal. The test animal was placed into the arena with an animal of the same sex and age from the same home pen. For 5 min, the pair of animals were allowed to interact. Parameters measured included approaching, and having an affectionate or agonistic interaction with the familiar animal.

Immunohistochemistry

Myelination and reactive astrocyte expression was examined in the CA1 region of the dorsal hippocampus, in addition to the adjacent subcortical white matter and dentate gyrus (**Figure 7**). Immunohistochemistry was performed on 8-μm sections of paraffin-embedded brains that were cut using a Leica RM2145 Microtome (Leica Microsystems Pty, North Ryde, Australia) (25,39). Tissues were dewaxed, incubated in citrate buffer (pH 6.0) and phosphate-buffered saline containing 3% hydrogen peroxide, and blocked for 1 h at room temperature in Bovine serum albumin blocking solution (0.5% w/v bovine serum albumin, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1M phosphate-buffered saline). Incubation in primary antibodies (MBP, M9434; and GFAP, G3893) and secondary antibodies (biotinylated anti-rat IgG, B7139; and anti-mouse IgG, B6649 respectively) were performed before tertiary incubation in streptavidin-biotin-horseradish peroxidase complex. To reveal the immunolabeling, incubation in 3,3'-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; ThermoFisher Scientific, Scoresby, Australia) occurred.

Stained slides were imaged using the Aperio imaging system (Leica Biosystems). Percent area coverage at 20× magnification was used to quantify staining. ImageJ version 1.47 (National Institutes of Health, Bethesda, MD) was used to calculate area coverage by converting to gray-scale and then binary, and manually adjusting threshold based on the original stained image (25,39). An overall average of area coverage staining was calculated by taking the average of three images captured from two consecutive sections per animal. Sections of only the highest standard were used for analysis, with poorer sections excluded from the study.

Real-Time PCR

RT-PCR was performed as previously described (25,36). Frozen hippocampal tissue was homogenized in RLT Plus Buffer (Qiagen RNeasy Plus Mini Kit; Qiagen Pty, Chadstone Centre, Australia) using a Precellys 24 dual tissue homogenizer (Bertin Technologies, Provence, France). RNA was extracted using the Qiagen RNeasy Plus Mini Kit. Samples with poor RNA A260/280 ratios and integrity on an RNA gel were not used for further analysis.

Superscript III Reverse Transcription kit (Invitrogen, Carlsbad, CA) was used to synthesize cDNA on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty, Mulgrave, Australia). RT-PCR was performed using a 7500 ABI real-time machine (Applied Biosystems), for primer pairs (δ, α5, and α6) and the housekeeping gene β-actin (25). Each sample was run in duplicate along with an associated negative control sample. Products were detected using the SYBR Green (Applied Biosystems) DNA binding dye method. Results were analyzed by Sequence Detection Software v2.01 (Applied Biosystems) and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate relative fold change. Controls included β-actin and a calibrator, which consisted of pooled brain samples and was used across all plates. Consistent Ct values were obtained for β-actin across the term/preterm and male/female samples.

Cortisol Enzyme-Linked Immunosorbent Assay

The Salimetrics Salivary Cortisol Assay (Salimetrics, State College, PA) was used to measure cortisol concentrations in guinea pig saliva samples. As previously described, the sensitivity of the assay is 0.012–3.0 μg/dl, with the inter- and intra-assay coefficients of variance 6.89 and 5.52% respectively (40).

Statistical Analysis

Data was analyzed by two-way ANOVA and unpaired *t*-tests in same-sex groups using Graphpad Prism software (version 6.01; Graphpad Software, La Jolla, CA). When a significant difference was found, Tukey *post-hoc* tests and corrections for multiple comparisons were performed. Unless otherwise stated, all data are expressed as mean ± SEM and significance considered as $P < 0.05$.

ETHICAL STANDARDS

The authors assert that all animal work performed complies with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Animal Ethics Advisory Committee of New Zealand, and the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes), and has been approved by the institutional committees (University of Otago, Wellington Animal Ethics Committee, and the University of Newcastle Animal Care and Ethics Committee).

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5.0 "Disruption of the cerebellar GABAergic system in juvenile guinea pigs following preterm birth"

This accepted manuscript teases out the effects of preterm birth on neurodevelopment and the inhibitory GABAergic system within the vulnerable cerebellum of the juvenile guinea pig.

Accepted manuscript

Author	Contribution	Signature
Julia C Shaw	Experimental design Animal protocols and tissue collections Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K Palliser	Experimental design Animal protocols and tissue collections Manuscript corrections	
Rebecca M Dyson	Experimental design Animal protocols and tissue collections Manuscript corrections	
Mary J Berry	Experimental design Animal protocols and tissue collections Manuscript corrections	
Jonathan J Hirst	Experimental design Manuscript corrections	

Professor Robert Callister Date: 14/09/17

Assistant Dean Research Training

Abstract

Background: Children that are born preterm are at an increased risk of developing cognitive problems and behavioural disorders, such as attention deficit hyperactivity disorder (ADHD). There is increasing interest in the role of the cerebellum in these processes and the potential involvement of GABAergic pathways in neurodevelopmental disorders. We propose that preterm birth, and the associated loss of the trophic intrauterine environment, alters the development of the cerebellum, contributing to ongoing neurobehavioral disorders.

Methods: Guinea pigs were delivered preterm (GA62) or spontaneously at term (GA69), and tissues collected at corrected postnatal day (PND) 28. Neurodevelopmental and GABAergic markers myelin basic protein (MBP), neuronal nuclei (NeuN), calbindin (Purkinje cells), and GAD67 (GABA synthesis enzyme) were analysed in cerebellar lobules IX and X by immunohistochemistry. Protein expression of GAD67 and GAT1 (GABA transporter enzyme) were quantified by western blot, whilst neurosteroid-sensitive $GABA_A$ receptor subunits were measured by RT-PCR.

Results: MBP immunostaining was increased in lobule IX of preterm males, and reduced in lobule X of preterm females when compared to their term counterparts. GAD67 staining was decreased in lobule IX and X of the preterm males, but only in lobule X in the preterm females compared to term cohorts for each sex. Internal granule cell layer width of lobule X was decreased in preterm cohorts of both sexes compared to terms. There were no differences for NeuN staining, GAD67 and GAT1 protein expression as measured by western blotting, or GABAA receptor subunits as measured by RT-PCR between preterm and term for either sex.

Conclusions: The present findings suggest that components of the cerebellar GABAergic system of the ex-preterm cerebellum are disrupted. The higher expression of myelin in the preterm males may be due to a deficit in axonal pruning, whereas females have a delay in myelination at 28 corrected days of age. Together these ongoing alterations may contribute to the neurodevelopmental and behavioural disorders observed in those born preterm.

Introduction

The incidence of moderate-late preterm birth (32-36 weeks of gestation) has significantly increased in developed nations over the past two decades[3, 4, 26, 28]. In line with this rise, it is also becoming increasingly evident that survivors of moderate-late preterm birth are at an increased risk of neurodevelopmental disorders in childhood and adolescence[2, 5-10]. Along with anxiety, attention deficit hyperactivity disorder (ADHD) is the most commonly diagnosed neurodevelopmental disorder in school-aged ex-preterm children[56, 57]. ADHD is characterized by a deficit in behavioural inhibition, inattention, impulsivity and social difficulties, and in a cohort of preterm/low birth weight children at 5 years old it was more commonly diagnosed in males following preterm birth compared to term birth[58]. In the same cohort, preterm females were more likely to be diagnosed with anxiety disorder than females born at term[58]. In a large Danish cohort of 2-18 year old children, preterm birth at 34-36 weeks of gestation was associated with an 80% increased risk of being diagnosed with hyperkinetic disorder (characterized by inattention, hyperactivity, and impulsivity) compared to children born after 37 weeks gestation, interestingly 90% of affected children were male[57]. Furthermore, there is a 33% increased chance of developing depression in childhood and adolescence in those born preterm compared to term, as reported by parents in the USA[60].

In addition to an increased incidence of neurodevelopmental disorders, ex-preterm children are more likely to have increased difficulty in learning and memory tasks beginning from a young age, as children born preterm who do not have evidence of major neuroanatomical injury (at the time of discharge home) may still have specific

difficulty in sustained attention, visuospatial processing, and spatial working memory when evaluated at ages 3-4[66]. At school-age, children born preterm have an increased need for special education, increased risk of repeating a grade, and lower reading and mathematics scores compared to term-born children[5]. Specifically, for moderate-late ex-preterm children many cohort studies have identified a 1-2 fold increased risk for requiring special education or repeating a grade at ages 5-10[61-64]. The development of these disorders and school-related difficulties can manifest in ex-preterm children who had no gross neuroanatomical injury in the newborn period[66, 138]; thus, absence of significant injury is not necessarily an indicator of later neurodevelopmental function and wellbeing. In this study we focused on identifying any subtle or diffuse differences in the cerebellum during childhood using our juvenile guinea pig model of preterm birth.

The posterior lobe of the cerebellum is interconnected with the prefrontal cortex, association cortices, and the limbic system, which allows for its involvement in higher executive functioning[269, 270]. Interestingly, it has been shown that disruptions to cerebellar development can have a major impact on the regions of the brain to which it projects[94]. Preterm survivors of cerebellar parenchymal injury (haemorrhage within the parenchyma) were shown to have impaired growth of the uninjured contralateral cerebral hemisphere in areas such as the dorsolateral prefrontal cortex and mid-temporal regions[95]. Furthermore, in a small study of age-matched 2-4 year olds 66% of survivors displayed social-behavioural deficits such as inattention and internalizing behaviour, as well as cognitive and language deficits[96]. Furthermore, it is also suggested that cerebellar injury in early life has more dramatic and long-lasting effects than damage late in life, presumably due to its role in skill

acquisition and lesser role in retention of learned behaviours[92, 93, 97]. Decreases in the white matter volumes of regions often impacted by preterm birth such as the hippocampus and cerebellum[11, 12, 135] are suggested to play a key role in the defects in neurodevelopment and behaviour following preterm birth[133]. In cases of moderate-late preterm birth, often the type of neuroanatomical injury is a subtle and diffuse delay in myelination compared to more marked decreases in brain volume seen in those born at lesser gestational ages[135, 138] when large-scale increases in brain structures are occurring. The cerebellum and hippocampus are particularly vulnerable to damage and developmental delay following preterm birth as they each continue to mature throughout late gestation[11-15], and this trajectory of maturation is inevitably altered following preterm birth.

Loss of the placental supply of the neurosteroid allopregnanolone occurs earlier than normal in neonates that are born preterm[155, 158]. In the guinea pig, we have demonstrated a dramatic drop in brain allopregnanolone concentrations following both term and preterm birth compared to fetal levels[21, 237]. We have also shown that preterm guinea pigs have significantly decreased myelination (as evidenced by less MBP expression) in the CA1 region of the hippocampus, the adjacent subcortical white matter, and the cerebellum 24 hours after delivery and at term equivalent age[21, 238, 271]. Furthermore, we then also identified a long lasting reduction in myelination of the subcortical white matter and CA1 region of the hippocampus in juvenile guinea pigs born preterm[272]. Juvenile ex-preterm guinea pigs also exhibited behavioural disturbances at the equivalence of childhood, supporting the clinical relevance of the model; specifically, the preterm male guinea pigs displayed a hyperactive phenotype (ADHD-type) compared to male term-born

animals[272]. Conversely, the preterm female guinea pigs exhibited anxiety-related behaviour compared to the female term-born animals[272]. These effects of preterm birth on long-term behaviour are potentially in part due to dysfunction of the GABAergic system.

The GABAergic system is integral to proper functioning of the nervous system as it plays a large role in essential processes including the regulation of neuronal activity levels, neuronal plasticity, and synapse formation[226]. GABA, the main neurotransmitter of the GABAergic system[273], is responsible for inhibition throughout the brain by hyperpolarizing the membrane and shunting excitatory inputs[274]. Dysfunction of the GABAergic system has been implicated in a number of psychiatric disorders including ADHD[228, 229], anxiety[234, 235], and depression[230-233], as well as myelin degenerative disorders such as Parkinson's disease[227]. Magnetic resonance spectroscopy in children aged between 8-12 with ADHD identified reduced concentrations of GABA in the brain of children with ADHD compared to age-matched controls[228], suggesting a reduced capacity for production of GABA and thus reduced inhibition throughout the brain. Deficits in the expression of $GABA_A$ receptors in the hippocampus and temporal areas have been observed in patients with major depressive disorder, post-traumatic stress disorder, and anxiety at postmortem[230, 231, 234, 235]. These deficits in human patients have also been confirmed in animal studies, with several studies demonstrating the increase in anxiety-like behaviour with a global deletion of the δ subunit of the $GABA_A$ receptor[209, 212, 222, 236]. These findings suggest that there is impairment of the GABAergic system, and an imbalance between inhibitory and excitatory arms may be involved in the development of neuropsychiatric disorders as a decrease in GABA

concentration and action is thus associated with an increase in glutamate and excitation[233]. In previous studies, we have identified an altered expression of $GABA_A$ receptors in the preterm guinea pig neonate and juvenile in both the cerebellum and hippocampus[271, 272], suggesting that the GABAergic system may also be deregulated following preterm birth in this species increasing the clinical relevance of the preterm guinea pig model. The aim of the current study was to determine the effects of preterm birth on the development of the cerebellum in the guinea pig. We hypothesized that preterm birth is associated with disruption of neurodevelopment in specific areas within the cerebellum as a result of early exposure to the *ex utero* environment resulting in impacts on the GABAergic pathway.

Methods

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, Australia).

Animals

Approval for all of the animal experiments and procedures carried out throughout the study was obtained prior to commencement from the University of Otago, Wellington and the University of Newcastle Animal Care and Ethics Committees.

Pregnant Dunkin Hartley female guinea pigs were obtained from the University of Otago Research Biomedical Research Unit. Guinea pigs were housed indoors and were supplied with a diet consisting of standard commercial guinea pig pellets, lucerne hay, fresh fruit and vegetables. Pregnant dams were randomly allocated to either preterm (GA62) delivery or term (GA69) delivery. Dams allocated to term delivery received no further interventions during pregnancy, with pups delivered spontaneously and receiving no respiratory or nutritional support. All pups (term and preterm) were handled at least daily from birth until weaning and serial growth measurements obtained. Preterm pups were born following preterm induction of labour as previously described[256, 272]. In brief, dams received Betamethasone 1mg/kg subcutaneously (Celestone chronodose; Merck, Sharp & Dohme, Auckland, New Zealand) 48 and 24 hours prior to delivery to accelerate fetal lung maturation and surfactant production. Aglepristone 10mg/kg subcutaneously (Provet, Auckland, New Zealand), a progesterone receptor antagonist, was also administered 24 hours prior to,

and on the morning of delivery to inhibit progesterone-based continuance of pregnancy. Oxytocin 3IU/kg intramuscular (Provet) was then administered to stimulate uterine contractions, beginning 1 hour after the second Aglepristone dose and repeated in 30-minute intervals until all pups and placentas were delivered.

Resuscitation and respiratory support of pups occurred as previously described[256, 272]. Briefly, immediately following delivery pups were placed on a heated pad under a heat lamp and respiration encouraged. Respiratory support was provided for at least 5 minutes to all preterm pups by continuous positive airway pressure (CPAP) support at 5cm H_2O using the "Neopuff" t-piece infant resuscitator (Fisher & Paykel, Auckland, New Zealand) with blended oxygen delivered at 5L/min. If spontaneous respiration was not achieved or sustained, positive pressure ventilation at 60 breaths/min with an inflation pressure of 12cm H_2O and expiratory pressure of 5cm H_2O was provided until spontaneous respiration was observed. Additionally, all preterm pups were also given an initial fractional inspired oxygen concentration of 30% that was adjusted based on the colour, heart rate, and respiratory activity of the pup. Once stable respiration was maintained, pups were housed with their mothers and littermates in a warm humidified human infant incubator (Dräger 8000 IC; Drägerwerk AG & Co., Lübeck, Germany); ambient temperature 33°C (titrated down to 28°C over the course of first 24 hours), and humidity at 60% (titrated down to 35% by 12 hours). Pups that were unable to achieve and maintain independent respiration, or showed other signs of distress (such as lack of weight gain) were excluded from the study and humanely euthanized.

Preterm pups received 0.3-0.5mL of replacement colostrum (Impact guinea pig colostrum replacement; Wombaroo Food Products, Adelaide, Australia) orally by a

1mL syringe within the first hour after birth and then every 3 hours until 24 hours old. Between postnatal days 1-7 the pups were fed 0.5-2.0mL of replacement milk (Impact guinea pig milk replacement; Wombaroo Food Products) every 3 hours or as needed to supplement independent suckling from the mother.

On PND7 (one day prior to term equivalence), pups were transferred with their mothers from the infant incubator into a standard single cage at room temperature, and on PND8/term equivalence age/corrected PND0 into the nursery pen with other dams and pups. Preterm and term neonates remained with their mother in the nursery pen until weaning occurred at corrected PND21 (calculated from term equivalence age), after which they were placed into floor pens with animals of the same sex.

These juveniles underwent behavioural testing in the open field, environment exploration, and social interaction tests at corrected PND25 (these results have been previously published[272] and thus are not presented here). Juveniles were then euthanized at corrected PND28 by sodium pentobarbitone (Lethabarb; Virbac, Milperra, Australia) 0.3mL for tissue collection. At this time body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the two hemispheres. Each left hemisphere was fixed for immunohistochemistry, whilst the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

Immunohistochemistry

Mature myelinating oligodendrocytes, reactive astrocytes, neuronal nuclei, Purkinje cells, and GABA synthesizing cell expression were examined in midsagittal sections of cerebellar lobules IX and X, and the deep white matter of the cerebellum.

Immunostaining for myelin basic protein (MBP) was used to quantify mature myelin area coverage, Calbindin to examine Purkinje cell number and cell layer thicknesses, neuronal nuclei (NeuN) to compare neuron area coverage, and GAD67 to quantify the number of GABA synthesizing cells. Immunohistochemistry was performed on 8µm sections of paraffin-embedded brains that were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, Australia) as previously described[109, 271, 272]. Tissues were dewaxed, incubated in citrate buffer (pH 6.0) and PBS containing 3% hydrogen peroxide, and then blocked for 1 hour at room temperature in BSA Blocking Solution (0.5% w/v BSA, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1M PBS). Incubation in primary antibodies [MBP, M9434; NeuN, MAB377 (Millipore); calbindin, ab49899 (Abcam); or GAD67 (K-87) ab26116] and secondary antibodies [biotinylated anti-rat IgG, B7139; anti-mouse IgG, B6649; or anti-rabbit IgG, RPN1004 (Amersham)] were performed before tertiary incubation in streptavidin-biotin-horseradish peroxidase complex. To reveal the immunolabelling, incubation in 3,3'-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; ThermoFisher Scientific, Scoresby, Australia) occurred.

Stained slides were imaged using the Aperio imaging system (Leica Biosystems, North Ryde, Australia). Percent area coverage at 20x magnification was used to quantify staining of MBP, GFAP, and NeuN. ImageJ version 1.47 (National Institutes of Health, Bethesda, Maryland) was used to calculate area coverage by converting to gray-scale and then binary, and manually adjusting threshold based on the original stained image[271, 272]. An overall average of area coverage staining was then calculated by taking the average of three images captured from each of the two consecutive sections per animal. For Purkinje cell and GABA synthesizing (GAD67) cell

number analysis, the number of positively stained cells were counted per lobule and expressed as number of cells/mm^2. Cerebellum layer thicknesses were measured as a percentage of total lobule width to account for differences in overall lobule size.

Immunoblotting

Total cerebellar protein expression of the GABA transporting enzyme GAT1 and the GABA synthesizing enzyme GAD67 was measured by western blot. Whole cell protein was extracted from frozen cerebellum by extraction in RIPA buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor Cocktail (Roche, North Ryde, NSW, Australia) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Homogenization was performed using the Precellys 24 dual tissue homogenizer (Bertin Technologies, Provence, France) followed by centrifugation and collection of supernatant. Protein concentrations were then determined by BCA assay (Thermo Fisher Scientific), which is a two-component, high-precision, detergent-compatible assay, and was performed by following the manufacturers instructions. The Bolt Mini Gel Western Blot system (Thermo Fisher Scientific) was used for electrophoresis of the protein in 4-12% Bis-Tris 1.0mm gels, and then transfer onto PVDF membrane (Thermo Fisher Scientific).

Membranes were then blocked overnight in 5% BSA/5% Skim milk in 0.1% TBS-T (Tris HCl, NaCl, Tween-20) at 4°C. Primary incubation was then carried out for 2 hours at room temperature in the appropriate antibody [Polyclonal anti-GAT1 ab426 (Abcam); or monoclonal anti-GAD67 ab26116 (Abcam)] and 5% BSA in 0.1% TBS-T. Following washes in 0.1% TBS-T the membranes were then incubated in secondary

antibody [goat anti-rabbit conjugated HRP 12-348 (Millipore); or goat anti-mouse conjugated HRP 65-6420 (Invitrogen) respectively] and 5% BSA in 0.1% TBS-T for 1 hour at room temperature. Detection of protein bands was then performed by ECL detection (Amersham, GE Healthcare) following washes in 0.1% TBS-T and TBS. Protein detection was imaged on the Amersham Imager 6000 (Amersham, GE Healthcare).

Following ECL detection the membranes were stripped by NaOH to allow for detection of the loading control (β-actin). Membranes were blocked in 3% skim milk in 0.1% TBS-T for 1 hour, followed by 1 hour in primary antibody [polyclonal anti- β-actin ab8227 (Abcam)] in 3% skim milk in 0.1% TBS-T at room temperature. Secondary antibody (goat anti-rabbit conjugated HRP) incubation occurred under the same conditions. Protein detection was then performed as described above.

Analysis of protein concentration for each antibody of interest was performed using the Amersham Imager 6000 software. The area of positive staining was measured by the software and then divided by the value obtained for the loading control (β-actin) of that sample. As all samples were run in duplicate, the average of these two resulting numbers was taken. To obtain the final value used for statistical analysis, each average per sample was then normalized to an internal control sample on each membrane.

Real time PCR

The real-time PCR was performed as previously described[271]. Frozen cerebellum tissue was homogenized in RLT Plus Buffer (Qiagen RNeasy Plus Mini Kit; Qiagen Pty Ltd, Chadstone Centre, Australia) using a Precellys 24 dual tissue homogenizer. RNA was extracted using the Qiagen RNeasy Plus Mini Kit following the manufacturer's directions. Samples with poor RNA A260/280 ratios (below 1.6) and integrity on an RNA gel (signified by smearing of bands) were not used for further analysis.

