Chapter Four: Prenatal stress alters hippocampal neuroglia and increases anxiety in childhood.

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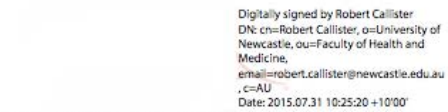
This Chapter contains a manuscript published in the Journal of Developmental Neuroscience (2015;37:533-545) and is a direct follow on study from the manuscript in Chapter two and therefore was submitted for publication to the same journal. This manuscript summarises the effect of late gestational prenatal stress on juvenile offspring using markers of neurodevelopment and also behavioural testing parameters to assess anxiety and neophobia. The role of the neurosteroid system here is also discussed. The format of the manuscript has been altered for the purposes of this thesis. The original manuscript if included in appendix A.

Statement of Author Contributions

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



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4.1 Abstract

Prenatal stress has been associated with detrimental outcomes of pregnancy, including altered brain development leading to behavioural pathologies. The neurosteroid allopregnanolone has been implicated in mediating some of these adverse outcomes following prenatal stress due to its potent inhibitory and anxiolytic effects on the brain. The aims of the current study were to characterise key markers for brain development as well as behavioural parameters, adrenocortical responses to handling and possible neurosteroid influences towards outcomes in guinea pig offspring in childhood.

Pregnant guinea pig dams were exposed to strobe light for 2 hours (9-11am) on gestational days 50, 55, 60 and 65, and were left to deliver spontaneously at term and care for their litter. Behavioural testing (open field, exploration) of the offspring was performed at post-natal day 18 (with salivary cortisol and DHEA measured) and brains were collected at post-mortem on day 21. Markers of brain development myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) were assessed via immunohistochemistry and the neurosteroid allopregnanolone and its rate limiting enzymes 5α reductase type 1 and 2 (5αR1/2) were measured in neonatal brains by radioimmunoassay, RT-PCR and western blot respectively. Brain-derived neurotrophic factor (BDNF) protein was measured as a marker of synaptic plasticity and $GABA_A$ receptor subunit expression was also assessed using RT-PCR.

Neonates born from mothers stressed during late pregnancy showed a reduction in both MBP ($p<0.01$) and GFAP ($p<0.05$) expression in the CA1 region of the hippocampus at 21 days of age. Pups of prenatally stressed pregnancies also showed higher levels of anxiety and neophobic behaviours at the equivalent of childhood ($p<0.05$). There were no significant changes observed in allopregnanolone levels, 5αR1/2 expression or $GABA_A$ receptor subunit expression in prenatally stressed neonates compared to controls.

This study shows alterations in markers of myelination and reactive astrocytes in the hippocampus of offspring exposed to prenatal stress. These changes are also observed in offspring who show increased anxiety behaviours at the equivalent of childhood, which indicates ongoing structural and functional postnatal changes after prenatal stress exposure.

4.2 Introduction

Maternal experiences during pregnancy can have profound and long lasting effects on the development of offspring; indeed, many diseases and pathologies which manifest in later life are now understood to have a prenatal origin (277). It has been proposed that prenatal maternal stress, particularly if experienced when the developing brain is susceptible to being perturbed, can disrupt normal brain development and thus result in neurobehavioural pathologies that appear later in life (278).

The normal response to stress has the effects of maintaining physiological homeostasis and this usually results in adrenal activation and the release of cortisol. However, repeated or chronic increases in stress, and hence chronically increased plasma cortisol, can entrain responses that have pathological and illness-inducing consequences for adults, including during pregnancy (279). Indeed, high levels of self-reported maternal stress and salivary cortisol concentrations in pregnant women predict poorer mental and motor development of the neonate and contribute to the determination of infant temperament (181, 280) including an increased risk for development of attention deficit disorders in childhood (281), schizophrenia (179) and depression in offspring (180). While these studies show prenatal stress can have effects on the fetus that persist after the end of pregnancy, the link between prenatal exposure to stress and altered postnatal behaviour is not fully understood. In rats, late gestation prenatal stress has been associated with altered reactive astrocyte expression and reduced synaptic density

in the frontal cortex, striatum, and hippocampus of adult offspring (120), as well as stress-induced, intensity-dependent alteration of hippocampal growth (127). Furthermore, prenatal stress was shown to be associated with reduced hippocampal levels of the important marker for neuroplasticity, brain-derived neurotrophic factor (BDNF), as well as social behavioural alterations in adult rat offspring (282) indicating prenatal stress may act through modifying hippocampal development as a mechanism of programming behaviour in later life. We have previously shown that late gestational prenatal stress in the pregnant guinea pig, a rodent with a relatively long gestation producing mature offspring at birth, results in sexually dimorphic reductions in markers for mature oligodendrocytes, reactive astrocytes and mature neurons, and with male fetuses showing profound neurodevelopmental deficits, particularly in the hippocampus (283). This study also showed a reduction in fetal circulating levels of the neuroactive steroid allopregnanolone following maternal neurosteroid administration in prenatal stress exposed pregnancies (283).

Neuroactive steroids are steroids that act in the brain with important neuromodulatory actions (188). The brain possesses the enzymes required for neurosteroid synthesis in astrocytes and oligodendrocytes (198, 199), indicating that it may be able to produce neurosteroids de novo *(284)*. In pregnancy a considerable source of neuroactive steroids is the placenta (270). Allopregnanolone, derived from the 5α 3α- reduction of progesterone, is perhaps the most important of these neuromodulators due to its action as a co-agonist with GABA at the $GABA_A$ receptor. $GABA_A$ receptors exist in a hetero-pentameric structure made up of a combination of subunits (285), the precise combination of subunits, which changes during development and is also influenced by stress, determines the binding affinity of agonists, including allopregnanolone (286). Social isolation as a stressor has been associated with reductions in tissue and plasma allopregnanolone and expression of the rate-limiting enzyme in allopregnanolone

production, 5α-reductase, indicating that stressful events may have a direct effect on neurosteroidogenesis (205, 287). Altered neurosteroid levels are also linked with various anxiety-like behaviours in both rodent models and humans (207, 288, 289), implicating this neurosteroid pathway in mediating some of the programming effects of prenatal stress exposure.

In the current study, we assessed the effect of prenatal stress on sustained changes in key hippocampal neurodevelopmental markers in guinea pig offspring at 21 days of age (equivalent to childhood in humans), together with its effects on postnatal growth and behaviour. Importantly, we assessed the effects of prenatal stress on neurosteroidogenesis and $GABA_A$ receptor subunit expression in the postnatal brain to determine if changes in this neuroprotective neurosteroid system are associated with altered brain development and/or behavioural patterns as well as prior prenatal stress exposure.

4.3 Methods

Animal stress protocol

The University of Newcastle supplied time-mated, outbred tricolour guinea pigs where all procedures were approved under the University of Newcastle Animal Care and Ethics Committee and undertaken in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. We used an established model of prenatal stress (91, 235, 283), in which pregnant dams were randomly assigned into either a stress (strobe light exposure; n=12) or control (no strobe light exposure; n=12) group. Briefly, dams allocated to the stress protocol were exposed to a strobe light for 2 hours (9am-11am) on gestational days 50, 55, 60 and 65 (term is ~71 days). The strobe light exposure events were conducted within the normal animal facility and after saliva

sampling; the dams were returned to their normal housing cages and placed into a darkened box (dimensions: 1metre wide X 1metre long X 0.7metre tall) for strobe light exposure whereas the control animals were returned in their housing cages to their normal room. This stress container was appropriately ventilated and maintained at similar temperature to the surrounding housing areas. The strobe light box was cleaned after every use to ensure no adverse reaction of the dams to residual smells within the box. Maternal saliva was collected by allowing the animals to chew on a cotton bud for approximately 30 seconds immediately before and after the stress protocol. Control animals were handled in the same manner as the stress exposed dams including saliva collection, but remained in their normal cage housing during the 2-hour test period until the second saliva sampling. Maternal saliva was analysed by methods previously described (283) to confirm a rise in cortisol concentrations following strobe light exposure, which was previously shown to markedly increase after each exposure (283), ensuring effectiveness of the stress protocol.

Neonatal protocol

All dams were allowed to deliver spontaneously, and on postnatal days (PND) 1 and 21 pups were weighed, measured for nose-rump-length (NRL), head length (HL), head circumference (HC) and abdominal circumference (AC). All procedures were carried out within visual and auditory contact of both the dam and remainder of the litter to minimise any stress to the neonate during handling (290). Dams were kept with their litters throughout the experiment. Data on physical growth parameters was analysed for all offspring used in analysis in this study (control n= 36: male n= 20; female n= 16, prenatal stress n=37: male n= 17, female n= 20).

Behavioural testing

On PND18, the behaviour of offspring (control n=23: male n=12, female n=11; prenatal stress n=24: male n=12, female n=12) was assessed using an open field and object exploration test. To achieve this, neonates were placed in the open field arena (40 x 40 x 30cm) for 10 minutes and their behaviour was recorded using the Stoelting ANY-Maze video tracking and analysis software (Stoelting Co., Wood Dale, IL, USA). Immediately following this open field test, neonates were subjected to an object exploration test for a further 10 minutes. This object exploration test involved placing two identical objects at fixed locations within the arena and allowing the animal to explore these objects for the full duration of the test. Using ANY-Maze tracking software, (Stoelting Co) the position of the animals' head (object exploration test) and also the whole body (open field) were used to determine the distance travelled and time spent in different zones within the arena. The outcome measures from the open field test include total distance travelled (locomotor activity), and time spent and crossings into the inner zone of the arena (inner 9 squares of the field divided into 49 equal squares; anxious behaviour). The object exploration test was used to determine the time spent exploring either object (object + 10% of object diameter as border for total zone) to further determine anxiety and neophobic behaviours as a function of the motivation to explore novelty in the arena or avoid the perceived danger of unknown objects (241). Saliva was collected by methods previously described immediately before and after the complete testing period. The arena was thoroughly cleaned between each test to ensure the removal of residual olfactory stimuli within the arena.

Tissue collection

Dams and neonates were euthanased at PND21 via inhalation of 100% CO_2. Neonatal body weight, crown-rump length (CRL), head length (HL), head circumference (HC) and

abdominal circumference (AC) measurements were recorded at the time of post mortem, as well as weights of organs, including the whole brain, heart, adrenal glands and liver. Brains were dissected in a sagittal plane with one half fixed via immersion in a 10% formalin neutral buffered solution (HT501128; Sigma Aldrich, Castle Hill, NSW, Australia) and the other half either further dissected to remove the hippocampus which was then weighed and snap frozen at -80^{o}C or the whole hemisphere was immediately snap frozen still containing the hippocampus. Fixed brains were used for immunohistochemical staining whilst frozen hippocampal samples were used for real-time PCR and western blotting with the frozen whole brains used for radioimmunoassay. The animal numbers for each technique are listed in parentheses.

Immunohistochemistry

The fixed brain tissues (control n= 16: male n= 8; female n=8; prenatal stress n=17: male n=9; female n=8) were embedded in paraffin wax and processed for immunohistochemical staining and analysis in methods previously described (243, 283). Briefly, two 8µm coronal brain sections per animal underwent a series of chemical washes for dewaxing, rehydration and finally inhibition of endogenous peroxidase activity. RevealIt Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia) was used for antigen retrieval followed by blocking with BSA in PBS (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide). The sections were then incubated with primary antibodies for myelin basic protein (MBP; Sigma Aldrich) and glial fibrillary acidic protein (GFAP; Sigma Aldrich) overnight at a concentration of 1:4000. On the following day, the sections were incubated with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK) for 2 hours. Subsequently, slides were then incubated in Streptavadin-Biotinylated HRP complex

(RPN1051, Amersham) for 3 hours and finally incubated in 3,3'-diaminobenzidine (DAB) concentrate with H_2O_2. Coverslips were fixed, and the slides were viewed using bright field microscopy on a Nikon Eclipse 90i microscope, and images were captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). Immunoreactivities were analysed by densitometry using ImageJ version 1.47v (National Institutes of Health, Bethesda, MD, USA) with expression presented as area coverage percentage. The brain regions analysed in the current study include the white matter area of the dorsal CA1 region of the hippocampus as well as the adjacent subcortical white matter (291). These two regions were imaged using four fields of view on two serial sections per animal (thus, eight fields of view for each animal, each region). Controls for specificity of primary antibodies were run using the appropriate IgG substitute for each primary antibody.

Western Blotting

The key neurosteroidogenic enzymes, 5α -Reductase types 1 and 2 (5αR-1/2) as well as brain-derived neurotrophic factor (BDNF) were all quantified at a protein level using western blotting by methods previously described (243).

Briefly, 20mg of brain tissue was homogenised in RIPA protein extraction buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with Complete Protease Inhibitor Cocktail (Roche Diagnostics Australia Pty Ltd) and PhosStop Phosphatase Inhibitor Cocktail Protein (Roche Diagnostics) with protein concentrations determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Subsequently, 70μg of total protein were separated on a precast NuPAGE Novex 12% Bis/Tris gel (Invitrogen) and transferred to a Hybond-P PVDF membrane (GE Healthcare, Sydney, NSW, Australia). Membranes were blocked using a BSA Blocking Solution (5% w/v BSA, 5% skim milk in 1X TBST-T; 25mM Tris-HCL, 12mM NaCl, 0.1% v/v Tween-20) and incubated

in primary antibody overnight against 5αR1/2 (Goat polyclonal antibody to 5αR1 NB100-1491- Novus Biologicals, Littleton, CO, USA; Goat polyclonal antibody to 5αR2 Ab27469-Abcam, Cambridge, UK) respectively and BDNF (Goat polyclonal antibody to BDNF (P-14) sc-33905- Santa Cruz Biotechnology Inc., Texas, USA). Immunoreactive protein was detected using ECL Western Blotting Detection kit (GE Healthcare) and the LAS-4000 Imaging System (Fuji Photo Film, Tokyo, Japan) following incubation with secondary antibody (anti-goat HRP P0449; DakoCytomation, Glostrup, Denmark). The relative protein levels (5αR1 ~26kDa; 5αR2 ~29kDa and BDNF ~32kDa) were determined using Multiguage v2.4 software (Fuji Photo Film), against β-actin loading control (ab8227-50 Abcam) and an internal loading control (guinea pig adrenal protein) on each gel. Blocking peptides specific to each primary antibody were used as control membranes to confirm specificity of each respective antibody (5αR1 NB100-1491PEP, Novus Biologicals; 5αR2 ab45681, Abcam; BDNF sc-33905 P, Santa Cruz). 5α Reductase type 1/2 and BDNF proteins were quantified in control (n=8: male n=4; female n=4) and prenatally stressed (n=8: male n= 4; female n= 4) offspring.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Hippocampal homogenates (control n=15: male n=7; female n=8, prenatal stress n=16: male n= 9; female n= 7) dissected from the sagittal frozen brain were extracted for RNA using an RNeasy miniprep kit (Qiagen, Clifton Hill, VIC, Australia) using methods previously described (73) and according to manufacturer's instructions. The quantity and quality of the RNA was determined using the ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and also by agarose gel electrophoresis. A total amount of RNA (1-3μg) was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Primer sequences and concentrations were selected and sourced from Life Technologies (Life Technologies, Mulgrave, VIC,

Australia) according to the mRNA sequence available on the guinea pig and are shown in Table 4.1. Real-time quantitative PCR was performed as previously described for $GABA_A$ receptor subunits α5 and δ and β-actin (292) and 5α R1 and 5α R2 (73). The abundance of the target sequences was determined relative to β-actin mRNA using the $\Delta\Delta$CT method using a guinea pig whole brain homogenate as the calibrator sample. There were no differences between the β-actin mRNA levels of control and prenatally stressed neonates (data not shown).

Allopregnanolone Radioimmunoassay

Allopregnanolone was determined by radioimmunoassay by methods previously described (73, 283). Briefly, 400mg of whole brain tissue (control n=16: male n=8; female n=8, prenatal stress n=16: male n= 8; female n= 8) was homogenised with 50% methanol and 1% acetic acid, respectively and after centrifugation the supernatants were applied to Sep-Pak C_{18} cartridges (Waters, Milford, Mass., USA). Tritium-labelled allopregnanolone (1,000-5,000 cpm, 5a-[9,11,12,3H(N)]; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) was used to calculate individual sample recovery concentrations. Average recovery of allopregnanolone was 75.5 ± 1.6% from brain homogenate with recoveries values used in the final calculation of allopregnanolone tissue concentration to adjust for extraction losses. Samples were then incubated with allopregnanolone antibody (AS04041, Agrisera AB, Vannas, Sweden) and radioactivity measure using β-counter (LS6500, Beckman Coulter Australia Pty Ltd, Lane Cove, Australia). The limit of detection was 25pg/mL and the inter- and intra- assay coefficients of variation were 9.39% and 2.75% respectively.

Table 4.1: Guinea pig specific primer sequences for quantitative real-time reverse transcriptase-polymerase chain reaction

Gene of interest	Forward primer sequence	Reverse primer sequence	Concentration
5α-Reductase type 1	CGA GGA GGG AAG CCA ACA	TAA CCA CAA GGC ACA ACC AGC	400nM
5α-Reductase type 2	TCA GAA AGC CTG GAG AAG TCA TC	CCG AGG AAA CAA AGC GTG AA	800nM
$GABA_A$ R subunit α5	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA	400nM
$GABA_A$ R subunit δ	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC	400nM
β-actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT	400nM

PRIMER SEQUENCES DESIGNED FOR GUINEA PIG 5α-REDUCTASE TYPES 1 AND 2, $GABA_A$ RECEPTOR A5 AND Δ SUBUNITS AND B-ACTIN GENE EXPRESSION. PRIMER SEQUENCES ARE DISPLAYED FROM 5'–3' FOR FORWARD AND REVERSE PRIMER.

Cortisol and DHEA Enzyme-Linked Immunoassay

Maternal salivary cortisol (control n=12; prenatal stress n=12) and neonatal (control n=16: male n=8; female n=8, prenatal stress n=17: male n= 8; female n= 9) salivary cortisol and DHEA levels were determined using a Salimetrics Cortisol Immunoassay kit and a Salimetrics DHEA Immunoassay kit respectively (Salimetrics Inc., State College, Pa., USA) according to manufacturer's instructions. Sensitivity of the cortisol assay was 0.012-3.0 μg/dL and inter- and intra-assay coefficients of variation were 12.04**%** and 5.52%, respectively. Sensitivity of the DHEA assay was 10.2-1000 pg/mL and inter- and intra-assay coefficients of variation were 2.89**%** and 2.16%, respectively.

Statistical Analysis

For all of the offspring outcome measures, a linear mixed model was used to characterise differences between prenatally stressed and control pups whilst adjusting for the sex of the pups and any correlation between pups from the same dams. In this way, stress exposure (group) and sex were used as independent fixed factors in the linear mixed model, and where more than one offspring was used from the same pregnancy, this was built into the model as a random factor (familial similarity) to account for any potential correlations between littermates and thus weight the analysis accordingly.

Repeated salivary cortisol concentrations were analysed using a repeated measures linear mixed model, which again adjusted for the sex of the pups and any correlation between pups from the same dams. Maternal salivary cortisol was analysed using a repeated measures two-way analysis of variance (ANOVA). All offspring data is expressed as β-coefficient values, 95% confidence intervals and $p<0.05$ considered significant. Unless otherwise specified, all data shown graphically is expressed as mean + SEM. Statistical analysis was performed using SPSS software (version 21, SPSS Inc. IBM,

Chicago Ill., USA) and graphs made using Graphpad Prism Software (version 6, Graphpad Software Inc., La Jolla, CA, USA).

4.4 Results

Maternal cortisol responses following strobe light exposure

Maternal salivary cortisol is presented as the fold change in salivary cortisol concentrations from samples collected immediately before and after the stress exposure or control-handling event (Figure 4.1). Dams who were exposed to prenatal stress in the form of strobe light exposure (n=12) demonstrated significantly increased salivary cortisol levels compared to controls (n=12) and thus showed a higher fold change in their cortisol concentrations (Figure 4.1). This significant rise in cortisol concentrations was observed after all exposure timepoints (GA 50 $p<0.0001$, GA55 $p<0.01$, GA60 $p<0.0001$ and GA65 $p<0.05$; Figure 4.1).

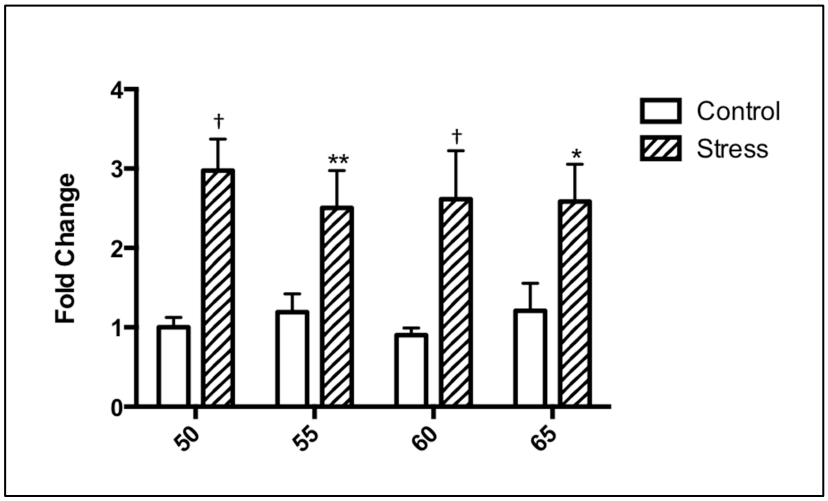

*FIGURE 4.1 MATERNAL SALIVARY CORTISOL CONCENTRATIONS IN PRENATAL STRESS EXPOSED (HATCHED BARS N=12) AND CONTROL (OPEN BARS N=12) DAMS. DATA IS EXPRESSED AS A TOTAL FOLD CHANGE IN CONCENTRATIONS BEFORE AND AFTER THE PRENATAL STRESS (OR CONTROL) EVENT ON GESTATIONAL DAYS 50, 55, 60 AND 65 (TERM 71 DAYS). * P = 0.05, * * P < 0.01 AND † P < 0.001 INDICATES SIGNIFICANCE LEVEL BETWEEN CONTROL AND PRENATAL STRESS EXPOSED GUINEA PIG DAMS.*

Effect of prenatal stress exposure on neonatal growth parameters

The offspring of both sexes of prenatally stressed guinea pigs displayed significantly increased head circumference (β=0.70, 95% CI= -1.21- -0.20, p=0.007; Figure 4.2A), abdominal circumference (β=1.01, 95% CI=-1.81- -0.20, p=0.01; Figure 4.2B), and greater nose-rump length (β=0.94, 95% CI=-1.87-0.001, p=0.04; Table 2) on postnatal day 21, despite no difference in body weight from birth to 21 days of age, compared to control (non-stressed group). Note: it is normal for the nose-rump length of females to be smaller than males, and this difference was observed in both treatment groups (β=-0.96, 95% CI= 0.05-1.86, p=0.03). At PND 21, kidney weight was significantly reduced in both male and female offspring after the prenatal stress treatment (β=-0.06, 95% CI=0.01-0.11, p=0.01; Table 4.2), as was the adrenal-to-body weight ratio (β=-0.08, 95% CI= 0.01-0.15, p=0.02; Table 4.2). Gestation length was not affected by the stress paradigm (control = 69.71 ± 0.27; stress = 70.46 ± 0.40), so the changes noted above were not due to preterm birth. Also, average birth weights were not different (control = 83.28g ± 3.20g; stress = 89.34g ± 2.26g). There was also no significant overall or sex-related effect of stress exposure on heart weight, brain weight, liver weight, brain-to-liver weight ratio, or hippocampal weight at the time of post mortem at 21 days of age.