Superscript III Reverse Transcription kit (Invitrogen, Carlsbad, California) was used to synthesize 33.3ng/µL of cDNA from 1µg of RNA per sample on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, Australia) according to the manufacturers instructions. RT-PCR was performed using a 7500 ABI real-time machine (Applied Biosystems), for primer pairs ($GABA_A$ receptor subunits δ, α5, and α6) and the housekeeping gene β-actin[271]. Each sample was run in duplicate along with an associated negative control sample where reverse transcriptase was absent during the reverse transcription process. Products were detected using the SYBR Green (Applied Biosystems) DNA binding dye method. Results were analysed by Sequence Detection Software v2.01 (Applied Biosystems) and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate relative fold change. Controls included β-actin and a calibrator, which consisted of pooled brain samples and was used across all plates. Consistent Ct values were obtained for β-actin across the term/preterm and male/female samples.

Statistical Analyses

Data was analysed and graphs made using Graphpad Prism Software (version 7, Graphpad Software Inc., La Jolla, CA, USA). Unpaired t-tests in same-sex groups were used to identify significant differences between delivery groups. The data is expressed as mean ± SEM and significance considered $p<0.05$. Each dam was represented by a maximum of 2 pups/sex.

Results

Physical characteristics

The average litter size across the term and preterm deliveries was three, and the number of pups that were either stillborn or were euthanized in the early neonatal period was 13.6% for term pups and 30% for preterm pups. Mean gestational ages at delivery were 62.0±0 and 68.9±0.3 for preterm and term pups, as expected this was significantly earlier for the preterm pups compared to the term ($p<0.0001$).

Mean birth, term equivalence age, and postmortem weights, as well as organ-to-body weight ratios following tissue collection at corrected postnatal day (PND) 28 for the juveniles used in the present study are presented in Table 1. Within each sex, birth weights of the preterm neonates were lower compared to term neonates (males $p=0.002$, females $p=0.004$). Preterm males weighed significantly more than term males at corrected PND28 ($p=0.002$), however there was no difference between the weights of the females at corrected PND28 or between the weights of preterm and term of either sex at term equivalence age. Brain-body weight ratio was significantly smaller for preterm males compared to term ($p=0.0009$). There were no other significant differences identified for brain and organ weight ratios between preterm and term cohorts.

Table 1. Juvenile physical characteristics

	Male		Female	
	Preterm	**Term**	**Preterm**	**Term**
Pups (n)	10	10	8	10
Dams (n)	8	8	8	9
Birth Weight (g)	76.3 ± 1.9*	94.2 ± 4.2	74.5 ± 5.2*	99.8 ± 5.5
Term Equivalence Weight (g)	96.0 ± 3.5	94.2 ± 4.2	92.6 ± 6.7	99.8 ± 5.5
Postmortem Weight (g)	322.0 ± 7.3*	270.1 ± 10.8	265.7 ± 21.7	286.7 ± 16.5
Brain-Body Weight	1.04 ± 0.03*	1.26 ± 0.04	1.3 ± 0.09	1.20 ± 0.06
Cerebellum-Brain Weight	13.6 ± 0.7	15.7 ± 1.5	13.2 ± 0.6	14.4 ± 1.2
Heart-Body Weight	0.35 ± 0.01	0.36 ± 0.02	0.33 ± 0.03	0.36 ± 0.01
Kidney-Body Weight	0.92 ± 0.03	0.86 ± 0.02	0.91 ± 0.04	0.84 ± 0.02
Liver-Body Weight	4.74 ± 0.2	4.50 ± 0.2	4.16 ± 0.2	4.01 ± 0.1
Adrenal-Body Weight	0.03 ± 0.0009	0.03 ± 0.001	0.04 ± 0.004	0.04 ± 0.002
Subcutaneous fat-Body Weight	1.15 ± 0.1	1.03 ± 0.1	0.99 ± 0.06	1.22 ± 0.2
Visceral fat-Body Weight	0.41 ± 0.06	0.41 ± 0.07	0.31 ± 0.05	0.32 ± 0.04

Values are displayed as mean ± SEM. Birth, term equivalence, and postmortem weights are in grams. All other measurements are organ-to-body weight ratios expressed as percentages. * denotes significance compared to term cohort within sex.

Immunohistochemistry

Quantification of mature myelin area coverage in lobule IX showed increased levels of myelin in preterm males (Figure 1a, p=0.002), whereas in lobule X preterm females had significantly less myelin coverage (Figure 1b, p=0.0008) when compared to their respective term counterparts. There were no significant differences identified in the deep white matter between preterm and term animals of either sex (refer to Supplementary Table 10.2).

Calbindin immunostaining for positive Purkinje cells identified no difference in the number of positive cells/mm2 (refer to Supplementary Table 10.2) between preterm and term animals within each sex. Preterm females had a thinner lobule X width compared to term females (Figure 2a, p=0.003), whilst both male and female preterm animals had smaller internal granule cell layer percentages compared to their term counterparts (Figure 2b, p=0.04 and p=0.0003 respectively). There were no significant differences identified in the molecular or Purkinje cell layers within each sex between preterm and term cohorts (refer to Supplementary Table 10.2).

There were no significant differences in neuronal expression as measured by area coverage of NeuN in lobule IX, lobule X, or the deep white matter within each sex between preterm and term cohorts (refer to Supplementary Table 10.2).

Preterm males had significantly less GAD67+ Purkinje cells in both lobules IX and X compared to term males (Figure 3a, p=0.01 and Figure 3b, p=0.01). Similarly, preterm females had significantly less GAD67+ cells in lobule X (Figure 3b, p=0.02) compared to term females whilst no difference was identified in lobule IX for either sex between preterm and term cohorts.

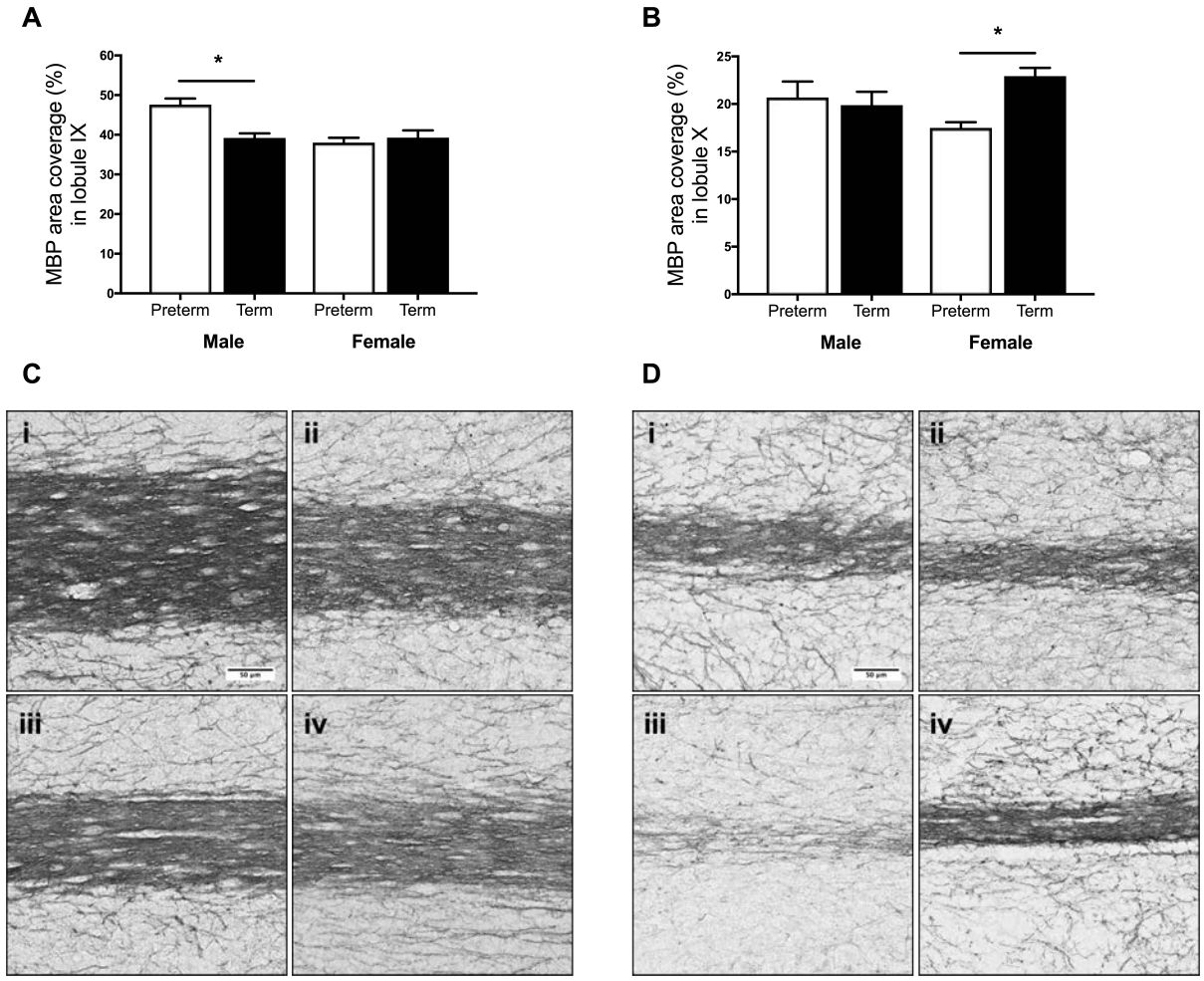

Figure 1. Immunostaining for myelin basic protein (MBP) in the cerebellum of guinea pigs at corrected postnatal day 28 in a) lobule IX and b) lobule X following preterm (open bars) or term (solid bars) delivery. Mean ± SEM, * indicates $p<0.05$, n=4-7. c) Representative photomicrographs of lobule IX and d) lobule X: i = preterm male, ii = term male, iii = preterm female, and iv = term female. Scale bar = 50μm.

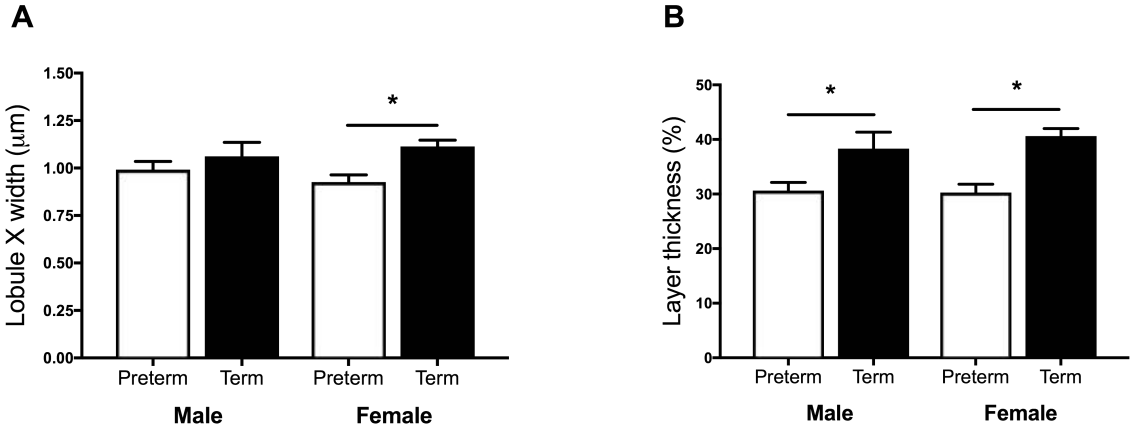

Figure 2. Total width of a) lobule X and b) the internal granule cell layer (as a percentage of total lobule width) of the cerebellum in guinea pigs at corrected postnatal day 28 following preterm (open bars) or term (solid bars) delivery. Mean ± SEM, * indicates $p<0.05$, n=4-7.

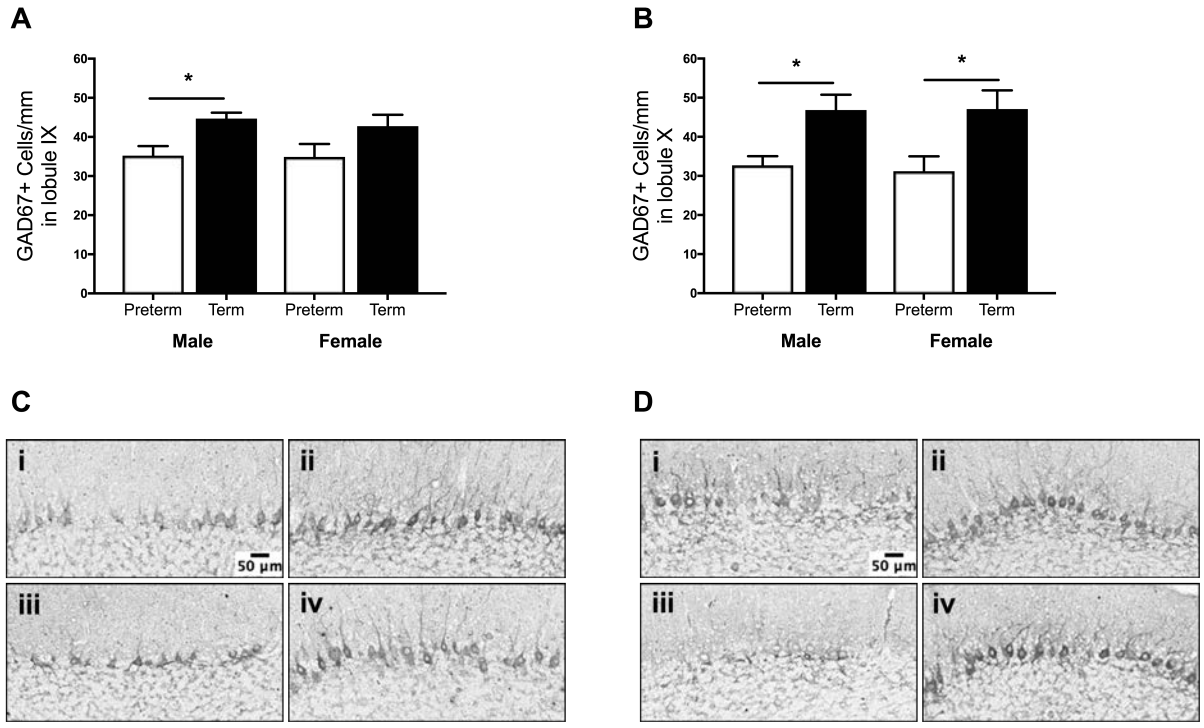

Figure 3. GAD67 positive Purkinje cells in lobule a) IX and b) X of the cerebellum in guinea pigs at corrected postnatal day 28 following preterm (open bars) or term (solid bars) delivery. Mean ± SEM, * indicates $p<0.05$, n=5-7. c) Representative photomicrographs showing GAD67+ Purkinje cells in lobule IX and d) lobule X: i = preterm male, ii = term male, iii = preterm female, and iv = term female. Scale bar = 50μm.

Immunoblotting

Whole cell protein expression of the GABA transporter protein (GAT1) and GABA synthesizing enzyme (GAD67) were measured in cerebellar tissue homogenate obtained at corrected PND28. There were no significant differences identified in total protein concentration for either GAT1 expression (Figure 4a), or for GAD67 expression (Figure 4b) within each sex between preterm and term cohorts.

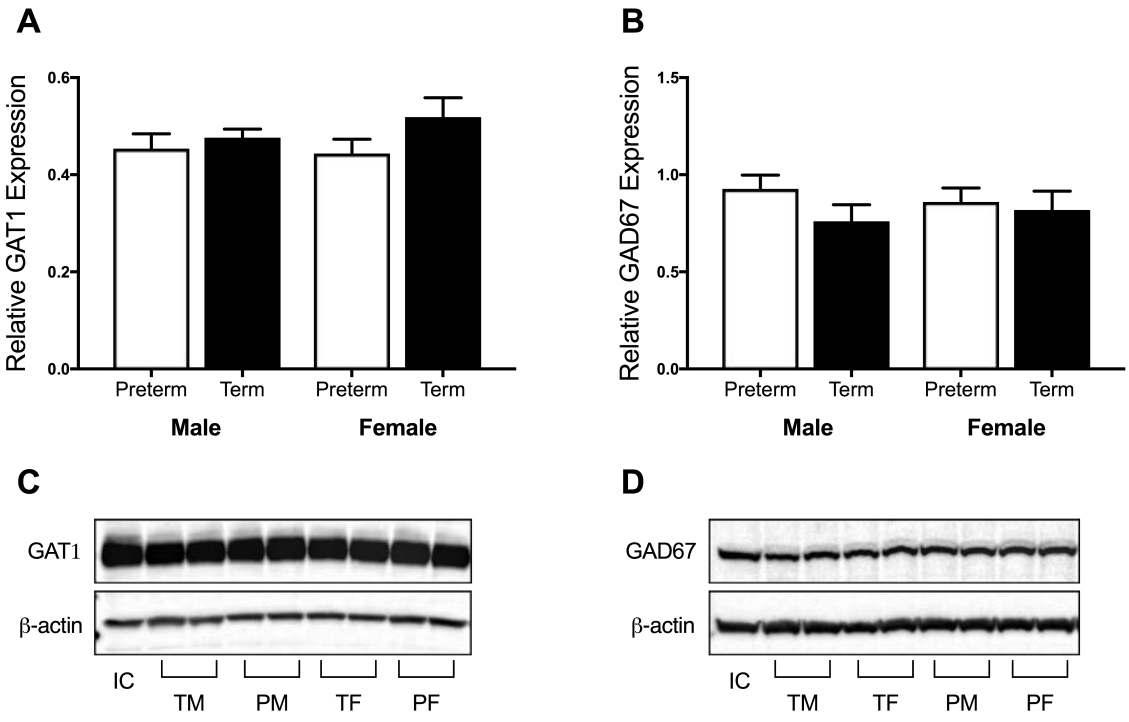

Figure 4. Relative protein expression of a) GAT1 (GABA transporter protein 1) and b) GAD67 (GABA synthesizing enzyme) in cerebellar tissue obtained from guinea pigs at corrected postnatal day 28 following preterm (open bars) or term (solid bars) delivery. Data is presented as mean ± SEM, n=8 for all groups. Representative western blots for c) GAT1 (67kDa) and loading control β-actin (42kDa) below, and d) GAD67 (67kDa) and β-actin below. IC = internal control, TM = term male, PM = preterm male, TF = term female, PF = preterm female.

$GABA_A$ Receptor Subunit mRNA Relative Expression

No effect of preterm delivery for either sex was found on the relative mRNA expression of the neurosteroid-sensitive $GABA_A$ receptor subunits α6 and δ (Figure 5a and 5b respectively) in the cerebellum.

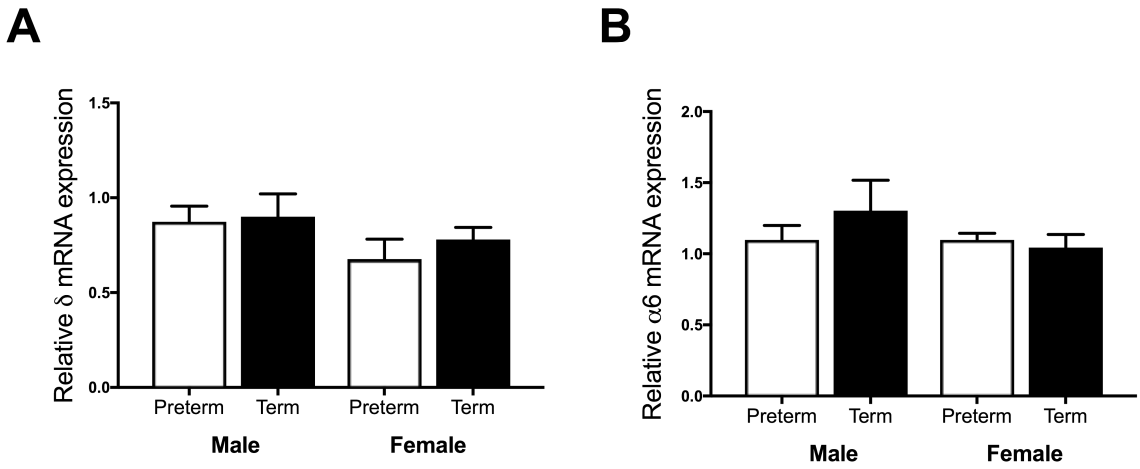

Figure 5. Relative mRNA expression of $GABA_A$ receptor a) δ and b) α6 subunits in cerebellar tissue obtained from guinea pigs at corrected postnatal day 28 following preterm (open bars) or term (solid bars) delivery. Data presented as mean ± SEM, n=8-10.

Discussion

This study identified disruptions to cerebellar development in ex-preterm juvenile male and female guinea pigs. The most striking differences were seen in myelination of cerebellar lobules, with preterm males having an abundance of myelin in lobule IX, and in contrast preterm females showing a deficit in lobule X. The absence of mature myelinating oligodendrocytes in lobule X of the female cerebellum is interesting, as it suggests an ongoing delay in mature myelin expression in this region. Previously we have identified reductions of lobule X MBP immunostaining in the cerebellum of the preterm neonate[271] therefore this data indicates that preterm-associated neuroanatomical differences are sustained through to childhood-equivalence at least in females in this model. Alternatively, the abundance of myelin in the preterm male cerebellum is unexpected. Whilst an increase in myelination may suggest that developmental catch-up or overcompensation has occurred, there is currently increasing interest in the importance of developmental axonal pruning and the impact of reduced pruning on functionality of the brain[275].

Normal brain development in late gestation is characterized by axonal growth and creation of various neural connections throughout the nervous system. Importantly, compared to the adult, in fetal and early postnatal life a larger number of axons extend to a single target during development[276]. Throughout childhood and adolescence there is a phase of developmental pruning that selectively removes unnecessary or unused neuronal branches and connections in the immature brain to ensure the optimal formation of functional connections[275]. As myelin encloses axons, the over-abundance of MBP immunostaining seen in the preterm male juvenile

guinea pigs here may suggest that this process of developmental pruning has not occurred sufficiently. Therefore, in lobule IX of the male cerebellum it is possible that excessive and inefficient neuronal connections may persist and quantitation of cell death and staining of neurofilaments may confirm that this mechanism is altered. Consistent with these observations, mapping of brain abnormalities in 10 year old males with autism identify an increase in volume of lobule IX compared to unaffected children, which may be due to a lack of axonal pruning and associated myelin[277].

Preterm males also had a decreased number of GAD67+ Purkinje cells in lobule IX which may suggest a reduction in GABAergic action as this enzyme is responsible for synthesizing GABA. As with myelination, GAD67+ Purkinje cells numbers in lobule IX were also unaffected in the preterm female cohort. Although the mechanism of these differences is unclear, this supports previous findings in the guinea pig that indicate female offspring are less vulnerable to neurological deficits caused by preterm birth[272]. The decrease in GAD67+ Purkinje cells numbers identified in lobule X in both the preterm males and females coincided with a reduction in the internal granule cell layer width in both sexes. The internal granule cell layer comprises the glutamatergic neurons of the cerebellum which excite Purkinje cells of the cerebellum, thereby resulting in an increase in GABAergic inhibitory transmission. Thus, in lobule X of the preterm juvenile cerebellum there is a decrease in GABA synthesizing cells in addition to a decrease in excitatory cells whose site of action are the GABA-releasing Purkinje cells, together this may suggest that there is a persistent reduction in inhibitory GABAergic tone at least to 28 corrected days of age in the guinea pig. The present findings therefore suggest that lobule X is particularly sensitive to damage due to preterm birth. This disruption of GABAergic inhibition may play a role in disorders

that are linked to the cerebellum as reductions in cerebellar GAD67 expression and GABA concentrations have previously been reported in adults with mood disorders[278], children with ADHD[279], and infants with epilepsy[280]. Despite these differences in the GABAergic pathway in lobule IX and X, the total cerebellar protein concentrations of GAD67 and GAT1 (responsible for transporting GABA out of the cell), as determined by immunoblotting, were not different. This may be due to regional differences in expression throughout the various lobules of the cerebellum, which could result in limited sensitivity of immunoblotting to show differences in specific areas.

We did not identify any differences in the relative expression of neurosteroid-sensitive $GABA_A$ receptors α6 or δ. Previously, we have shown that these subunits are particularly vulnerable to preterm delivery in the guinea pig as there is no change or adaptive increase in expression following birth as occurs following term birth[271]. Our previous studies in the guinea pig have shown that these subunits undergo a large increase in expression after term delivery in response to falling levels of substrate[271]. However, the preterm guinea pig neonate had the same levels of neurosteroid-sensitive $GABA_A$ receptors as the preterm fetus despite a large reduction in neurosteroid concentrations[271]. The data in the current study suggests that sometime between the early postnatal period and juvenile age there is either a large increase in expression of these subunits in the ex-preterm brain, or more likely that levels in the term brain have dropped to lower levels that are developmentally appropriate. Subunits of the $GABA_A$ receptor go through age-related changes in expression, with early development often a period of high expression, and down regulation of expression occurring as adulthood is approached[281]. This age-related

change in expression follows the maturation profile of the brain. Therefore, if the expression of neurosteroid-sensitive receptors in the preterm brain remains reduced throughout the early neonatal period, as is suggested here, this may be contributing to the long–term disturbances in neurodevelopment often present following preterm birth.

Overall this study has identified key alterations to the juvenile cerebellum following preterm birth that persisted to 28 corrected days of age. Disruptions of the GABAergic pathway and myelination following preterm birth suggest a role for the premature loss of neurosteroids post birth in these alterations. The presence of these abnormalities in the juvenile brain following preterm birth and associated premature exposure to the *ex utero* environment, followed by their manifestation since the time of birth, indicate that the neurological consequences of preterm birth are apparent at least up until childhood-equivalence in the guinea pig. This study demonstrates possible targets for further investigation into the mechanisms leading to, and therapies to prevent, the manifestation of neurodevelopmental and behavioural disorders caused by preterm delivery.

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Disclosure statement:

The authors confirm that there are no financial ties to products, or conflicts of interest to disclose.

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Ethical Standards:

The authors assert that all animal work performed complies with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Animal Ethics Advisory Committee of New Zealand, and the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes), and has been approved by the institutional committees (University of Otago, Wellington Animal Ethics Committee, and the University of Newcastle Animal Care and Ethics Committee).

6.0 "ADMINISTRATION OF PROGESTERONE THROUGHOUT PREGNANCY INCREASES MATERNAL STEROIDS WITHOUT ADVERSE EFFECT ON MATURE OLIGODENDROCYTE IMMUNOSTAINING IN THE GUINEA PIG"

This published manuscript uncovers the effects of maternal progesterone administration on both the maternal and fetal progesterone, allopregnanolone, and cortisol steroid profiles in addition to myelination of the vulnerable fetal hippocampus.

Published manuscript

Author	Contribution	Signature
Julia C Shaw	Experimental design Animal protocols and tissue collections Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K Palliser	Experimental design Manuscript corrections	
Kerrin Palazzi	Statistical support Manuscript corrections	
Jonathan J Hirst	Experimental design Manuscript corrections	

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Assistant Dean Research Training

Original Article

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Administration of Progesterone Throughout Pregnancy Increases Maternal Steroids Without Adverse Effect on Mature Oligodendrocyte Immunostaining in the Guinea Pig

Julia C. Shaw, BBiomedSc (Hons)[1,2], Hannah K. Palliser, PhD[1,2], Kerrin Palazzi, PhD[3], and Jonathan J. Hirst, PhD[1,2]

Abstract

Progesterone is administered to pregnant women at risk of premature labor, despite systematic reviews showing conflicting outcomes regarding its use, highlighting doubt over the effectiveness of the therapy. Progesterone can be rapidly metabolized into a number of steroids, but to date, there has been a lack of investigation into the fetal steroid profiles following administration and whether this impacts fetal neurodevelopment. The objective of this study was to determine the effect of progesterone treatment on allopregnanolone and cortisol levels in the fetus and on a marker of myelination in the fetal brain. We used a guinea pig model where pregnant dams were administered vehicle (β-cyclodextrin) or progesterone orally throughout pregnancy (GA29-61). Maternal and fetal fluids and tissues were collected at both preterm (GA61) and term (GA68) ages. Maternal and fetal progesterone and cortisol were analyzed by enzyme immunoassay and allopregnanolone by radioimmunoassay. Measurement of myelination of fetal brains (hippocampus, cingulum, and subcortical white matter) at preterm and term ages was performed by immunohistochemistry staining for myelin basic protein. We found that dams receiving progesterone had significantly elevated progesterone and cortisol concentrations, but there was no effect on allopregnanolone. Interestingly, the increased cortisol concentrations were not reflected in the fetuses, and there was no effect of progesterone treatment on myelination. Therefore, we conclude that in our guinea pig model, maternal administration of progesterone has no effect on cortisol levels or markers of mature oligodendrocytes in the fetus and suggest this is potentially due to the protective cortisol barrier in the placenta.

Keywords

neurodevelopment, maternal, fetal, neurosteroid

Introduction

The incidence of preterm birth (defined as <37 completed weeks gestation) accounts for 8.6% of all live births in developed regions and up to 13.6% in developing regions.[1] Vaginal progesterone and 17α-hydroxyprogesterone caproate (17-OHPC) use has dramatically increased over the last decade in developed countries in an effort to reduce the incidence of preterm delivery.[2,3] Despite this increasing use, however, the effectiveness of these therapies is heavily debated. A number of studies with small sample sizes have suggested that progesterone use in singleton pregnancies in women with a history of preterm birth, or a short cervix, can be effective in reducing the risk of preterm labor, provided onset of labor has not occurred at doses ranging from 100 to 400 mg/d from mid-gestation onward.[4-10] Once arrested preterm labor begins, progesterone is no longer effective at delaying birth.[2,3,11] Recently, a large double-blind, placebo-controlled trial known as the OPPTIMUM trial with 587 neonates in the placebo versus 589 in the progesterone groups showed that there was no effect of vaginal progesterone on gestational age at the time of delivery in singleton pregnancies.[12] Similarly, the PREDICT trial, also double

[1] School of Biomedical Sciences and Pharmacy, University of Newcastle, New South Wales, Australia
[2] Mothers and Babies Research Centre, Hunter Medical Research Institute, New South Wales, Australia
[3] Clinical Research Design, Information Technology and Statistical Support, Hunter Medical Research Institute, New South Wales, Australia

Corresponding Author:
Julia C. Shaw, Mothers and Babies Research Centre, Hunter Medical Research Institute, School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle 2305, New South Wales, Australia.
Email: julia.shaw@uon.edu.au

blind and placebo controlled, found that vaginal progesterone treatment had no effect on preterm delivery in twin pregnancies, which is in line with previous studies in twin studies.[10,13]

Prophylactic use of progestogens during pregnancy continues to be popular, despite the conflicting results from previous trials and the lack of knowledge regarding the effects on the unborn fetus, in particular the effects on fetal steroid profiles. In vivo cholesterol is metabolized into pregnenolone and then into progesterone by the enzymes p450scc and 3β-hydroxysteroid dehydrogenase. Progesterone then has the potential to metabolize to a number of steroids in the glucocorticoid, mineralocorticoid, estrogen, and androgen families via steroid biosynthesis enzymes known to be present in amniotic fluid and fetuses.[14,15] These include cortisol and the neuroactive steroid allopregnanolone both of which can markedly influence fetal neurodevelopment.[16,17] Metabolism to cortisol occurs following conversion of progesterone to 17α-hydroxyprogesterone, followed by 11-deoxycortisol and then cortisol, via the 17α-hydroxylase, 21-hydroxylase, and 11β-hydroxylase enzymes, respectively. To form allopregnanolone, progesterone is first converted to 5α-dihydroprogesterone by the rate-limiting enzymes 5α-reductases type I and II and then to allopregnanolone by the enzyme 3α-hydroxysteroid oxidoreductase.[18,19] The placenta readily metabolizes progesterone to allopregnanolone; however, in addition to this production, there is also high levels of allopregnanolone produced and maintained within the fetal brain itself.[18,20]

Previous studies have demonstrated that the neuroprotective and promyelinating effects of progesterone are mediated at least partially by its conversion to the neuroactive metabolite allopregnanolone. For example, in rat astrocyte and oligodendroglial progenitor primary cell cultures, in vitro progesterone exposure upregulated expression of the promyelinating factor insulin-like growth factor 1.[21] Similarly, in organotypic slice cultures of rodent cerebellum, myelination was stimulated by progesterone following its metabolism into allopregnanolone and action on $GABA_A$ receptors.[22] In vivo progesterone treatment has also been shown to attenuate neuropathological changes in a murine model of spinal cord motorneuron degeneration and improve outcomes.[23] The use of progesterone to treat patients following traumatic brain injury is also proving to be promising, with the ProTECT trial proceeding into phase III and a large clinical trial in China showing similar therapeutic benefits following progesterone therapy.[24,25] Hence, the neuroprotective roles of progesterone and its metabolite allopregnanolone have been well established in both the literature and the clinical setting. These studies, however, administer progesterone to either the cell/tissue or adult patient directly, rather than via the maternal circulation, which could potentially introduce other effects. In vitro administration of 17-OHPC showed that it can transfer from the maternal circulation to the fetal and is detectable in fetal plasma up to 44 days following the final injection.[26,27] Further studies not only showed that progesterone transferred to the fetal circulation but that it also bound to nuclear progesterone receptors in the developing fetal brain.[28] These data and the ability of progesterone to transfer from the maternal to the fetal circulation suggest that increased progesterone exposure can affect fetal steroid levels and the fetal brain. This work highlights the need to further investigate the effects that progesterone therapy during pregnancy may have on the vulnerable developing fetal brain. Thus, the action of elevated levels of progesterone at abnormal times, compared to normal endogenous exposure, may have unforeseen implications on neural development.

In our laboratory, we have previously shown that exposure to progesterone in the early neonatal period increases allopregnanolone concentrations in female guinea pigs. Males, in contrast, showed a marked rise in cortisol levels that was associated with reduced markers of the oligodendrocyte lineage and reduced myelin coverage in the cerebellum.[29] This exposure occurred postnatally, which raises the question of whether increased prenatal exposure via maternal supplementation as is the case with clinical therapy, undergoes similar metabolism and has downstream negative effects on neurodevelopment and cognition. In a neonatal rat study, daily injections of 17-OHPC from postnatal day (PND)1 to PND14, the period of medial prefrontal cortex development in the rat, resulted in a juvenile phenotype indicative of impaired synaptic pruning and cognitive flexibility of the prefrontal cortex.[30] Together, these studies demonstrate the unexpected detrimental effects of progestins on brain development in the early neonatal period. The aim of this study was to examine the effect of prenatal exposure to progesterone therapy on fetal allopregnanolone levels and the effect this may have on fetal brain development. Specifically, we hypothesized that a clinically relevant dose of progesterone administered maternally would be metabolized into other steroids, either in the maternal or in the fetal circulation and thus would have an adverse effect on brain development.

Materials and Methods

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, Australia).

Animals

Prior to beginning this work, approval for all of the animal experiments and procedures carried out throughout the study was obtained from the University of Newcastle Animal Care and Ethics Committee. In addition, all experiments and procedures were carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Pregnant, time-mated outbred tricolor guinea pigs were obtained from the University of Newcastle Research Support Unit. Guinea pigs were housed indoors with a 12-hour light–dark cycle and were supplied with a diet consisting of commercial guinea pig pellets, Lucerne hay, and water supplemented with ascorbic acid. Pregnant dams received either vehicle (45% β-cyclodextrin) or progesterone (5 mg/kg in 45%

β-cyclodextrin) orally each morning from gestational age (GA) 29-61. Women primarily receive progesterone as a vaginal pessary in the clinic; however, the logistics to administer a pessary to a guinea pig posed a challenge. Due to oral and vaginal administration having similar bioavailabilities,[31] we chose to provide progesterone orally to the pregnant dams to avoid the logistical challenge.

Pregnant dams were euthanized by carbon dioxide inhalation at either GA61 (preterm) or GA68 (term). Once euthanized, the fetuses were immediately removed from the uterus, and fetal tissues were collected. At the time of tissue collection, body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the 2 hemispheres. Each left hemisphere was fixed for immunohistochemistry.

Steroid Analyses

Salivary progesterone and cortisol. Maternal salivary progesterone concentrations were measured throughout gestation using salivary progesterone enzyme immunoassay kits (Salimetrics LLC, State College, Pennsylvania).[29] Assays were performed according to the manufacturers' instructions, with samples diluted in assay buffer as needed. The inter- and intra-assay coefficients of variance of this assay were 9.76% and 16.5%, respectively. Cortisol was measured in maternal saliva collected at GA58, 24 hours after the previous progesterone or vehicle administration, using a Salimetrics salivary assay kit (Salimetrics) according to the manufacturers' instructions. As previously described, the sensitivity of this assay is 0.012 to 3.0 μg/dL, with the inter- and intra-assay coefficients of variance 3.55% and 5.52%, respectively.[16,32]

Plasma allopregnanolone. Maternal and fetal plasma allopregnanolone concentrations were measured by radioimmunoassay, as described in detail previously.[16,33,34] In brief, plasma steroids were isolated using solid-phase Sep-Pak C18 cartridge (Waters, Rydalmere, Australia) extraction with graded, acidified, methanol washes. To reduce cross-reactivity, the extracts from all samples were then treated with potassium permanganate to oxidize the excess progesterone. Tritium-labeled allopregnanolone (1000-5000 cpm, 5a-[9,11,12,3H(N)]; Perkin Elmer Life and Analytical Sciences, Boston, Massachusetts) was used to determine individual sample recovery concentrations following manual tissue extraction. The average extraction recovery was 83.5% ± 1.4%, and individual sample recoveries were used for calculation of allopregnanolone concentrations in plasma samples. Radioactivity of the allopregnanolone was measured using a β-counter (LS6500, Beckman Coulter Australia Pty Ltd, Lane Cove, Australia), with the polyclonal antiallopregnanolone antibody (AS04041, Agrisera AB, Vannas, Sweden). The limit of detection was 25 pg/mL. All fetal or maternal samples were run in a single assay with an inter- and intra-assay coefficient of variance of 9.13% and 5.52%, respectively.

Plasma progesterone and cortisol. Pathology North (John Hunter Hospital, Newcastle, NSW, AUS) measured fetal plasma progesterone and cortisol concentrations, using quantitative chemiluminescent microparticle immunoassay kits (Architect, Abbott Laboratories, Abbott Park, Illinois) specific for cortisol and progesterone and by following the manufacturers' instructions. Briefly, the sample and anticortisol (or antiprogesterone)-coated paramagnetic microparticles were combined. Conjugate solution was then added followed by a series of washes and finally a pretrigger and trigger solution added to the reaction tube. The resulting chemiluminescent reaction was measured as relative light units using the Beckman Coulter UniCel Dx1800 Access Immunoassay System (Beckman Coulter). The intra-assay coefficients of variance were 7.9% and 4.3% for progesterone and cortisol, respectively. Inter-assay coefficients were 6.1% and 6.6%, respectively.

Immunohistochemistry

A marker of mature oligodendrocytes was examined in a number of brain regions, including the cornu ammonis 1 (CA1) region of the dorsal hippocampus, the adjacent subcortical white matter, and cingulum. Immunohistochemistry was performed on 8-μm sections of paraffin-embedded brains that were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, Australia) as described previously.[35,36] Tissues were dewaxed, incubated in citrate buffer (pH 6.0), and phosphate-buffered saline (PBS) containing 3% hydrogen peroxide and blocked for 1 hour at room temperature in bovine serum albumin (BSA) blocking solution (0.5% w/v BSA, 0.05% w/v saponin, 0.05% v/v sodium azide in 0.1 mol/L PBS). Incubation in primary (myelin basic protein [MBP], M9434) and secondary (biotinylated anti-rat immunoglobulin G, B7139) antibodies were performed before tertiary incubation in streptavidin–biotin–horseradish peroxidase complex. To reveal the immunolabeling, sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride solution (metal-enhanced diaminobenzidine substrate kit; ThermoFisher Scientific, Scoresby, Australia).

Stained slides were imaged using the Aperio imaging system (Leica Biosystems, North Ryde, Australia). Percent area coverage at 20× magnification was used to quantify staining. ImageJ version 1.47 (National Institutes of Health, Bethesda, Maryland) was used to calculate area coverage by converting to grayscale and then binary, and manually adjusting threshold based on the original stained image.[32] An overall average of area coverage staining was calculated by taking the average of 4 images captured from 2 consecutive sections per animal.

Statistical Analyses

Maternal salivary progesterone data was measured over time (categorized into: pre- [before commencement of treatment], early- [GA35-44], mid- [GA45-54], late- [GA55-60], and post-treatment [after treatment has ceased]). Differences between vehicle and progesterone groups (overall and for each time

category) were examined using linear-mixed modeling to account for correlation of repeated measures (random intercept for mother) and including an interaction term between treatment and time category. Treatment effect for maternal allopregnanolone and cortisol data was analyzed using 2-way analysis of variance (Treatment × Delivery) and independent *t* test, respectively.

The association between treatment and each fetal outcome measure was examined by linear-mixed modeling, including a 3-way interaction term (Delivery × Treatment × Sex), all 2-way interactions, and main effects. A mixed model was used to account for correlation of errors for multiple pups per mother (random intercept for mother) with Bonferroni correction to adjust for multiple comparisons. Analysis in this study is exploratory due to the small sample size and low power, and therefore the reported results are conservative.

Analyses were performed using SPSS (IBM SPSS Statistics for Windows) version 2.3 (IBM Corp, Armonk, New York), with statistical significance set a priori as $P < .05$. Significance for interaction terms was set at $P < .2$, as this study was not powered to detect significant 3-way interactions; if P value for interaction was <.2, relevant 2-way comparisons were compared. All results from mixed modeling are expressed as mean $\pm$ standard error of the mean. For all specific mean and P values, please refer to relevant supplementary tables.

Results

Fetal Characteristics

The average litter size across the deliveries was 3, with no effect of treatment. Mean GA at delivery and organ weights for fetuses collected at preterm and term are depicted in Table 1, with P values for main effect, 2-way, and 3-way interactions reported in supplementary Table 1. There were no significant differences identified between treatment groups (within age and sex) in any of the parameters investigated. However, progesterone-treated males at both preterm and term had significantly higher brain weights than corresponding females (preterm; $P = .04$ and term; $P = .009$). Hippocampal weight was also higher for preterm males from progesterone-treated pregnancies compared to females ($P = .04$). None of these differences were present in the vehicle-treated groups.

Maternal Steroid Concentrations

Progesterone concentrations were measured in the saliva of pregnant dams over the course of the experiment. During mid- and late treatment (GA45-60), the concentration of progesterone was higher in the dams receiving progesterone treatment, compared to those receiving vehicle (Figure 1A, $P = .003$ and $P = .005$, respectively). Dams receiving progesterone treatment had significantly higher levels of salivary cortisol compared to vehicle controls at GA58 (Figure 1B, $P = .003$). Circulating maternal allopregnanolone concentrations were measured following euthanasia at preterm and term GAs. There was no effect of progesterone administration on the concentration of allopregnanolone in maternal plasma at these GAs (Figure 1C).

Fetal Steroid Concentrations

Circulating levels of progesterone, cortisol, and allopregnanolone were measured in the fetuses from pregnancies where the dams received either progesterone or vehicle. Plasma progesterone was higher in female fetuses from progesterone-treated dams at term (Figure 2A, $P = .01$). Male fetuses from progesterone-treated pregnancies showed a trend that did not quite reach significance (Figure 2A, $P = .06$). Progesterone was also significantly higher in the term progesterone-treated females compared to the preterm progesterone-treated females (Figure 2A, $P = .02$). There were no significant differences in either of the preterm cohorts for circulating progesterone. Fetal plasma cortisol was not significantly different between treatment groups in the preterm or the term cohort; however, within each treatment and sex group, the term concentrations were significantly higher than the preterm concentrations (Figure 2B, $P < .001$ for each). There were no significant differences in circulating plasma allopregnanolone levels between fetuses treated prenatally with vehicle or progesterone in either GA cohort (Figure 2C).