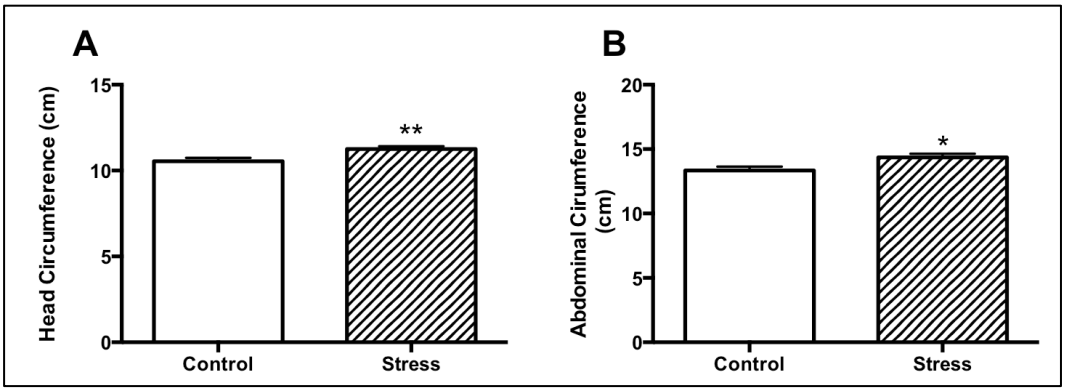

*FIGURE 4.2 GROWTH PARAMETERS IN OFFSPRING. HEAD CIRCUMFERENCE (A) AND ABDOMINAL CIRCUMFERENCE (B) OF OFFSPRING MEASURED ON POSTNATAL DAY (PND) 21 (CHILDHOOD). TREATMENT GROUPS ARE: PRENATAL CONTROL GUINEA PIGS (OPEN BARS, N =3: MALE N=18; FEMALE N=15) AND PRENATAL STRESS EXPOSED GUINEA PIGS (HATCHED BARS, N=36: MALE N=16; FEMALE N=20). ** P<0.01, *P<0.05.*

Table 4.2: Neonatal Physical Growth Parameters at 21 Days

Sex	Treatment Group	Body Weight	Brain to Body weight	Nose-Rump Length	Heart to Body weight	Liver to Body weight	Kidney to Body Weight	Adrenal Gland to Body Weight	Hippocampus to Body Weight	Brain-to-Liver-Ratio	Birth Weight
Male	Control (n=20)	224.3 + 10.9	1.3 + 0.06	21.2 + 0.4	0.44 + 0.02	4.06 + 0.10	1.12 + 0.03	0.04 + 0.003	0.09 + 0.007	0.33 + 0.02	82.6 + 4.0
	Stress (n=17)	230.7 + 8.6	1.2 + 0.04	22.8 + 0.4^{A}	0.43 + 0.02	4.24 + 0.18	1.09 + 0.02^{A}	0.04 + 0.002	0.10 + 0.009	0.31 + 0.02	87.7 + 6.4
Female	Control (n=16)	213.6 + 9.5	1.4 + 0.10	20.8 + 0.4^{B}	0.48 + 0.05	4.32 + 0.25	1.20 + 0.07	0.05 + 0.003	0.11 + 0.006	0.33 + 0.01	86.6 + 6.8
	Stress (n=20)	220.0 + 7.6	1.3 + 0.04	21.2 + 0.4^{A,B}	0.41 + 0.01	3.78 + 0.07	1.09 + 0.02^{A}	0.04 + 0.001	0.09 + 0.018	0.35 + 0.01	90.7 + 6.6

ALL VALUES ARE REPRESENTED AS A PERCENTAGE OF BODY WEIGHT AT THE TIME OF POST MORTEM WITH THE EXCEPTION OF NOSE-RUMP LENGTH, WHICH IS EXPRESSED IN CENTIMETRES (CM), BIRTH WEIGHT WHICH IS EXPRESSED IN GRAMS (G) AND BRAIN TO LIVER WEIGHT RATIO, WHICH IS A RATIO VALUE. THIS VALUE IS INDICATIVE OF GROWTH RESTRICTION AND BRAIN SPARING, WHEREBY A VALUE OF >0.9 IS USED TO CLASSIFY GROWTH RESTRICTED OFFSPRING. ^{A} *INDICATES SIGNIFICANT (P<0.05) EFFECT OF STRESS AND* ^{B} *INDICATES SIGNIFICANT (P<0.05) EFFECT OF SEX. VALUES ARE EXPRESSED AS THE MEAN PERCENTAGE + SEM AND ARE CALCULATED FOR ANIMAL NUMBERS SHOWN IN PARENTHESES.*

Effect of prenatal stress exposure on Myelin Basic Protein (MBP) expression

Following prenatal stress exposure, offspring at 21 days of age show a markedly reduced level of MBP expression in the CA1 region of the hippocampus in both males and females (β=-6.23, 95% CI= 1.32-11.13, p=0.015; Figure 4.3), compared to the control group. There was no effect of prenatal stress exposure on MBP expression in the subcortical white matter, nor was there an effect of the sex of the offspring on MBP expression (area coverage) in this region (male control = 51.4% ± 3.3%, male stress = 44.6% ± 3.1%, female control = 44.7% ± 3.7%, female stress = 42.1% ± 3.7%).

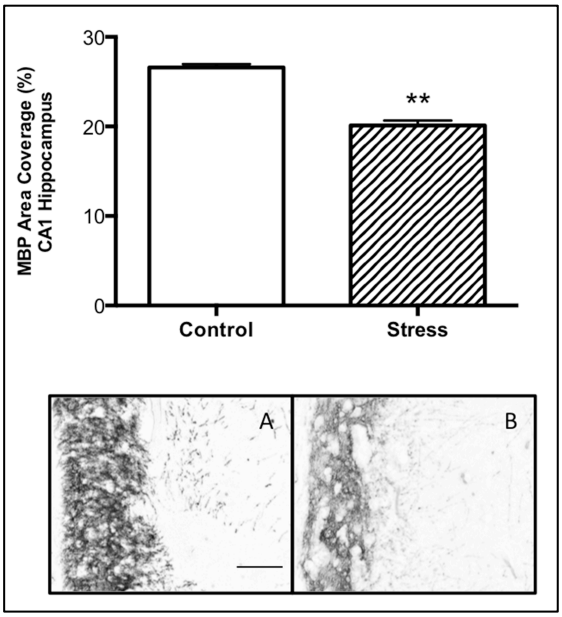

*FIGURE 4.3 EFFECTS OF PRENATAL STRESS ON MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS IN PUPS AT 21 DAYS OF AGE (CONTROL- OPEN BARS, N=16: MALE N=8, FEMALE N=8; PRENATAL STRESS- HATCHED BARS, N=17: MALE N=9, FEMALE N=8). REPRESENTATIVE IMAGES OF MBP IMMUNOSTAINING FOR CONTROL GUINEA PIG OFFSPRING (A) AND PRENATALLY STRESSED GUINEA PIG OFFSPRING (B). MBP EXPRESSION CALCULATED AS COVERAGE AREAS OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS). **P=0.01. SCALE BAR =100μM.*

Effect of prenatal stress exposure on Glial Fibrillary Acidic Protein (GFAP) expression

At 21 days of age, pups exposed to prenatal stress show significantly reduced expression of GFAP in the CA1 region of the hippocampus compared to pups from control pregnancies (β=-3.52, 95% CI=0.07-6.96, p=0.04; Figure 4.4). This effect was not present in the subcortical white matter of the cortex, immediately adjacent to the CA1 region of the hippocampus however, nor did the sex of the offspring affect MBP expression (area coverage) in this region (male control = 17.1% ± 1.9%, male stress = 13.1% ± 2.2%, female control = 16.7% ± 1.9%, female stress = 15.5% ± 2.0%).

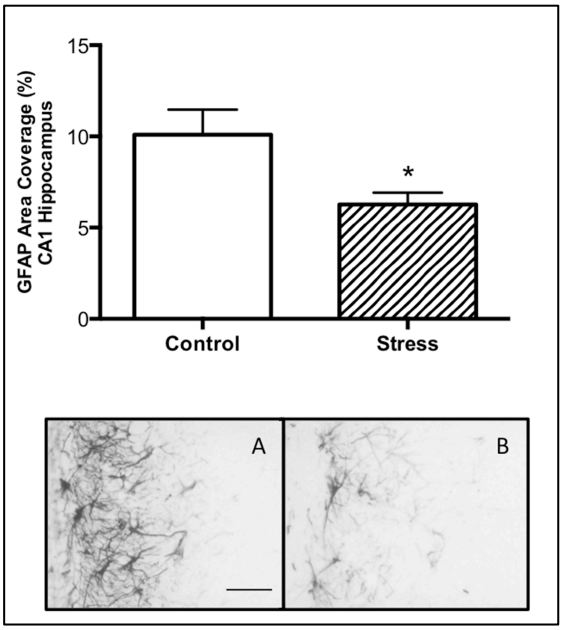

*FIGURE 4.4 EFFECTS OF PRENATAL STRESS ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS IN PUPS AT 21 DAYS OF AGE (CONTROL- OPEN BARS, N=16: MALE N=8, FEMALE N=8; PRENATAL STRESS- HATCHED BARS, N=17: MALE N=9, FEMALE N=8). REPRESENTATIVE IMAGES OF MBP IMMUNOSTAINING FOR CONTROL GUINEA PIG OFFSPRING (A) AND PRENATALLY STRESSED GUINEA PIG OFFSPRING (B). MBP EXPRESSION CALCULATED AS COVERAGE AREAS OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS). *P=0.05. SCALE BAR =100µM.*

Neurosteroidogenic capacity of neonates following prenatal stress

5α Reductase type 1/2 mRNA (Figure 4.5A and B) and protein in the hippocampus (Figure 4.5C and D) were not affected by prenatal stress exposure at 21 days of age. There were no differences in the amount of allopregnanolone extracted from brain homogenates of either sex, or of either treatment group at 21 days of age (Figure 4.6).

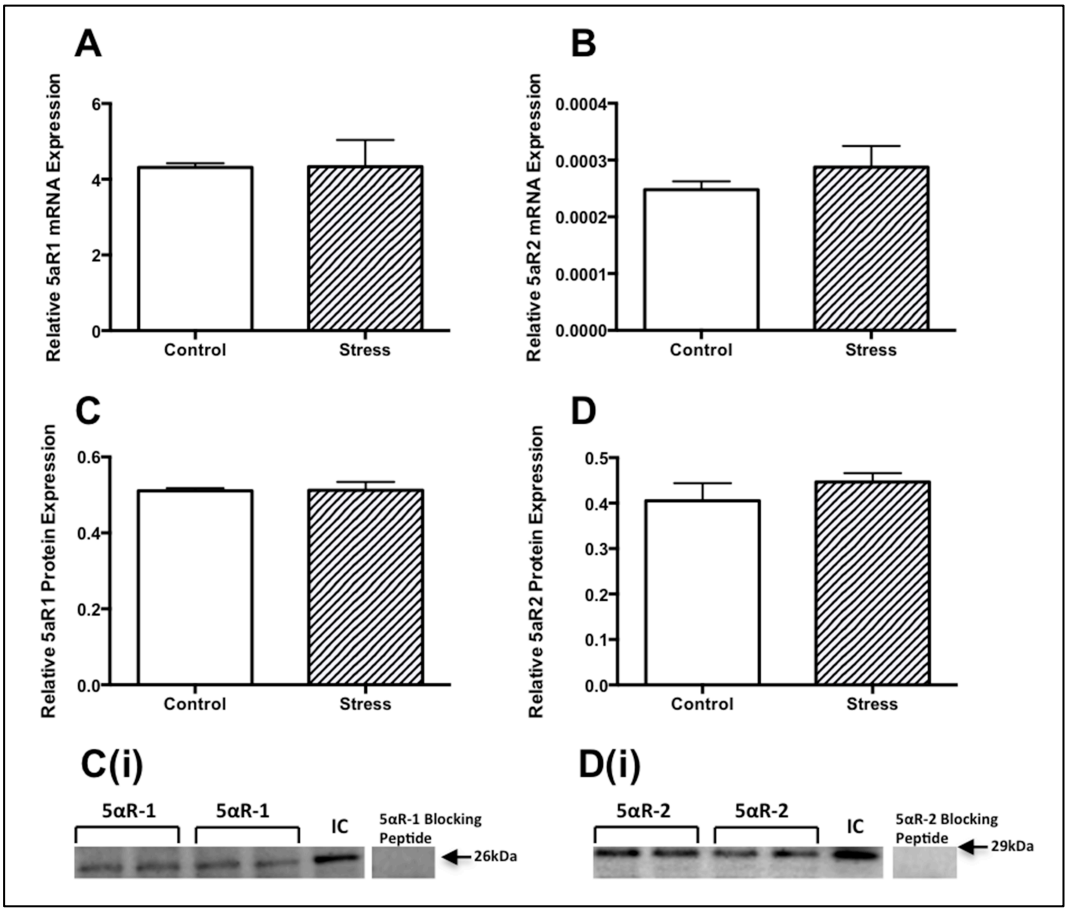

FIGURE 4.5 HIPPOCAMPAL NEUROSTEROIDOGENIC CAPACITY OF PRENATALLY STRESSED ADOLESCENT OFFSPRING. RELATIVE MRNA AND PROTEIN EXPRESSION OF 5α REDUCTASE TYPES 1 (A,C) AND 2 (B,D) IN THE HIPPOCAMPUS OF 21-DAY-OLD PUPS. PRENATAL CONTROL GUINEA PIGS (OPEN BARS, N=15: MALE N=7; FEMALE N=8) AND PRENATAL STRESS EXPOSED NEONATES (HATCHED BARS, N= 16: MALE N=9; FEMALE N=7). REPRESENTATIVE WESTERN BLOTS SHOW TWO PAIRED GUINEA PIG HIPPOCAMPAL TISSUE SAMPLES AND GUINEA PIG ADRENAL GLAND INTERNAL CONTROL (70 μG TOTAL PROTEIN PER LANE); 26 KDA BAND) AND 5AR2 (D(I); 29 KDA BAND). PRE-INCUBATION OF PRIMARY 5AR1 AND 5AR2 ANTIBODY WITH RESPECTIVE BLOCKING PEPTIDES BLOCKED SPECIFIC BINDING AT 26 KDA AND 29 KDA RESPECTIVELY IN GUINEA PIG HIPPOCAMPAL SAMPLE.

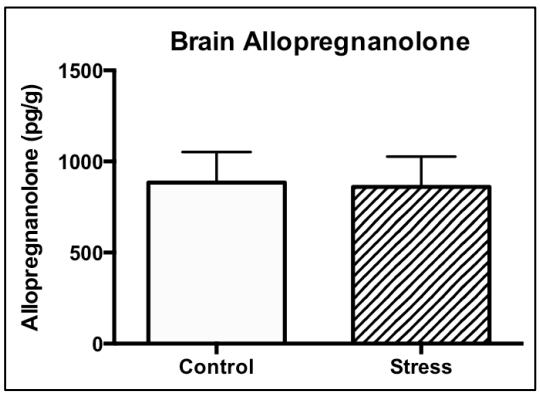

FIGURE 4.6 BRAIN ALLOPREGNANOLONE CONCENTRATIONS IN ADOLESCENT OFFSPRING. ALLOPREGNANOLONE LEVELS AS MEASURED BY RADIOIMMUNOASSAY IN BRAIN HOMOGENATE COLLECTED AT 21 DAYS OF AGE. PRENATAL CONTROL GUINEA PIGS (OPEN BARS, N=16: MALE N=8; FEMALE N=8) AND PRENATAL STRESS EXPOSED OFFSPRING (HATCHED BARS, N=16: MALE N=8; FEMALE N=8).

BDNF protein expression in guinea pig hippocampus

Relative expression of BDNF in the hippocampus of offspring exposed to prenatal stress did not differ from that of unaffected controls, in either sex (Figure 4.7).

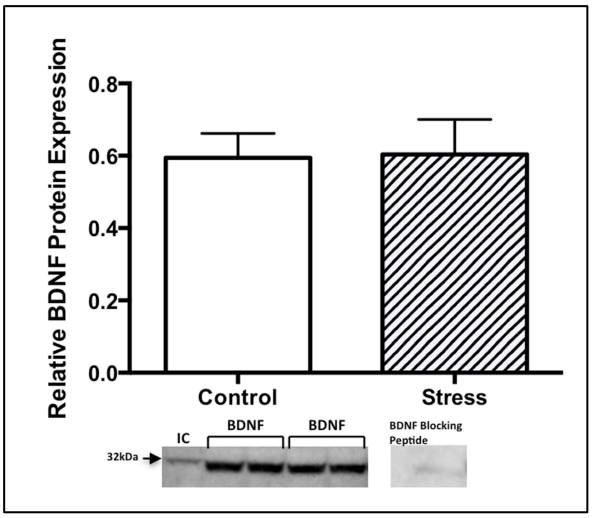

FIGURE 4.7 RELATIVE PROTEIN EXPRESSION OF BDNF IN THE HIPPOCAMPUS OF 21-DAY-OLD GUINEA PIG PUPS. PRENATAL CONTROL GUINEA PIGS (OPEN BARS, N=8: MALE N=4; FEMALE N=4) AND PRENATAL STRESS EXPOSED NEONATES (HATCHED BARS, N=8: MALE N=4; FEMALE N=4). REPRESENTATIVE WESTERN BLOTS SHOW TWO PAIRED GUINEA PIG HIPPOCAMPAL TISSUE SAMPLES AND GUINEA PIG ADRENAL GLAND INTERNAL CONTROL (70 µG TOTAL PROTEIN PER LANE). PRE-INCUBATION OF PRIMARY BDNF WITH BLOCKING PEPTIDE BLOCKED SPECIFIC BINDING AT 32 KDA IN GUINEA PIG HIPPOCAMPAL SAMPLE.

$GABA_A$ Receptor subunit expression in guinea pig offspring

There was no significant effect of prenatal stress exposure on $GABA_A$ receptor subunit δ or α5 relative mRNA expression in the hippocampus of either sex (Figure 4.8A and B respectively).

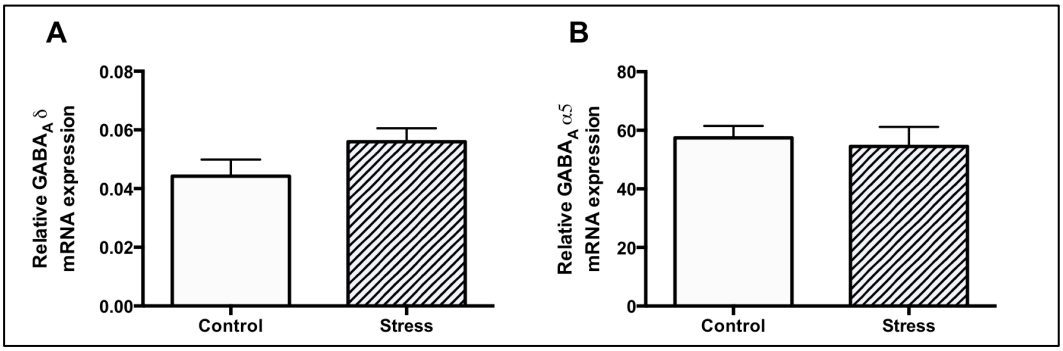

FIGURE 4.8 $GABA_A$ RECEPTOR SUBUNIT EXPRESSION. $GABA_A$ RECEPTOR SUBUNIT (A) δ AND α5 (B) EXPRESSION IN THE HIPPOCAMPUS OF PRENATAL CONTROL (OPEN BARS, N=14: MALE N=6; FEMALE N=8) AND PRENATAL STRESS OFFSPRING (HATCHED BARS, N=11: MALE N=5; FEMALE N=6).

Effect of prenatal stress on behaviour

At 18 days of age, pups from prenatal control and prenatally stressed pregnancies were tested in an open field to determine their innate activity levels, preference for inner/outer zones of the field (Figure 4.9A) and their propensity to explore objects placed in the field (Figure 4.9B). In the open field test, both male and female pups born to a prenatally stressed pregnancy spent significantly less time in the inner zone of the area (β=-32.68, 95% CI= 1.09-64.27, p=0.04; Figure 4.9A) compared to controls. Prenatally stressed offspring of both sexes also spent less time exploring the objects (β=-21.97, 95% CI= 1.83-42.10, p=0.03; Figure 4.9B) compared to their control counterparts. There were no significant differences between male and females in any parameter measured during behavioural testing.

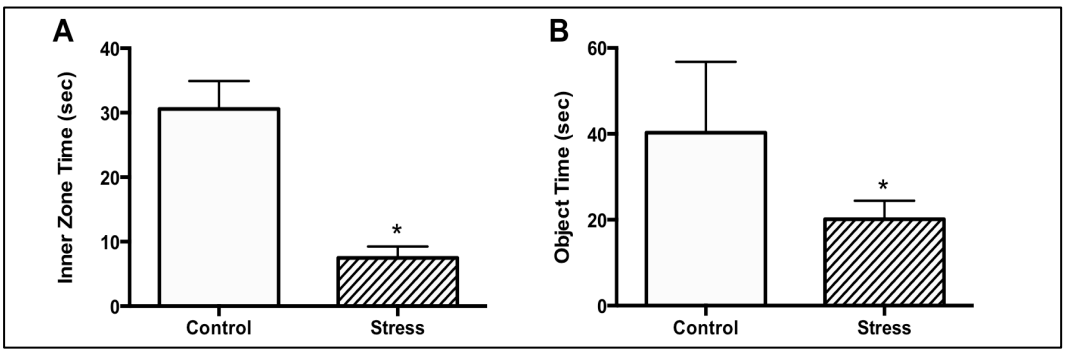

*FIGURE 4.9 BEHAVIOURAL OUTCOMES IN ADOLESCENT OFFSPRING. TIME SPENT IN INNER ZONE OF OPEN FIELD ARENA (A) AND TIME SPENT EXPLORING AN OBJECT (B) DURING BEHAVIOURAL TESTING AT 18 DAYS OF AGE IN PRENATAL CONTROL (OPEN BARS, N=23: MALE N=12; FEMALE N=11) AND PRENATALLY STRESSED OFFSPRING (HATCHED BARS, N=24: MALE N=12; FEMALE N=12). *P<0.05.*

Neonatal salivary cortisol and DHEA levels

Serving as an indirect measure of the neonatal stress response, the salivary cortisol concentrations measured immediately before and after behavioural testing did not show a significant difference between prenatally stressed and control animals, despite most neonates exhibiting a modest rise in cortisol following the behavioural testing irrespective of prenatal treatment group (Figure 4.10A). There was also no effect of sex on the levels of salivary cortisol measured at the time of behavioural testing. There was no effect of prenatal stress exposure or sex on salivary DHEA concentrations either before or after behavioural testing (Figure 4.10B), nor was there any change in the cortisol/DHEA ratio in offspring (Figure 4.10C).

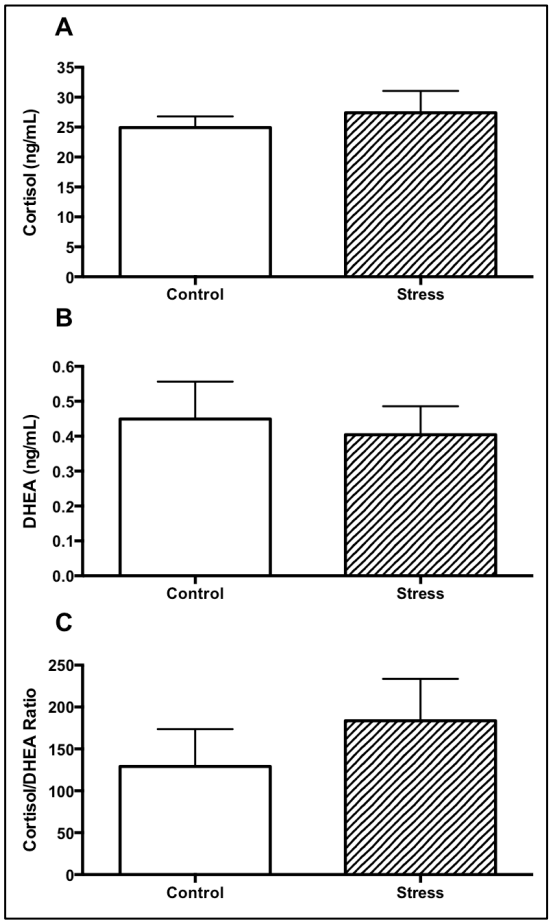

FIGURE 4.10 STRESS RESPONSIVENESS OF OFFSPRING DURING BEHAVIOURAL TESTING. SALIVARY CORTISOL CONCENTRATIONS (A), SALIVARY DHEA CONCENTRATIONS (B) AND CORTISOL/DHEA RATIO (C) IN PRENATAL CONTROL (OPEN BARS, N=16: MALE N=8; FEMALE N=8) AND PRENATALLY STRESSED OFFSPRING (HATCHED BARS, N=17: MALE N=8; FEMALE N=9) MEASURED AFTER BEHAVIOURAL TESTING.