Fetal Oligodendrocyte Marker Immunostaining

Immunostaining of mature oligodendrocytes was measured in the CA1 region of the hippocampus, adjacent subcortical white matter, and cingulum of fetuses from progesterone- and vehicle-treated pregnancies by calculating MBP area coverage. There was no significant effect of treatment on MBP coverage between vehicle and progesterone groups in any of the brain regions investigated. There were, however, significant differences in area coverage in the CA1 region of the hippocampus between delivery groups, with term progesterone-treated males having increased MBP compared to preterm (Figure 3A, $P = .006$), in addition to both the term vehicle- and progesterone-treated females having increased MBP compared to respective preterm cohorts (Figure 3A, $P = .03$ and $P = .02$). In the cingulum, term vehicle-treated males and females had increased MBP compared to the preterm cohorts (Figure 3C, $P < .001$ and $P = .003$, respectively). Additionally, the female progesterone-treated term fetuses exhibited greater coverage of MBP compared to the preterm cohort ($P = .002$). There were no significant differences in MBP levels in the subcortical white matter (Figure 3D).

Discussion

In this study, we found that maternal administration of progesterone did not have an overt effect on the steroid profile or a mature oligodendrocyte marker in the hippocampus of the fetus in an animal model. As expected, maternal levels of progesterone were increased following administration of exogenous

Table I. Fetal Physical Characteristics at Preterm and Term GAs.[a]

Delivery	Sex	Treatment	Pups (n)	Dams (n)	GA	Body weight	Brain weight	Hippocampus Weight	Heart Weight	Liver Weight	Kidney Weight	Adrenal Weight	Placenta Weight	BLR
Preterm	Male	Vehicle	10	8	60.6 ± 0.2	72.7 ± 4.0	2.15 ± 0.04	0.090 ± 0.004	0.42 ± 0.03	3.30 ± 0.3	0.65 ± 0.04	0.029 ± 0.004	5.48 ± 0.3	0.69 ± 0.05
		Progesterone	8	7	60.7 ± 0.2	72.1 ± 4.4	2.17 ± 0.04[b]	0.100 ± 0.005[b]	0.46 ± 0.03	3.49 ± 0.3	0.66 ± 0.04	0.024 ± 0.005	5.76 ± 0.4	0.66 ± 0.05
	Female	Vehicle	6	5	60.6 ± 0.2	66.8 ± 5.1	2.09 ± 0.04	0.081 ± 0.006	0.37 ± 0.04	2.82 ± 0.3	0.61 ± 0.05	0.032 ± 0.006	4.61 ± 0.4	0.75 ± 0.06
		Progesterone	11	7	60.7 ± 0.2	65.3 ± 4.0	2.09 ± 0.04	0.088 ± 0.004	0.37 ± 0.03	2.95 ± 0.3	0.60 ± 0.04	0.024 ± 0.004	5.00 ± 0.4	0.75 ± 0.05
Term	Male	Vehicle	8	5	67.7 ± 0.2	93.8 ± 4.7	2.34 ± 0.04	0.093 ± 0.005	0.47 ± 0.03	4.24 ± 0.3	0.72 ± 0.04	0.026 ± 0.005	5.56 ± 0.4	0.61 ± 0.05
		Progesterone	10	7	68.0 ± 0.4	89.8 ± 4.2	2.34 ± 0.04[b]	0.095 ± 0.004	0.53 ± 0.03	4.02 ± 0.3	0.71 ± 0.04	0.036 ± 0.005	5.80 ± 0.4	0.61 ± 0.05
	Female	Vehicle	8	6	67.9 ± 0.2	90.7 ± 4.5	2.35 ± 0.04	0.099 ± 0.005	0.50 ± 0.03	4.23 ± 0.3	0.74 ± 0.04	0.029 ± 0.005	5.31 ± 0.4	0.58 ± 0.05
		Progesterone	8	7	68.0 ± 0.2	90.9 ± 4.4	2.23 ± 0.04	0.090 ± 0.005	0.52 ± 0.03	4.10 ± 0.3	0.67 ± 0.04	0.031 ± 0.005	5.34 ± 0.4	0.56 ± 0.05

Abbreviations: GA = gestational age; BLR = brain-to-liver ratio that is used as an indicator of growth restriction.
[a]All values are from mixed modeling and expressed as mean ± SEM, calculated for the animal numbers indicated. Body weight and all organ weights are in grams
[b]Significant pairwise comparison between sexes within a treatment/delivery group. For 3-way interaction *P* values, please refer to supplementary data table 1.

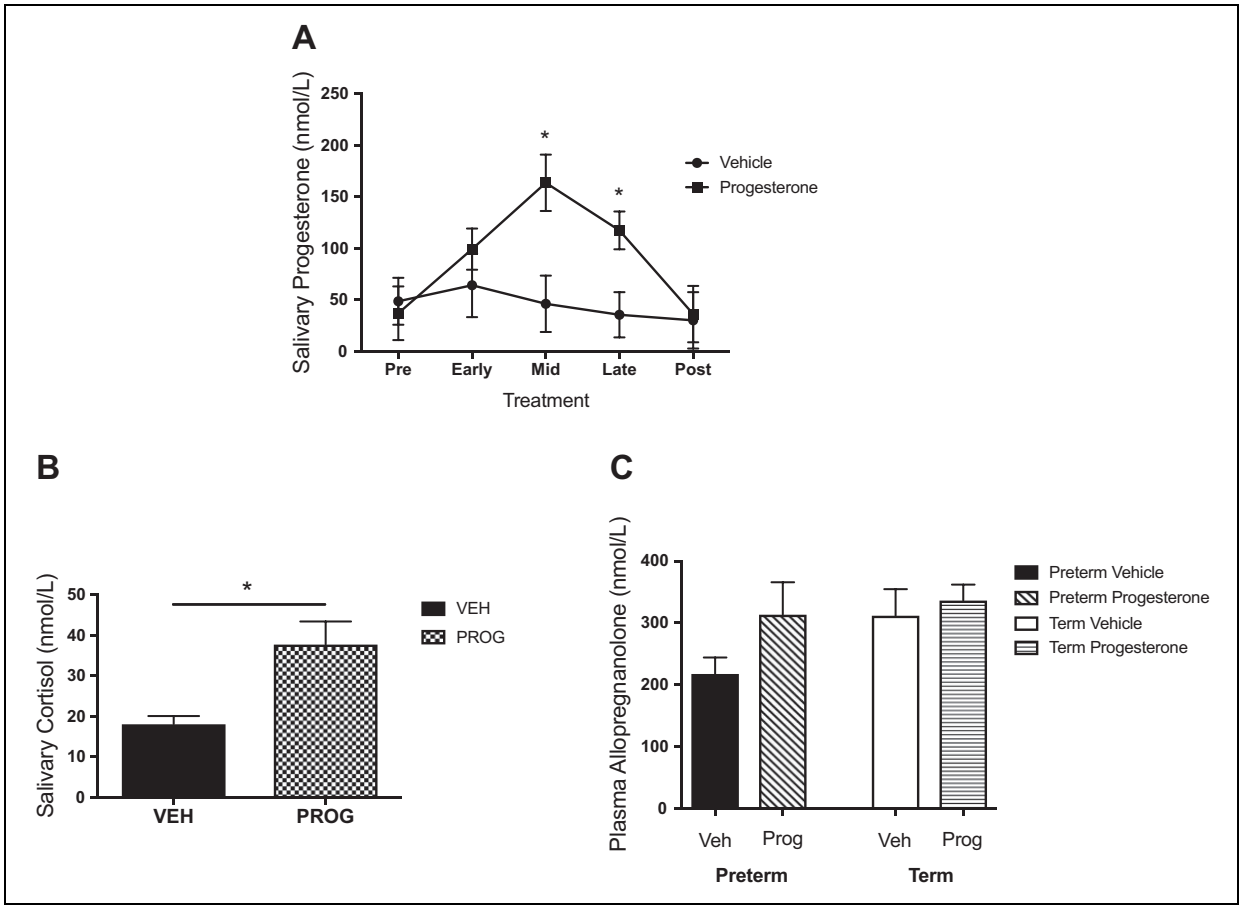

Figure 1. Maternal salivary: (A) progesterone, (B) cortisol, and (C) plasma allopregnanolone concentrations. For (A), salivary values were obtained prior to progesterone (n = 16 circles) or vehicle (n = 15 squares) treatment and then throughout the treatment period, followed by 7 days after final dose (post). For (B), salivary cortisol values following progesterone (n = 16 checked bar) or vehicle (n = 15 solid bar) treatment were obtained during late treatment at GA58. For (C) plasma allopregnanolone values were obtained following progesterone (Prog) or vehicle (Veh) treatment at preterm (solid and hatched bars, n = 8-10) or term (open and lined bars, n = 7-8) GAs. All values are mean ± SEM. * $P < .05$. GA indicates gestational age; SEM, standard error of the mean.

progesterone, and there was a rise in fetal levels at term. These findings indicate the dose of progesterone used was sufficient to raise levels in the fetal circulation of females at term, despite placental metabolism. The raised levels of progesterone in both the maternal and the fetal circulation were not associated with an ongoing rise in allopregnanolone levels, although we cannot exclude the possibility that levels were higher at peak progesterone exposure during treatment. Interestingly, we saw a significant rise in cortisol concentrations in dams assigned to progesterone treatment compared to those receiving vehicle. One of our key concerns leading into this study was the possibility of progesterone being metabolized to cortisol in the fetus. The fetus is protected from increased maternal cortisol by feto-placental expression of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme, which is responsible for the rapid metabolism of active cortisol to the less potent cortisone, before passing into the fetal circulation.[37] This enzymatic barrier is not total, however, and markedly elevated maternal cortisol during maternal stress, for example, can lead to increased passage of active cortisol to the fetal circulation and have detrimental effects on fetal development.[16,38] The present findings suggest that elevated progesterone, as a result of exogenous progesterone administration, and the excess cortisol produced were not sufficient to affect fetal levels. This further indicates the placenta is effective at protecting the fetus from the levels of maternal cortisol seen when progesterone levels are supplemented. Further investigation is required to determine whether placental levels of 11β-HSD2 are increased in the progesterone-treated pregnancies as an adaptive response to further protect against the rise in maternal cortisol or whether normal gestational levels are sufficient.

We examined expression of MBP as a marker of mature oligodendrocytes in a number of brain regions that are developing during mid-late gestation and are known to be vulnerable

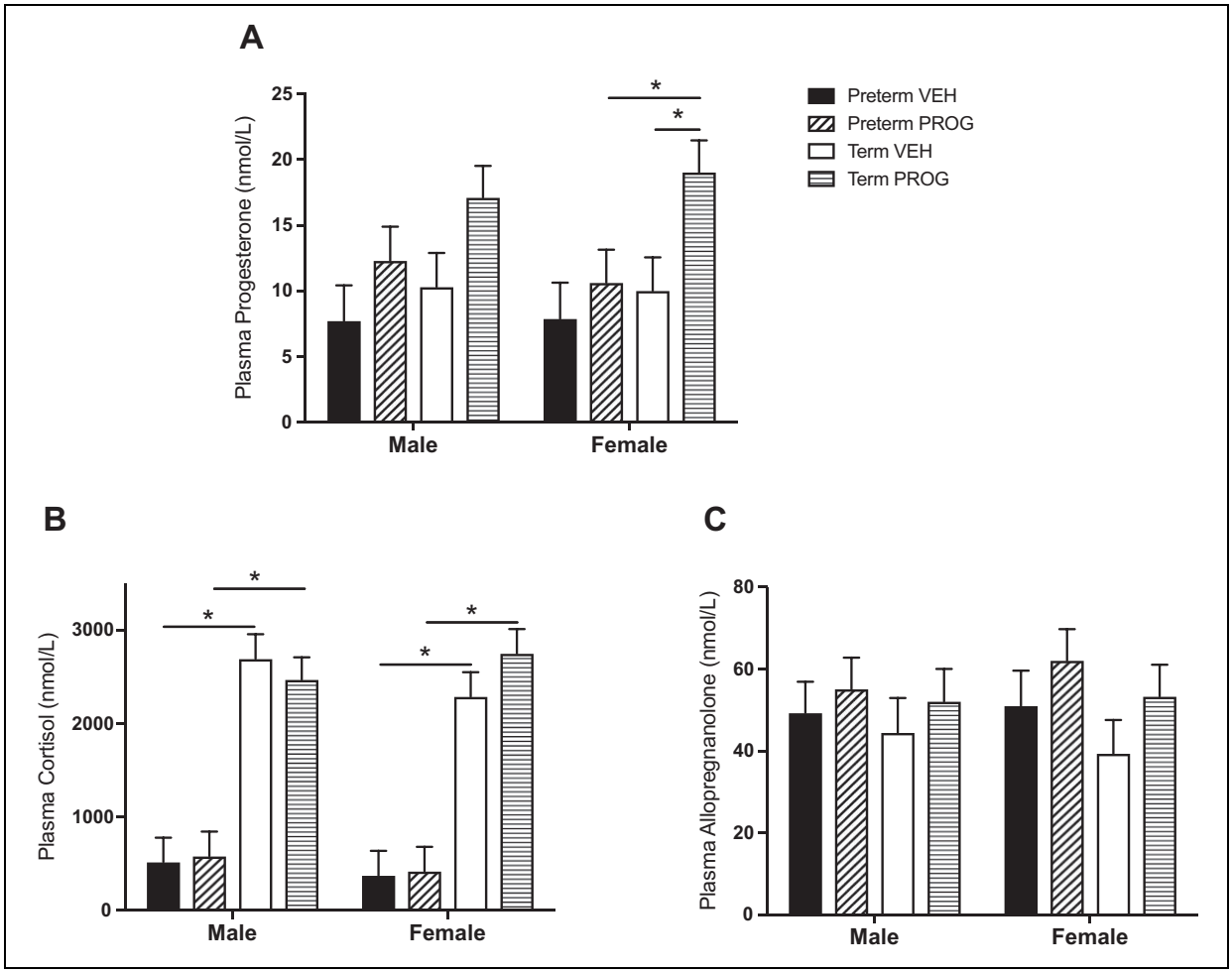

Figure 2. Fetal plasma: (A) progesterone, (B) cortisol, and (C) allopregnanolone concentrations obtained at 24 hours (preterm) and 7 days (term) after final treatment dose. For (A), progesterone levels were significantly higher in female fetuses collected at term from progesterone-treated pregnancies compared to preterm, in addition to those treated with vehicle. For (B), cortisol was not significantly different between fetuses collected from progesterone- or vehicle-treated pregnancies in either the preterm or term cohort. Concentrations at term were significantly higher than at preterm within each treatment and sex. For (C), allopregnanolone was not significantly different for male or female fetuses collected from vehicle- or progesterone-treated pregnancies. For males and females receiving vehicle or progesterone at preterm (solid and hatched bars n = 5-8), and term (open and lined bars n = 5-8). All values are mean ± SEM obtained from mixed modeling. *$P < .05$. SEM indicates standard error of the mean.

to insults. The finding of no significant differences between treatments is consistent with the absence of any changes in steroids other than progesterone in the fetal circulation and further indicates that the levels of progesterone reached in the fetal circulation do not affect oligodendrocyte markers in the hippocampus. As MBP is a marker of mature myelinating oligodendrocytes, the present findings suggest there are no differences in the mature oligodendrocytes following progesterone administration from mid-gestation onward. As these cells are the product at the end of the oligodendrocyte linage, these present findings also suggest it is unlikely there were major differences in earlier progenitor cells following treatment. Previously, we have shown that progesterone administered subcutaneously to premature neonates after birth until term equivalence, to compensate for the loss of placental progesterone supply, significantly increased circulating and brain allopregnanolone levels in female neonates,[29] an effect that we did not see in the current study. This may be due to the markedly lower levels of allopregnanolone in neonates compared to levels prior to delivery and that the substrate progesterone may be limited in these neonates. In contrast, regulation of enzyme expression may limit conversion to allopregnanolone when progesterone levels are elevated. In addition, the progesterone dose used in the previous study was higher (16 mg/kg). This higher dose aimed to be neuroprotective, however, was found to be detrimental, as it resulted in markedly elevated cortisol

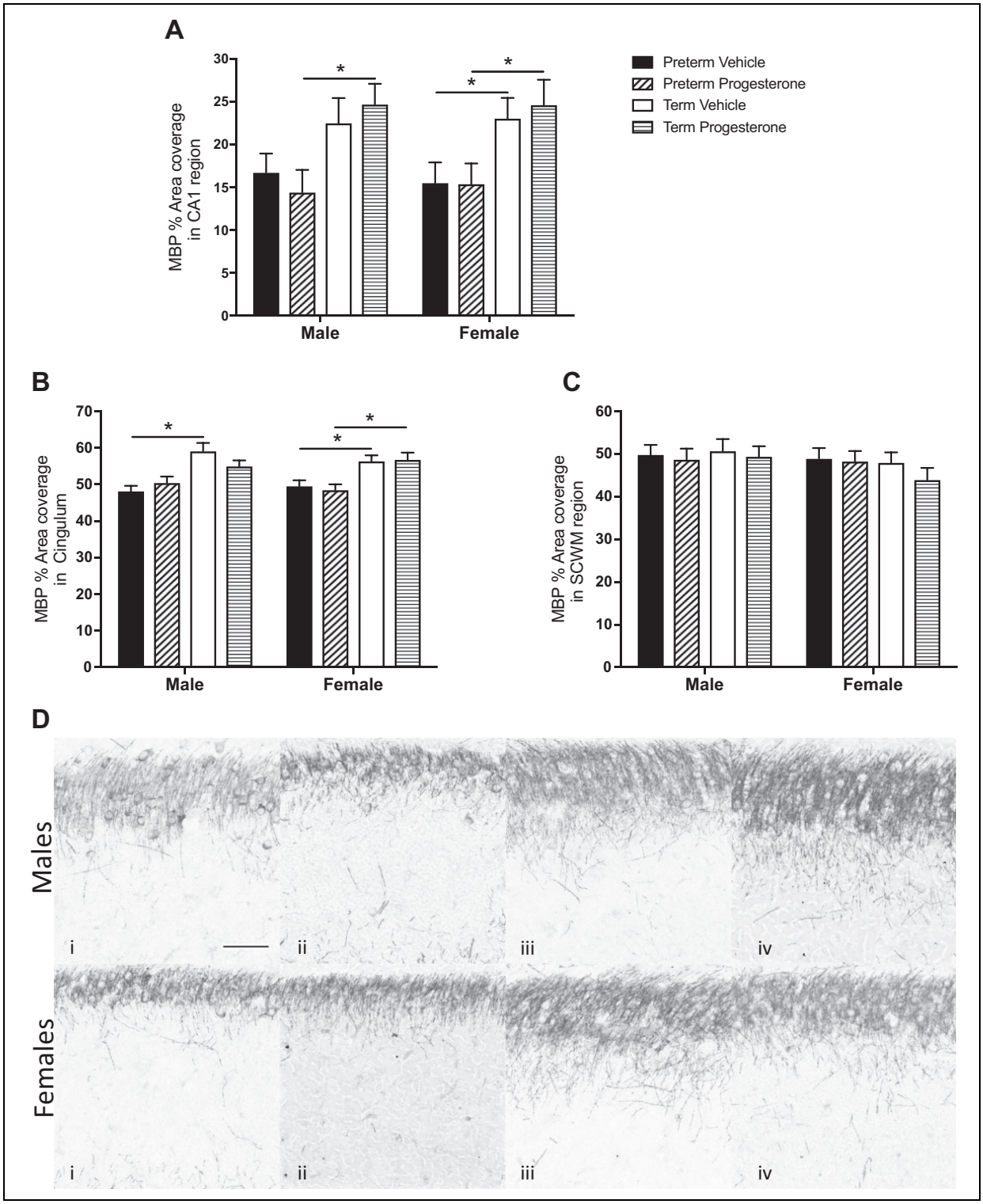

Figure 3. Fetal oligodendrocyte marker in (A) CA1 region of hippocampus, (B) cingulum, and (C) subcortical white matter measured by percent area coverage of myelin basic protein (MBP) staining 24 hours (preterm) and 7 days (term) after final treatment dose. A) MBP in the CA1 region of the hippocampus was not significantly different between fetuses obtained from progesterone- or vehicle-treated pregnancies in either

concentrations and associated impairment at all stages of the oligodendrocyte lineage[29] in preterm male neonates. Unlike the previous study, the present findings that cortisol levels did not rise in the fetal circulation suggest maternal progesterone treatment does not cause this side effect.

The finding that female fetal progesterone levels were elevated 1 week after the final progesterone dose rather than at the time of dosing is interesting. One possibility to explain this delayed rise is the ability of exogenous progesterone to be stored in fat and undergo delayed release into the circulation. Alternatively, a recent study found that administration of exogenous progesterone to mothers increased fetal serum progesterone levels within 2 hours of dosing[28]; based on this, it is possible that we missed measuring our peak progesterone levels, as our measurement of fetal plasma in the preterm cohort was 24 hours after the final dose. Similarly, our measurements of salivary progesterone in the mother taken throughout gestation were obtained 24 hours after each daily dose, so the levels we see are not indicative of peak levels but rather closer to the baseline level achieved. Another possible explanation for the lack of an overall rise in progesterone levels in the fetus is metabolism by members of the aldo-keto reductase (AKR) 1C family. There are 3 members of this family (AKR1C1, AKR1C2, and AKR1C3) that eliminate progesterone, 5α-dihydroprogesterone, and 5β-dihydroprogesterone by catalyzing the formation of inactive progestin metabolites.[39-41] The most catalytically efficient of these enzymes is the AKR1C1 enzyme, also known as 20α-hydroxysteroid dehydrogenase, and is highly active in placental tissues.[42,43]

In the clinical context, the present findings are reassuring as in the last decade progesterone therapy to prevent preterm labor is being used more frequently. There is currently growing concern in the literature regarding the safety of progesterone therapy in pregnant women due to this potential of progesterone to metabolize to a number of different steroids. This study has shown for the first time that progesterone therapy during pregnancy does not increase circulating cortisol levels in the fetus and that a mature oligodendrocyte marker in some of the most vulnerable regions of the developing brain is not impaired either at term or preterm fetal ages. Furthermore, this study has suggested the protective placental barrier is adequately robust to handle cortisol levels that are raised by increased levels of progesterone in the maternal circulation. This study has paved the way for further studies regarding effects on the fetus, and there are numerous additional analyses to perform, most notably to assess if any effects are present at neonatal age and onward following progesterone exposure throughout pregnancy. Although we cannot yet draw conclusions on the effect of progesterone in later life, we have demonstrated that progesterone has not been metabolized into excessive levels of cortisol in the fetus and has not led to a delay in the maturation of oligodendrocytes in the hippocampus.

Authors' Note

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes) and has been approved by the institutional committee (University of Newcastle Animal Care and Ethics Committee).

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Declaration of Conflicting Interests

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Supplementary Material

Supplementary material is available for this article online.

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Figure 3. (continued) the preterm or term cohort. Male progesterone-treated term fetuses had significantly more MBP than preterm, while female vehicle- and progesterone-treated term fetuses had significantly more MBP than respective preterm cohorts. B) MBP in the cingulum was not affected by treatment in either the preterm or term cohort but male and female vehicle-treated and female progesterone-treated, term fetuses had significantly more MBP expression than preterm. C) MBP in the adjacent subcortical white matter was not significantly different between fetuses obtained from progesterone- or vehicle-treated pregnancies in either the preterm or term cohort. For males and females receiving vehicle or progesterone at preterm (solid and hatched bars n = 5-7) and term (open and lined bars n = 4-6). All values are mean ± SEM obtained from mixed modeling.* $P < .05$. CA indicates cornu ammonis; SEM, standard error of the mean. D) Representative photomicrographs of the CA1 for males and females: i = preterm vehicle, ii = preterm progesterone, iii = term vehicle, and iv = term progesterone. Scale bar = 50 μm.

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7.0 "NEUROSTEROID REPLACEMENT THERAPY USING THE ALLOPREGNANOLONE-ANALOGUE GANAXOLONE FOLLOWING PRETERM BIRTH IN THE GUINEA PIG"

This manuscript is prepared for submission and details the outcomes following neonatal neurosteroid-replacement therapy following preterm birth, and explores the effects of this therapy on myelination and behaviour at juvenile age in the male guinea pig.

Prepared manuscript

Author	Contribution	Signature
Julia C Shaw	Experimental design Animal protocols and tissue collections Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Hannah K Palliser	Experimental design Animal protocols and tissue collections Manuscript corrections	
Rebecca M Dyson	Experimental design Animal protocols and tissue collections Manuscript corrections	
Mary J Berry	Experimental design Animal protocols and tissue collections Manuscript corrections	
Jonathan J Hirst	Experimental design Manuscript corrections	

Professor Robert Callister Date: 14/09/17

Assistant Dean Research Training

Abstract

Background: Prematurely born males are at risk of developing attention deficit hyperactivity disorder (ADHD) and learning difficulties during childhood. We propose that neurosteroid-replacement therapy with ganaxolone (GNX) following preterm birth may prevent the deficits in neonatal development that contribute to these disorders.

Methods: Preterm guinea pigs (delivered 62d gestation), preterm-receiving ganaxolone (2.5mg/kg subcutaneously twice daily until term equivalence), and term-born (spontaneously delivered) animals underwent behavioural testing at corrected postnatal age (CPNA) 25. Brain tissue was collected at CPNA28 and mature myelinating oligodendrocytes of the hippocampus and subcortical white matter quantified by immunostaining of myelin basic protein (MBP). Relative expressions of neurosteroid-sensitizing $GABA_A$ receptor subunits (α4, α5, δ) were measured by RT-PCR.

Results: Whilst ganaxolone treatment impaired weight gain and imposed sedation during the therapy period, it returned the hyperactive behavioural phenotype of preterm born male juveniles to a term-born phenotype. Deficits in the MBP immunostaining of preterm hippocampus and subcortical white matter were also ameliorated in animals receiving ganaxolone. Hippocampal expression of the δ subunit was decreased following ganaxolone therapy.

Conclusion: Ganaxolone improved neurobehavioural outcome suggesting that neonatal treatment may be an option for reducing the risk of long-term neurodevelopmental deficits and disorders in childhood. Further dosing studies are required to reduce the sedation and its consequences on preterm neonates.

Introduction

Preterm birth, defined as birth at less than 37 completed weeks of gestation, comprises approximately 10% of births each year[26]. The majority of preterm births occur between weeks 34-36, and in Australia these late preterm births now account for up to 70% of preterm births[2]. Preterm birth is the leading cause of death and neurodevelopmental related disability in neonates, accounting for up to 70% of neonatal deaths and with approximately 50% of survivors developing a long-term neurodevelopmental disability[4, 54]. In addition to well studied preterm birth related disorders such as cerebral palsy and sensory deficits, there is now a growing body of evidence suggesting that preterm infants are much more likely to develop neurodevelopmental morbidities and learning disorders that become apparent at school age[2, 5, 6]. The incidence of anxious and hyperactive behaviours is markedly increased in school-aged ex-premature children[56, 57]. Furthermore, ex-premature children have an increased need for special education, increased risk of repeating a grade, and lower reading and mathematics scores compared to full term children[5]. Currently, there are no preventive or therapeutic approaches for these babies as the disorders are recognized later in life and treated long after causative processes have occurred, and yet they result in significant social, family, and economic burdens.

The brain is vulnerable following preterm birth as it is not only removed early from the placental precursor supply of fetal neurosteroids and other placentally supplied nutrients, but it is also prematurely exposed to a stimulating environment and excitotoxic damage. The neurosteroid 3α-hydroxy,5α-pregnane-20-one (allopregnanolone) is found in very high concentrations in both the fetal plasma and

fetal brain compared to concentrations following birth[21, 151, 152]. *In utero,* the elevated concentration of allopregnanolone provides neuroprotection for the developing fetal brain and a reduction in the normal fetal neurosteroid environment is associated with adverse outcomes such as excessive excitation of the fetal brain and an increase in potentially damaging seizures, which can lead to permanent neurodevelopmental damage[109, 153]. Allopregnanolone has an inhibitory action over the brain and maintains the fetal 'sleep' state, which is necessary for correct neurodevelopment and encourages myelination[19]. Neurosteroids, including allopregnanolone, specifically enhance $GABA_A$ receptor mediated inhibition via their agonist action at the extrasynaptic gamma-aminobutyric acid A ($GABA_A$) receptors[186-188]. These neurosteroid sensitive receptors are highly expressed on cells such as oligodendrocytes (myelinating cells) throughout the fetal brain from mid-gestation onwards[158, 196]. We have shown that the birth-associated loss of allopregnanolone also occurs after preterm birth such that neonates born prematurely have markedly reduced exposure to allopregnanolone concentrations in the brain compared to those delivered at term[21, 237]. Preterm neonates that prematurely lose allopregnanolone exposure have significantly decreased myelination in the hippocampus and adjacent subcortical white matter, key areas involved in memory and learning[21, 271]. Further studies by our group have identified white matter deficits in preterm animals at term equivalence and at juvenility [corrected postnatal age 28 (CPNA28)] in various, high-functioning, and vulnerable regions of the brain such as the hippocampus and cerebellum[238, 272]. Interestingly, the deficits observed are associated with disturbances in behaviour, supporting a role for reduced allopregnanolone in the behavioural changes seen in this model[272].

Neurosteroids have been shown to exert neuroprotective effects following damage to neurons and glia by preventing necrosis, apoptosis, and inflammation, and by also increasing re-myelination and regenerative mechanisms. In addition, the therapeutic benefit of neurosteroid supplementation treatment in cases of brain injury has been demonstrated in rats where administration of progesterone (the pre-cursor of allopregnanolone) following traumatic brain injury resulted in reduced brain edema, neuronal loss, and lipid peroxidation [240-242]. Allopregnanolone administration has been shown to reduce memory deficits and loss of neurons in the frontal cortex of rats following bilateral injury[240]. In humans, patients administered progesterone following traumatic brain injury in a randomized clinical trial had a lower 30-day mortality risk and were more likely to have a moderate to good outcome than those receiving placebo[246].

Whilst the neuroprotective effects of progesterone and allopregnanolone are becoming established, there is little data relating to their possible administration in neonates to prevent the onset of neurodevelopmental disorders following preterm birth. We have previously investigated the use of neurosteroid-based therapy for protecting against neurodevelopmental deficits following preterm birth, originally using progesterone and allopregnanolone supplementation between preterm delivery and term-equivalent age in order to restore prematurely lost *in utero* levels of these neurosteroids. Progesterone administration was found to have detrimental effects on neurodevelopment in the neonatal guinea pig, particularly the male offspring, which contrasted to studies on traumatic brain injury in rats[237]. One possible explanation for this unexpected outcome is increased cortisol concentrations following postnatal progesterone administration. This increase in cortisol, observed only in male neonates,

was associated with a decrease in myelination of the cerebellum[238], potentially explaining the lack of a positive rescue of neurodevelopment. Preliminary findings following the use of allopregnanolone also suggested effectiveness may be limited due to the short half-life of ~ 30 minutes, and/or interactions with the placenta (data unpublished). To avoid these issues with allopregnanolone and the metabolism of progesterone into cortisol, in the current study we have trialed postnatal therapy with ganaxolone. Ganaxolone is a β-methylated analogue of allopregnanolone with a methyl group that prevents metabolism into other active steroids[248]. Ganaxolone binds to the neurosteroid-binding site of $GABA_A$ receptors with similar affinity and efficacy to allopregnanolone, but has a much longer half-life of ~12 hours[249]. Ganaxolone is currently being used in a phase 2 trial for infantile spasms and epilepsy, due to its actions in increasing activation of the $GABA_A$ receptor mediated pathways[25]. In the current study, we hypothesized that postnatal neurosteroid-based therapy using ganaxolone would ameliorate the adverse effects of a premature loss of allopregnanolone on neurodevelopment and behaviour in a guinea pig model of preterm birth.

Methods

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, Australia).

Animals

Approval for all of the animal experiments and procedures performed throughout the study was obtained prior to the commencement of the study from both the University of Otago and the University of Newcastle Animal Care and Ethics Committees.

Mature breeding Dunkin Hartley female guinea pigs were obtained from the University of Otago Research Biomedical Research Unit. Guinea pigs were housed indoors under a 12hour light/dark cycle and were supplied with a diet consisting of standard commercial guinea pig pellets, lucerne hay, fresh fruit and vegetables. Pregnant sows were randomly allocated to either preterm delivery or term delivery. Sows allocated to term delivery received no further interventions during pregnancy, with pups delivered spontaneously and receiving no respiratory or nutritional support. Preterm (GA62 of a 71 day pregnancy) pups were born following preterm induction of labour as previously described[256, 272]. In brief, sows received Betamethasone 1mg/kg subcutaneously (Celestone chronodose; Merck, Sharp & Dohme, Auckland, New Zealand) 48 and 24 hours prior to delivery to accelerate fetal lung maturation and surfactant production. Aglepristone 10mg/kg subcutaneously (Provet, Auckland, New Zealand), a progesterone receptor antagonist, was also administered 24 hours prior to,

and on the morning of delivery to inhibit progesterone-based continuance of pregnancy. Oxytocin 3IU/kg intramuscular (Provet) was then administered to stimulate uterine contractions, beginning 1 hour after the second Aglepristone dose and repeated in 30-minute intervals until all pups and placentas were delivered.

Resuscitation and respiratory support of pups occurred as previously described[256, 272]. Briefly, immediately following delivery, pups were placed on a heated pad under a heat lamp and respiration encouraged. Respiratory support was provided for at least 5 minutes to all preterm pups by continuous positive airway pressure (CPAP) support at 5cm H_2O using the "Neopuff" t-piece infant resuscitator (Fisher & Paykel, Auckland, New Zealand) with blended oxygen delivered at 5L/min. If spontaneous respiration was not achieved or sustained, positive pressure ventilation at 60 breaths/min with an inflation pressure of 12cm H_2O and expiratory pressure of 5cm H_2O was provided until spontaneous respiration was observed. Additionally, all preterm pups were also given an initial fractional inspired oxygen concentration of 30% that was adjusted based on the colour, heart rate, and respiratory activity of the pup. Once stable respiration was maintained, pups were housed with their mothers and littermates in a warm humidified human infant incubator (Dräger 8000 IC; Drägerwerk AG & Co., Lübeck, Germany); ambient temperature 33°C (titrated down to 28°C over the course of first 24 hours), and humidity at 60% (titrated down to 35% by 12 hours). Pups that were unable to achieve and maintain independent respiration were excluded from the study and humanely euthanized.

Preterm pups received 0.3-0.5mL of replacement colostrum (Impact guinea pig colostrum replacement; Wombaroo Food Products, Adelaide, Australia) orally by a 1mL insulin syringe within the first hour after birth and then every 3 hours until 24

hours old. Between postnatal days 1-7 the pups were fed 0.5-2.0mL of replacement milk (Impact guinea pig milk replacement; Wombaroo Food Products) every 3 hours or as needed to supplement independent suckling from the mother. Pups assigned to the neurosteroid-based therapy group received 2.5mg/kg ganaxolone (Prem-GNX) twice daily by subcutaneous injection from birth until term equivalence age (TEA), whilst those in the control group (Prem-CON) received vehicle (45% β-cyclodextrin in sterile water).

On postnatal day 6 (one day prior to term equivalence), all pups were transferred with their mothers from the infant incubator into a standard single cage at room temperature, and on postnatal day 7 into the nursery pen with other sows and pups. Preterm and term neonates remained with their mother in the nursery pen until weaning occurred at corrected GA21, after which they were placed into floor pens with animals of the same sex.

Behavioural tests

All juveniles underwent behavioural testing at CPNA25 as previously described[272]. A pre-test saliva sample was obtained from each animal immediately prior to the first test by allowing the pup to chew a cotton bud, and a post-test sample following the completion of the environment exploration testing. ANY-maze tracking software version 4.7 (Stoelting Co., Wood Dale, Illinois) was used to analyse the videos obtained from each test. All salivary samples and behavioural testing was performed by staff familiar to the animals, in a designated space free from the sight, sound, or

scent of other animals. Assessments were always made with the observer blinded to the treatment group.

Open field and environment exploration test

The open field test was used as a measure of anxiety and locomotion and was performed as previously described[265, 272]. Guinea pig pups were allowed to explore the arena (40cm x 40cm) for 10 minutes, with entries into the inner zone (an area 20cm x 20xm in the centre of the arena) recorded using ANY-maze tracking software.

The environment exploration test was used as a measure of the animals' exploratory behaviour and anxiety[272]. Following the open field test the animal was removed from the arena and two identical objects were placed in the center of the top two quadrants of the arena. The animal was then placed back into the arena and allowed to investigate the objects for 10 minutes.

Parameters measured included distance travelled in the entire arena and total time mobile, in addition to distance travelled and time spent mobile within the inner zone, and time spent investigating the foreign objects.

Familiar social test

The familiar social test was used to measure social interactions towards a familiar animal[272]. The test animal was placed into the arena (same as the open field and environment exploration arena) with an animal of the same sex and age from the same home pen. For 5 minutes the pair of animals were allowed to interact.

Parameters measured included approaching, and having an affectionate or agonistic interaction with the familiar animal.

Tissue Collection

Juveniles were euthanized at CPNA28 by sodium pentobarbitone (0.3mL administered IC; Lethabarb; Virbac, Milperra, Australia) 0.3mL for tissue collection. At this time body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the two hemispheres. Each left hemisphere was fixed for immunohistochemistry, whilst the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

Cortisol ELISA

A commercially available salivary cortisol assay (Salimetrics Inc., State College, Pennsylvania) was used to measure the concentration of cortisol in guinea pig saliva samples, and was performed by following the manufacturer's instructions as previously described[272]. The sensitivity of the assay is specified by the manufacturer as 0.012 – 3.0μg/dl, and the assays performed in our laboratory had inter- and intra-assay coefficients of variances of 10.89% and 2.58% respectively.

Immunohistochemistry

Mature myelinating oligodendrocyte expression was examined in CA1 region of the hippocampus, cingulum, and the adjacent subcortical white matter. Immunohistochemistry was performed on 8µm sections of paraffin-embedded brains that were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, Australia) as previously described[109, 271, 272]. Tissues were dewaxed, incubated in citrate buffer (pH 6.0) and PBS containing 3% hydrogen peroxide, and then blocked for 1 hour at room temperature in BSA Blocking Solution (0.5% w/v BSA, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1M PBS). Incubation in primary antibody (myelin basic protein [MBP] M9434) and secondary antibody (biotinylated anti-rat IgG B7139) were performed before tertiary incubation in streptavidin-biotin-horseradish peroxidase complex. Incubation in 3,3'-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; ThermoFisher Scientific, Scoresby, Australia) was used to reveal the immunolabelling.

Stained slides were imaged using the Aperio imaging system (Leica Biosystems, North Ryde, Australia). Percent area coverage at 20x magnification was used to quantify staining. ImageJ version 1.47 (National Institutes of Health, Bethesda, Maryland) was used to calculate area coverage by converting to gray-scale and then binary, and manually adjusting threshold based on the original stained image[271, 272]. An overall average of area coverage staining was then calculated by taking the average of four images captured from two consecutive sections per animal.

Real time PCR

The real-time PCR was performed as previously described[271, 272]. Frozen hippocampal tissue was homogenized in RLT Plus Buffer (Qiagen RNeasy Plus Mini Kit; Qiagen Pty Ltd, Chadstone Centre, Australia) using a Precellys 24 dual tissue homogeniser (Bertin Technologies, Provence, France). RNA was extracted using the Qiagen RNeasy Plus Mini Kit following the manufacturer's directions. Samples with poor RNA A260/280 ratios and integrity on an RNA gel were not used for further analysis.

Superscript III Reverse Transcription kit (Invitrogen, Carlsbad, California) was used to synthesize cDNA on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, Australia) according to the manufacturers instructions. RT-PCR was performed using a 7500 ABI real-time machine (Applied Biosystems), for primer pairs (α5, α6, and δ) and the housekeeping gene β-actin[271]. Each sample was run in duplicate along with an associated negative control sample which were treated the same throughout the reverse transcription process but in the absence of reverse transcriptase. Products were detected using the SYBR Green (Applied Biosystems) DNA binding dye method. Results were analysed by Sequence Detection Software v2.01 (Applied Biosystems) and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate relative fold change. Controls included β-actin and a calibrator, which consisted of pooled brain samples and was used across all plates. Consistent Ct values were obtained for β-actin across the term/preterm and male/female and samples.

Statistical Analyses

Data was analysed and graphs made using Prism v7.0 software (Graphpad Software Inc., La Jolla, California). All data are presented as mean ± SEM for each group and significance considered $p<0.05$. In order to identify differences between groups the data was first analyzed by one-way ANOVA. Post-hoc tests with Tukey corrections for multiple comparisons was performed when the ANOVA was $p<0.05$. When data were not normally distributed, non-parametric Kruskal-Wallis test with post-hoc tests with Dunn's correction for multiple comparisons were performed. The exception to this was the fractional weight gain and supplemental feeding data, which was analysed by repeated measures two-way ANOVA, with corrections for multiple comparisons by Bonferroni.

Results

Physical Characteristics

Animal weights and organ-body weight ratios are detailed in Table 1, and body measurements are detailed in Table 2. The term animals (68.9±0.3) were born significantly later than the Prem-CON (62.0±0, $p<0.0001$) and Prem-GNX animals (62.0±0, $p<0.0001$). The preterm animals also weighed significantly less at the time of birth ($p<0.0001$, and $p=0.02$ respectively), which was to be expected. At TEA and CPNA28 (postmortem) there were no significant differences in body weights across the three groups. Kidney-body weight ratio was increased in Prem-CON animals compared to term ($p=0.03$), this also appeared to be increased in the Prem-GNX animals however did not reach significance. Visceral fat-body weight ratio however was significantly decreased in the Prem-GNX animals compared to term ($p=0.05$) suggesting that the Prem-GNX animals were leaner. There were no other significant differences identified in organ-body weight ratios. Crown-rump length was significantly longer for term animals compared to Prem-CON ($p=0.02$), whilst head circumference was smaller ($p=0.02$). There were no other significant differences identified in body measurements at CPNA28.

Nutritional support for preterm animals and weight gain

Additional nutritional support required in the week leading up to term equivalence for the Prem-CON and Prem-GNX animals was recorded and daily averages are depicted in Figure 1a. On CPNA-7 (birth), CPNA-6, CPNA-1 and CPNA0

(term equivalence) there was no significant difference in additional feeding amounts required. Prem-GNX animals however required significantly more nutritional support than Prem-CON animals on CPNA-5 ($p=0.001$), CPNA-4 ($p<0.0001$), CPNA-3 ($p=0.0001$), and CPNA-2 ($p=0.008$) but this was not reflected in fractional weight gain where Prem-GNX animals gained significantly less weight at CPNA-5 and CPNA-4 compared to Prem-CON animals (Figure 1b, $p=0.02$ and $p=0.003$ respectively).

Mortality rates

The number of pups that were either stillborn or euthanized in the early neonatal period was approximately 10% for term pups and was greater in the preterm pups with a loss of 30% for Prem-CON pups and 60% for the Prem-GNX pups. The increased mortality rate in the Prem-GNX animals were due to apnea and respiratory depression, aspiration of fluids during feeding and an inability to move out from under the dam, therefore resulting in asphyxiation.

Table 1. Body and organ weights

Treatment Group	n	Birth Weight	TEA Weight	CPNA 28 Day Weight	Brain-Body Weight	Hippocampus-Brain Weight	Liver-Body Weight	Adrenal-Body Weight	Heart-Body Weight	Kidney-Body Weight	Sub. Fat-Body Weight	Vis. Fat-Body Weight
Term	14	96.8 ± 3.4	96.8 ± 3.4	284.6 ± 10.4	1.2 ± 0.04	3.6 ± 0.1	4.3 ± 0.2	0.034 ± 0.002	0.36 ± 0.02	0.84 ± 0.02	0.97 ± 0.07	0.34 ± 0.05
Prem-CON	15	76.2 ± 1.7*	95.7 ± 2.8	302.7 ± 8.7	1.1 ± 0.03	3.8 ± 0.2	4.4 ± 0.2	0.035 ± 0.001	0.41 ± 0.02	0.91 ± 0.02*	0.88 ± 0.11	0.29 ± 0.05
Prem-GNX	4	80.1 ± 5.3*	86.2 ± 2.9	258.2 ± 9.2	1.3 ± 0.05	3.8 ± 0.2	3.7 ± 0.1	0.035 ± 0.002	0.39 ± 0.03	0.93 ± 0.02	0.73 ± 0.07	0.11 ± 0.02*

Values presented are mean ± SEM and are calculated for animal numbers with birth, term equivalence age (TEA), and corrected postnatal age (CPNA) 28 day weights in grams. Body weights are in grams and organ weights are displayed as organ-to-body weight ratio. Data is presented as mean ± SEM, * indicates significance $p<0.05$ from term.