4.5 Discussion

This study has demonstrated a persistent effect of prenatal stress exposure on the brain development and behavioural outcomes of offspring in postnatal life. We have previously shown a detrimental effect of prenatal stress exposure on fetal brain development (283) and the current study extends these observations by showing that there are ongoing deficits in key markers of mature oligodendrocyte development and reactive astrocyte expression in the postnatal brain, notably, the hippocampus. Furthermore, indicators of anxiety were increased in pups born from pregnancies that

experienced late gestational prenatal stress, and these pups were also less attentive (inquisitive) of objects placed in an open field environment. However, basal neurosteroidogenic pathways of these offspring as well as the systemic adrenocortical responsiveness (cortisol and DHEA) to handling during the behaviour testing regimens, were both not affected by prior in utero stress exposure in childhood, potentially implicating persistent structural neural changes, rather than a disturbance in neurosteroid pathways or immature adrenal functioning, in leading changes that result in altered behavioural functioning at this age. In prenatally stressed offspring in this study, the decreased drive to explore new objects within their environment is an indication of heightened avoidance and neophobic actions, which suggests a shift in these offspring towards more anxious behaviour. This study also found both sexes exposed to prenatal stress spent less time in the inner zone of the open field arena during behavioural testing which supports a role for prenatal stress in increasing vulnerability to disorders leading to anxiety-like behaviours (276, 293). With an increasing number of studies now supporting the striking association between prenatal stress exposure and the development of anxiety related disorders in offspring (294-296), there appears to be a role for the sex of the offspring in determining the severity of the outcomes associated with prenatal stress exposure (293, 297, 298). Interestingly, this was not the case in the current study design. Indeed, there is now a growing body of knowledge highlighting the impact of prenatal stress exposure on the outcomes of offspring; however the particular stressor used and behavioural parameters assessed in offspring are varied between studies and thus the outcomes within the current literature are diverse (299). Key differences in the species used as well as the type and timing of stressor used are all major variables in determining the outcome of offspring and these factors must be considered when interpreting findings. In the current study, stress was induced in late gestation in a transient manner and was shown to lead to

marked rises in maternal cortisol, which has also been shown by our group previously and others using the same model (235, 283). Prenatal stress was induced in the third trimester of pregnancy in this study design, which correlates to around 70% of gestation. This is a time of considerable cholesterol accumulation and myelination in the fetal brain (100) as well as glial cell proliferation, synaptogenesis, and a number of other neuronal and receptor maturational processes, (107, 108) making late gestation a susceptible period for neurodevelopmental damage by the effects of prenatal stress exposure. In human pregnancy, this period of brain growth occurs at around 32 weeks of gestation (0.8), whereas maximal brain growth in rats does not occur until after birth around postnatal day 7 (112, 256). These differences support the greater relevance of the guinea pig model in assessing the effects of prenatal perturbations on human behavioural outcomes (91).

The hippocampus has been implicated in many of the behavioural changes observed in offspring associated with prenatal stress exposure and is highly susceptible to adverse adaptations during fetal development and early life (116, 122, 126, 273, 300). It is understood that while the hippocampus has a large population of pyramidal cells and efferent projections to the cortex, thalamus and hypothalamus which are important for a variety of functions, notably memory and learning, it also has a high level of expression of the glucocorticoid receptor (123) potentially leading to its vulnerability to effects of stress and thus, increased glucocorticoid exposure. Previously, we have shown a sex specific effect of prenatal stress on the expression of mature oligodendrocytes and reactive astrocytes in the hippocampus, such that male fetuses exposed to stress in utero had severe reductions in their markers of brain development at term compared to both control fetuses and females (283). The findings of the current study however showed a reduction in these markers in both sexes (in a similar magnitude to that reported previously) indicating that after birth males are unable to recover their levels

of oligodendrocyte maturity or reactive astrocytes to the level of unaffected controls, but perhaps more strikingly, that females may develop a delayed reduction in their expression of neurodevelopmental markers that was not present before birth (with no changes observed in hippocampal weight in any group).

The influence of early life maternal care has also been suggested to be an important factor in determining the developmental outcomes of offspring, with the suggestion that stressed dams may perform worse at early life care of their pups, potentially exacerbating any deficits present at birth (185, 186, 293). This effect has even been shown transgenerationally, with prenatal stress being associated with altered maternal care in subsequent generations (187). However, whilst guinea pig pups demonstrate strong attachments to their mothers (290), maternal care itself is very passive in nature, limited primarily to active licking of the pups over the first postnatal week (301). In this study, there were no differences observed in neonatal birth-weight and weight gain trajectories over the 21 days after birth following prenatal stress exposure, which is indicative of normal feeding and maternal care compared to controls. However, in a model of early life stress using fragmented (removed) maternal care, the neurosteroid system, which is normally neuroprotective, becomes ineffective due to increased excitatory input in the brains of offspring (302) and therefore early life stress and the quality of maternal care following prenatal stress exposure may challenge vulnerable developing offspring and cause further neurodevelopmental, behavioural and neurosteroid deficits. In this regard, further studies assessing enriched maternal care in early life as a treatment method are warranted to assess whether optimal maternal care at this age and throughout early life may help to counteract detrimental programming effects of prenatal stress before birth. In this context, it is important to consider that previous studies have found increased excitatory input in the brains of offspring, potentially as a programming effect of prenatal stress and elevated glucocorticoid

exposure (156, 257), may be overwhelming any neuroprotective effects of normal basal neurosteroid levels in this cohort. Furthermore the finding of decreased levels of oligodendrocytes and reactive astrocytes, both of which may be neurosteroid producing (270), in this prenatally stressed population may lead to reduced levels of neuroprotection, both in the face of 'programmed' increased excitatory input and at times of stress, potentially explaining some of the behavioural deficits seen in this population despite the absence of changes in overall neurosteroid levels. This study has also shown no effect of prenatal stress on BDNF expression in the hippocampus, indicating that neurotrophic factors may not be susceptible in the hippocampus in this model of prenatal stress. However, as BDNF is often considered a marker for synaptic plasticity, this may suggest that the changes seen following prenatal stress in this study and previously (283) could be permanent structural deficits.

The $GABA_A$ receptor subunits $\alpha 5$ and δ assessed in this study are dominant within the hippocampus however no changes in $GABA_A$ receptor subunit expression were observed following prenatal stress exposure in the current study and whilst we cannot rule out changes to other $GABA_A$ receptor subunits, those measured in this study are important for neurosteroid binding. As such, whilst no changes were observed in the current study with respect to basal neurosteroidogenesis or receptor expression, any potential deficits in neurosteroid levels during a postnatal stress event cannot be ignored, with the need for further study into the ability of these offspring to cope with stress throughout the lifespan (childhood, sexual maturity, adulthood). Furthermore, in the current study allopregnanolone levels were measured from total brain homogenates and therefore any differences in the hippocampus, or other regions, could not be detected and may have affected outcomes. Although, given the lipid solubility of this steroid, production in one brain region is likely to influence adjacent regions (303). Considering the involvement of the hippocampus in the adult stress response (304), its population of

$GABA_A$ receptors (305), neuronal and glial cells (300), as well as its vulnerability to the effects of stress (116), assessing allopregnanolone concentrations specifically in this region warrants further investigation.

This study extends a growing body of knowledge highlighting the disruptive role for prenatal stress during important periods of fetal brain development. Although the role of additional brain regions contributing to outcomes in prenatally stressed offspring were not examined in the current study, the finding of marked deficits in mature oligodendrocyte development and reactive astrocyte expression in the hippocampus, indicates the effects of prenatal stress observed in this model are sustained beyond birth and into early life. This suggests that persisting prenatal stress-induced changes likely contributed to the anxious behaviour observed in guinea pigs at the equivalent of childhood in this study. This is a time in postnatal development when many neuropathologies manifest, and deficits in brain development may be important for susceptibility to further stress-induced damage and may also be potential targets for amelioration therapies.

4.6 Acknowledgements

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Chapter Five: Prenatal stress has persistent effects on the development of the cerebellum and $GABA_A$ receptor expression.

Greer A. Bennett, Hannah K. Palliser, Julia C. Shaw, David Walker, Jonathan Hirst.

This Chapter contains a manuscript in preparation for submission to the Journal of Stress. This paper analyses the effect of prenatal stress exposure on perinatal cerebellar development and also on circulating neurosteroid levels, $GABA_A$ receptor subunit expression and neurosteroidogenesis in the cerebellum.

Statement of Author Contributions

Author	Contribution	Signature
Greer Bennett	Experimental design, animal stress protocols and tissue collection, laboratory procedures, data analysis and manuscript preparation.	
Hannah Palliser	Experimental design, animal stress protocols and tissue collection and manuscript corrections.	
Julia Shaw	Laboratory procedures and manuscript corrections.	
David Walker	Experimental design.	
Jon Hirst	Experimental design and manuscript corrections.	

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Date: 2015.07.31 10:25:00 +10'00'

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

31/7/15
Date:

5.1 Abstract

Prenatal stress is associated with perturbations in fetal brain development, which may predispose offspring to neurodevelopmental and endocrine alterations later in life. Many of the behavioural pathologies often considered to have a developmental origin such as prenatal stress can be linked with the structure and function of the cerebellum. Effects on GABAergic inhibitory signalling may involve changes in levels of the subunit composition of $GABA_A$ receptors in the cerebellum, which can also be linked to disorders of hyperactivity and attention that may have a prenatal stress component in their pathogenesis. This study assessed determinants of brain growth and development and found that fetal offspring exposed to prenatal stress showed decreased expression of mature oligodendrocytes (MBP) and reactive astrocytes (GFAP) within the cerebellum (MBP $p<0.02$, GFAP $p=0.03$) however these deficits are able to return to control levels by 21 days of age. Females demonstrated increased 5α-reductase type 1 mRNA in the cerebellum ($p=0.009$), which may be a neuroprotective response to increase neural inhibition. $GABA_A$ receptor subunit δ was decreased ($p=0.005$) in prenatally stressed fetal females but this difference was resolved by 21 days of age. The α6 $GABA_A$ receptor subunit significantly increased expression from fetal life to 21 days of age ($p<0.001$). There were no differences in circulating allopregnanolone levels in prenatally stressed offspring compared to controls however as expected, circulating allopregnanolone levels dropped significantly after birth ($p<0.001$). These observations indicate that prenatal stress exposure has ongoing effects on the development of the cerebellum. GABAergic signalling in the cerebellum may be vulnerable to the effects of prenatal stress and the contribution this system has on behavioural outcomes in each sex warrants further investigation.

5.2 Introduction

Prenatal stress has been shown to have many long-term programming effects on offspring including behavioural, neuroanatomical and endocrine alterations. Indeed, many neurodevelopmental disorders and psychopathologies have developmental origins which can be linked to maternal stress during pregnancy (299). Programming can occur by altering the developing brain (306) in response to environmental cues, potentially predisposing the offspring to behavioural problems later in life (307). Previous studies have highlighted the association between prenatal stress and anxiety disorders in adult offspring (295), as well as schizophrenia (179), autism (176, 308) and attention deficit hyperactivity disorder (281, 309). Interestingly, these behavioural abnormalities and psychopathologies are also associated with alterations in cerebellar structure and/or function (150-152, 310). Furthermore, the cerebellum, once thought largely to have roles in motor functioning, has now been shown to be one of the key structures influencing emotional and cognitive functions (147). This is perhaps not surprising given the extensive afferent and efferent connections to the prefrontal cortex and limbic system (149). The cerebellum has further been shown to have important reciprocal mechanisms of activation with the hypothalamic-pituitary-adrenal axis, through peptide secretions and high expression of glucocorticoid receptors (148, 311), supporting the potential vulnerability to damage from the effects of prenatal stress. Despite these observations, and that the cerebellum continues neurogenesis throughout late pregnancy until after birth (102), there has been limited study of the effects of prenatal stress on the developing cerebellum. In rats, prenatal stress exposure has been shown to alter granule and Purkinje cell morphology and cause deficits in interneuronal connectivity in the cerebellum (153, 155) as well as reduce cell proliferation in the cerebellum after birth (312). These observations indicate the potential for persistent

alterations in cerebellar development and support the need for further understanding of the effects of prenatal stress on neurodevelopment.

Excitability of the fetal brain is an important factor in determining proper neurodevelopment (313) such that critical processes including myelination may be dependant upon it (314). Levels of GABAergic inhibition are key in regulating overall excitability, which in turn involves signalling through the $GABA_A$ receptors. Neurosteroids, potent $GABA_A$ receptor agonists, are synthesised peripherally and in the brain by oligodendrocytes and glia. $GABA_A$ receptors are made up of five subunits in a pentameric structure with the subunit composition determining the sensitivity to neurosteroids and other agonists. Subunits are highly adaptive, with expression changing with development and conditions during pregnancy, with such changes leading differences in the binding affinity of ligands, including neurosteroids. The granule cells of the cerebellum have been found to strongly express $GABA_A$ receptors in late gestation (217, 315) and acute prenatal stress has been linked with alterations in the organisation of $GABA_A$ receptor subunits (316). An increase in the δ subunit after prenatal stress has been proposed to raise the level of tonic inhibition in the prefrontal cortex and act as a compensatory mechanism to stress (317). Adult rats exposed to stress also show a decrease in $GABA_A$ receptor function and the levels of neurosteroids in the brain (205). Extrasynaptic located $GABA_A$ receptors are responsible for tonic neural inhibition. These receptors typically include the δ and $\alpha 6$ subunits and these receptors are the most highly expressed isoforms within the cerebellum (195) however to the best of our knowledge, there have been no studies assessing changes in cerebellar $GABA_A$ receptor subunit expression following prenatal stress. Therefore one of the major objectives of the current study is to determine the effect of late gestational prenatal stress on markers of cerebellar development and on the expression of key $GABA_A$ receptor subunits responsible for $GABA_A$ receptor signalling.

5.3 Methods

Prenatal stress protocol

Under a protocol approved by the University of Newcastle Animal Care and Ethics Committee and undertaken in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, pregnant outbred guinea pigs were time-mated and randomly allocated to the prenatal stress exposure group (strobe light exposure) or control group (no strobe light exposure), as previously described (283). Briefly, dams were exposed to strobe light for 2 hours (9am-11am) on gestational days 50, 55, 60 and 65 (term approximately 71 days) if allocated to the stress group. Saliva was collected from dams via mastication on a cotton bud for approximately 30 seconds immediately before and after the strobe light exposure to confirm rises in salivary cortisol (data not shown, see 283). Control allocated dams (no strobe light exposure) were also subjected to saliva collection and cortisol analysis to ensure no rise in cortisol concentrations as a result of handling.

Animal Tissue Collection

Fetal tissue collected was conducted as previously described (283). Briefly, at term, dams were euthanased via inhalation of 100% CO_2. Fetal brains were immediately removed and dissected in a sagittal plane with one half of the cerebellum snap frozen at -80^{o}C and the other half fixed via immersion in a formalin solution (4% w/v Paraformaldehyde in 0.1M Phosphate Buffer (Na_2PO_4; $NaH_2PO_4H_2O$) (Sigma Aldrich, Castle Hill, NSW, Australia).

For collection of 21-day old (juvenile) tissues, dams were allowed to spontaneously deliver at term and pups were kept with their litter for the duration of the experiment. At 21 days of age, juvenile offspring were euthanased in the same manner previously described, via 100% CO_2 inhalation. Again, juvenile brains were immediately removed

and dissected in a sagittal plane with one half of the cerebellum snap frozen at -80^{o}C and the other half fixed via immersion in a formalin solution (4% w/v Paraformaldehyde in 0.1M Phosphate Buffer (Na_2PO_4; $NaH_2PO_4H_2O$) (Sigma Aldrich).

Immunohistochemistry

The fixed cerebellums from both fetal offspring and juvenile offspring were embedded in paraffin wax and processed for immunohistochemical staining and analysis by methods previously described (243, 283) with a few minor alterations in protocol. Briefly, three serial 8μm brain sections per slide underwent a series of chemical washes for dewaxing, rehydration and inhibition of endogenous peroxidase activity before incubation in the RevealIt Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia) for antigen retrieval for 10 minutes at 80-90^{o}C. The sections were then blocked with a solution of BSA in PBS (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide) before incubation with primary antibodies for myelin basic protein (MBP; Sigma Aldrich) and glial fibrillary acidic protein (GFAP; Sigma Aldrich) overnight at a concentration of 1:4000. On the following day, secondary antibodies were incubated at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK) for 2 hours. Subsequently, slides were incubated in the VECTASTAIN® Elite® ABC System (Vector Laboratories Inc., Burlingame, CA, USA) for immunodetection at room temperature for 1 hour before incubation with 3,3'-diaminobenzidine (DAB) concentrate with H_2O_2. Finally, one section on the slide was stained with 1% cresyl violet counterstain to allow for easy orientation under the microscope and measurement of layer widths. Slides were fixed with coverslips using Microscopy DPX (Merck Australia, Kilsyth, VIC) and viewed on a Nikon Eclipse 90i microscope and images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon,

Australia). Positive immunostaining was determined using densitometry methods on ImageJ version 1.47v (National Institutes of Health, Bethesda, MD, USA). In this way, the percentage area of coverage was recorded for three fields of view per region analysed (lobe VIII, lobe X and deep white matter of the cerebellum) on two sections per animal (thus, six fields of view for each region of the cerebellum per animal). Controls for specificity of primary antibodies were run using the appropriate IgG substitute for each primary antibody.

Structural Analysis

To assess the morphology of the cerebellum, cresyl violet counterstained sections were used to measure the widths of the external granule layer, molecular layer containing the purkinje cell layer, internal granule layer, and white matter in lobes VIII and X. The layers measured have been described previously in the guinea pig brain (318). This analysis was performed serially on the sections with two measurements per layer, per lobe using the ImageJ program v1.47 (National Institutes of Health) in fetal and 21 day old offspring.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Cerebellar homogenates were extracted for RNA using an RNeasy miniprep kit (Qiagen, Clifton Hill, VIC, Australia) according to manufacturer's instructions with quantity and quality of the RNA ensured using an ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and also by agarose gel electrophoresis. RT-PCR was performed using methods and conditions previously described (73, 292) with total RNA (1-3μg) reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia). Primers were selected

and sourced from Life Technologies (Life Technologies, Mulgrave, VIC, Australia), with sequences as previously described (73, 292).

Real-time quantitative PCR was performed on a 7500 ABI real-time machine (Applied Biosystems), and results were analysed by Sequence Detection Software v2.01 (Applied Biosystems). The comparative Ct method ($2 - \Delta\Delta Ct$) was used to calculate relative fold changes in the mRNA levels of each gene relative to the house-keeping gene (β-actin) and a calibrator sample of whole guinea pig brain homogenate was used as controls in the comparative Ct method of analysis. There were no differences between the β-actin mRNA levels of control and prenatally stressed neonates (data not shown).

Allopregnanolone Radioimmunoassay

Allopregnanolone was determined by radioimmunoassay by methods previously described (73, 283). Briefly, 60μL (fetal plasma) and 600μL (21 day plasma) of relevant plasma was combined with 50% methanol and 1% acetic acid, centrifuged and the supernatants applied to Sep-Pak C_{18} cartridges (Waters, Milford, Mass., USA). Tritium-labelled allopregnanolone (1,000-5,000 cpm, 5a-[9,11,12,3H(N)]; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) was added to determine individual sample recovery concentrations accounting for sample loss during extraction. Using a β-counter (LS6500, Beckman Coulter Australia Pty Ltd, Lane Cove, Australia), radioactivity was measured using incubation with allopregnanolone antibody (AS04041, Agrisera AB, Vannas, Sweden). Average recovery of allopregnanolone was 73.1 ± 1.8% from plasma with recoveries values used in the final calculation of allopregnanolone plasma concentration to adjust for extraction losses. The limit of detection was 25pg/mL and the inter- and intra- assay coefficients of variation were 9.39% and 2.75% respectively.

Statistical Analysis

For all perinatal outcome measures, a linear mixed model was used to characterise differences between prenatally stressed and control pups using prenatal stress exposure as a fixed factor in the model as well as the sex of the pups. For pups from the same pregnancy, a random factor was created to account for any possible familial correlation in the mixed model. Furthermore, a Bonferroni correction was used to determine multiple comparisons between the groups within each sex and perinatal age cohort. All data is expressed as β-coefficient values, 95% confidence intervals and $p<0.05$ considered significant. Unless otherwise specified, all data shown graphically is expressed as mean $\pm$ SEM. Statistical analysis was performed using SPSS software (version 21, SPSS Inc. IBM, Chicago Ill., USA) with graphs made using Graphpad Prism Software (version 6, Graphpad Software Inc., La Jolla, CA, USA).

5.4 Results

Structural analysis of cerebellums

There was no significant effect of prenatal stress exposure, sex or perinatal age (fetal or juvenile) on the width of lobes VIII and X in the cerebellum (Table 5.1). The external granule layer (expressed as a percentage of total lobe width) in both lobes was significantly increased in the juvenile population compared to fetuses, irrespective of prenatal stress exposure or sex (Lobe VIII β=4.372, 95% CI=3.718-5.025, $p<0.0001$, Table 5.1; Lobe X β=3.006, 95% CI=2.610-3.402, $p<0.0001$; Table 5.1). This decrease in the external granule was reflected with a significant increase in the molecular layer containing the purkinje cell layer from the fetal to the juvenile cohort in both lobes, regardless of stress exposure or sex of the offspring (Lobe VIII β=6.192, 95% CI=3.816-

8.568, p<0.0001, Table 5.1; Lobe X β=7.454, 95% CI=5.055-9.853, p<0.0001; Table 5.1).

There was no effect of

Table 5.1: Cerebellar lobes VIII and X layer widths

		Lobe VIII Width	*Lobe VIII EGL/Lobe Width*	*Lobe VIII ML/Lobe Width*	*Lobe VIII IGL /Lobe Width*	Lobe X Width	*Lobe X EGL/Lobe Width*	*Lobe X ML/Lobe Width*	*Lobe X IGL/Lobe Width*
Control Male	Fetus n=9	1293.25 ± 189.01	6.36 ± 0.78	48.36 ± 2.10	37.59 ± 1.70	1213.39 ± 149.07	5.03 ± 0.35	46.89 ± 1.38	40.63 ± 0.63
	21 Day n=8	1292.03 ± 191.12	2.01± 0.15[a]	51.91 ± 1.77[a]	34.88 ± 0.87	1201.00 ± 153.24	2.19 ± 0.16[a]	54.10 ± 0.79[a]	36.56 ± 1.38
Control Female	Fetus n=7	1038.61 ± 57.78	5.66 ± 0.41	48.85 ± 1.44	36.88 ± 0.79	971.86 ± 66.12	4.75 ± 0.28	47.16 ± 0.92	40.56 ± 1.19
	21 Day n=8	1108.74 ± 68.40	1.68 ± 0.14[a]	53.68 ± 1.18[a]	36.18 ± 0.61	990.09 ± 53.95	1.85 ± 0.19[a]	52.59 ± 1.72[a]	40.68 ± 2.55
Stress Male	Fetus n=7	1213.39 ± 149.07	6.72 ± 0.67	46.37 ± 1.14	36.26 ± 1.04	1216.64 ± 149.62	5.31 ± 0.43	44.10 ± 2.84	37.64 ± 1.80
	21 Day n=9	1198.28 ± 62.16	1.50 ± 0.15[a]	55.35 ± 0.92[a]	34.82 ± 0.79	996.54 ± 45.51	2.15 ± 0.30[a]	52.13 ± 1.67[a]	40.00 ± 1.67
Stress Female	Fetus n=8	976.69 ± 31.21	6.08 ± 0.46	50.03 ± 1.04	35.49 ± 1.01	895.96 ± 57.44	5.34 ± 0.38	45.13 ± 1.42	43.28 ± 1.52
	21 Day n=6	1176.94 ± 52.82	2.24 ± 0.17[a]	57.18 ± 2.96[a]	36.96 ± 1.24	976.20 ± 73.67	2.15 ± 0.23[a]	54.89 ± 2.09[a]	34.98 ± 2.94

VALUES ARE REPRESENTED AS A PERCENTAGE OF TOTAL LOBE WIDTH FOR EXTERNAL GRANULE LAYER (EGL/LOBE), MOLECULAR LAYER CONTAINING THE PURKINJE CELL LAYER (ML/LOBE) AND INTERNAL GRANULE LAYER (IGL/LOBE) IN CEREBELLAR LOBES VIII AND X RESPECTIVELY. 'A' INDICATES SIGNIFICANT (P<0.05) EFFECT OF POSTNATAL AGE. ANIMAL NUMBERS SHOWN IN PARENTHESES.

prenatal stress exposure, perinatal age or the sex of the offspring on internal granule cell layer width (Table 5.1).