Table 2. Body measurements

Treatment Group	n	Head Length	Crown-Rump Length	Hind Limb Length	Hock-Toe Length	Head Circum.	Neck Circum.	Chest Circum.	Abdominal Circum.
Term	14	6.62 ± 0.14	19.7 ± 0.31	4.91 ± 0.14	4.00 ± 0.03	10.79 ± 0.29	9.76 ± 0.42	13.7 ± 0.34	17.02 ± 0.45
Prem-CON	15	6.86 ± 0.15	18.2 ± 0.49*	4.85 ± 0.12	4.02 ± 0.12	11.83 ± 0.23*	9.0 ± 0.36	14.04 ± 0.26	16.98 ± 0.37
Prem-GNX	4	6.22 ± 0.06	18.13 ± 0.10	4.5 ± 0.04	3.92 ± 0.08	11.38 ± 0.19	9.7 ± 0.23	13.03 ± 0.19	16.15 ± 0.49

Values presented are mean ± SEM and are calculated for animal numbers with all measurements in centimeters. Measurements are in centimetres. * denotes significance from term.

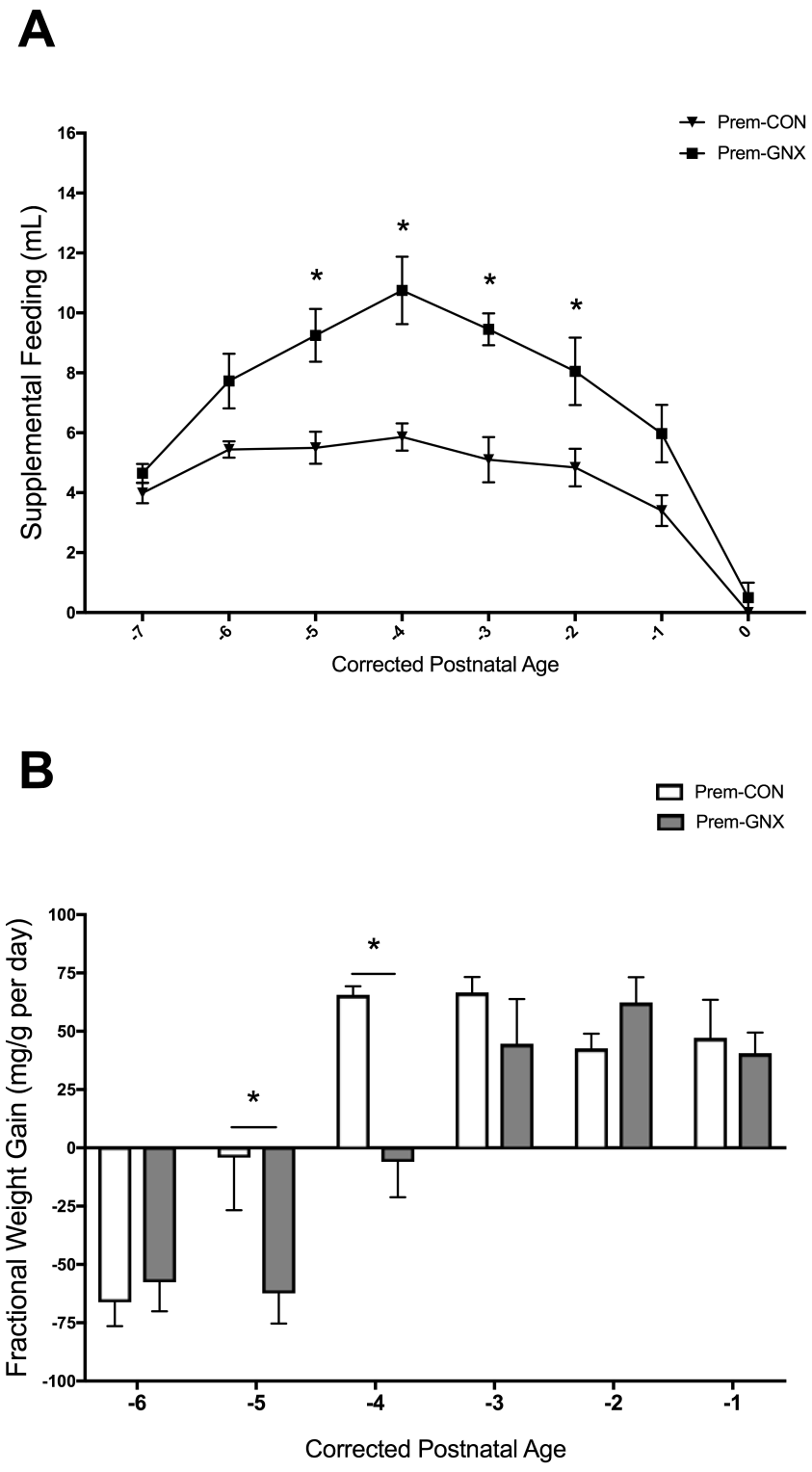

Figure 1. Supplemental feeding until TEA in a) and fractional weight gain until term equivalence in b) of preterm guinea pigs receiving vehicle therapy (Prem-CON; inverted triangles and white bars n=5 n=5), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; squares n=4 and grey bars n=4). Data is presented as mean ± SEM, * indicates significance $p<0.05$.

Behavioural outcomes and associated cortisol levels at juvenility

Results of open field-testing are shown in Figure 2, foreign object exploration in Figure 3a and 3b, and social interaction test in Figure 3c and 3d. In the open field test, the Prem-CON animals had significantly higher activity compared to the term animals which was measured by increased distances travelled in the total arena (Figure 2a, p=0.01) and inner zone (Figure 2c, p=0.002), and more time spent mobile in both total arena (Figure 2b, p=0.02) and inner zone (Figure 2d, p=0.005). Furthermore, the Prem-CON animals also spent significantly more time in the inner zone when compared to the term animals (Figure 2f, p=0.05) despite there being no difference in the number of entries into this inner zone (Figure 2e). Prem-GNX animals showed no differences compared to the term or Prem-CON animals.

In the foreign object exploration test, the Prem-CON animals had significantly more interactions with the foreign objects (Figure 3a) and spent significantly longer interacting with the objects (Figure 3b) when compared to term animals (p=0.02, and p=0.04 respectively). The number of interactions with the objects by the Prem-CON animals were also significantly higher than that exhibited by the Prem-GNX animals (p=0.05). There was no difference in behaviour between the Prem-GNX and term animals.

Lastly, in the social interaction test the Prem-CON animals approached the familiar animal (Figure 3c) and exhibited a higher frequency of interactions with the familiar animal (Figure 3d) when compared to term animals (p=0.02, and p=0.02 respectively). Both of these behaviours' were also significantly higher than that

exhibited by the Prem-GNX animals (p=0.04 and p=0.03 respectively). Behaviour exhibited by Prem-GNX animals was not significantly different to term animals.

Saliva samples were obtained immediately prior the open field test, and following the foreign object exploration test. There were no significant differences identified in the salivary cortisol concentrations of the animals before or after behavioural testing between any of the groups (data not presented). Furthermore, there were no significant differences identified in the change in concentration following the foreign environment exposure measured as a percentage of baseline concentration (Figure 4).

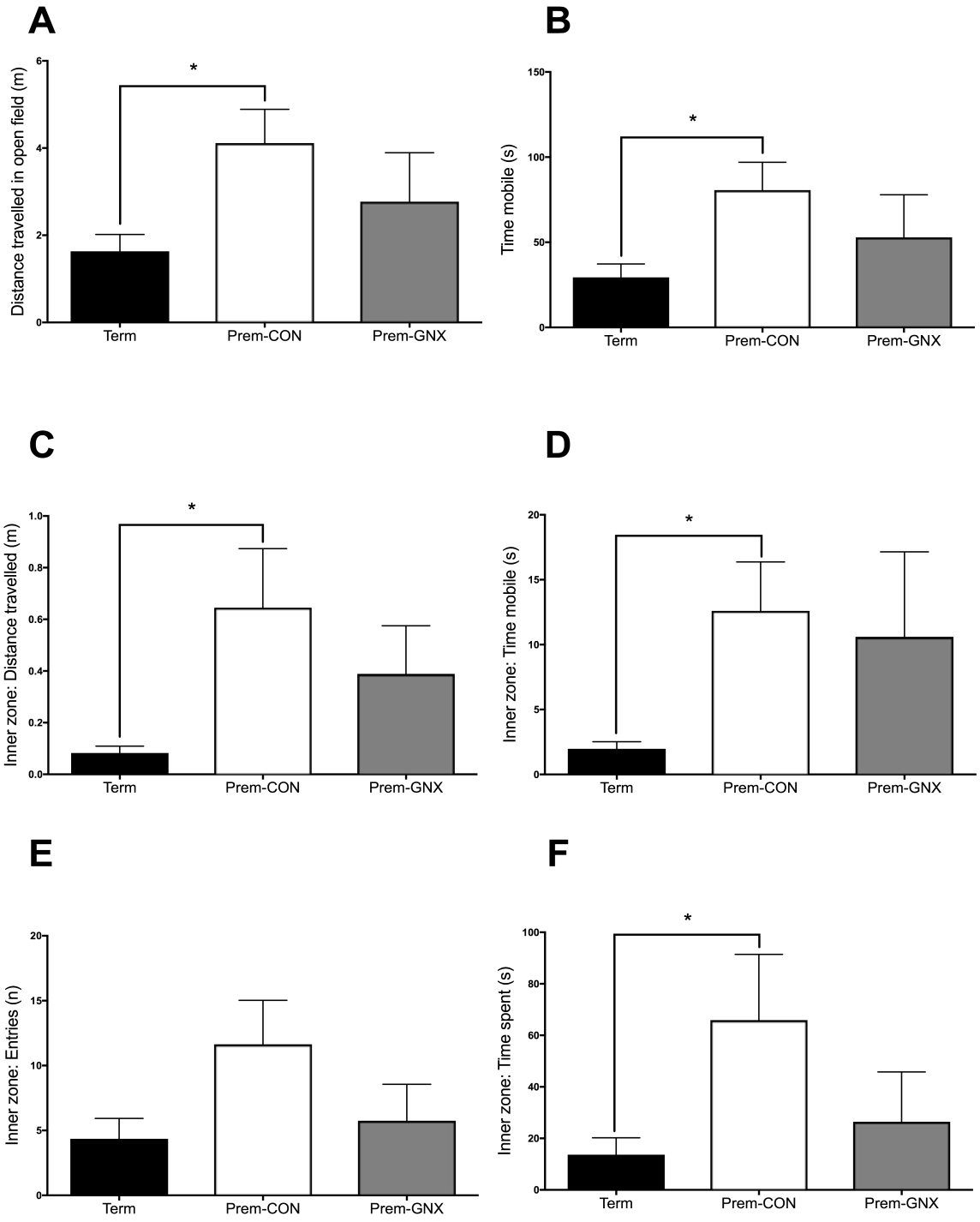

Figure 2. Open field exploration outcomes for term control (black bars, n=14), preterm guinea pigs receiving vehicle therapy (Prem-CON; white bars, n=11), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bars, n=4) at corrected postnatal age 25. Parameters measured included a) distance travelled and b) time mobile in the open field arena, c) distance travelled and d) time mobile in the inner zone, and e) number of entries and f) time spent in the inner zone. Data is presented as mean ± SEM, * indicates significance $p<0.05$.

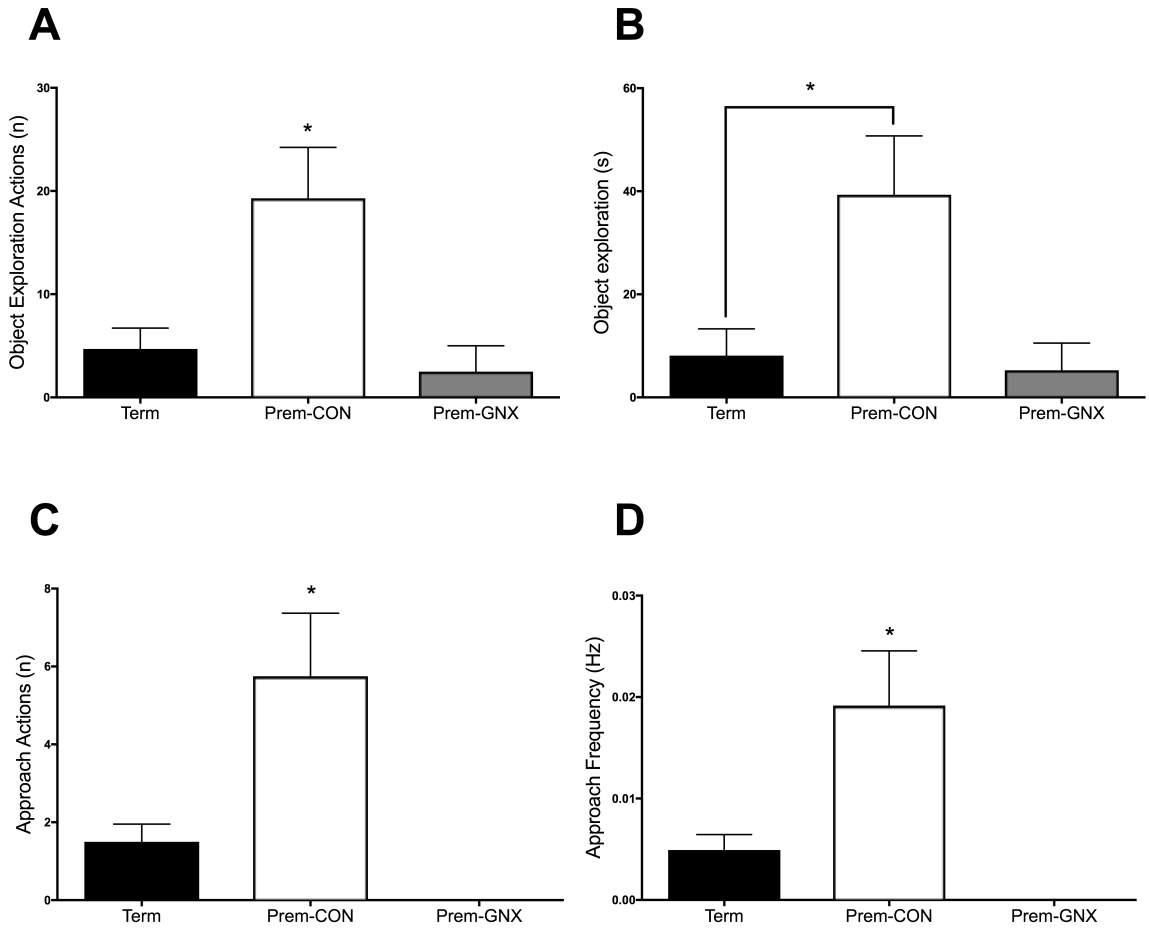

Figure 3. Foreign object investigation and social interaction by term control (black bar, n=10), preterm guinea pigs receiving vehicle therapy (Prem-CON; white bar, n=10), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bar, n=4) at corrected postnatal age 25. In the foreign object investigation behavioural test the parameters measured were a) the number of interactions with a foreign object, and b) the time spent interacting with the objects. In the social interaction test c) the number of approach actions and d) the frequency of actions were recorded. Data is presented as mean ± SEM, * indicates significance $p<0.05$.

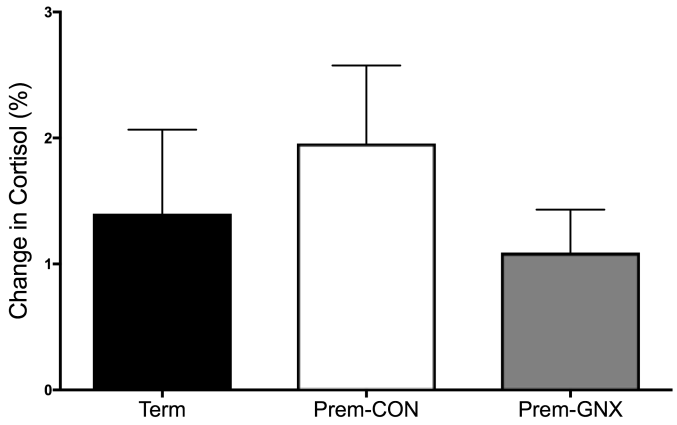

Figure 4. Cortisol concentration in saliva measured pre (baseline) and post behavioural testing and expressed as a percentage of the baseline concentration for term control (black bar, n=12), preterm guinea pigs receiving vehicle therapy (Prem-CON; white bar, n=15), and the preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bar, n=4) at corrected postnatal age 25. Data is presented as mean ± SEM.

$GABA_A$ receptor subunit expression in the hippocampus at juvenility

There were no significant differences identified between the three groups for either the α4 or α5 subunits (Figure 5a and 5b respectively). Interestingly, the δ subunit relative expression was significantly decreased in Prem-GNX animals compared to Prem-CON (Figure 5c, p=0.03). There were no other significant differences identified.

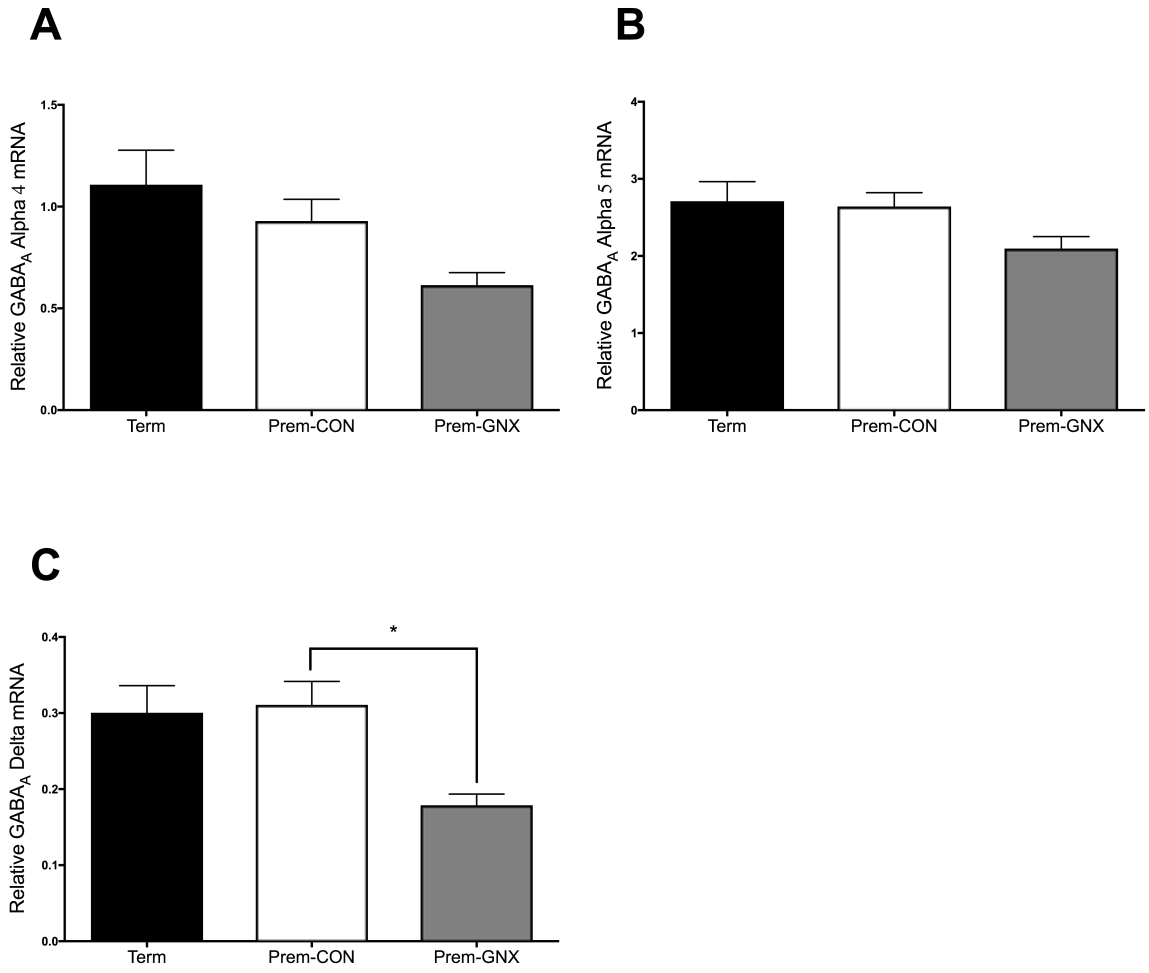

Figure 5. Relative mRNA expression of $GABA_A$ receptor subunits in the hippocampus of term control (black bar, n=11), preterm guinea pigs receiving vehicle therapy (Prem-CON; white bar, n=15), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bar, n=4) at corrected postnatal age 28 was quantified using PCR. The relative expression of a) the α4, b) the α5 subunit, and c) the δ subunit were measured. Data is presented as mean ± SEM, * indicates significance $p<0.05$.

Myelination in guinea pig brains at juvenility

Prem-CON animals had significantly less immunostaining in the CA1 region of the hippocampus compared to both the term control (Figure 6a, p=0.003) and the Prem-GNX animals (p<0.0001), furthermore the Prem-GNX animals had significantly more immunostaining compared to the term animals (p=0.002). Similarly, in the

subcortical white matter the Prem-CON animals also had significantly less immunostaining compared to the term (Figure 6b, p=0.02) and Prem-GNX animals (p=0.04), and there was no difference in immunostaining between the term and Prem-GNX animals. There were no significant differences identified in the cingulum (data not shown).