Effect of prenatal stress on Myelin Basic Protein (MBP) expression

In lobe VIII of the cerebellum, a significant reduction was seen in the area coverage of MBP immunostaining in fetal offspring exposed to prenatal stress compared to controls in the fetal cohort (β= -4.268, 95% CI=-7.176- -1.360, p=0.005; Figure 5.1A). Fetal levels of MBP positive immunostaining were also significantly less than those observed in the 21-day old cohort (β= -8.657, 95% CI=-12.096- -5.217, p<0.0001; Figure 5.1A) however no differences were observed between stress exposed or control offspring at 21 days of age in either sex.

In lobe X of the cerebellum, fetuses of both sexes exposed to prenatal stress showed significantly decreased expression of MBP compared to controls (β=-3.606, 95% CI=-6.651- -0.562, p=0.02; Figure 5.1B), however this was again not the case in the 21-day old population (Figure 5.1B). Fetal levels of MBP positive immunostaining were significantly less than those observed in the 21-day old cohort in both sexes (β=-5.451, 95% CI=-8.401- -2.501, p<0.0001; Figure 5.1B). There was no significant effect of the sex of the offspring on MBP expression in lobe X of the cerebellum, nor was there any significant effect of prenatal stress exposure or post natal age on MBP expression in the deep white matter of the cerebellum (data not shown).

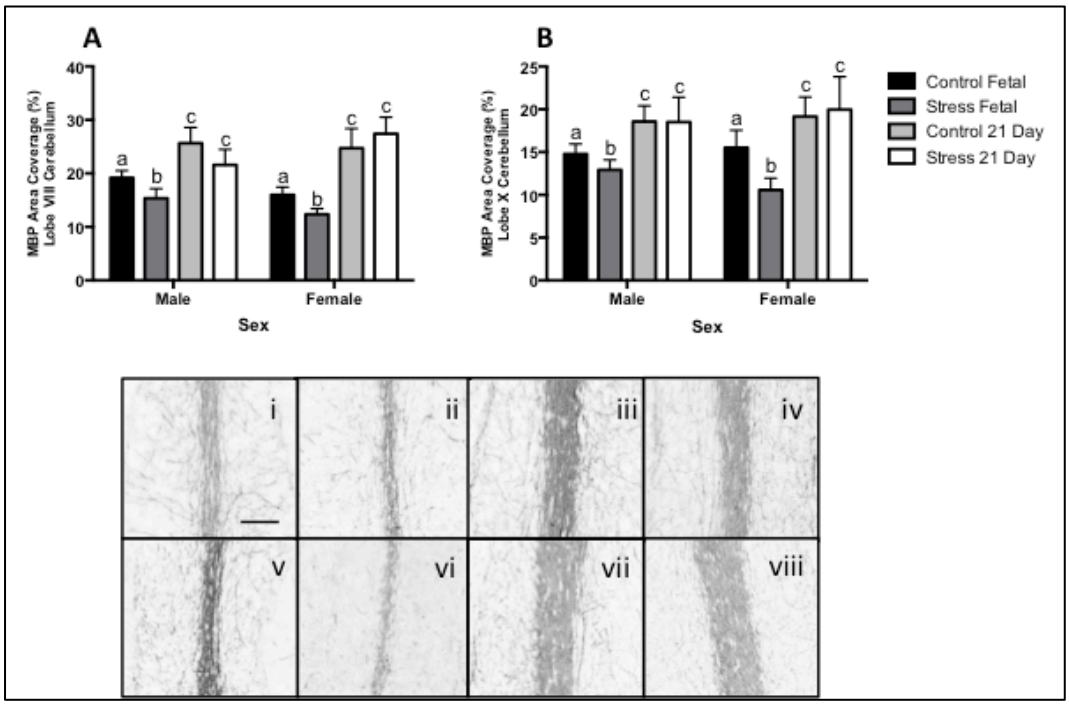

FIGURE 5.1: EFFECTS OF PRENATAL STRESS ON EXPRESSION OF MYELIN BASIC PROTEIN (MBP) IN LOBE VIII (A) AND LOBE X (B) OF THE CEREBELLUM. PRENATAL CONTROL FETUSES ARE SHOWN IN BLACK BARS (MALE N=9, FEMALE N=7), PRENATAL STRESS FETUSES ARE SHOWN IN DARK GREY BARS (MALE N=5, FEMALE N=8), PRENATAL CONTROL 21 DAY OLD OFFSPRING ARE SHOWN IN LIGHT GREY BARS (MALE N=8, FEMALE N=8) AND PRENATAL STRESS 21 DAY OLD OFFSPRING ARE SHOWN IN WHITE BARS (MALE N=9, FEMALE N=6). REPRESENTATIVE IMAGES FROM LOBE VIII ARE SHOWN IN PRENATAL CONTROL FETUSES (MALE (I), FEMALE (V)), PRENATAL STRESS FETUSES (MALE (II), FEMALE (VI)), PRENATAL CONTROL 21 DAY OLD OFFSPRING (MALE (III), FEMALE (VII)), PRENATAL STRESS 21 DAY OLD OFFSPRING (MALE (IV), FEMALE (VIII)). MBP EXPRESSION CALCULATED AS PERCENTAGE AREA COVERAGE OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS). MEANS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE $p<0.05$ LEVEL. MIXED MODELLING WAS USED TO DETERMINE THE MAIN EFFECTS WITHIN THE MODEL WITH A BONFERRONI ADJUSTMENT FOR MULTIPLE COMPARISONS.

Effect of prenatal stress on Glial Fibrillary Acidic Protein (GFAP) expression

Positive immunostaining for GFAP was reduced in the male prenatal stress exposed group at 21-days of age compared to their control counterparts in lobe VIII (β= -3.882, 95% CI= -7.393- -0.371, p=0.03; Figure 5.2A) and the deep white matter (β= -10.028, 95% CI= -19.192- -0.864, p=0.03; Figure 5.2B) of the cerebellum with no effect of prenatal stress exposure on fetal or 21-day old females or on the fetal male cohort (Figures 5.2A and B). Control males also showed significantly reduced expression of GFAP in lobe VIII of the cerebellum compared to their female control counterparts at 21

days of age that was not apparent fetally (β= -6.132, 95% CI= -9.951- -2.313, p=0.005; Figure 5.2A).

There was also no significant effect of prenatal stress exposure, irrespective of the sex of the pups, on the expression of GFAP in lobe X of the cerebellum either as a fetus or at 21 days of age (data not shown).

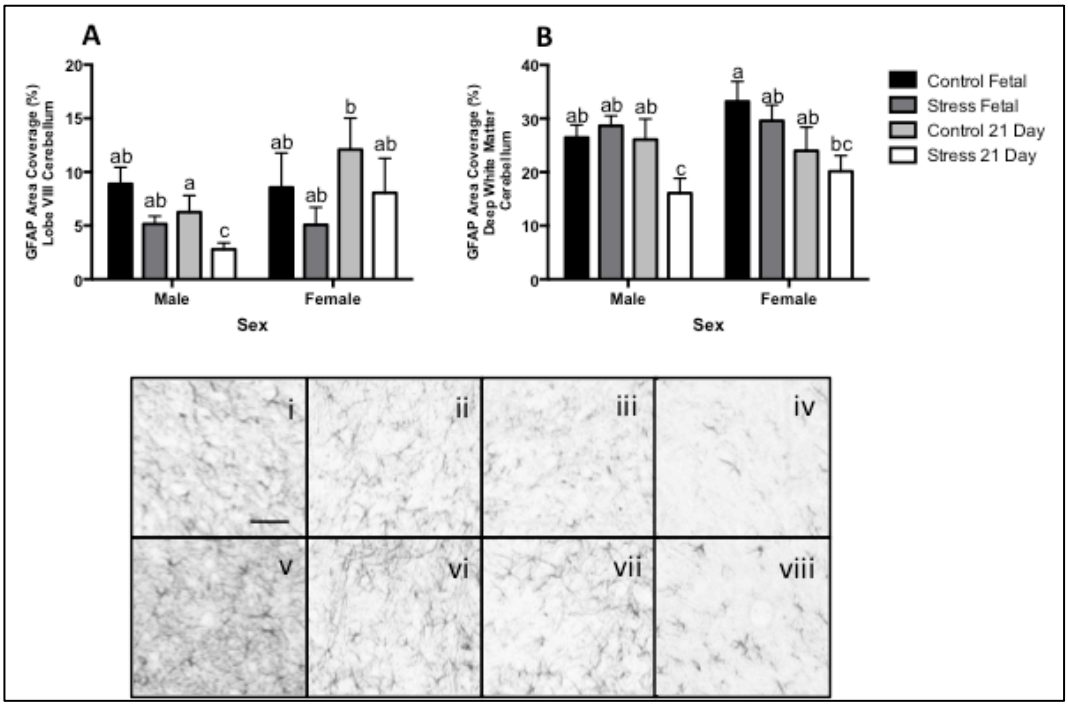

FIGURE 5.2: EFFECTS OF PRENATAL STRESS ON EXPRESSION OF GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) IN LOBE VIII (A) AND THE DEEP WHITE MATTER (B) OF THE CEREBELLUM. PRENATAL CONTROL FETUSES ARE SHOWN IN BLACK BARS (MALE N=9, FEMALE N=7), PRENATAL STRESS FETUSES ARE SHOWN IN DARK GREY BARS (MALE N=5, FEMALE N=8), PRENATAL CONTROL 21 DAY OLD OFFSPRING ARE SHOWN IN LIGHT GREY BARS (MALE N=8, FEMALE N=8) AND PRENATAL STRESS 21 DAY OLD OFFSPRING ARE SHOWN IN WHITE BARS (MALE N=9, FEMALE N=6). REPRESENTATIVE IMAGES FROM THE DEEP WHITE MATTER ARE SHOWN IN PRENATAL CONTROL FETUSES (MALE (I), FEMALE (V)), PRENATAL STRESS FETUSES (MALE (II), FEMALE (VI)), PRENATAL CONTROL 21 DAY OLD OFFSPRING (MALE (III), FEMALE (VII)), PRENATAL STRESS 21 DAY OLD OFFSPRING (MALE (IV), FEMALE (VIII)). MBP EXPRESSION CALCULATED AS PERCENTAGE AREA COVERAGE OF IMMUNOHISTOCHEMICAL STAINING (SEE METHODS). MEANS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL. MIXED MODELLING WAS USED TO DETERMINE THE MAIN EFFECTS WITHIN THE MODEL WITH A BONFERRONI ADJUSTMENT FOR MULTIPLE COMPARISONS.

$GABA_A$ Receptor Subunit composition in prenatally stressed offspring

There was no effect of prenatal stress exposure on $GABA_A$ receptor subunit α6 mRNA expression in the fetal or 21 day old cerebellum however there was a significant increase in relative expression from fetal levels to the expression seen at 21 days of age

(β=1.531, 95% CI= 0.845-2.216, p<0.0001; Figure 5.3A). Within the juvenile cohort, females also showed an overall increase in expression of the α6 subunit compared to males (β=1.276, 95% CI= 0.130-2.421, p=0.03; Figure 5.3A).

In the δ $GABA_A$ receptor subunit, female fetuses showed a significant reduction in relative expression compared to control fetuses (β=-0.857, 95% CI=-1.280- -0.430, p=0.002; Figure 5.3B), however this was not significant by 21 days of age (Figure 5.3B).

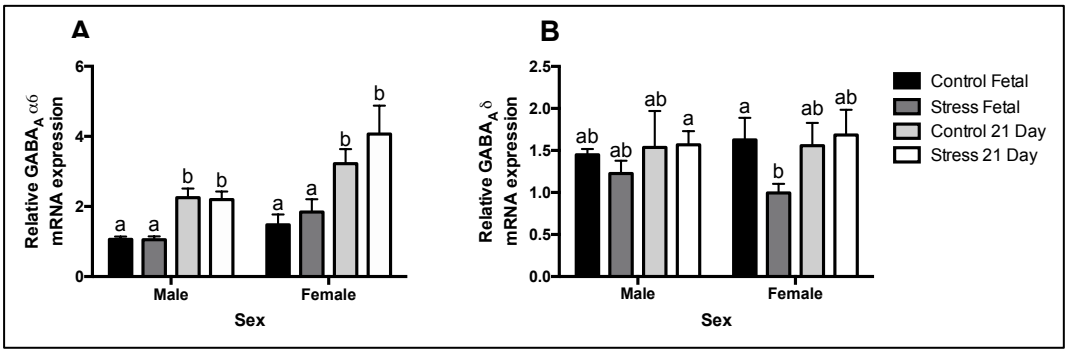

FIGURE 5.3: RELATIVE MRNA EXPRESSION OF $GABA_A$ RECEPTOR SUBUNIT α6 (A) AND δ (B) IN THE CEREBELLUM OF PRENATAL CONTROL FETUSES (BLACK BARS, MALE N=8, FEMALE N=6), PRENATAL STRESS FETUSES (DARK GREY BARS, MALE N=8, FEMALE N=10), PRENATAL CONTROL 21 DAY OLD OFFSPRING (LIGHT GREY BARS, MALE N=7, FEMALE N=11) AND PRENATAL STRESS 21 DAY OLD OFFSPRING (WHITE BARS, MALE N=8, FEMALE N=12). EXPRESSION IS SHOWN RELATIVE TO β-ACTIN USING THE COMPARATIVE CT METHOD OF CALCULATION. MEANS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL. MIXED MODELLING WAS USED TO DETERMINE THE MAIN EFFECTS WITHIN THE MODEL WITH A BONFERRONI ADJUSTMENT FOR MULTIPLE COMPARISONS.

Effect of prenatal stress on neurosteroid pathways

Female offspring exposed to prenatal stress at 21-days of age show increased 5α-Reductase type 1 mRNA levels in the cerebellum compared to controls at the same age (β= 67.13, 95% CI= -115.16 - -19.15, p=0.009; Figure 5.4). There was no significant effect of prenatal stress exposure or sex on relative 5α-Reductase type 1 mRNA levels in the fetal and male 21-day old population (Figure 5.4). *5*α-Reductase type 2 mRNA could not be detected in the cerebellum of either cohort due to low expression levels.

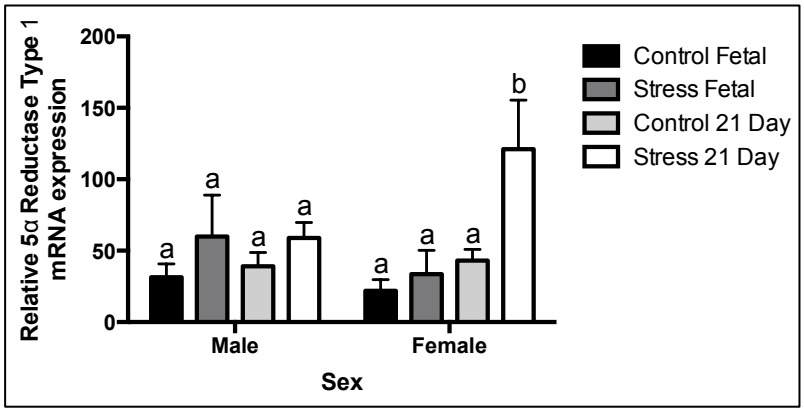

FIGURE 5.4: RELATIVE MRNA EXPRESSION OF 5α REDUCTASE TYPE 1 IN THE CEREBELLUM OF PRENATAL CONTROL FETUSES (BLACK BARS, MALE N=8, FEMALE N=6), PRENATAL STRESS FETUSES (DARK GREY BARS, MALE N=8, FEMALE N=6), PRENATAL CONTROL 21 DAY OLD OFFSPRING (LIGHT GREY BARS, MALE N=6, FEMALE N=6) AND PRENATAL STRESS 21 DAY OLD OFFSPRING (WHITE BARS, MALE N=7, FEMALE N=7). EXPRESSION IS SHOWN RELATIVE TO β-ACTIN USING THE COMPARATIVE CT METHOD OF CALCULATION. MEANS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL. MIXED MODELLING WAS USED TO DETERMINE THE MAIN EFFECTS WITHIN THE MODEL WITH A BONFERRONI ADJUSTMENT FOR MULTIPLE COMPARISONS.

Plasma concentrations of allopregnanolone were not altered by prenatal stress exposure or sex of the offspring however as expected, fetal levels were significantly higher than those at 21 days of age (β= 56.385, 95% CI= 45.22-67.55, p<0.0001; Figure 5.5).

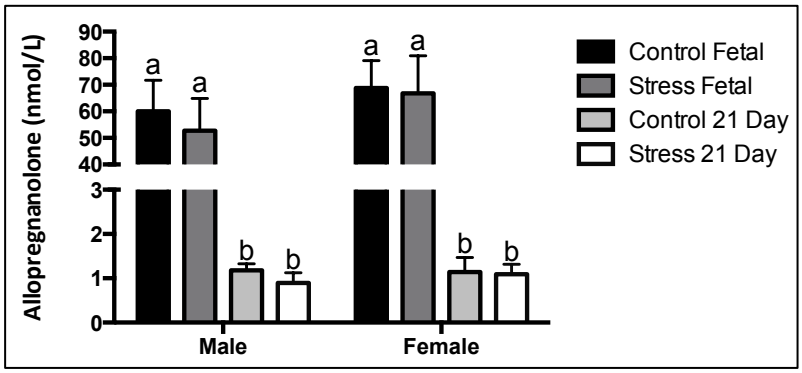

FIGURE 5.5: PLASMA ALLOPREGNANOLONE CONCENTRATIONS (NMOL/L) IN PRENATAL CONTROL FETUSES (BLACK BARS, MALE N=4, FEMALE N=5), PRENATAL STRESS FETUSES (DARK GREY BARS, MALE N=5, FEMALE N=5), PRENATAL CONTROL 21 DAY OLD OFFSPRING (LIGHT GREY BARS, MALE N=7, FEMALE N=5) AND PRENATAL STRESS 21 DAY OLD OFFSPRING (WHITE BARS, MALE N=7, FEMALE N=5). MEANS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL. MIXED MODELLING WAS USED TO DETERMINE THE MAIN EFFECTS WITHIN THE MODEL WITH A BONFERRONI ADJUSTMENT FOR MULTIPLE COMPARISONS.

5.5 Discussion

The main finding of this study is that prenatal stress is associated with specific alterations in cerebellar development that are largely sexually dimorphic. This is the first study to show that prenatal stress exposure affects the neurosteroidogenic capacity of the cerebellum in female offspring. This study has also shown a stress-induced alteration in mature oligodendrocyte and reactive astrocyte marker expression in the cerebellum, indicating that this brain region is particularly susceptible to the effects of prenatal stress, with males being somewhat more vulnerable to these alterations than females.

In the current study, posterior lobes VIII and X were analysed due to their respective time of development (lobe VIII early developing, lobe X late developing 111) in order to further understand any stress-induced perturbations in cerebellar development. As expected, fetal offspring in this study had larger external granule cell layers compared to their 21-day-old counterparts, a finding that confirms postnatal migration of granule cells inward in the cerebellum to ultimately form a mature adult brain (110, 111). This is further confirmed by the increased width of the molecular layer in both lobes analysed from fetal to early life, again suggesting a functional inward migration of granule cells.

An increased level of mature oligodendrocyte expression in the 21 day old cerebellums compared to fetal expression also confirms postnatal maturation in these brains.

In response to prenatal stress, males and females show a reduction in myelinating oligodendrocytes in both the early developing lobe VIII of the cerebellum and the late developing lobe X of the cerebellum, however these reductions in oligodendrocyte expression are able to return to levels of unaffected controls by 21 days of age. A reduction in mature oligodendrocytes early in life however may have downstream effects on offspring later in life whereby reduced myelination during childhood could affect later behaviours despite a catch up in expression by juvenility.

In contrast, reactive astrocyte expression is reduced in a number of cerebellar regions in male offspring only at 21 days of age compared to controls. Indeed, many studies now agree that male offspring are particularly vulnerable to the effects of prenatal stress with both human and animal studies reporting specific differences in outcomes between males and females (81, 262). This decrease in reactive astrocyte expression in prenatally stressed male offspring was significant in lobe VIII despite control males showing already reduced expression compared to their female counterparts at 21 days of age. Previous studies using a model of maternal deprivation in rats found reactive astrocyte cell number was increased in male offspring, indicative of astrocytic gliosis (319). Although maternal cortisol concentrations were significantly raised as a result of the stress protocol in this study (283), prenatal stress was induced in a mild-moderate transient manner and therefore gliosis would not be expected to be extensive as a result. Astrocytes have been shown to have critical neuroprotective and neuro-supportive functions (263), thus decreased astrocyte activation following prenatal stress in this study may indicate reduced neuronal stability in a population of vulnerable cells within the male cerebellum which could confer increased risk for the development of neuropathologies later in life. Previously, using the same model of prenatal stress, we have shown reduced reactive astrocyte expression in the CA1 region of the hippocampus in prenatally stressed males that was further associated with altered behavioural development by childhood/juvenility (283, 320).

The GABAergic neuro-signalling pathway is the main inhibitory pathway in the cerebellum and is comprised of Purkinje cells, Golgi cells and basket cells, among others (321). Appropriate levels of inhibition (and excitation) are imperative for normal neurodevelopment, both pre and postnatally (322). The $GABA_A$ receptor, which regulates neural inhibition in the cerebellum, is stimulated by the neurosteroid allopregnanolone, which has also been implicated in the regulation of prenatal stress

and other affective disorders (226, 323). In this study, as expected, circulating allopregnanolone levels were significantly reduced at 21 days of age compared to the fetal cohort as a result of birth and the removal of the neurosteroid producing placenta (203). However interestingly, prenatally stressed females at 21 days of age showed increased mRNA levels of the key neurosteroid producing enzyme, 5α Reductase type 1, in their brains suggesting a stress-induced alteration in neurosteroidogenesis pathways at this age. Specifically, juvenile females appear to have increased 5α Reductase type 1 levels as well as showing no effect of prenatal stress on markers of brain development indicating that this selective increase in the neurosteroidogenic capacity of the female brain may be neuroprotective. Indeed, rats subjected to swim stress show increases in 5α-reduced metabolites within just 5 minutes of stress, highlighting the importance of this system as an endogenous neuroprotectant (201). Despite circulating allopregnanolone levels remaining the same in prenatally stressed and control cohorts in this study, an alteration in cerebellar 5α Reductase mRNA suggests that any effect on allopregnanolone is likely to be region specific and not evident in broader, total body concentrations as measured in the circulation. Unfortunately due to the small size of the guinea pig cerebellum, measurement of allopregnanolone in this region was not possible.

The most abundant cerebellar $GABA_A$ receptor subunits are δ and α6. In this study, the δ subunit was decreased in females fetally in response to prenatal stress however these levels matched controls by 21 days of age. The α6 subunit increased significantly in expression from the fetal to the 21-day-old cohort and this increase parallels a significant reduction in allopregnanolone, suggesting that the α6 subunit is important in postnatal life for regulation of the $GABA_A$ receptor to increase sensitivity, compensating for reduced levels of the ligand itself. An increase in the α6 receptor subunit has previously been shown in guinea pigs after birth, confirming that an increase in this

subunit is typical of postnatal brain growth (292). The δ subunit of the $GABA_A$ receptor has often been linked with stress exposure, both changing in response to stress and also acting on CRH neurons themselves (316, 324).

Previous studies have found an increase in excitatory input in the brains of offspring, potentially as a programming effect of prenatal stress and elevated glucocorticoid exposure (156, 257). Importantly for the current study, this effect may overwhelm a normally balanced neurosteroid system. Thus, although this study has shown that prenatal stress does not alter $GABA_A$ receptor subunit composition at juvenility (δ and α6) or basal circulating allopregnanolone levels, if prenatal stress results in increased basal excitatory input, then it is possible that these offspring may already be predisposed to further damage if they themselves face a stressful challenge. Moreover, given the sex-specific reduction in reactive astrocyte expression in the cerebellum at juvenility, it is possible that this male phenotype could confer reduced levels of endogenous neuroprotection, particularly at times of stress.

The cerebellum, whether in human imaging or animal studies, has been linked to various neuropathologies including autism, schizophrenia, mood and anxiety disorders and attention-deficit-hyperactivity disorder (325, 326), conditions which can also be linked to prenatal stress exposure (83). Very few studies have assessed the effect of prenatal stress on cerebellar development however one previous study in primates has shown that early life stress (peer-rearing instead of mother-rearing) is associated with an enlarged cerebellar vermis (327) and that this alteration may confer increased risk for neuropathologies. Another study assessing maternal pregnancy anxiety at 19 weeks of gestation found that grey matter was reduced in the cerebellum in offspring at 6-9 years of age, suggesting a long-term alteration in cerebellar structure following maternal anxiety (328). Therefore the implications of the current study are to confirm that prenatal stress alters specific layers and markers of brain development in the

cerebellum, which may contribute to behavioural pathologies later in life. This study has also shown for the first time, that prenatal stress exposure is associated with alterations in the neurosteroidogenic pathway in female offspring, an adaptation that appears to be neuroprotective. Whilst the contribution of other $GABA_A$ receptor subunits or *de novo* synthesis of allopregnanolone in the cerebellum cannot be excluded, the current study provides novel data on $GABA_A$ receptor subunits in the cerebellum following prenatal stress, which warrants further investigation.

Chapter Six: Timing matters: The effect of early, late and acute prenatal stress on neurodevelopment, behavioural and neurosteroid responses in offspring.

Greer A. Bennett, Hannah K. Palliser, Julia C. Shaw, David Walker, Jonathan Hirst.

This Chapter contains a manuscript in preparation for submission to the Journal of Psychoneuroendocrinology. The effect of prenatal stress at different times in gestation is investigated using markers of neuroglia as well as circulating neurosteroid levels in both fetal and juvenile populations of offspring. Anxiety and neophobia are also assessed in the juvenile cohort.

It is of note in this Chapter that in order to assess the effect of the timing of prenatal stress exposure on outcomes, additional stress timing groups were included to that previously reported in Chapters 3,4 and 5. For reference, the 'mid' stress group in this Chapter includes some animals and data previously reported.

Statement of Author Contributions

Author	Contribution	Signature
Greer Bennett	Experimental design, animal stress protocols and tissue collection, laboratory procedures, data analysis and manuscript preparation.	
Hannah Palliser	Experimental design, animal stress protocols and tissue collection and manuscript corrections.	
Julia Shaw	Laboratory procedures and manuscript corrections.	
David Walker	Experimental design.	
Jon Hirst	Experimental design and manuscript corrections.	

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Date: 2015.07.31 10:24:40 +10'00'

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

Date: 31/7/15

6.1 Abstract

Prenatal stress has been associated with a variety of developmental changes in offspring, notably those associated with brain development and subsequent risk for neuropathologies later in life. Recently, the importance of the timing of the stressor during pregnancy has been emphasized, with specific outcomes for offspring dependent on the systems undergoing development during the stressor. Neurosteroids including allopregnanolone have been implicated in the regulation of stress and also for endogenous neuroprotection in offspring.

Prenatal stress was induced using strobe light exposure in time-mated pregnant guinea pigs in three defined stress exposure groups (early, mid and late stress). Those allocated to an early stress group were subjected to 2 hours of strobe light (9-11am) every 5 days beginning from gestational day 35 with term being approximately 69 days in the guinea pig. Dams in the mid pregnancy stress group were subjected to strobe light beginning on gestational day 50 every 5 days and those allocated to late stress group exposed to strobe light on gestational days 60 and 65. A fetal cohort were euthanized at term with fetal brains and plasma collected. A separate cohort of offspring were spontaneously delivered and subjected to behavioural testing (open field and object recognition) to measure anxiety at 18 days of age with brains and plasma collected at 21 days of age. Neurodevelopment was measured using markers for mature oligodendrocytes and reactive astrocytes in the CA1 region of the hippocampus and the subcortical white matter and the neurosteroid allopregnanolone was measured by radioimmunoassay in fetal and postnatal plasma.

In the CA1 region of the hippocampus, fetuses from all stress-timing groups showed reduced ($p<0.05$) expression of mature oligodendrocytes and reactive astrocytes. By juvenility, all male stress exposure groups had recovered to levels of unaffected controls with the exception of the early stress group. In females in juvenility, mature

oligodendrocyte marker expression did not recover in any stress group to control levels however reactive astrocyte expression was reduced in the early and late stress groups. Increased reactive astrocyte expression was also apparent in the subcortical white matter in both sexes both at term and at juvenility. Offspring of both sexes also displayed anxious behaviour as seen by wall hugging and reduced inquisitive behaviour at juvenility. Circulating allopregnanolone concentrations were significantly reduced in both the early stress exposed and late stress exposed offspring with those in the early stress group remaining reduced by 21 days of age.

This study has shown the profound effects of prenatal stress on neurodevelopment by altering mature oligodendrocytes and reactive astrocytes in the CA1 region of the hippocampus and the subcortical white matter, effects that persisted from fetal life into juvenility with the most prominent effects being on those offspring subjected to stress beginning early in gestation with the longest duration of exposure. Prenatally stressed offspring also showed increased anxious behaviour at 18 days of age. Pups born to a pregnancy stressed from early pregnancy also showed persistent reductions in allopregnanolone concentrations, indicating that this group is particularly vulnerable to the effects of prenatal stress.

6.2 Introduction

Prenatal stress has been linked with altered pregnancy outcomes such as preterm birth and low birth weight (74), as well as alterations in fetal brain development (262) that may contribute to neuropathologies later in life in offspring (329). An increasing number of studies also highlight the importance of fetal sex, the type and the timing of the prenatal stressor experienced in determining the outcomes for offspring. Indeed, the timing at which the stressor is experienced during pregnancy not only affects the extent to which the maternal hypothalamic-pituitary-adrenal axis (HPA axis) response is activated, but also the fetal organs and systems undergoing rapid development at the time. For example, in the fetal brain, neuronal differentiation and migration occurs early in gestation and myelination beginning in the last trimester in humans with synaptic maturation and apoptotic pruning both occurring throughout late pregnancy and into adolescence (99). Therefore, the pathogenesis of many detrimental neurological and behavioural outcomes associated with prenatal stress may be attributable to time-dependent perturbations in fetal brain development that could confer increased vulnerability for altered health outcomes later in life.

Stress and the activation of the HPA axis is an adaptive mechanism where the mother's HPA responsiveness is thought to 'program' the fetus for ex utero life, however when there are discrepancies between what is experienced prenatally and postnally, stress during pregnancy could be considered maladaptive. One hypothesised mechanism of action underlying prenatal stress-induced fetal programming involves elevated maternal circulating glucocorticoids in response to the stressor and through placental exchange, potential perturbations in cortisol exposure of the growing fetal brain (262), potentially resulting in excitotoxicity (330) and developmental changes. However, despite this hypothesised link between maternal glucocorticoid exposure and fetal programming, there have been very few studies actually linking maternal stress with elevated levels of

cortisol and altered perinatal outcomes. Davis et al found that rises in maternal cortisol and maternal pregnancy specific anxiety predicted infant outcomes independently of each other and no positive association was found between the level of maternal cortisol and the psychological state of the mother (280). This study did however show differing outcomes in terms of infant mental development when maternal cortisol concentrations were increased in early vs. late gestation. In addition, pregnancy specific anxiety was only associated with poor outcomes in infants when it was reported early in pregnancy, highlighting the importance of the timing of the stressor on perinatal outcomes. Indeed, previous studies have assessed the effect of the timing of prenatal stress exposure on the outcomes of human offspring, however a wide variation have been reported. In one observational study which used the experience of war whilst pregnant as a measure of maternal psychosocial stress, fetuses affected in the first trimester showed a 2 to 5 fold increase in risk for mood disorders and bipolar disorder later in life (182). First trimester prenatal stress has also been shown to predict higher scores for autism spectrum disorder at $6^{1/2}$ years of age in offspring (183). Others have found that prenatal stress exposure in the second trimester of pregnancy is associated with an increased risk for producing a small-for-gestational-age baby (SGA) (76) as well as a 2 fold increased risk for development of ADHD symptoms at 4-5 years of age, particularly in male offspring (184). Pregnancy-specific anxiety at 19 weeks of gestation has also been associated with reductions in grey matter volume in the brains of offspring at 6-9 years of age, with maternal anxiety reported at 25 and 31 weeks showing no change in the offspring (328), indicating the highly specific effects of the type and timing of prenatal stress on outcomes. In non-human primates, prenatal stress in both early and late pregnancy has been shown to decrease neurogenesis in the hippocampus and produce emotional alterations in offspring (119), highlighting the importance of differences between species as well as type and timing of prenatal stressor. In the precocious guinea pig,

prenatal stress in mid gestation was associated with anxious behaviour in male offspring (91) and we have previously shown, using the same model of prenatal stress, that prenatal stress may affect key inhibitory neurosteroid systems in offspring (283, 320). Neurosteroids, including allopregnanolone, have been shown to be important for optimal neurodevelopment and are also responsive to stressors during pregnancy, indicating that neurosteroids may provide endogenous neuroprotection against prenatal stress (196). The synthetic glucocorticoid betamethasone, when administered to pregnant guinea pigs, has been shown to result in a male-specific reduction in the neurosteroid synthesising enzyme which is further associated with reduced markers of brain development (73), again highlighting the potential importance of this system in providing protection to the vulnerable fetal brain, particularly in male offspring. To the best of our knowledge however, there have been no studies assessing the impact of the timing of prenatal stress exposure on neurodevelopment, behavioural parameters and their interaction on the neurosteroid system in a translational guinea pig model of stress in pregnancy.

This study assesses the effect of prenatal stress on pregnant guinea pigs beginning from early (0.5 gestation), mid (0.7 gestation) and late (0.8 gestation) pregnancy on markers of two main cell types in the growing brain: oligodendrocytes and glia. These cells are capable of producing neurosteroids *de novo* within the brain (270) and therefore levels of allopregnanolone, the most potent neurosteroid, were also assessed. Developmental markers were examined to allow for comparison of neurodevelopment and endocrine changes in fetal offspring before birth and also in offspring in childhood/juvenility, which has been identified as a vulnerable period for development of future pathologies following prenatal stress (331). Measures of offspring anxiety and exploration were assessed in juvenility, which is comparable with the emergence many adverse human behavioural outcomes.

6.3 Methods

Prenatal stress protocol

The University of Newcastle Animal Care and Ethics Committee approved and oversaw the protocol, which was also conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Under this protocol, guinea pigs were time-mated and palpated at approximately gestational day 21 to confirm pregnancy. Once confirmed to be pregnant, dams were randomly allocated to either a prenatal stress exposure or control group. Three prenatal stress groups were established: early stress, mid stress and late stress exposure. In these groups, prenatal stress was induced using strobe light exposure by methods previously described (91, 234, 235, 283) with control dams being removed from their holding rooms and handled but not being exposed to strobe light. Those dams allocated to an 'early stress' group were exposed to strobe light beginning on gestational day 35 and repeated every 5 days until gestational day 65 (7 incidents) with term being approximately gestational day 69. Similarly, dams allocated to the 'mid stress' group were exposed to strobe light beginning on gestation day 50 until 65 (4 incidents) and finally those allocated to the 'late stress' group were exposed to just 2 strobe light incidents on gestational days 60 and 65. Maternal saliva was collected via mastication of a cotton bud for approximately 1 minute immediately before and after the strobe light exposure or control incidents, as previously described (283), for measurement of salivary cortisol concentrations.

It is to be noted that control dams and those is the 'mid stress' group were collected as a part of concurrent studies. Maternal salivary cortisol data, offspring weights and measurements as well as markers of brain development, behavioural parameters and allopregnanolone concentrations have been reported in previous publications for these groups (283, 332).

Fetal Tissue Collection

Dams allocated to an early, mid or late stress exposure group as well as controls were further divided into fetal or 21 day tissue collection groups. For those allocated to a fetal collection timepoint, tissues were obtained as previously described (283). Briefly, at term dams were euthanased using inhalation of 100% CO_2 with fetal plasma and brains immediately removed. Brains were dissected in a sagittal plane to separate the hemispheres with one half snap frozen at -80^{o}C or formalin fixed in (% w/v Paraformaldehyde in 0.1M Phosphate Buffer (Na_2PO_4; $NaH_2PO_4H_2O$), (Sigma Aldrich, Castle Hill, NSW, Australia). Organ weights for the brain, adrenal glands, liver, and kidney were all recorded at the time of post mortem (Table 6.1 and 6.2).

Postnatal behavioural testing and tissue collection

Pregnancies that were allocated to a 21-day-old tissue collection group were allowed to spontaneously deliver at term and upon birth, weights of pups were recorded. All offspring were maintained with their litter and dam for the duration of the experiment. On postnatal day 18, pups were subjected to behavioural testing by methods previously described (320)**.** Briefly, offspring were placed in an open field arena for 10 minutes with their exploratory activity and any anxious behaviour (wall hugging) recorded using the Stoelting ANY-Maze behavioural testing software via tracking of the animals head (Stoelting Co., Wood Dale, IL, USA). Upon the completion of this test, a further 10 minutes was recorded with two identical objects placed in the arena to determine the animals' willingness to explore as another measure of anxiety and neophobic behaviours.

At 21 days of age, these pups were euthanased in the same manner previously described, via 100% CO_2 inhalation. Plasma and brains were immediately collected, processed and stored as above.

Immunohistochemistry

The fixed brains from both fetal offspring and those collected at 21 days of age were embedded in paraffin wax and processed for immunohistochemical staining and microscopy by methods previously described (243, 283). Briefly, three serial 8µm brain sections were de-waxed, rehydrated and blocked for endogenous peroxidase activity using serial chemical washes. Reveal It Solution (ImmunoSolutions Pty Ltd, NSW, Australia) was used for antigen retrieval. The brain sections were then incubated in a blocking solution of BSA in PBS (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide) before incubation with primary antibodies overnight for myelin basic protein (MBP; Sigma Aldrich) and glial fibrillary acidic protein (GFAP; Sigma Aldrich) at a concentration of 1:4000. Secondary antibodies (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK) were then applied for 2 hours with the final incubation in Streptavadin-Biotinylated HRP complex (RPN1051, Amersham). The 3,3'-diaminobenzidine (DAB) concentrate containing 3% H_2O_2 was used to visualise the immunostaining before one section was counterstained with 1% cresyl violet to allow for easy orientation under the microscope. Slides were then fixed with coverslips using Microscopy DPX (Merck Australia, Kilsyth, VIC) and viewed on a Nikon Eclipse 90i microscope with images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). Positive immunostaining was determined using densitometry methods with ImageJ version 1.47v (National Institutes of Health, Bethesda, MD, USA). In this way, the percentage area of coverage was recorded for four fields of view per region analysed (CA1 region of the hippocampus and adjacent subcortical white matter) on two sections per animal (thus, eight fields of view for each animal, each region). Controls for specificity of primary antibodies were run using the appropriate IgG substitute for each

primary antibody.

Allopregnanolone Radioimmunoassay and Cortisol Immunoassay

Allopregnanolone was determined by radioimmunoassay by methods previously described (73, 283). Briefly, 60μL (fetal plasma) and 600μL (21 day plasma) of relevant plasma were combined with 50% methanol and 1% acetic acid and the supernatants applied to Sep-Pak C_{18} cartridges (Waters, Milford, Mass., USA). Tritium-labelled allopregnanolone (1,000-5,000 cpm, 5a-[9,11,12,3H(N)]; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) was used to determine individual sample recovery concentrations following manual tissue extraction. Average recovery of allopregnanolone was 78.6 ± 0.6% from plasma with recoveries values used in the final calculation of allopregnanolone plasma concentration to adjust for extraction losses. Radioactivity of the allopregnanolone was measured using a β-counter (LS6500, Beckman Coulter Australia Pty Ltd, Lane Cove, Australia), with the allopregnanolone antibody (AS04041, Agrisera AB, Vannas, Sweden). The limit of detection was 25pg/mL and the inter- and intra- assay coefficients of variation were 6.4% and 6.7% respectively. Cortisol was measured in maternal saliva collected immediately before and after each strobe light exposure or control incident using a Salimetrics salivary assay kit (Salimetrics Inc., State College, Pa., USA), according to manufacturers instructions. Sensitivity of the assay was 0.012-3.0μg/dL and the inter- and intra-assay coefficients of variation were 9.39% and 5.52% respectively.

Statistical Analysis

For all outcome measures, a linear mixed model was used with stress exposure, sex and postnatal age as fixed independent factors in the model. A random factor was also included in the mixed model to adjust for familial correlations between pups from the same dams where more than one pup was included in the analysis from the same

pregnancy. Furthermore, a Bonferroni correction was used to determine multiple comparisons between the stress exposure groups within each sex and postnatal age cohort. All data is expressed as β-coefficient values, 95% confidence intervals and $p<0.05$ considered significant. Unless otherwise specified, all data shown is expressed as mean ± SEM. Statistical analysis was performed using SPSS software (version 21, SPSS Inc. IBM, Chicago Ill., USA) and graphs made using Graphpad Prism Software (version 6, Graphpad Software Inc., La Jolla, CA, USA).

6.4 Results

Effect of strobe light exposure on maternal salivary cortisol concentrations

Exposure of pregnant dams to strobe light caused a significant increase in maternal salivary cortisol concentrations (expressed as fold change in pre to post concentrations, Figure 6.1) compared to controls in all groups (early, mid and late stress exposure) at stress incidents from gestational day 50 until 65 ($p<0.05$). There was no significant difference in salivary cortisol concentrations between stress exposed and control dams at the earlier strobe light exposure incidents (gestational days 35, 40 and 45, Figure 6.1), although trends were seen at each age examined.

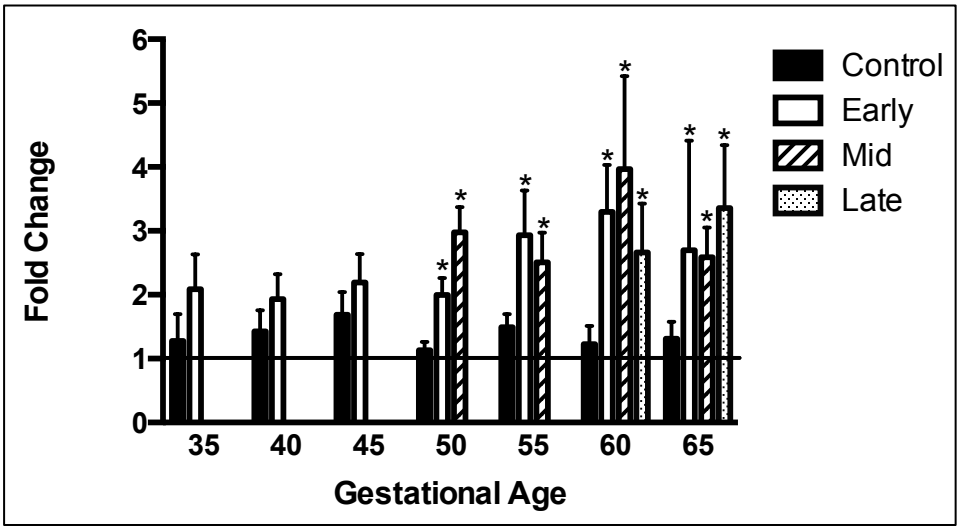

FIGURE 6.1. MATERNAL SALIVARY CORTISOL CONCENTRATIONS IN CONTROL (BLACK BARS N=22), EARLY STRESS EXPOSED DAMS (OPEN BARS N=18), MID STRESS EXPOSED DAMS (HATCHED BARS N=22) AND LATE STRESS EXPOSED DAMS (SPOTTED BARS N=12). DATA IS EXPRESSED AS A TOTAL FOLD CHANGE IN CONCENTRATIONS BEFORE AND AFTER THE PRENATAL STRESS (OR CONTROL) INCIDENT ON GESTATIONAL DAYS 35, 40, 45, 50, 55, 60 AND 65 (TERM 69 DAYS) WITH THE BLACK HORIZONTAL LINE REPRESENTING A FOLD CHANGE VALUE OF '1' BEING NO CHANGE FROM PRE TO POST CONCENTRATIONS. * P = 0.05 INDICATES SIGNIFICANCE LEVEL BETWEEN CONTROL AND STROBE LIGHT EXPOSED GUINEA PIG DAMS.

Table 6.1: Fetal physical characteristics

Sex	Treatment Group	Body weight (g)	Brain:Body weight	Adrenal Gland:Body Weight	Heart:Body weight	Liver:Body weight	Kidney:Body Weight	Brain:liver ratio
Male	Control (n=11)	99.36 ± 4.25	2.49 ± 0.07	0.029 ± 0.002	0.51 ± 0.02	5.15 ± 0.09	0.82 ± 0.02	0.49 ± 0.02
	Early Stress (n=12)	84.50 ± 5.42	2.79 ± 0.14	0.029 ± 0.005	0.53 ± 0.04	4.47 ± 0.62	0.67 ± 0.09	0.50 ± 0.02
	Mid Stress (n=10)	86.10 ± 4.25	2.71 ± 0.12	0.029 ± 0.002	0.59 ± 0.05	4.66 ± 0.22	0.84 ± 0.01	0.61 ± 0.06[A]
	Late Stress (n=9)	82.33 ± 4.57	2.89 ± 0.14	0.042 ± 0.004	0.55 ± 0.08	5.35 ± 0.22	0.86 ± 0.07	0.55 ± 0.05
Female	Control (n=7)	91.21 ± 4.87	2.81 ± 0.11	0.037 ± 0.002	0.53 ± 0.03	5.01 ± 0.20	0.86 ± 0.03	0.55 ± 0.03
	Early Stress (n=8)	93.75 ± 5.91	2.23 ± 0.34	0.026 ± .0006	0.47 ± 0.07	4.25 ± 0.67	0.67 ± 0.11	0.53 ± 0.04
	Mid Stress (n=10)	87.51 ± 5.17	2.84 ± 0.18	0.069 ± 0.034	0.52 ± 0.03	3.88 ± 0.25	0.79 ± 0.08	0.71 ± 0.05[A]
	Late Stress (n=10)	90.80 ± 6.55	2.68 ± 0.17	0.034 ± 0.006	0.51 ± 0.02	5.31 ± 0.18	0.83 ± 0.04	0.51 ± 0.03

[A] *INDICATES A SIGNIFICANT EFFECT OF EARLY STRESS EXPOSURE COMPARED TO CONTROLS.*

Table 6.2: Postnatal (juvenile) physical characteristics

Sex	Treatment Group	Birth weight	Body	Brain to Body weight	Adrenal Gland to Body Weight	Heart to Body weight	Liver to Body weight	Kidney to Body Weight	Brain to liver ratio
Male	Control (n=11)	87.10 ± 6.12	220.59 ± 10.56	1.31 ± 0.58	0.06 ± 0.02	0.44 ± 0.02	4.08 ± 0.09	1.13 ± 0.03	0.33 ± 0.02
	Early Stress (n=6)	100.67 ± 3.53[B]	263.83 ± 14.12[B]	1.13 ± 0.59	0.03 ± 0.01	0.53 ± 0.09	3.54 ± 0.71	0.96 ± 0.20	0.27 ± 0.02
	Mid Stress (n=12)	90.50 ± 4.31	234.00 ± 8.57	1.26 ± 0.04	0.04 ± 0.001	0.42 ± 0.15	4.24 ± 0.15	1.09 ± 0.19	0.31 ± 0.16
	Late Stress (n=11)	88.19 ± 4.02	221.08 ± 12.83	1.37 ± 0.08	0.04 ± 0.007	0.37 ± 0.04	3.63 ± 0.15	1.11 ± 0.02	0.39 ± 0.03
Female	Control (n=10)	80.90 ± 3.56	211.23 ± 8.29	1.36 ± 0.05	0.19 ± 0.08	0.46 ± 0.03	4.11 ± 0.17	1.15 ± 0.03	0.34 ± 0.01
	Early Stress (n=6)	99.67 ± 4.57[B]	260.33 ± 10.68[B]	1.16 ± 0.04	0.04 ± 0.007	0.34 ± 0.07	3.32 ± 0.68	0.91 ± 0.18	0.29 ± 0.01
	Mid-Stress (n=12)	88.67 ± 2.65	227.35 ± 7.01	1.29 ± 0.03	0.04 ± 0.001	0.41 ± 0.01	3.85 ± 0.06	1.07 ± 0.02	0.34 ± 0.01
	Late Stress (n=9)	84.31 ± 4.20	226.00 ± 9.98	1.28 ± 0.04	0.04 ± 0.002	0.42 ± 0.01	4.12 ± 0.09	1.12 ± 0.02	0.32 ± 0.11

[B] *INDICATES A SIGNIFICANT EFFECT OF MID-LATE STRESS EXPOSURE COMPARED TO ALL OTHER GROUPS.*

Effect of prenatal stress timing on physical growth

There was no significant effect of the timing of stress exposure on the gestational age of delivery (control = 69.7 ± 0.3, early stress = 69.4 ± 0.4, mid stress = 70.2 ± 0.5, late stress = 69.4 ± 0.4), indicating that stress exposure did not affect gestational length in this study. There was also no significant effect of the timing of stress exposure on litter size (control = 3.2 ± 0.2, early stress = 2.4 ± 0.3, mid stress = 2.9 ± 0.3, late stress = 3.0 ± 0.4) or sex ratio of offspring (see animal numbers in results Sections).