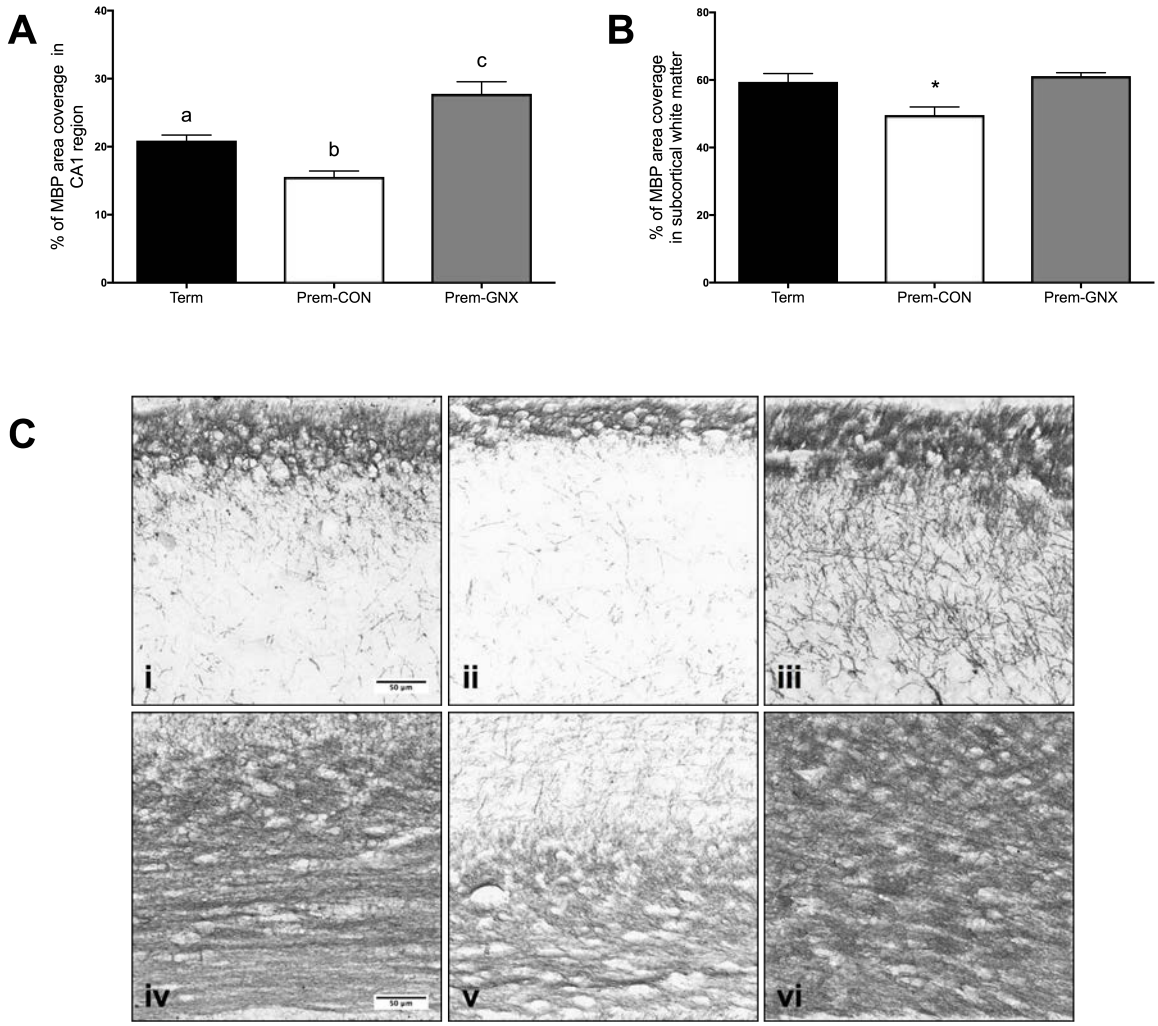

Figure 6. Myelin basic protein (MBP) immunostaining in the brain of term control (black bars, n=7), preterm guinea pigs receiving vehicle therapy (Prem-CON; white bars, n=8), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bars, n=4) at corrected postnatal age 28 was measured by area coverage. MBP immunostaining was quantified in a) the CA1 region of the hippocampus, and b) the subcortical white matter. Data is presented as mean ± SEM, different letters or * indicates significance $p<0.05$. c) Representative photomicrographs of the CA1 region (i = term, ii = preterm, iii = GNX), and the subcortical white matter (iv = term, iv = preterm, vi = GNX). Scale bar = 50µm.

Discussion

The key finding of this paper was that neurosteroid replacement therapy with ganaxolone reversed some of the effects observed when animals were prematurely exposed to the *ex utero* environment. As we have previously shown, male juvenile preterm born guinea pigs exhibit hyperactive behaviour signified by greater distances travelled in the foreign arena, more time spent in an open environment, and more investigation of foreign objects as opposed to 'safer' behaviour such as staying closer to the walls of the arena. In addition to this hyperactive phenotype, the ex-preterm males have a disinhibited social response, i.e. the persistent approaching of the other animal. This behavioural pattern in the guinea pig pup is similar to those of hyperactivity seen in children with ADHD and suggests the deficits we observe after preterm birth may contribute to the increased risk of the development of this disorder. Importantly, we observed a reversal of these phenotypes in the preterm animals treated with ganaxolone in the immediate neonatal period. The effects of ganaxolone and natural analogue allopregnanolone, on GABAergic activity and behaviour have been previously reported, with ganaxolone shown to increase inhibitory action of GABAgeric pathways in a similar manner to allopregnanolone[251, 252, 254].

Fetal sheep studies involving the inhibition of allopregnanolone synthesis show an increase in arousal and seizure-like activity *in utero* compared to when allopregnanolone supply is restored[161]. We have previously shown that suppression of allopregnanolone concentrations has detrimental effects on neurodevelopment and that this has adverse effects on long–term behaviour, including increased anxiety in female guinea pigs[168]. The anti-anxiolytic effects of ganaxolone have been

demonstrated in socially isolated mice where anxiety-like behaviour was successfully reduced following treatment[252]. The anti-seizure/anti-convulsion properties of ganaxolone have also been verified in a neonatal rat model of infantile spasms where treatment with doses of 25mg/kg and 50mg/kg both decreased the occurrence and delayed the onset of spasms[254].

The dose used in the current study was markedly lower than those used in the anti-seizure anti-convulsive studies, however, anecdotally, the preterm pups receiving ganaxolone appeared heavily sedated compared to their control counterparts which indicated that the dose used was too high. There was also decreased independent suckling from the dam, which resulted in the need for additional feedings. Despite the additional feeding the weight gain in the period leading up to term equivalence was reduced for those receiving ganaxolone, and this may be due to effects on the immature gastrointestinal tract as well as the lack of active suckling. While the sedation associated with treatment is troublesome in a small animal model in which the animals must maintain breathing unaided and interact with the dams, contributing to the high mortality in the treated group compared to preterm controls, large animal models have shown that the sedative effects of ganaxolone are useful in suppressing seizures *in utero*. Indeed Yawno et al. (2016) have reported that ganaxolone supresses seizures in neonatal sheep[282]. Whilst sedative effects have been observed in clinical trials using ganaxolone[249, 255], it was rather unexpected in this present study as the dose used does not produce sedation when administered to neonatal guinea pigs born at term (unpublished data). The disparities indicate there are considerable differences in metabolism and dose requirements between the term born and preterm born

animals and indicates the need for further animal studies of optimal dosing after preterm birth.

Previously we have identified lasting deficits in mature myelinating oligodendrocytes of the ex-preterm male juvenile hippocampus and subcortical white matter[272]. The hippocampus and adjacent subcortical white matter are known to be particularly vulnerable to damage and delays following exposure to the *ex utero* environment and in the absence of the pro-myelinating neurosteroid allopregnanolone[21, 109]. The finding that ganaxolone therapy successfully restored myelination levels to that of term-born animals in both of these areas is consistent with the trophic action of allopregnanolone on brain development during late gestation. This action involves upregulation of $GABA_A$ receptor signalling and therefore the challenge will be to find an optimal dose suitable for chronic supplementation therapy following preterm birth, which maintains $GABA_A$ receptor mediated trophic actions and yet minimises sedative effects.

Allopregnanolone exerts its pro-myelinating effects via the $GABA_A$ receptors of the central nervous system. The sensitivity of these receptors to different ligands depends on the makeup of subunits. Neurosteroid-sensitive $GABA_A$ receptors within the hippocampus predominantly feature the α4, α5, and especially the δ subunits. The observation that the expression of the α4 or α5 subunits did not change, but that the δ subunit was decreased in the animals receiving ganaxolone is interesting as this receptor has a major role in regulating neurosteroid sensitivity of the $GABA_A$ receptors. $GABA_A$ receptors can undergo feedback changes depending on chronic neurosteroid exposure and we have previously demonstrated that suppression of allopregnanolone

levels results in an adaptive increase in the δ subunit in term-born guinea pigs[271]. The present finding suggests that ganaxolone treatment at the dose used lowers receptor expression levels, which further suggests that the dose used was higher than required to produce a positive outcome on neurodevelopment.

Despite there being substantial evidence that neurosteroids, in particular allopregnanolone, are integral to fetal neurodevelopment and are subsequently lost prematurely in the event of preterm birth[21], this is the first study to replace an analogue of allopregnanolone and demonstrate therapeutic benefits. Our previous studies have examined the use of both allopregnanolone and its precursor progesterone in an attempt to restore neurosteroid levels to which the fetus is exposed *in utero*. The half-life of allopregnanolone of approximately 30 minutes – 1 hour, is not sufficient to achieve a sustained action at the $GABA_A$ receptors. Additionally, allopregnanolone can be oxidised to its immediate precursor, 5α-dihydroprogesterone, which has very low activity for the $GABA_A$ receptor, and furthermore can be metabolised to $GABA_A$ receptor antagonist steroids. Relatively high doses of progesterone were also previously examined in relation to restoring neurosteroid deficits in the neonatal preterm guinea pig. Whilst we observed raised allopregnanolone following treatment in the female neonates, the male neonates showed elevated cortisol concentrations[238] which may in itself have negative effects and highlights the potential of progesterone to be metabolised to steroids with unknown effects on development. The advantages of using the synthetic analogue ganaxolone include low metabolism to other steroid metabolites and the longer half-life of 12-20 hours in humans[249] allows for sustained stimulation of $GABA_A$

receptors, with convenient dosing intervals, which is likely to be important for trophic processes.

Overall, the present results show that neurosteroid-replacement therapy using ganaxolone resulted in myelination levels that were closer to levels seen in term delivered pups. The therapy also reversed the potentially adverse hyperactive behaviour patterns observed in preterm delivered pups at a postnatal age the equivalent to human childhood. The sedation seen in the pups, which resulted in an increased mortality rate, and the downregulation of one neurosteroid-sensitive receptor subunit highlights the requirement of determining optimal doses that lead to the steady enhancement of trophic process in the brain yet minimises the sedation of the pups.

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Disclosure statement:

The authors confirm that there are no financial ties to products, or conflicts of interest to disclose.

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Ethical Standards:

The authors assert that all animal work performed complies with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Animal Ethics Advisory Committee of New Zealand, and the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes), and has been approved by the institutional committees (University of Otago, Wellington Animal Ethics Committee, and the University of Newcastle Animal Care and Ethics Committee).

8.0 DISCUSSION

The overarching aims of this body of work were to uncover the differences present in the juvenile brain following preterm birth compared to normal term birth; how this may be involved in the altered behaviour that these ex-premature offspring exhibit, how prenatal steroids may affect the development of these irregularities, and ultimately a mechanism of preventing these deficits from occurring. Until recently, neonates born moderate-late preterm were not thought to be at risk of adverse outcomes, but it is becoming more widely accepted that this is not the case. In the absence of overt brain damage at the time of birth, subtle disturbances in myelination combined with the loss of allopregnanolone and in the face of excessive *ex utero* excitotoxicity, these neonates experience a slow developing impairment that often only becomes obvious during childhood. Currently, the mechanisms of these late presenting neurodevelopmental disorders are largely unknown and treatment involves management once the conditions present themselves, as opposed to prevention during the early neonatal period. Through use of the guinea pig model of preterm birth we were able to address these gaps in the knowledge of neurodevelopment following late gestation premature birth, and suggest key pathways involved, targets for intervention, and a therapy for moving forwards to prevent preterm birth-associated neurodevelopmental disorders from occurring.

8.1 Effects of premature exposure to the *ex utero* environment on neurodevelopment and behaviour

This thesis examined the effects of premature exposure to the *ex utero* environment on neurodevelopment at two ages; neonatal (PND1) and juvenile age (corrected PND28), in addition to the preterm fetus. Reductions in white matter is suggested to be a key component in the development of neurobehavioural disorders in children that are born preterm[11] and may stem from the birth-associated loss of allopregnanolone, as the pro-myelinating effects of this neurosteroid are evident *in vitro* on rat cerebellar slice cultures[166], and protection against cell death has been demonstrated in an *in vivo* mouse model of neurodegeneration[283] and in a sheep model of acute fetal hypoxia[153]. In the current studies, preterm neonates have reduced myelination in the CA1 region (which affected females only) and adjacent subcortical white matter, in addition to lobule X of the cerebellum. Previously, our group has shown that preterm male and female neonates at term equivalence age exhibit deficits in MBP immunostaining of the CA1 region[21]. In a pharmacological model of reduced allopregnanolone supply by finasteride therapy there are similar reductions in myelination also present in the CA1 region of fetuses[168]. Likewise, reduced allopregnanolone supply as a result of intrauterine growth restriction also impairs myelin development of the CA1 in male fetuses[110]. Taken together, these data emphasize the importance of gestational allopregnanolone concentrations for myelination to occur in fetal and neonatal life, and the deficits we see in myelination following preterm birth can be largely accounted for by the loss of allopregnanolone supply. Complementing these studies are our investigations of reduced

allopregnanolone synthesis *in utero* and lasting effects on offspring. Previously, studies have shown that chronic stress during pregnancy increases circulating cortisol, and decreases allopregnanolone in the fetus and juvenile offspring with lasting deficits in myelination of the CA1 region in male and female guinea pigs[284, 285]. In a similar way, the preterm juvenile male offspring in the studies presented here also had lasting deficits in myelination of the CA1 region and subcortical white matter[272]. Furthermore, circulating cortisol was also increased in these males, implying a common link between increased cortisol and decreased allopregnanolone resulting in impaired myelination. Interestingly, male offspring from preterm deliveries showed greater vulnerability at juvenility than females, which suggests a sex-dependent, and robust response by female offspring. The support structure for oligodendrocytes involves astrocytes, and in a mouse model of spinal cord injury astrocytes were shown to reduce white matter damage and preserve myelination following trauma, and several astrocyte knockout mouse models highlight the myelin repair properties of astrocytes[102-104]. Based on these data the reduction in astrocytes that is present in the preterm neonate following birth may play a role in the ongoing damage to myelin of preterm offspring, and we also see deficits in astrocyte expression in the ex-preterm male juvenile.

The studies presented here also identified that the cerebellum is particularly vulnerable to the insults associated with preterm delivery. Studies in humans have shown that preterm infants have smaller cerebellar volume and white matter, and at school age are described as ‘clumsier’ and with worse fine motor control, such as hand dexterity, than those born at term age[286-288]. Recent studies also support previously unidentified roles of the cerebellum in cognitive functions. Functional

imaging and clinical studies have shown that the cerebellum is involved in language processing and reading, working memory and associative learning[270, 289, 290]. It is also now well documented that children born preterm function more poorly in school, with learning difficulties and lowers IQs compared with term born children[5, 62], and this may in part be due to cerebellar defects. In this study, as with the CA1 region of the hippocampus, reductions in myelination of cerebellar lobule X were evident in preterm neonates (PND1)[271]. Furthermore, at term equivalence age our group has demonstrated that not only is the expression of the mature oligodendrocyte reduced, but also that reductions are present throughout the oligodendrocyte lineage thereby lessening the potential of catch-up growth to occur[291]. In fact, at juvenile age sex dependent alterations were observed in myelination as well as components of the GABAergic pathway. Juvenile females following preterm delivery had lasting reductions in myelination of lobule X and rather unexpectedly the males had an increase in myelination of lobule IX. The deficit present in females is likely to be a result of interrupted development following preterm birth and highlights the lasting impact that early life insults can have on the brain. This disruption to development of the male cerebellum however is potentially due to a deficit in axonal pruning.

There is increasing interest in the importance of developmental axonal pruning and the impact of reduced pruning on functionality of the brain[275]. Gestational development is characterized by axonal growth and the creation of various neural connections throughout the nervous system and, compared to the adult, a larger number of axons extend to a single target during development[276]. Throughout childhood and adolescence there is a phase of developmental pruning that selectively removes unnecessary or unused neuronal branches and connections in the immature

brain to ensure the correct formation of functional connections[275]. Excess connections that are formed during development are removed and the remaining necessary connections made stronger during this process. We suggest that axonal pruning has not occurred correctly in the male cerebellum following preterm birth, and as myelin encloses axons, this results in an overexpression of myelin as well as inefficient and unnecessary synaptic connections remaining. Indeed this finding is consistent with observations from mapping of brain abnormalities in children with autism, which show increased volumes of white matter in lobule IX[277], which is suggested to represent a deficit in axonal pruning. Functional imaging studies suggest that lobule IX as part of the posterior lobe is particularly involved in cognition and emotion[270], as it is interconnected with the prefrontal cortex, association cortices, and the limbic system, which allows for its involvement in higher order executive functioning[269]. Therefore, the altered development of this area within the male brain may be having a role in some of the neurobehavioural disorders that are more common following premature birth such as ADHD and autism.

Additional lasting alterations of the Purkinje cells were observed from term equivalence age until juvenility. In neonates, Purkinje cell area and diameters were smaller following preterm delivery[291], and as a juvenile the expression of the GABA synthesizing enzyme GAD67 was reduced in lobule IX (males only) and X which indicates an impairment in their ability to carry out one of their primary roles, the synthesis of the inhibitory neurotransmitter GABA. Furthermore, the studies performed here suggest that excitatory input onto Purkinje cells is also reduced following preterm delivery as the relative width of the internal granule cell layer was reduced in both sexes. The internal granule cell layer comprises the glutamatergic

neurons of the cerebellum whose primary role is excitation of Purkinje cells. These granule cells excite Purkinje cells of the cerebellum, which results in an increase in GABAergic inhibitory transmission. Thus, in lobule X of the preterm cerebellum there is a decrease in GABA synthesizing cells in addition to a decrease in excitatory cells whose site of action are the GABA-releasing Purkinje cells, which may suggest that there is a persistent reduction in GABAergic tone until juvenility at least. In fact, reduced GABA concentrations are a common feature in mood disorders[278], children with ADHD[279], and infants with epilepsy[280]. The varied nature of the differences identified in the ex-preterm cerebellum again highlights the sex dependent manner in which preterm birth affects development. Specifically, in lobule IX where both myelination and GABA synthesizing capacity was unaffected in the preterm female cohort, the data indicates that whilst the mechanisms of the sex differences are unclear it is undeniable that female offspring are less vulnerable to the neurological deficits caused by preterm birth. It is interesting to note that whilst we identified an impact of preterm delivery on the GABAergic pathway within lobule IX and X, when we analysed protein expression of GAD67 and GAT1 in total cerebellar homogenate, we failed to detect a difference. The most likely explanation for this is the presence of regional differences in expression throughout the various lobules of the cerebellum, which may stem from their varied roles, and hence the limited sensitivity of immunoblotting to show differences in specific areas. The rationale for focusing on lobules IX and X for the region-specific analyses is due to their involvement in higher order executive functions (as part of the posterior lobe), in addition to their late developing nature and therefore likelihood of being impacted by late gestation preterm birth[93, 292, 293].

Whilst altered GABAergic pathway development is evident at juvenility in the cerebellum, there was no difference in the mRNA expression of allopregnanolone sensitive $GABA_A$ receptor subunits α6 and δ. This is despite overt reduced expression of both subunits in the preterm fetal and neonatal cerebellums[271]. The global cerebellar composition of neurosteroid sensitive $GABA_A$ receptor subunits was altered by preterm birth in this model at these early ages, where preterm neonates also demonstrated seizure-like behaviour and ataxia, suggesting impairment of cerebellar motor function. Despite the limited data concerning $GABA_A$ receptors subunits and motor function, a mutant mouse strain that exhibits ataxia and excessive head tossing has similar reductions in both the δ and α6 subunits in the cerebellum, providing evidence for the role of $GABA_A$ receptors in this region[294]. Taken together our data suggests that sometime between birth and juvenile age (PND28) there is either a catch-up in expression, or more likely that levels in the term brain have dropped to lower levels. Subunits of the $GABA_A$ receptor are reported to go through age-related changes in expression, with early development often a period of high expression, and down regulation of expression occurring as adulthood is approached[281]. This age-related change in expression follows the maturation profile of the brain and therefore if the neurosteroid-sensitive receptors in the preterm brain do not undergo any 'catch-up' between birth and juvenility this may be contributing to the disturbances in neurodevelopment often present in offspring of preterm deliveries. An additional vulnerability that we discovered preterm neonates face in relation to the $GABA_A$ receptors is the lack of a birth-related adaptive increase in the cerebellar mRNA levels of the α6 and δ $GABA_A$ receptor subunits after birth[271]. This potentially reduces the effect of allopregnanolone postnatally and may contribute to neurodevelopmental

delays in preterm neonates by exposing the immature brain to damaging excitotoxicity. Additionally, knockout studies of the δ subunit (which is known to commonly group with the α6 subunit) in rats and mice frequently link a lack of these subunits to the manifestation of multiple neurodevelopmental phenotypes such as anxiety-like behaviour and pro-epileptic behaviour[209, 212, 213], thus suggesting that reduced neurosteroid sensitivity plays a role in the etiology of these behaviours which are common to those born premature. Interestingly, receptor changes are present in human brain tissue in disorders that primarily affect myelination such as Alzheimers disease, Parkinsons disease, and multiple sclerosis[295], and whilst their role in disease progression is unknown the neurosteroid sensitive $GABA_A$ receptors present a common link between proper myelination and normal behaviour. Conversely, the hippocampal $GABA_A$ neurosteroid sensitive receptor subunits appear to be largely unaffected by preterm delivery with the exception of a decrease in the expression of the α5 subunit mRNA at juvenility[272]. This particular subunit is known to mediate tonic inhibition in the CA1 of the hippocampus, is required for associative learning as demonstrated by a mouse model knockout, and furthermore is known to be reduced in response to increased levels of cortisol, which were present in these animals[296-298]. Thus, a reduction in α5 subunit expression in childhood may be reducing tonic inhibition and therefore increasing excitation in the hippocampus, which may be contributing to the hyperactivity often exhibited by ex-preterm male children.