Despite no significant changes in body weight within the fetal cohort, pups born following spontaneous labour to a mother from the early stress group had on average approximately a 17 gram increase in their birth weight compared to their control counterparts (β=17.079, p=0.007, 95% CI= 3.888-30.271; Table 6.2) and this was extended to, on average, approximately a 50 gram increase by juvenility (β=50.669, p=0.03, 95% CI= 3.110-98.227; Table 6.2), irrespective of sex. Fetuses from mid stress exposed dams showed a modest but significant increase in their brain-to-liver ratio (BLR) compared to all other groups (β=0.138, p=0.003, 95% CI= 0.236-0.041; Table 6.1) however this was not seen in juvenility, indicating a spontaneous recovery from any reduction in liver growth to levels that match controls 21 days after birth. There was no significant effect of the timing of stress exposure or the sex of the pup on ratios of the brain, adrenal gland, heart, liver or kidney to body weight (Table 6.1 and 6.2).

Prenatal stress alters expression of mature oligodendrocytes

Male fetuses exposed to stress from early in pregnancy show on average, a 12% reduction in MBP expression compared to controls (β=-12.788, 95% CI= -19.209- -6.366, p<0.0001; Figure 6.2A) where females showed an average 8% reduction in this group (β=-8.900, 95% CI= -16.963- -0.836 p=0.02; Figure 6.2A). Males also showed a similar level of reduction in MBP expression levels in the mid stress group (β=-12.675, 95% CI= -

19.855- -5.496 p<0.0001; Figure 6.2A) however; there was no difference between female control and stress exposed levels as fetuses in this mid gestation group (Figure 6.2A). Male fetuses in the late stress group showed the largest reductions in MBP expression at, on average, 14% less than controls (β=-14.639, 95% CI= -21.060- -8.217 p<0.0001; Figure 6.2A) with females again showing on average 8% less expression than their control counterparts (β=-8.725, 95% CI= -17.132- -0.372 p=0.03; Figure 6.2A).

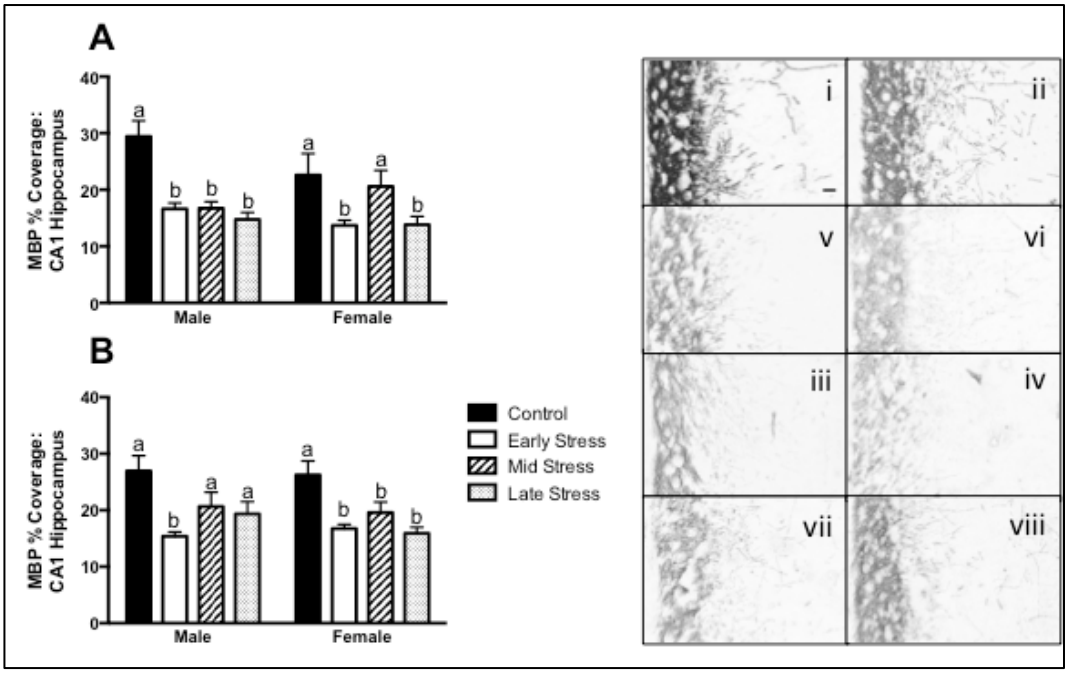

FIGURE 6.2. MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS OF FETAL (A) AND 21 DAY OLD OFFSPRING (B). CONTROL OFFSPRING ARE SHOWN IN BLACK BARS (FETAL MALE N=6, FEMALE N=4; 21 DAY MALE N=8, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-I AND 21 DAY-II). EARLY STRESS EXPOSED OFFSPRING ARE SHOWN IN OPEN BARS (FETAL MALE N=6, FEMALE N=6; 21 DAY MALE N=4, FEMALE N=5) AND REPRESENTED IN IMAGES (FETAL-III AND 21 DAY- IV). MID STRESS EXPOSED OFFSPRING ARE SHOWN IN HATCHED BARS (FETAL MALE N=4, FEMALE N=4; 21 DAY MALE N=9, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-V AND 21 DAY- VI). LATE STRESS EXPOSED OFFSPRING ARE SHOWN IN SPOTTED BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=5, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL- VII AND 21 DAY- VIII). BARS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL. SCALE BAR =30MM.

Interestingly, by 21 days of age hippocampal MBP expression in both the mid stress and late stress group had recovered to levels of unaffected controls within the male cohort, however expression remained reduced in the early stress group by 21 days of age (β=-11.563, 95% CI= -16.963- -0.836 p=0.02; Figure 6.2B), indicating a lack of catch-up

growth in this group. In the female cohort, all stress-exposed groups were significantly lower than controls at 21 days of age (early- β=-9.476, 95% CI= -16.955- -1.997 p=0.007; mid-late- β=-6.660, 95% CI= -13.220- -0.100 p=0.04; late- β=-10.320, 95% CI= -16.880- -3.701 p=0.001; Figure 6.2B).

In the subcortical white matter, male fetuses exposed to mid gestation prenatal stress showed the lowest levels of MBP expression (on average, 26% less than controls), this level also being significantly less than all other stress groups as well as controls (compared to early- β=-18.944, 95% CI= -26.575- -11.313, p<0.0001; compared to late- β=-15.960, 95% CI= -23.591- -8.329, p<0.0001; Figure 6.3A). However, by 21 days of age, there were no significant differences in MBP expression in the subcortical white matter in any of the stress groups (Figure 6.3B). Female offspring did not show any effect of prenatal stress exposure in the subcortical white matter either as fetuses or at 21 days of age (Figure 6.3).

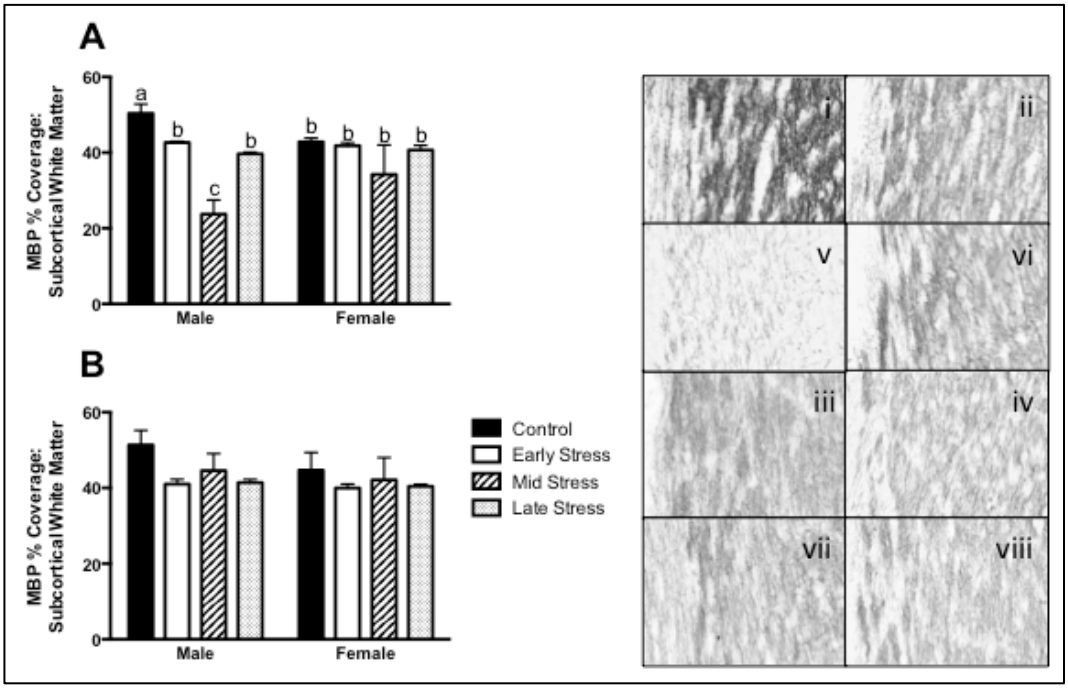

FIGURE 6.3. MYELIN BASIC PROTEIN (MBP) EXPRESSION IN THE SUBCORTICAL WHITE MATTER OF FETAL (A) AND 21 DAY OLD OFFSPRING (B). CONTROL OFFSPRING ARE SHOWN IN BLACK BARS (FETAL MALE N=6, FEMALE N=4; 21 DAY MALE N=8, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-I AND 21 DAY-II). EARLY STRESS EXPOSED OFFSPRING ARE SHOWN IN OPEN BARS (FETAL MALE N=6, FEMALE N=6; 21 DAY MALE N=4, FEMALE N=5) AND REPRESENTED IN IMAGES (FETAL-III AND 21 DAY- IV). MID STRESS EXPOSED OFFSPRING ARE SHOWN IN HATCHED BARS (FETAL MALE

N=4, FEMALE N=4; 21 DAY MALE N=9, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-V AND 21 DAY- VI). LATE STRESS EXPOSED OFFSPRING ARE SHOWN IN SPOTTED BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=5, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL- VII AND 21 DAY- VIII). BARS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL.

Reactive astrocyte expression is affected by prenatal stress exposure

Within the male cohort, all fetuses exposed to prenatal stress demonstrated reduced expression of GFAP in the CA1 region of the hippocampus compared to controls (early- β=-4.636, 95% CI= -7.921- -1.352, p=0.003; mid- β=-3.825, 95% CI= -7.497- -0.153, p=0.03; late- β=-4.227, 95% CI= -7.512- -0.943, p=0.007; Figure 6.4A) with offspring in the early stress group showing the largest reduction with, on average, 4.6% less expression than their control counterparts. By 21 days of age, this reduction in GFAP levels was not significantly different from controls however this could reflect subtle changes in the control population at this age (Figure 6.4B).

Female fetuses did not show any significant effect of prenatal stress exposure on GFAP expression in the CA1 region of the hippocampus however those in the early and also the late stress groups had reduced GFAP expression at 21 days of age (early- β=-7.169, 95% CI= -14.227- -0.112, p=0.04; late- β=-7.016, 95% CI= -13.164- -0.869, p=0.01; Figure 6.4B), indicating a delayed response to prenatal stress exposure in this female cohort.

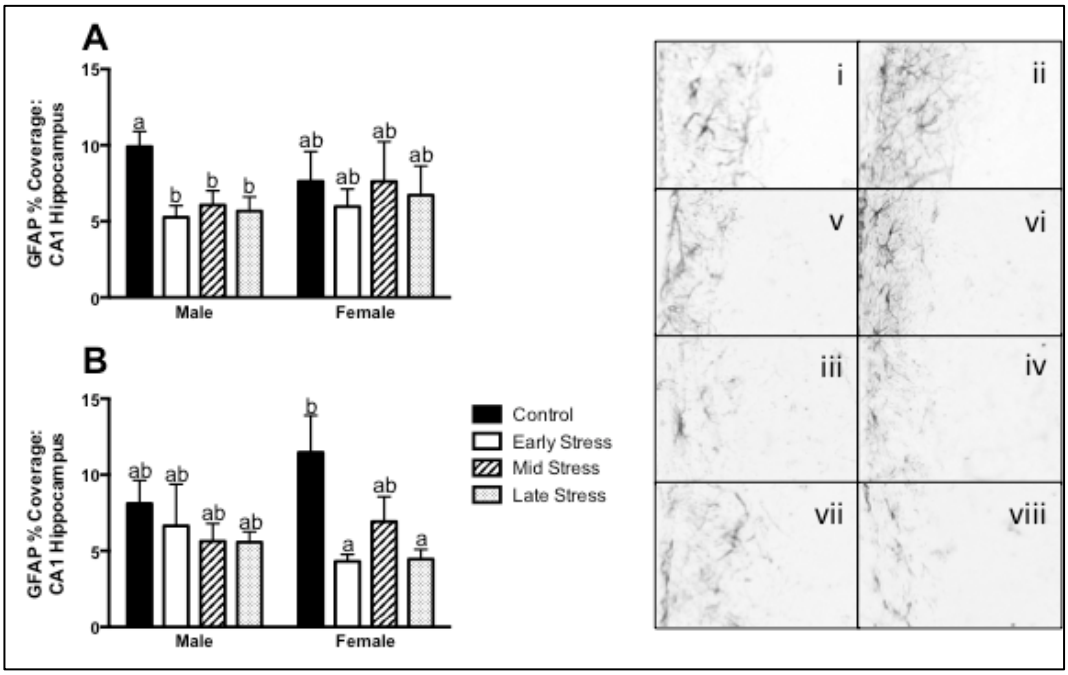

FIGURE 6.4. GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE CA1 REGION OF THE HIPPOCAMPUS OF FETAL (A) AND 21 DAY OLD OFFSPRING (B). CONTROL OFFSPRING ARE SHOWN IN BLACK BARS (FETAL MALE N=6, FEMALE N=4; 21 DAY MALE N=9, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-I AND 21 DAY-II). EARLY STRESS EXPOSED OFFSPRING ARE SHOWN IN OPEN BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=4, FEMALE N=5) AND REPRESENTED IN IMAGES (FETAL-III AND 21 DAY- IV). MID STRESS EXPOSED OFFSPRING ARE SHOWN IN HATCHED BARS (FETAL MALE N=4, FEMALE N=4; 21 DAY MALE N=8, FEMALE N=9) AND REPRESENTED IN IMAGES (FETAL-V AND 21 DAY- VI). LATE STRESS EXPOSED OFFSPRING ARE SHOWN IN SPOTTED BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=5, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL- VII AND 21 DAY- VIII). BARS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE $P<0.05$ LEVEL.

In the adjacent subcortical white matter, male fetuses in the early stress group show increased levels of GFAP expression compared to those in the mid stress group (β=9.554, 95% CI= 0.907-18.202, p=0.02; Figure 6.5A). In the female fetal cohort, it was the offspring in the late stress group that showed increased levels of GFAP compared to those in the mid stress group (β=11.873, 95% CI= 0.764-22.982, p=0.03; Figure 6.5A). By 21 days of age, male offspring from the early stress group maintained their increase in GFAP levels compared to the mid stress group and also to controls at this age (compared to mid stress- β=15.358, 95% CI= 4.913-25.802, p=0.002; compared to control- β=11.390, 95% CI= 1.376-21.404, p=0.01; Figure 6.5B). Male offspring in the late stress group also showed higher expression of GFAP than those in the 50 stress group at 21

days of age (β=12.710, 95% CI= 2.952-22.467, p=0.006; Figure 6.5B). Female offspring from the late stress group at 21 days of age also maintained their increase in GFAP expression in the subcortical white matter compared to the mid stress group (β=11.046, 95% CI= 3.311-18.782, p=0.002; Figure 6.5B) and also increased expression relative to offspring in the control group at this age (β=9.784, 95% CI= 2.311-17.258, p=0.005; Figure 6.5B).

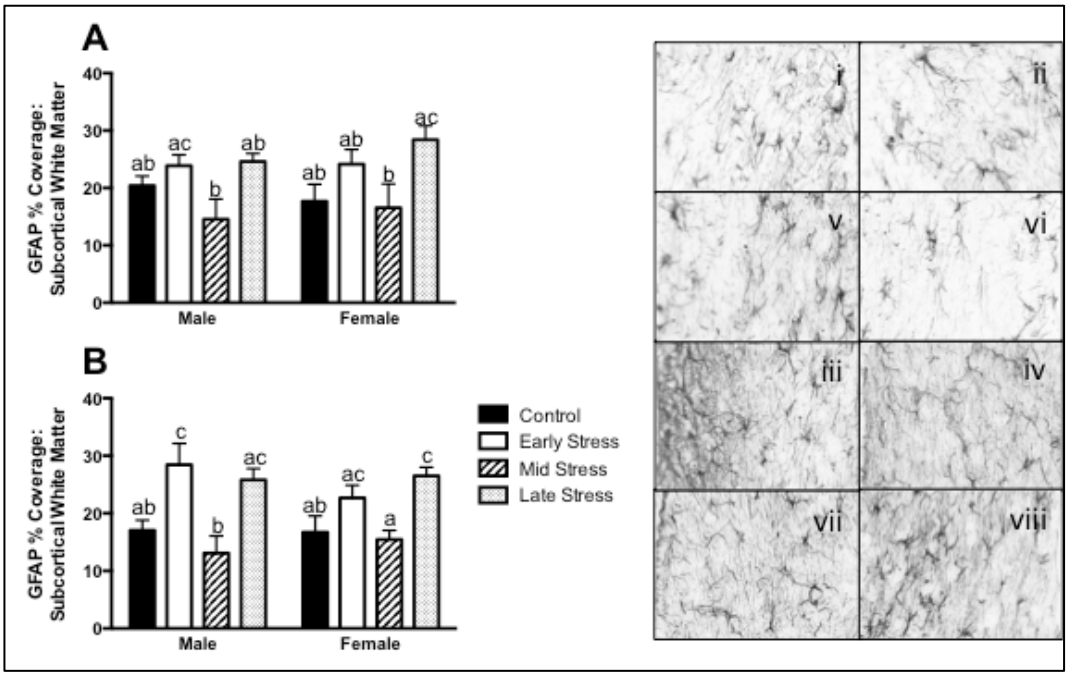

FIGURE 6.5. GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) EXPRESSION IN THE SUBCORTICAL WHITE MATTER OF FETAL (A) AND 21 DAY OLD OFFSPRING (B). CONTROL OFFSPRING ARE SHOWN IN BLACK BARS (FETAL MALE N=6, FEMALE N=4; 21 DAY MALE N=9, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL-I AND 21 DAY-II). EARLY STRESS EXPOSED OFFSPRING ARE SHOWN IN OPEN BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=4, FEMALE N=5) AND REPRESENTED IN IMAGES (FETAL-III AND 21 DAY- IV). MID STRESS EXPOSED OFFSPRING ARE SHOWN IN HATCHED BARS (FETAL MALE N=4, FEMALE N=4; 21 DAY MALE N=7, FEMALE N=7) AND REPRESENTED IN IMAGES (FETAL-V AND 21 DAY- VI). LATE STRESS EXPOSED OFFSPRING ARE SHOWN IN SPOTTED BARS (FETAL MALE N=6, FEMALE N=5; 21 DAY MALE N=5, FEMALE N=8) AND REPRESENTED IN IMAGES (FETAL- VII AND 21 DAY- VIII). BARS WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL.

Behavioural outcomes in juvenility following prenatal stress

Pups exposed to prenatal stress in all groups spent significantly less time exploring an object in the arena (early stress- β=-26.70, 95% CI= -50.091- -3.305, p=0.02; mid stress- β=-19.892 95% CI= -39.561- -0.222, p=0.04; late stress- β=-21.379, 95% CI= -41.962- -

0.797, p=0.04, Figure 6.6A), with no effect of the sex of the offspring on time spent exploring (Figure 6.6A).

Similarly, offspring exposed to prenatal stress also spent significantly less time in the inner zone of the open field arena compared to controls at 21 days of age (early stress- β=-65.288, 95% CI= -120.890- -9.685, p=0.02; mid stress- β=-51.96 95% CI= -94.070- -9.850, p=0.01; late stress- β=-65.84, 95% CI= -112.333- -19.369, p=0.006, Figure 6.6B). There was no effect of the sex of the offspring on time spent in the inner zone of the arena in open field-testing (Figure 6.6B).

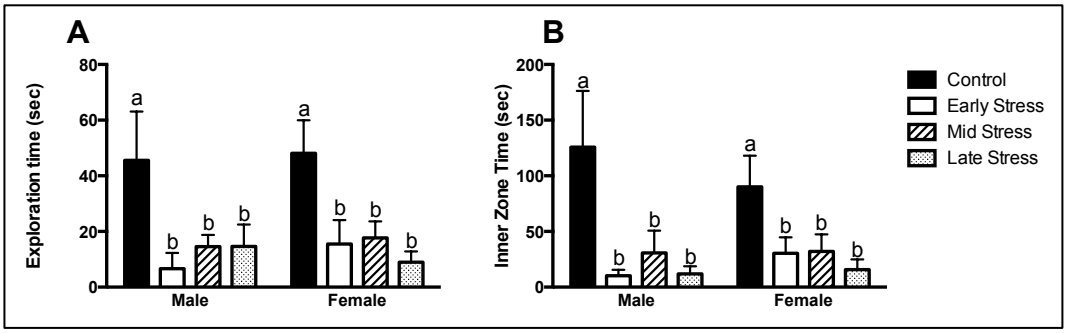

FIGURE 6.6. BEHAVIOURAL TESTING OUTCOMES FROM OBJECT EXPLORATION (A) AND OPEN FIELD (B) IN OFFSPRING FROM CONTROL PREGNANCIES (BLACK BARS; MALE N=10, FEMALE N=9), EARLY STRESS EXPOSED (OPEN BARS; MALE N=6, FEMALE N=6) MID STRESS EXPOSED (HATCHED BARS; MALE N=12, FEMALE N=11) AND LATE STRESS EXPOSED PREGNANCIES (SPOTTED BARS; MALE N=10, FEMALE N=9). BARS SHOWN WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL.

Effect of prenatal stress timing on circulating allopregnanolone levels

Both male and female fetuses exposed to prenatal stress late in pregnancy showed reduced circulating allopregnanolone concentrations compared to controls (males β=-35.695, 95% CI= -71.081- -0.310, p=0.04; Figure 6.7A, females β=-36.111, 95% CI= -64.55- -7.660, p=0.01; Figure 6.7A). Female fetuses exposed to stress from early in pregnancy also showed a trend towards a reduction in allopregnanolone concentrations compared to controls (p=0.06; Figure 6.7A).

In both sex cohorts by 21 days of age, only those offspring who were exposed to stress from early in pregnancy showed sustained reductions in their circulating

allopregnanolone levels compared to controls (β=-0.462, 95% CI= -0.921- -0.004, p=0.04; Figure 6.7B). Despite only male offspring exposed to stress from early in pregnancy showing significantly reduced plasma allopregnanolone concentrations compared to controls (β=-0.480, 95% CI= -0.932- -0.030, p=0.03; Figure 6.7B), control females also demonstrated increased variance at this age, which likely contributed to the lack of significance in the female cohort.

As expected, fetal allopregnanolone concentrations were significantly higher than the 21-day-old population by approximately 50 fold (β=49.729, 95% CI= 41.278- 58.180, p<0.0001; Figure 6.7).

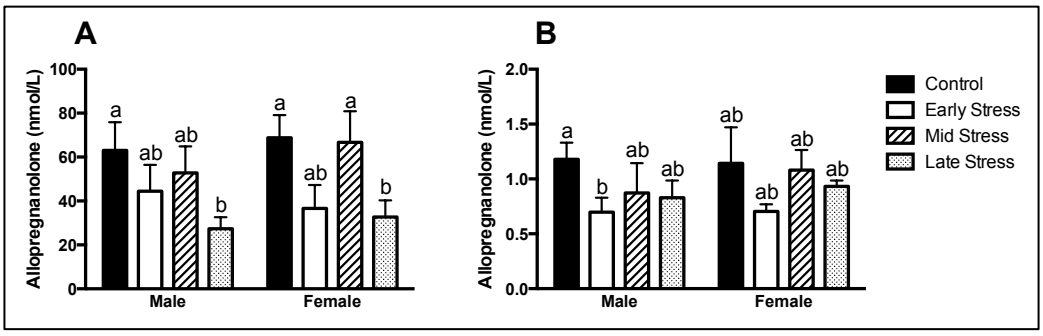

FIGURE 6.7. CIRCULATING ALLOPREGNANOLONE CONCENTRATIONS IN PLASMA FROM FETAL (A) AND 21 DAY OLD OFFSPRING (B). CONTROL PREGNANCIES ARE SHOWN IN BLACK BARS (FETAL MALE N=5, FEMALE N=5; 21 DAY OFFSPRING MALE N=6, FEMALE N=6), EARLY STRESS EXPOSED PREGNANCIES SHOWN IN OPEN BARS (FETAL MALE N=6, FEMALE N=6; 21 DAY OFFSPRING MALE N=6, FEMALE N=6), MID STRESS EXPOSED PREGNANCIES SHOWN IN HATCHED GREY BARS (FETAL MALE N=5, FEMALE N=5; 21 DAY OFFSPRING MALE N=6, FEMALE N=6) AND LATE STRESS EXPOSED PREGNANCIES ARE SHOWN IN SPOTTED BARS (FETAL MALE N=6, FEMALE N=6; 21 DAY OFFSPRING MALE N=6, FEMALE N=6). BARS SHOWN WITH DIFFERENT LETTERS ARE CONSIDERED SIGNIFICANT AT THE P<0.05 LEVEL.

6.5 Discussion

The major finding of this study was that prenatal stress has long lasting effects on neurobehavioural development in guinea pig offspring that are specific to the timing at which the stressor was experienced. This study is also the first to show that prenatal

stress beginning in early pregnancy can have prolonged effects on circulating allopregnanolone concentrations in juvenile offspring, which may confer increased vulnerability to further neuropathologies later in life.

The present result indicate that maternal HPA responsiveness (salivary cortisol concentration) in the pregnant guinea pig was only increased from 50 days gestation until term in response to a strobe light stressor and no increases were seen earlier in pregnancy compared to controls. However it is noteworthy that in the early stress exposed group, the average fold change in salivary cortisol in response to stress incidents occurring on GA35, 40 and 45 was 2 fold, indicating that these dams did show trends to significant increases in cortisol concentrations. Control dams at these ages showed a moderate increase in cortisol concentrations in response to handling (average 1.5 fold increase) which was reduced to an average 1.3 fold increase during the last 4 handling timepoints. The reduction in cortisol increases in response to handling from GA50 onwards in control dams may reflect reduced HPA responsiveness as pregnancy progresses (333), however this was not the case for stress exposed dams in the current study.

Although there is a link between the maternal HPA axis dysregulation and adverse perinatal outcomes, there is very little evidence specifically linking maternal cortisol concentrations, prenatal stress exposure and altered outcomes in offspring (334, 335). This suggests that despite extensive work examining the programming effects of maternal stress during pregnancy on offspring, the mechanisms of action by which the maternal psychological state influences fetal growth, are not fully understood. This study has shown an association between induced maternal stress, increases in salivary cortisol concentrations and altered perinatal outcomes which are dependent on the timing of the stressor, however it is clear that quantifying stress during pregnancy is

complex, and that maternal cortisol levels alone may not adequately serve as a marker for stress during pregnancy.

The guinea pig is a precocious species with similar growth trajectories to that of humans in pregnancy and early life (112). These rodents have a relatively long gestation compared to rats and mice (approximately 71 days), which allows for diverse manipulations in fetal life. In the guinea pig, relative brain to body weight reaches its peak at around 29-35 gestational days of age and declines with increasing gestational age which is characteristic of many mammals during development (256). A peak in brain growth by wet weight occurs around gestational day 50 in this species, with maximal cholesterol and DNA polymerase (glial cell multiplication) accumulation occurring shortly before birth at around 60 days of gestation (256). Therefore, prenatal stress in this study beginning at gestational day 35 (termed early stress although it represents 0.5 of gestation), gestational day 50 (0.7 gestation) and gestational day 60 (0.8 gestation) target vulnerable windows of neurodevelopment in the guinea pig which are similar to that of human pregnancy (112). Furthermore, body weight in guinea pigs reaches its fastest growth peak at 3 weeks postnatal age (256), indicating that this time may be important for determining developmental changes in the preceding time.

Previous work using the same stressor as the current study (strobe light exposure) in guinea pigs has shown differential effects of prenatal stress on gestational day 50-52 to that of 60-62, with stress experienced in the earlier of the two time points associated with behavioural indicators of anxiety in juvenility and adulthood together with significantly increased brain-to-liver ratios in these offspring, suggesting brain sparing as a result of prenatal stress exposure (91). This study has also found an increase in brain-to-liver ratio in fetuses exposed to stress from gestational day 50 however there was no difference compared to controls by 21 days of age, indicating a spontaneous recovery from brain sparing by juvenility in this cohort. Despite this, prenatally stressed offspring

from all three groups (early, mid, late stress) in the current study showed anxious behaviour (less entries into the inner zone of the open field (wall-hugging behaviour) and were less inquisitive of objects within their environment) at 21 days of age, however the duration of testing was just a third of that reported previously (91, 235). The method of behavioural testing in the current study may therefore may provide a sensitive approach to determining behaviour in guinea pigs, as a shorter duration of testing is not likely to result in maternal separation anxiety (290). Wall-hugging and exploratory/inquisitive behaviour are known indicators of anxiety in rodents (336) and have previously been shown to result from prenatal stress exposure (295).

This study has also found an effect of stress beginning early in pregnancy on whole body growth in offspring, resulting in increased body weight both at birth and in juvenility. This is consistent with current literature suggesting that offspring who are affected by prenatal stress earlier in pregnancy may possess more efficient energy utilization processes as a result of programming for a difficult ex utero environment (337). In this way, the anxious behaviour observed in the current study may be viewed in the same manner: to produce offspring who are adapted to a stressful environment. The discrepancies between in utero and ex utero life cannot be ignored however and although postnatal stress testing of offspring using the current model would be warranted, deficits in key markers of brain development in this study indicate a maladaptive response to prenatal stress.

Indeed, markers of mature oligodendrocytes and reactive astrocytes were severely affected by prenatal stress exposure in this study. Both markers were reduced most profoundly in prenatally stressed offspring as term fetuses and in juvenility in the CA1 region of the hippocampus, highlighting the vulnerability of this brain region to the effects of prenatal stress which do not reach levels of unaffected controls after birth. This is consistent with very low levels of neurogenesis after birth in the guinea pig (338),

although some catch-up growth is possible. In male offspring born to a pregnancy that was subjected to prenatal stress beginning in mid and late gestation, mature oligodendrocyte expression in the CA1 region of the hippocampus is able to recover to levels that match controls by juvenility, whereas those born to a pregnancy affected by stress from early in gestation maintain a deficit in oligodendrocyte marker expression, suggesting a more profound and potentially irreversible effect of stress on these early developing brains. Males subjected to prenatal stress from early, mid and late pregnancy also show deficits in reactive astrocytes at term, which match control levels by juvenility, however it is interesting to note that reactive astrocyte expression did not change between fetal and 21 day male offspring in the hippocampus and therefore the 'recovery' to control levels in this brain region is a reflection of a small (but not significant) reduction in expression within the control cohort.

Females show different patterns of expression in their makers of brain development where mature oligodendrocyte expression was reduced in the hippocampus with prenatal stress exposure (early, mid and late stress) at term and were still reduced in juvenility, indicating a lesser capacity of these females to recover from stress after birth compared to males from mid and late stress groups. Reactive astrocyte expression however is reduced only after birth in the CA1 region of the hippocampus in female offspring of early and late stress groups and this is coupled with a dramatic increase in reactive astrocyte expression in the subcortical white matter. This increase in reactive astrocyte expression is seen in both sexes, both at term and in juvenility, as a result of prenatal stress beginning early and late in utero but not from gestational day 50. As such, offspring from the early stress group received the most stress incidents, followed by those from a mid stress group and finally the late stress group. Increased astrocyte expression present in offspring from a pregnancy subjected to stress beginning earlier in gestation suggests that chronic prenatal stress throughout pregnancy may result in

impaired neuroplasticity and reparative capabilities in these offspring throughout development (339) as these pups show some of the most dramatic changes in their neurodevelopment observed in the current study. The stress paradigm used in this study however cannot determine whether the more severe effects observed in the early stress group are due to the earlier onset of stress exposure or the increased duration of stress exposure.

Concurrently, offspring from the late stress group also show similar reductions in markers of brain development to those in the early stress group, as well as increased astrocyte expression in the subcortical white matter both at term and in juvenility, suggesting a specific yet independent mechanism of damage in these pregnancies. It is possible that by targeting the specific window of glial cell growth at 60 days gestation, that the effects of stress on reactive astrocytes are substantial in this group. Although this does not explain why offspring of the mid stress group did not show the same effects, it has previously been shown that strobe light exposure results in a larger rise in maternal cortisol at gestational day 60 compared to gestational day 50 (91, 283), as well as increased fetal adrenal sensitivity (340) and reduced 11β-hydroxysteroid dehydrogenase 2 in the guinea pig placenta at this age (341), suggesting that even relatively small bouts of prenatal stress during this particularly vulnerable window of development may perturb normal development in a more detrimental manner than at other gestational ages.

The discrepancies between expression of markers for mature oligodendrocytes and reactive astrocytes in the brains of offspring and their behaviour in the current study cannot be ignored. Although striking differences are seen, clearly one does not fully explain the other. It is possible that more subtle, rather than overall changes, are responsible for alterations in behaviour such as synaptic connectivity and regional distribution of neurons and glia within the early developing brain. In 2013, Xu et al

showed prenatal stress induced reductions in synaptophysin (a marker of synaptogenesis) were associated with disrupted behaviour and abnormal ultrastructural changes in neurons and myelin sheaths in rats (129). It is therefore likely, given these specific findings, that measuring overall expression of markers for highly populous cell types such as astrocytes, whilst important an important aspect, may not be the only link between a developmental insult and a programmed behaviour after birth.

Nonetheless, offspring born to pregnancies subjected to stress beginning early and late in gestation were also more susceptible to changes in the neurosteroid system with reductions in circulating allopregnanolone present in fetal life in males and females of the late stress group and reductions apparent at juvenility in early stress exposed males. Allopregnanolone has previously been shown to have potent anxiolytic effects (289) and has also been implicated in the regulation of chronic stress, with treatment preventing anxious and depressive behaviours as a result of increased hippocampal neurogenesis in rodents following chronic isolation stress (207). We have also previously shown a disruption in circulating allopregnanolone levels in prenatally stressed offspring whose mothers received exogenous treatment of the neurosteroid (283). In the current study, we have demonstrated a reduction in allopregnanolone levels in prenatally stressed offspring from both early and late in pregnancy. These deficits in circulating allopregnanolone may contribute to the anxious phenotype observed in the current study in early stress exposed male offspring and also predispose to further neuropathologies later in life, particularly when coupled with reduced mature oligodendrocyte and reactive astrocyte expression in the hippocampus. Furthermore, reduced systemic allopregnanolone may also impair stress responsiveness in early stress exposed male offspring with allopregnanolone known to have important functions in the regulation of HPA axis and opioid-mediated stress responses in adulthood (342). Given the anxiolytic and neuroprotective effects of allopregnanolone on the fetal brain and in

times of stress, postnatal administration as a restorative treatment to affected neonates in this model is a worthwhile future direction.

This study has shown that prenatal stress induced from different time points in gestation has significant enduring effects on the neurodevelopmental, behavioural and neurosteroid ontogenesis in guinea pig offspring in fetal life and juvenility, particularly if stress was induced repeatedly from early in gestation. This study contributes to a growing body of knowledge elucidating the effects of prenatal stress on the developing fetus and the subsequent risk for neuropathologies later in life. This work further suggests a potential neurosteroid link between prenatal stress and ongoing changes in behaviour. Lastly, this study shows that prenatal stress may result in diverse developmental changes in offspring which must be considered in light of the type and timing of the prenatal stressor as well as the sex of the offspring.

Chapter Seven: General Discussion

It has been over two decades since David Barker first observed the effects of developmental influences on adult health and proposed the developmental origins of health and disease hypothesis (8). Since this time, the importance of the early life development of offspring has become increasingly recognised as a determinant for lifelong health (6, 8, 343). The perinatal brain is particularly vulnerable to perturbations during fetal and early life and thus changes in the brain that may contribute to altered behaviour or neural functioning can have long-lasting impacts on the development and maturation of the offspring throughout their lifespan. The studies in this thesis confirm, using an established model of prenatal stress and HPA axis programming, that stress during pregnancy alters normal developmental processes in the perinatal brain and contributes to anxiety later in life. These studies also show for the first time, that the timing of prenatal stress experience in pregnancy can specifically alter circulating allopregnanolone levels in both fetal and juvenile offspring, indicating a programming effect of stress on the neurosteroid system. These findings elucidate a potential contributory neurosteroid mechanism by which stress exposure in utero may alter the normal growth and development of the brain, endocrine and behavioural outcomes in offspring. These studies specifically identify vulnerable cell populations and brain regions to the effects of prenatal stress and as such, may be targets for treatment in the future. Such treatments may include supplementing the neurosteroid system and raising its role in providing perinatal neuroprotection.

7.1 The effect of prenatal stress on perinatal brain development

A major aim of this thesis was to explore how prenatal stress exposure may alter key neurodevelopmental processes that detrimentally affect perinatal brain development. The studies presented in this thesis show that both mature oligodendrocytes and

reactive astrocytes in the brains of offspring are affected by maternal stress. In agreement with previous studies which highlight the hippocampus as a particularly vulnerable brain region to the effects of prenatal stress (262), these studies have shown that the CA1 region of the hippocampus is indeed susceptible to developmental alterations by maternal stress exposure.

Consistent with a hypothesised difference between male and female offspring, many of the changes in outcome measures observed in the current studies have shown sexually dimorphic results. For instance, male offspring have shown the ability for oligodendrocyte catch up growth postnatally in the hippocampus (when stress was induced prenatally in the mid and late stress groups). When stress was induced from early in gestation, males showed a sustained reduction in expression of oligodendrocyte markers in the hippocampus, which did not improve or recover by juvenility. In females, offspring from all stress groups showed marked reductions in mature oligodendrocyte expression at juvenility which was not present at term. This finding suggests that females demonstrate a delayed reduction (or failure to develop after birth) in oligodendrocyte cell expression. In 2013, Xu et al found similar results in rats, where maternal restraint stress in mid-late or late pregnancy was associated with disrupted myelin sheaths in the hippocampus (129). These investigators also found that the greatest alterations in myelin sheath organisation were in male offspring exposed to stress in mid-late gestation (129). Due to a different trajectory of prenatal brain development between rats and the more mature guinea pigs and humans (112), it is difficult to compare these findings with mid-late stress in pregnancy in the current studies. However, it is possible to draw comparisons between prenatal stress induced earlier in pregnancy in the current model in which males showed the largest reduction (a sustained 12%) in oligodendrocyte expression from term until juvenility.

In the guinea pig, relative brain-to-body weight reaches a peak at 29-35 days gestation (256), indicating that this is an important time for fetal brain growth. By around 35 days gestation in the guinea pig, the fetal brain is undergoing a rapid process of cell generation and differentiation which is to be followed by growth of the rest of the body and organs (256). This time of rapid brain growth includes the development of the hippocampus which increases substantially in size from GA40 (112, 344). A stress-induced perturbation at this age may prevent the generation of cells, such as oligodendrocyte precursors, or arrest cells and prevent differentiation into mature myelinating oligodendrocytes, which are therefore not able to regenerate after birth. In experiments which assess myelination in 'shiverer' mice who have a genetic deletion of the myelin basic protein (MBP) gene, animals show reduced compact myelin around large neurons, leading to abnormal maturation of the neuronal cytoskeleton in large CNS neurons (345). In this study by Brady et al, it is suggested that a disruption in MBP expression could lead to an immature axonal cytoskeleton, and therefore unstable synaptic connectivity and decreased neuronal plasticity, particularly if the cause of the decrease in myelination occurs during critical developmental windows of brain development (345). Further studies indicate that 'shiverer' mice lacking MBP expression show altered locomotor activity in open field testing (346), which is consistent with findings in guinea pigs in this thesis.

The data herein suggests that oligodendrocytes are particularly vulnerable to the effects of stress and therefore given their important role in behavioural functions such as locomotor activity (347), these data have functional implications for offspring.

To date, the effects of prenatal stress on myelination and the oligodendrocyte lineage are not well characterised however a number of studies have assessed oligodendrocyte maturation in other models of pregnancy compromise such as preterm birth and intrauterine growth restriction (IUGR). In preterm birth, term equivalent male guinea pig

offspring (delivered preterm and assessed at the equivalent of term delivery age) show alterations at every stage of the oligodendrocyte lineage (from precursors to mature myelinating oligodendrocytes) in the cerebellum (348). This observation indicates that pregnancy compromises can affect the formation of myelin with male offspring having a particular sensitivity to this effect. Interestingly, the same study also hypothesised that the alterations in male oligodendrocyte lineage may result from overexposure to cortisol (which was increased in males following progesterone administration)(348). In support of this suggestion, one study in rats administered exogenous cortisol to neonates and found that the amount of myelin and the number of myelinated axons were reduced (349), suggesting a direct relationship between cortisol and myelination. In a study of the effects of IUGR in guinea pigs the oligodendrocyte lineage was again altered by a prenatal insult however in this study, all deficits were resolved by 8 weeks of age in the brains of the offspring (244). This work suggested that offspring affected by IUGR may have increased reparative processes compared to those in offspring exposed to prenatal stress. It is important to note that this previous study assessed postnatal development at a substantially more mature time point than in the current studies.

In the guinea pig, GA50 has also been identified as a time of maximal brain growth (wet weight) and GA60 as a period of maximal cholesterol accumulation and myelination (256). MR and GR expression also reach a peak in gestation around GA40-50 (340, 350), which suggests that maturation of these important regulators of the HPA stress response undergo a potentially vulnerable period of development at this age. This is similar to human pregnancy in which many developmental processes in the brain such as myelination and synaptogenesis overlap towards the end of pregnancy (100). Mature oligodendrocyte expression in the hippocampus is also reduced in the current studies in offspring who were exposed to prenatal stress on GA60 and 65 only (late stress group) and these deficits persisted to juvenility in both males and females. These data suggest

that the period of maximal myelination in the guinea pig brain is indeed a vulnerable window of development and thus stress-induced perturbations can affect late myelination processes. Previous studies have also shown that the placental barrier to cortisol (11β-HSD2) decreases in capacity from GA50 to 60 in the guinea pig (341), thereby increasing the amount of active cortisol that may reach the fetus. Furthermore, fetal adrenal sensitivity in the guinea pig is dramatically increased as gestation progresses (340, 351) and therefore an increased effect of glucocorticoid programming at this later gestational age is likely. Consistent with other studies in guinea pigs which have shown a 3-fold increase in maternal salivary cortisol concentrations in response to strobe light exposure at GA60 (352), the current studies showed the largest increase in maternal salivary cortisol at the latest stress timepoints which again suggests that the fetus may be exposed to high levels of cortisol at this time. Notwithstanding, maternal salivary cortisol concentrations did not rise, as expected, with every stress episode in the current studies, particularly at the earlier stress timepoints, and this is discussed in Section 7.4.

Alterations in mature oligodendrocytes were observed in the cerebellum in fetal life with both males and females exposed to mid-late gestational prenatal stress showing reduced expression compared to controls (Chapter 5). By juvenility, there were no changes between prenatal stress and control offspring, indicating that offspring have a high regenerative potential in the cerebellum in early life. Given the relatively late development of the cerebellum in gestation and its known potential for postnatal neurogenesis (102), early life catch up growth in the cerebellum is expected and indeed has been shown by others (244).

Reactive astrocytes were also shown to be susceptible to the effects of prenatal stress exposure in the current studies. In the hippocampus, male offspring from all stress groups showed reduced expression of astrocytes as fetuses however all stress exposed

groups also recovered to control levels by juvenility. In females, a delayed reduction was again seen in juvenility (similar to that observed in mature oligodendrocyte expression in the hippocampus), where pups from a pregnancy subjected to stress from early and late in gestation showed reduced astrocyte marker expression in the hippocampus after birth. This is consistent with studies in adult tree shrews which showed a significant decrease in in both the number and soma volume of reactive astrocytes following chronic stress, however this study only assessed outcomes in males (353). Astrocytes are the most abundant cell type within the human brain, once thought to only provide stability for neurons (354), are now known to have critical functions in the brain including regulation of neuronal activity (355). For example, astrocytes have been shown to uptake excess glutamate in order to prevent excitotoxicity in the brain (356). This is particularly important for the current studies as a reduction in reactive astrocyte expression could confer increased risk for stress-induced increases in excitatory signalling and neural damage. Conversely, increased astrocyte expression has also been associated with detrimental outcomes for offspring. In the current studies, increases in reactive astrocytes were observed specifically in the subcortical white matter in male and female offspring, particularly in the early and late stress exposed groups. Astrocytes have previously been shown to be increased in the hippocampus, cortex and striatum of adult rat offspring exposed to prenatal stress in late pregnancy (120), suggesting that prenatal stress may dynamically affect the activity of astrocytes. Recently, exogenous dexamethasone treatment has been shown to affect astrocyte morphology in the hippocampus in adult rats without a loss in astrocyte number which indicates that glucocorticoid action on astrocytes may also be structural (357). The adverse effects of increased astrocyte activation are thought to result from hypertrophied processes which form a physical barrier for axons to pass through, thereby acting as scar tissue (358). This function has also been hypothesised to prevent the spread of further brain injury

(359) and therefore astrocytes may perform two simultaneous, yet conflicting functions. As well as regulating excess glutamate levels in the brain, astrocytes help to maintain the blood brain barrier, support neurons and secrete various neurotransmitters, including pro-inflammatory cytokines (358). The functions of astrocytes are diverse and therefore depending on the outcomes observed, their expression may be interpreted as either protective or detrimental to recovery. Given that offspring in the early and late stress exposed groups showed similar behavioural outcomes to those in the mid stress exposed group in the current studies, the possibility of prenatal stress inducing severe brain trauma that elicits astrogliotic scaring seems unlikely. The functional implications of increased astrocyte expression in the subcortical white matter is therefore unclear however the role of astrocytes in the regulation of various neuropathologies, particularly stress (360), is becoming increasingly recognised. Assessing additional and comprehensive markers of reactive astrocytes such as ALDH1L1 may help to better understand their function and distribution. The current studies however offer further insight into the changes in astrocyte activation during development and after prenatal stress exposure and further suggest that astrocyte expression (increase or decrease) is regionally specific, as well a dynamic in nature. The role of astrocytes in brain development and stress responses required further investigation however the studies in this thesis suggest that regulation of astrocyte expression in the hippocampus and subcortical white matter may be related to altered behavioural phenotypes after birth. In the cerebellum, female offspring from stressed pregnancies showed no significant effect of stress on reactive astrocyte marker expression, indicating that astrocytes in the female perinatal cerebellum may not be vulnerable to perturbations due to prenatal stress. Conversely in males, reactive astrocytes appear to be selectively reduced in lobe VIII and the deep white matter of the cerebellum continuing into juvenility, which indicates that these cells are vulnerable to stress-induced damage. Given the

involvement of the cerebellum and in particular, lobe VIII, in conditions such as schizophrenia (151), autism (150) and ADHD (152), these postnatal deficits may contribute to alterations in the behaviour of males as they develop. In fact, males have frequently shown higher susceptibility to the development of schizophrenia, autism and ADHD following prenatal stress (175, 179, 183). These data further implicate reductions in reactive astrocyte expression in the cerebellum of male offspring with the development of behavioural pathologies later in life.