Clinical studies indicate that behaviour during childhood is affected by premature birth in a sex-dependent manner and we were able to model that in these studies. Our data indicates that juvenile males demonstrate a hyperactive phenotype following preterm birth[272] and exhibit behaviour similar to that observed in mouse

models of ADHD where, as with our study, the distance travelled and time spent mobile in open field testing is markedly higher for the affected mice compared to the controls[299]. This hyperactive type behaviour has parallels with clinical studies on ex-premature male children that show an increased incidence of disorders that to some extent involve hyperactivity[56, 57]. An underlying factor involved in the development of this phenotype may be increased cortisol. In these studies we have observed increased circulating cortisol levels in preterm offspring at birth[271], PND1[271], and juvenility[272]. In humans, one study has found that as birth weight and gestational age decreases, there is an increase in cortisol[300], and early life stress has also been shown to negatively impact hippocampal development with long-term effects into adolescence[301]. Interestingly at juvenility, male preterm offspring had increased baseline concentrations of circulating cortisol that were unaffected by exposure to foreign situations (in the form of behavioural testing), whilst females experienced a substantial rise in cortisol in response to foreign situations suggesting that they have an anxious phenotype compared to term females. Previous studies by our group suggest prenatally increased cortisol may program behaviour in childhood, for example maternal stress exposure was shown to result in increased anxious behaviours in female offspring at juvenility[265] in the absence of a parallel change in cortisol concentrations suggesting that early exposure to increased cortisol has programmed an altered behavioural response to stress-inducing situations.

These behaviour-altering affects of cortisol may be due to interactions between cortisol and allopregnanolone. Glucocorticoids, including cortisol and corticosterone, are known to interfere with allopregnanolone production and our group has previously demonstrated this using repeated administration of betamethasone (a synthetic

glucocorticoid) to pregnant guinea pig dams. Repeated glucocorticoid exposure reduced the allopregnanolone synthesizing capacity of both the placenta and the brain as demonstrated by a reduction in the expression of the rate-limiting enzyme 5α-reductase type 2[118]. Interestingly expression of this enzyme is also decreased in the brain of preterm neonates[21], which may be a result of increased cortisol exposure. Our group has also shown that both late gestation maternal stress and pharmacological inhibition of allopregnanolone synthesis by finasteride both result in a reduction of allopregnanolone concentrations in the fetus, and the presentation of an anxious phenotype in female juvenile offspring[168, 265]. Therefore, in light of this data and the findings of the studies presented here we suggest that in addition to the lack of protection by allopregnanolone against excitotoxic damage, and the raised levels of cortisol present following early exposure to the *ex utero* environment, that cortisol hinders the synthesis and action of any locally derived allopregnanolone in the preterm neonate. These changes have lasting implications on neurodevelopment and behaviour.

8.2 Effects of prenatal progesterone and neurosteroid-replacement therapies on neurodevelopment and behaviour

This thesis modeled the clinical use of maternal progesterone administration during pregnancy to delay onset of preterm labour to investigate the previously undetermined effect of progesterone administration on the developing fetus, in addition to the use of a neurosteroid-replacement therapy to ameliorate the neurobehavioural effects of preterm birth on offspring at juvenility. The rationale

behind investigating the effects of maternal progesterone administration on steroid profiles and fetal neurodevelopment stemmed from a previous study by our group utilizing progesterone for postnatal neurosteroid-replacement therapy[291]. Additionally, there is the potential of progesterone to metabolize to a number of steroids in the glucocorticoid, mineralocorticoid, estrogen, and androgen families via steroid biosynthesis enzymes that are known to be present in amniotic fluid, placenta, and fetal brain[302, 303]. These include cortisol and allopregnanolone, both of which can markedly influence fetal neurodevelopment[158, 284]. In a neonatal rat study, daily injections of 17-OHPC from PND1 to PND14, the period of medial prefrontal cortex development in the rat, resulted in a juvenile phenotype indicative of impaired synaptic pruning and cognitive flexibility[304]. Whilst the progesterone administered in the previous study successfully increased circulating allopregnanolone concentrations in the preterm female offspring, an unexpected increase in circulating cortisol concentrations was observed in the males[291]. Furthermore, the increased cortisol was associated with deficits at all stages of the oligodendrocyte lineage in the cerebellum; again, emphasizing the detrimental effects that raised cortisol has on neurodevelopment that were discussed above. Following the differing results obtained from this previous study we investigated the steroid profiles and fetal neurodevelopment following prenatal progesterone, as women at risk of preterm labour are frequently prescribed prophylactic progesterone therapy, which as the previous study suggests may have adverse consequences on fetal development. Fortunately there were no apparent effects on fetal cortisol profiles[305]. In addition, of the steroids examined, the only change in fetal steroid profiles was an increase in circulating progesterone in the term female fetuses, one week following the end of

treatment. Despite postnatal progesterone being able to increase allopregnanolone concentrations in the female preterm neonate, the lack of an increase in allopregnanolone following maternal administration in the fetus may be due to the markedly lower levels of allopregnanolone in neonates compared to levels prior to delivery[21] and that the substrate progesterone may be limited in these neonates, both of which are not the case in the fetus. In contrast, regulation of enzyme expression may limit conversion to allopregnanolone when progesterone levels are elevated. One possibility to explain the delayed rise in progesterone in this study is the ability of exogenous progesterone to be stored in fat and undergo delayed release into the circulation. Studies administering 17-OHPC have shown that it can transfer from the maternal circulation to the fetal and is detectable in fetal plasma up to 44 days following the final injection[306, 307], and furthermore that it can be found bound to nuclear progesterone receptors in the developing fetal brain[308]. Alternatively, our measurements were 24 hours after dosing and we may have missed measuring the peak levels and rather have measured baseline levels as exogenous progesterone administration to mothers has been shown to increase progesterone levels in the fetal serum within 2 hours of dosing[308]. It is also possible that a rise in fetal progesterone across the groups may not have occurred due the enzymatic activity of the aldo-keto reductase (AKR) 1C family, which features the AKR1C1, AKR1C2, and AKR1C3 enzymes whom are responsible for conversion of progesterone, 5α-dihydroprogesterone, and 5β-dihydroprogesterone into inactive metabolites[309-311]. Of these enzymes, the most catalytically efficient against progesterone is AKR1C1, also known as 20α-hydroxysteroid dehydrogenase, which is highly expressed in placental tissues[312, 313]. Consistent with absences in alterations to steroid profiles, there was no effect of

prenatal progesterone on myelination of the fetal hippocampus, and considering we used a marker of mature oligodendrocytes and the fetal environment was unchanged it is unlikely there would be alterations in cells of the early oligodendrocyte lineage.

Maternal levels of progesterone were increased following administration of exogenous progesterone but there was no effect on allopregnanolone concentrations[305]. Conversely, maternal cortisol was raised instead and as previously mentioned this can have damaging effects on the neurodevelopment of the fetus, as well as program for behavioural disorders later in life. Fortunately the rise in maternal cortisol was not enough to raise fetal levels due to the protective enzymatic 11βHSD2 barrier of the placenta, which converts cortisol into less cortisone[314]. The activity of this enzymatic barrier may have been increased to account for the excess cortisol in the maternal circulation however our data suggests that the placental expression of 11βHSD2 was not affected by progesterone therapy (see appendix 10.1) indicating basal levels were adequate. Therefore, from this study we could conclude that maternal administration of progesterone does not alter fetal allopregnanolone or cortisol concentrations and thus does not have either beneficial or detrimental effects on brain development and that this is most likely due to actions of the placental barrier.

The final component of this body of work was neurosteroid-replacement therapy in preterm neonates. Following results in the progesterone therapy studies, and the obvious potential of progesterone to metabolize to other active steroids in the neonate, a synthetic analogue of allopregnanolone was utilized. Unpublished data from our group has shown that allopregnanolone itself is unsuitable for replacement

therapy due to its very short half-life, and thus inability to have sustained effects on target receptors. The benefits of using the analogue of allopregnanolone, ganaxolone, include its inability to metabolize to active metabolites and have off target effects, its long half life of approximately 12 hours[249] meaning that twice daily doses should sustain appropriate levels for therapeutic effects to be achieved, and as an analogue it has similar actions to allopregnanolone on $GABA_A$ receptors[248]. Ganaxolone is already being used in multiple clinical trials for epilepsy management in infants and adults[249, 255].

We found that neurosteroid-replacement therapy with ganaxolone reversed some of the effects observed when animals were prematurely exposed to the *ex utero* environment. Previously we observed the greatest negative impact on male neurodevelopment and behaviour following preterm birth and thus in this pilot study of ganaxolone therapy we treated male offspring only and saw a reversal of the hyperactive behavioural phenotype and amelioration of deficits in myelination. This is the first study to show that neurosteroid-replacement therapy can replicate the *in utero* neurosteroid environment and that this restores neurodevelopment to a normal, term-born, trajectory. By combining our groups research over the past few years on pregnancy compromises and the impact of disturbances in allopregnanolone supply on the developing fetus, neonate, and the long-term effects on the juvenile, we have now shown that reestablishment of this supply in the period between birth and term equivalence is a prospective therapy for future clinical use. Whilst there is still much to investigate, this study has paved the path for future preclinical trials using neurosteroid-replacement therapy following preterm birth, and in fact this therapy may also be useful following other pregnancy compromises discussed previously

where a major contributing factor to deficits in neurodevelopment is a lack of allopregnanolone.

Unfortunately, the adverse side effect of treatment was sedation during the ganaxolone therapy period. As a result of the sedation, independent suckling from the dam was reduced and thus pups receiving therapy were provided additional feedings throughout the treatment period, despite this however they did not manage to gain sufficient weight and overall failed to thrive. Following the treatment period weight gain increased to be on par with untreated pups, suggesting that ganaxolone was interfering with metabolism of food by potentially having interactions with the gastrointestinal tract as periods of diarrhea were observed in some of the pups receiving therapy. Treatment-induced sedation was likely to have been the major contributing factor to the higher mortality rate in the treated group, as death was often as a result of being squashed by the dam, and not having the strength or awareness to move, or respiratory failure associated with aspiration of feeding fluids. Clinical trials using ganaxolone state that the most common adverse side effect is sedation and sleepiness[249, 255]. Alternatively, the suppression of excitability may have a beneficial effect in raising the seizure threshold, a major problem for human preterm neonates. Importantly, double the dose used here did not result in sedation or mortality of term born pups (unpublished data) which highlights the vast difference in metabolism and dose requirements between term born and preterm born animals which is likely to be similar for humans. This indicates the need for preclinical drug dose studies before transitioning therapies from term born babies to preterm.

8.3 CONCLUSION

This thesis has resulted in a greater understanding of the mechanisms leading to neurodevelopmental, behavioural, and learning disorders in children whom are born preterm. By utilizing our guinea pig model of preterm birth, we have identified key contributing factors related to adverse outcomes following preterm birth such as decreased allopregnanolone supply, persistently increased cortisol, an inadequate mRNA expression profile of $GABA_A$ receptors, altered myelination patterns, and deregulation of the GABAergic pathway (see Figure 8.1). We have also demonstrated that maternal progesterone therapy, whilst raising maternal cortisol, does not overtly impact the fetal steroid profile or myelination in the short term, and thus does not present an additional vulnerability (or benefit) to preterm offspring. Finally, this thesis also featured the first study using ganaxolone for neurosteroid-replacement therapy, and despite the adverse side effects, has uncovered a pathway that, following further optimization, can re-establish the *in utero* environment and prevent the development of preterm birth related neurodevelopmental and behavioural disorders.

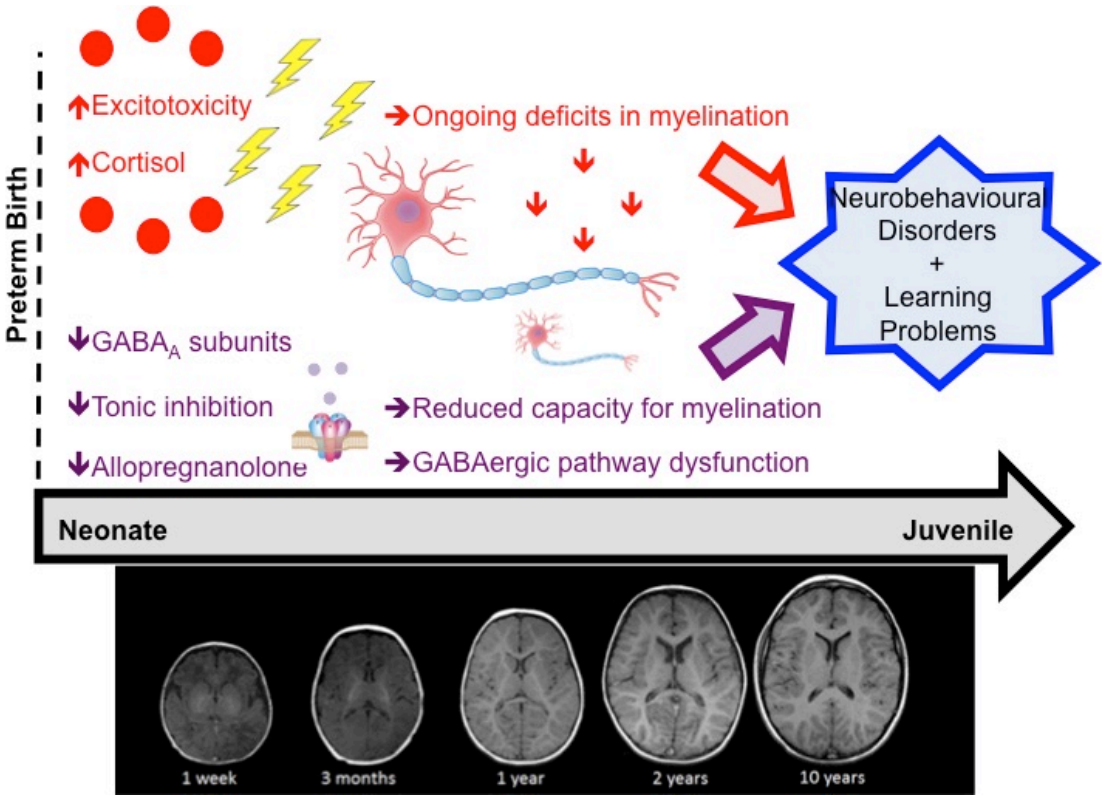

Figure 8.1. Proposed mechanisms leading to neurobehavioural and learning problems following preterm birth. We have demonstrated that preterm birth, and the associated early exposure to the *ex utero* environment, results in a dramatic drop in allopregnanolone and in the absence of an appropriate $GABA_A$ receptor subunit profile, a reduction in the levels of tonic inhibition required for proper neurodevelopment to occur. Combined with these reductions, there is an increase in excitotoxicity and cortisol levels, which actively prevent myelination from occurring at a normal rate. Approaching juvenility, and as the brain continues to grow, there is an imbalance of inhibition and excitation which results in a reduced capacity for myelination to occur and a dysfunction of the GABAergic pathway. Ultimately, this myriad of impairments leads to the appearance of neurobehavioural disorders and learning problems in the ex-preterm juvenile.

8.4 Future Directions

Whilst many questions were answered by the studies performed as part of this thesis, it has also identified further avenues for investigation. These studies focused on three age groups; the fetus, neonate, and juvenile, and thus lacking from these studies are adolescent/aged cohorts, therefore limiting the conclusions we can draw regarding

long-term alterations in behaviour and neurodevelopmental damage. Furthermore, we have identified an interesting relationship between behaviour, myelination, and cortisol in this study and further exploration of the roles of increased cortisol, such as following maternal separation which is commonplace following preterm birth, may compound this effect and provide a greater understanding of this relationship. In the future, it will also be essential to perform neurosteroid-replacement therapy with an altered dose, potentially a titrated dose, throughout the week leading to term equivalence in a preterm cohort and examine effects on neurodevelopment at term equivalence as in the current study we omitted this group in favour of observing effects on behaviour at juvenility. Finally, optimization of a method to analyse ganaxolone, possibly by liquid chromatography mass spectrophotometry (LCMS), in body fluids and tissues will provide an accurate depiction of the amount that is readily distributed and available for action at $GABA_A$ receptors following administration and excretion.

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10.0 APPENDIX

10.1 IMMUNOBLOTTING OF PLACENTAL 11BHSD2

The data presented below was analysed as part of the maternal progesterone administration study (chapter six) but was not submitted as part of the publication. Total protein expression of the placental enzyme 11βHSD2, which is responsible for the conversion of active cortisol into the less potent cortisone, was measured by immunoblotting in placentas obtained from male and female fetuses where the dam received either progesterone or vehicle therapy from mid-gestation to late-gestation. There were no significant differences identified between the treatment groups at either preterm or term gestational ages (see Figure 10.1).

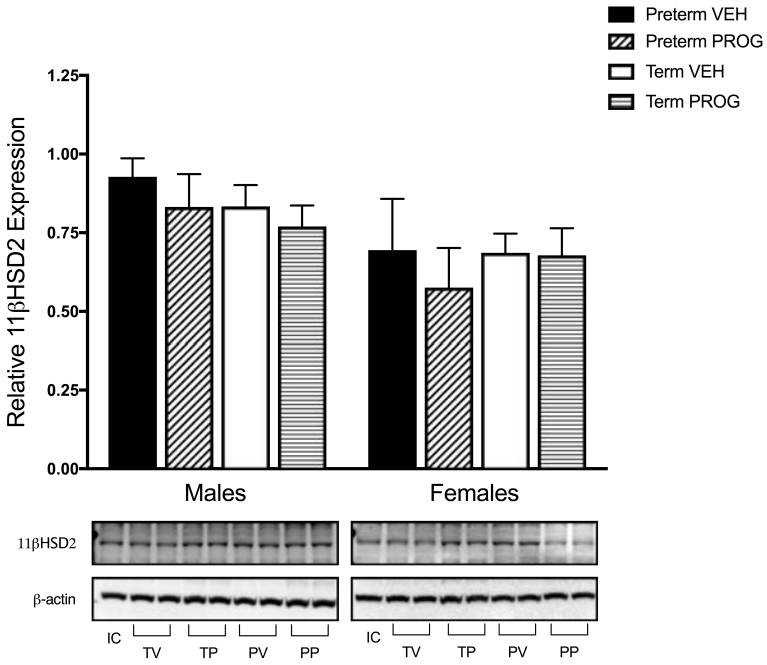

Figure 10.1 Relative protein expression of 11βHSD2 enzyme in the placenta of fetuses from progesterone or vehicle treated pregnancies. Protein expression was measured in placentas collected at preterm (solid and hashed bars) and term (open and striped bars) gestational ages from progesterone (hashed and striped bars) or vehicle (solid and open bars) treated pregnancies by immunoblotting. Data is expressed as mean SEM, with n=4-5 in each group. Representative images are displayed where IC=internal control, TV=term vehicle, TP=term progesterone, PV=preterm vehicle, and PP=preterm progesterone for 11βHSD2 and β-actin.

10.2 SUPPLEMENTARY DATA TABLE FOR CHAPTER FIVE

Supplementary Data Table 10.2 Additional immunohistochemical analyses

Parameter	Preterm Male	Term Male	Preterm Female	Term Female
Deep white matter MBP	60.7 ± 0.8	61.5 ± 0.4	60.2 ± 1.0	60.0 ± 1.5
Purkinje cells/mm^2	16.8 ± 1.9	17.3 ± 1.4	15.6 ± 1.3	16.8 ± 0.7
Lobule IX NeuN	78.2 ± 3.7	84.8 ± 1.1	80.2 ± 0.7	81.2 ± 1.8
Lobule X NeuN	72.5 ± 4.3	79.4 ± 1.2	77.5 ± 1.8	76.5 ± 1.4
Molecular layer relative thickness	38.6 ± 1.5	40.8 ± 5.2	33.7 ± 2.2	37.3 ± 1.8
Purkinje cell layer relative thickness	4.9 ± 0.2	5.6 ± 0.4	5.0 ± 0.4	5.7 ± 0.3

Data represented as mean ± SEM.

10.3 SUPPLEMENTARY DATA TABLES FOR CHAPTER SIX

Supplementary Data Table 10.3.1 Maternal outcome values

Group	Treatment	Progesterone (nmol/L)	Cortisol (nmol/L)	Allopregnanolone (nmol/L)
Pre-treatment	Vehicle	15.3 ± 7.2	-	-
	Progesterone	11.7 ± 8.2	-	-
Early-treatment	Vehicle	20.2 ± 9.8	-	-
	Progesterone	31.2 ± 6.3	-	-
Mid-treatment	Vehicle	14.6 ± 8.6	-	-
	Progesterone	51.4 ± 8.6	-	-
Late-treatment	Vehicle	11.2 ± 6.9	-	-
	Progesterone	36.9 ± 5.8	-	-
Post-treatment	Vehicle	9.5 ± 8.6	-	-
	Progesterone	11.4 ± 8.6	-	-
GA58	Vehicle	-	18.1 ± 1.9	-
	Progesterone	-	37.8 ± 5.6	-
Preterm	Vehicle	-	-	217.9 ± 26.1
	Progesterone	-	-	313.6 ± 52.1
Term	Vehicle	-	-	312.2 ± 42.4
	Progesterone	-	-	336.7 ± 25.2

Data represented as mean ± SEM.

Supplementary Data Table 10.3.2 Fetal outcome values

Sex	Group	Treatment	Progesterone (nmol/L)	Cortisol (nmol/L)	Allopregnanolone (nmol/L)	MBP CA1	MBP Cingulum	MBP SCWM
Male	Preterm	Vehicle	7.7 ± 2.7	520.2 ± 260.0	49.3 ± 7.6	16.7 ± 2.2	42.9 ± 2.7	49.9 ± 2.3
		Progesterone	12.3 ± 2.8	583.8 ± 260.0	55.2 ± 7.6	14.4 ± 2.6	44.9 ± 2.6	48.7 ± 2.5
	Term	Vehicle	10.3 ± 2.6	2696.8 ± 260.0	44.6 ± 8.4	22.5 ± 2.9	52.5 ± 3.3	50.7 ± 2.8
		Progesterone	17.1 ± 2.4	2473.0 ± 237.4	52.1 ± 7.9	24.7 ± 2.4	50.4 ± 2.7	49.4 ± 2.4
Female	Preterm	Vehicle	7.9 ± 2.7	377.6 ± 260.0	51.0 ± 8.6	15.5 ± 2.4	46.5 ± 3.2	48.9 ± 2.5
		Progesterone	10.6 ± 2.5	422.2 ± 260.0	62.1 ± 7.6	15.4 ± 2.4	44.2 ± 2.6	48.3 ± 2.4
	Term	Vehicle	10.0 ± 2.5	2290.6 ± 260.0	39.4 ± 8.1	23.1 ± 2.4	49.2 ± 2.7	48.0 ± 2.4
		Progesterone	19.0 ± 2.4	2753.0 ± 260.0	53.4 ± 7.7	24.6 ± 2.9	51.2 ± 3.2	44.0 ± 2.8

Data represented as mean ± SEM.