Neurogenesis has also been shown to be reduced following prenatal stress in the current studies (Chapter 3), with both male and female fetal offspring showing reduced mature neuron marker expression compared to controls. Neurogenesis is an active process in the hippocampus beginning in gestation and continuing throughout the life of the offspring (338). It has been implicated in learning and memory functions of the hippocampus and also in synaptic plasticity (361, 362). Prenatal stress has been shown to attenuate neurogenesis in the hippocampus and is also associated with deficits in learning and memory in adult offspring (115, 119, 127). Indeed, the effects of prenatal stress on neurogenesis in the hippocampus have been extensively studied, with long-term potentiation and reduced learning recently reported in guinea pigs using a similar prenatal stress protocol (363). The current studies therefore strengthen data implicating neurogenesis as a vulnerable process to damage by prenatal stress.

Interestingly, the current studies did not show a dramatic and consistent reduction in either mature oligodendrocytes or reactive astrocytes in offspring exposed to stress from early in pregnancy, as expected. With the exception of oligodendrocyte cell marker expression in the hippocampus of juvenile males, all other measures of brain development were similar to levels reported in the mid and late stress groups. Involvement of cell proliferation and apoptotic processes may have had a role in determining outcomes in the offspring in this thesis and indeed, given the dynamic

results seen herein, further investigation into other developmental processes is warranted. Previous studies in rats have shown a dramatic (50%) reduction in cell proliferation and a 36% increase in apoptosis (activated caspase 3) in the hippocampus following prenatal stress, which indicates that gross brain development could be contributing to the outcomes seen in the current model (312). It is also possible that the upregulation of adrenal (maternal and fetal) production of cortisol (48), increased placental transfer (43, 44) of cortisol and increased GR expression (24) in the fetal brain in late pregnancy may contribute to a particularly vulnerable window of development from GA50 in the guinea pig. In this way, stress induced during the vulnerable period of mid-late pregnancy may be sufficient to perturb fetal brain development to a similar extent to that induced with an increased duration of stress exposure as seen in the early stress group. The present studies have therefore supported the hypothesis that prenatal stress affects perinatal brain development and that the hippocampus is particularly vulnerable to perturbations in neurodevelopment.

7.2 Prenatal stress results in increased anxious behaviour in juvenility

The current studies indicate there is an increase in anxious and neophobic behaviours in offspring exposed to prenatal stress with no effects of sex on outcomes. Offspring of pregnancies affected by maternal stress in early, mid and late stress groups all showed similar behavioural parameters in open field and object exploration tests, indicating that the timing of prenatal stress did not determine the behavioural outcomes in these studies.

Perhaps the most commonly reported outcomes in animal studies following prenatal stress include increased anxiety and fear responses in offspring (329, 364). Variable results are seen however that may result from differences in the timing of prenatal stress and/or the timing of assessment of offspring and the behavioural test employed.

The open field test and object exploration test were therefore used in the current studies to specifically measure anxiety and fear/neophobia, which have been linked with stress and glucocorticoid exposure previously (91, 365, 366). Unlike most studies however which use rats or mice to assess behaviour, the guinea pig has not been extensively studied, or even well optimised, for behavioural testing. One of the major hurdles in these studies therefore was to optimise behavioural testing for tricoloured guinea pigs (i.e. all with different markings) who do not have a tail for easy video tracking. Guinea pigs are also highly social animals who demonstrate strong attachments to their mothers early in life (290, 367). The open field and object exploration tests were chosen in the current study as they could be conducted in short timeframes (10 minutes per test) and therefore would not confound the study by inducing maternal separation anxiety which occurs over a longer period of time (290). The open field test is also a well-established means of identifying anxious behaviour (368), and which is commonly used to assess behaviour after prenatal stress exposure (295, 369). The time spent in the inner zone of the open field arena was significantly less in stress-exposed offspring in the current studies, which suggests anxious wall-hugging behaviour (thigmotaxis). Previous studies using a similar model to that used in the present work have shown that both males and females exposed to late gestational prenatal stress demonstrate reduced entries into the inner zone of the open field arena, again indicating anxious behaviour (91, 235). These studies, conducted in older offspring, showed that females showed anxious behaviour based on the estrous cycle (235) and that male offspring show reduced testosterone in parallel with anxious behaviour (91). Sex hormones can have major actions on the developing brain (370), and therefore the effect of hormones such as estrogen and testosterone on the behaviour of offspring may be a consideration for future behavioural studies assessing outcomes which may have a developmental origin. Indeed, estrogen and testosterone have both

been shown to play a role in anxious behaviour (whether it be anxiolytic or anxiogenic; 371, 372). Therefore around the time of sexual maturity (3-6 weeks of age in guinea pigs) the role of sex hormones should be investigated to determine what impact they have on behaviour and how prenatal stress may modulate these responses. To prevent such confounding factors in the current studies however, behavioural testing was performed before sexual maturity at 18 days of postnatal age. Learning and memory tests, as performed by other researchers in older guinea pigs (363), may be inappropriate in guinea pigs of pre-weaning age due to the strong attachment to the mother (367) and hence these behavioural parameters and their association with changes in the hippocampus were unable to be assessed in the current studies.

The object exploration test was used in the current studies to assess neophobic (fear of novelty) behaviour. Neophobia is the aversive reaction to novel stimuli and has been studied in laboratory species for over 100 years (373). Neophobia has been shown to elicit activation of the HPA axis stress response to increase circulating cortisol/corticosterone levels (374). Higher baseline levels of endogenous corticosteroids have also been associated with increased fearfulness (375), suggesting that stress and neophobia may be linked. The natural avoidance behaviour associated with unknown objects in a behavioural test of neophobia has been shown recently in mice and is linked with anxiety-like behaviour (241). The interconnections of the limbic system (amygdala, hippocampus and PFC) are all activated upon exposure to anxiogenic stimuli (376). The amygdala plays an important role in the regulation of fear and anxiety as lesions in this area have been shown to attenuate anxiety-like behaviours and stimulation of the amygdala produces fearful behaviours (377). The hippocampus is also implicated in anxiogenic and fearful responses with experimental activation of inhibitory $GABA_A$ receptors shown to abolish anxious behaviour in rats (378). Indeed, the hippocampus is known to play a central role in terminating the HPA stress response,

primarily by cell populations that reside in the CA1 region (379), highlighting the critical role that the hippocampus plays in stress reactivity. Recently, chronic maternal stress and/or exogenous cortisol treatment in rats has been shown to alter extinction of learned fear in offspring, which was coupled with a decrease in GR expression in the mPFC, hippocampus and hypothalamus (380). This previous study therefore suggests that high levels of maternal stress or cortisol during pregnancy may have an effect on fear-related behaviours in offspring that are associated with decreased GR expression in the brain. A decrease in GR expression may have downstream effects on the HPA axis, causing an upregulation in stress responsiveness due to reduced negative feedback at the level of the GR. This in turn, may increase neophobic responses to stimuli in offspring which has been shown to increase corticosterone levels (374). GR expression has been shown in human brains to be abundantly expressed in the hippocampus with almost all nuclei and approximately 50% of astrocytes expressing GR in the CA regions of the hippocampus (381). Hippocampal GR expression is critical in mediating the stress response and has a distinct profile of expression throughout gestation, primarily to regulate and respond to levels of glucocorticoids during development. In the rat brain, glucocorticoids are present in the hippocampus and pituitary from GA13 (0.6 gestation) however in the guinea pig, MR levels reach a peak at GA40 (0.5) in the fetal hippocampus and then decreases until term (125). GR levels however are high in the guinea pig hippocampus throughout gestation with expression rising to reach a peak at term (125), highlighting the importance of glucocorticoid actions during late pregnancy for fetal development (39). Prenatal stress may therefore increase HPA axis activity through decreased expression of GR and/or MR in the hippocampus (382). Prenatal social stress in rats has shown a selective reduction in MR expression in the hippocampus of male and female offspring (139), suggesting that glucocorticoids may bind to GR in the absence of MR and therefore activate the HPA axis. In guinea pig brain

cell cultures, exposure to dexamethasone and cortisol result in an increase in GR expression with no change in MR (383) however differing results have also been noted in guinea pig brains when measured at different stages of gestation (350, 384), suggesting that GR and MRs may be expressed at different levels during gestation, depending on the functional requirements of the fetus. A stress-induced alteration in GR/MR expression in the brains of offspring may therefore help to explain why prenatally stressed offspring commonly show increased stress responsiveness after birth and therefore altered behaviours that may have a stress-related origin such as neophobia. Further study is therefore warranted to investigate how GR and MR expression may contribute to the structural and behavioural deficits observed in this thesis using the guinea pig as a developmentally relevant animal model to human pregnancy.

The present findings have important implications for informing future studies which aim to characterise behavioural outcomes in the guinea pig. These data have shown for the first time, that the guinea pig is a useful model for studying the effects of prenatal stress on neophobic behaviour using an object exploration test. Furthermore, these studies have shown an association between prenatal stress and anxious, fearful behaviour in juvenile offspring, irrespective of the gestational age of onset. Therefore, the neurodevelopmental deficits observed in conjunction with these altered behavioural outcomes may implicate perturbed hippocampal development, with increased anxious behaviour in juvenility.

7.3 Neurosteroids, endocrine development and prenatal stress

There is a substantial body of evidence implicating the neurosteroid system in the stress response and prenatal brain development. A reduction in circulating allopregnanolone concentrations was observed particularly in the early stress exposed group, which was

maintained from fetal life to juvenility, with a parallel drop in concentrations due to birth observed in both groups. Furthermore, a disruption in fetal allopregnanolone levels was observed following maternal exogenous treatment in stressed pregnancies (Chapter 3), suggesting that prenatal stress may lead to the alteration of neurosteroid transfer to the fetus. Interestingly, in a concurrent study which assessed neurosteroid metabolising enzymes in the placentas of these same prenatally stressed pregnancies, whilst no structural changes were observed, 5α-reductase expression was reduced in both male and female placentas who were exposed to mid-late gestational stress and exogenous maternal allopregnanolone treatment (data not shown). Specifically in male placentas of these stress exposed, allopregnanolone treated pregnancies, allopregnanolone degrading enzymes were also increased (data not shown), suggesting that this male group may be particularly vulnerable to stress-induced perturbations due to reduced levels of the neuroprotective allopregnanolone. Females did not show the same effects in the placenta and also did not show reductions in oligodendrocyte or reactive astrocyte expression in the fetal hippocampus following mid gestational stress (Chapter 3).

By juvenility, there were also no effects of prenatal stress exposure seen on hippocampal neurosteroidogenesis, brain allopregnanolone levels or expression of the most abundant $GABA_A$ receptor subunits in the hippocampus, δ or α5, in either sex cohort. This suggests that the perinatal hippocampal neurosteroid system is resilient to the effects of prenatal stress. Additional $GABA_A$ receptor subtypes and local expression of allopregnanolone however may contribute to the alterations in structural features of the hippocampus noted in the current studies. In the cerebellum, a reduction in the key $GABA_A$ receptor subunit δ was observed in prenatally stressed female fetuses, which suggests that the composition of these receptors in the cerebellum may confer reduced tonic GABA inhibition (317). The functional outcomes of reduced tonic inhibition in

these fetuses may be particularly important during times of adversity, where endogenous neuroprotection in required. The α6 $GABA_A$ receptor subunit was also increased in the cerebellum after birth, which is consistent with studies in preterm guinea pigs suggesting that the function of this receptor subunit after birth is to increase $GABA_A$ receptor binding of allopregnanolone to compensate for the loss of circulating levels with the removal of the placenta (292). Therefore the current studies have shown alterations in the circulating and local neurosteroid systems, which may confer increased risk for neural damage later in life. In line with a recommendation to further characterise the effect of sex hormones on the postnatal behaviour of offspring in the current model, the role of the complete neurosteroid system in these offspring may help to elucidate how neurosteroids may be altered by stress exposure. Due to the structural similarities of many of these steroid hormones, techniques with high sensitivity such as liquid chromatography mass spectrometry (LCMS-MS) are required as they are able to differentiate and quantify many similar substances in one sample. This would be of particular use in physically small species such as rats and guinea pigs where obtaining large volumes of plasma, sera or specific brain regions are not possible.

The HPA axis of offspring has been shown previously to be vulnerable to alterations due to prenatal stress and exogenous glucocorticoids (24). Assessing the stress responses of offspring was not a major aim of the current studies in this thesis however as HPA axis functioning plays a central role in many physiological conditions it is therefore inextricably linked to many functional outcomes in offspring. Neonatal salivary cortisol concentrations in response to behavioural testing was not significantly different to controls in this thesis. However, the behavioural tests employed in these studies lasted for 20 minutes in total and were not intended to induce a stress response in the neonates. In this way, the innate behaviour of the offspring could be determined independent of any programming effects maternal stress may have had on HPA

functioning in offspring. Salivary DHEA levels were not affected by prenatal stress in these studies (Chapter 4), which indicates that prenatal stress did not affect basal functioning of the adrenal gland in offspring.

The role of other neurotransmitters such as serotonin and dopamine have previously been implicated in models of prenatal stress and maternal anxiety. In rats, prenatal exposure to stress in conjunction with the common anxiolytic drug, fluoxetine (a selective serotonin reuptake inhibitor; SSRI), has been shown to cause a reduction in hippocampal serotonin levels and also dopamine levels in the hippocampus of offspring (385), suggesting a role for prenatal stress in regulating development of these neurotransmitter systems. Interestingly these drugs have been shown to interact with the neurosteroid system in the rat brain suggesting a part of their action may involve changes to allopregnanolone levels (386). Prenatal stress has also been shown in rats to affect the development of serotonin neurons (387) which may have a functional implication on behaviour later in life through altered establishment of the serotonin system in utero. However, maternal administration of another SSRI, escitalopram, as well as chronic mild prenatal stress was shown in rats to have no effect on maternal care after birth (388) which suggests that the type of anxiolytic used may determine its effect on offspring. This emerging field of research is important for the understanding of how maternal stress and anxiety interacts with neurotransmitter systems in the fetus and also how current anxiolytic medications can affect fetal development. Serotonin depletion has been associated with reduced locomotion and increased neophobia in rats (389) and therefore its involvement in mediating some of the behavioural effects seen after prenatal stress is worth investigation.

7.4 Prenatal stress and maternal cortisol

There is vast amount of literature linking maternal stress (in many forms, see Section 1.2.1) with altered outcomes in her progeny. Although the mechanisms that underlie the programming of disease by prenatal stress are largely unknown, overexposure to glucocorticoids is one of the most widely accepted hypotheses. Maternal urinary cortisol levels have been shown to be predictive of fetal weight and fetal cortisol levels in human pregnancies (390). Maternal cortisol has even been shown to be higher in women with a mental illness such as depression and in these cases (391), cortisol is predictive of infant temperament after birth (392). Many of these studies however fail to account for the effect that a maternal mental illness may have on the early life care of the infant and therefore postnatal outcomes of infants may not be exclusively linked with maternal prenatal cortisol. In a recent study assessing mothers who were depressed or anxious after childbirth, Feldman et al showed that infants had lower social engagement, negative emotionality and increased cortisol responsiveness in the first year of life (393), which are similar to effects observed after prenatal stress exposure. Therefore, the maternal psychological state before and after birth may be important determinants for infant health.

Despite the strong independent links between maternal mental illness, cortisol levels and pregnancy outcomes, data is lacking regarding the specific relationship between maternal stress, rises in cortisol and perinatal outcomes. Even in animal studies where collection of regular biological samples and measurement of cortisol or corticosterone is readily available, studies have failed to show a consistent link between prenatal stress, cortisol and outcomes in offspring.

In fact, during pregnancy, there is limited correlation between a woman's psychological state and her cortisol levels (334). In human studies, maternal reports of stress and anxiety are unrelated to cortisol levels in both maternal plasma and amniotic fluid (394).

This may be particularly true towards the end of pregnancy as the HPA axis of the mother becomes less responsive to stressful stimuli (28). This lack of consistent data supporting a rise in maternal cortisol during times of stress has lead to a number of hypotheses regarding the mode of transfer of maternal psychological stress to the fetus. Some have suggested that the maternal diet and protein load can affect infant outcomes (395), whilst others have suggested a role for altered uterine placental blood flow (396). Perhaps some of the most well supported hypotheses include those that find prenatal stress decreases 11β-HSD2 in the placenta (43) and therefore increases the amount of cortisol reaching the fetal circulation without showing a rise in maternal levels. Due to the high proportional difference between maternal and fetal cortisol levels (397), this could potentially explain some of the effects consistent with glucocorticoid programming in fetuses despite a lack of a consistent and reliable increase in maternal cortisol. Interestingly, there is evidence that low maternal protein intake can reduce 11β-HSD2 expression in the placenta, potentially increasing active cortisol levels in fetal circulation (398), highlighting the interplay between multiple maternal factors and pregnancy outcomes.

As mentioned previously, rises in guinea pig salivary cortisol have been reported previously following prenatal stress (352) however as demonstrated in the current studies, cortisol levels may not accurately reflect the stress status of the mother. In rats, a variable stressor induced from 0.6 gestation has shown an increase in maternal plasma corticosterone levels compared to controls but only when induced at 0.9 gestation (399). When measured earlier in pregnancy (0.7), no rise in maternal corticosterone was observed compared to both controls and to baseline levels measured prior to the onset of stress exposure 2 days earlier (399). These studies suggest that maternal HPA stress responses are not consistently shown to be associated with prenatal stress exposure. The present studies have shown that maternal HPA responsiveness (salivary cortisol

concentrations) in the pregnant guinea pig was only increased from 50 days gestation until term in response to a strobe light stressor and no increases were seen earlier in pregnancy compared to controls. As mentioned in Chapter 6, salivary cortisol concentrations in stress-exposed dams did, on average, increase following strobe light exposure (2-fold) however so did those of control-handled dams (average 1.5-fold). In control animals, some reduced responsiveness to handling was evidence as pregnancy progresses, however this did not appear to be the case in stress-exposed dams. Reduced maternal HPA responsiveness has been reported with advancing gestational age (333). However it is possible in dams who are exposed to stress previously in pregnancy, that HPA activity may remain high throughout gestation in anticipation of repeated stress events. Although this hypothesis has not been specifically investigated in the current studies, there is some evidence to support this theory in the literature. In 2006, women who were awaiting amniocentesis were assessed for state and trait anxiety and also provided blood samples for cortisol measurement. In anticipation of the amniocentesis, pregnant women showed higher cortisol levels in conjunction with increased state anxiety (400), indicating that the anticipation of a stressful event can increase cortisol levels in pregnant mothers.

Offspring exposed to prenatal stress often present with outcomes that have a likely association with overexposure to glucocorticoids, and thus there is a link between the maternal HPA axis and perinatal outcomes. There is very little evidence to date specifically linking prenatal stress exposure with maternal cortisol concentrations and altered outcomes in offspring (334, 335). This suggests that despite the decades of research into the programming effects of maternal stress during pregnancy on offspring, the mechanisms of action by which the maternal psychological state informs fetal growth, are largely unknown. The results presented in this thesis offer novel information regarding the potential alterations of the maternal HPA axis in response to prenatal

stress and also further adds to the growing body of knowledge which supports the use of salivary cortisol as a non-invasive and successful measure of unbound cortisol in pregnant dams.

7.5 Conclusions and future directions

The effect of the maternal psychological state on perinatal outcomes has been a topic of much scientific interest for many years. Our understanding of stress and how it may perturb fetal development and lead to neuropathologies later in life has grown over time, however there are still many aspects which remain unknown. The present studies provide novel information regarding the types of brain regions and cell types within those regions that are particularly vulnerable to the effects of prenatal stress. These studies have also characterised how such perturbations in fetal brain development may influence the development of behavioural disorders such as anxiety in postnatal life. However, as behaviour is affected by prenatal stress onset at all ages tested in this thesis, further studies are required to elucidate the functional significance of alterations in mature oligodendrocyte and reactive astrocyte expression as well as changes in allopregnanolone concentrations which are affected by the timing of prenatal stress and the sex of the offspring.

Altogether, the studies presented in this thesis contribute to a growing body of knowledge which aims to understand how conditions during pregnancy can shape the development of offspring throughout their entire life. Maternal stress has been shown to be one of the most potent programming factors for the postnatal functioning of offspring and therefore understanding the mechanisms which underpin this association are important determinants to improving health for all affected babies.

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Effects of Prenatal Stress on Fetal Neurodevelopment and Responses to Maternal Neurosteroid Treatment in Guinea Pigs

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Key Words
Stress · Neurosteroid · Cortisol · Fetus · Myelin basic protein · Glial fibrillary acidic protein · Microtubule-associated protein 2 · Neonate

Abstract
Background: Maternal psychosocial stress during pregnancy is associated with adverse neonatal outcomes. These outcomes result from changes in fetal brain development and lead to disrupted cognitive, behavioural and emotional development. The neurosteroid allopregnanolone has been shown to reduce neural excitability and aid in protecting the fetal brain from excitotoxic insults. The objectives of this study were to assess the effect of prenatal maternal stress on fetal brain development with and without maternal allopregnanolone treatment. ***Methods:*** Pregnant guinea pigs were subjected to stress induced by exposure to a strobe light at 50, 55, 60 and 65 days gestation. Salivary cortisol levels were measured before and after each exposure. Fetal brains were assessed for markers of brain development using immunohistochemistry and plasma allopregnanolone was measured by radioimmunoassay. ***Results:*** Female, but not male prenatal stress-exposed fetuses demonstrated higher brain-to-liver ratios (BLR). Male fetuses showed significantly reduced expression of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and both males and females showed reduced expression of microtubule-associated protein 2 (MAP2). These markers were not affected by maternal allopregnanolone treatment. However, maternal allopregnanolone treatment resulted in an increase in fetal plasma allopregnanolone concentrations in control pregnancies but concentrations were not raised after prenatal stress exposure. ***Conclusions:*** These findings indicate that the effects of prenatal stress on fetal brain development are sexually dimorphic with more pronounced negative effects seen on male neurodevelopment. Allopregnanolone treatment was not effective in raising fetal plasma concentrations after prenatal stress suggesting a stress-induced dysregulation of neurosteroid pathways during gestation. Interestingly, this study directly implicates prenatal stress in the disruption of fetal neurosteroid levels, such that it may mediate some of the deleterious effects on fetal neurodevelopment by facilitating a deficit in normal endogenous neuroprotective mechanisms.

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Introduction

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

Stress can be thought of as the adaptive response of an organism to ready itself for a threat to survival. The stress response is often classified by the increased release of cortisol and the downstream effects this glucocorticoid produces. There is now a substantial body of evidence highlighting the association between maternal psychosocial stress during pregnancy and a number of adverse perinatal outcomes. Some of the strongest associations in epidemiological studies include those relating to cognitive, behavioural and emotional development of offspring [1–3]. Maternal stress during pregnancy is associated with increased incidences of childhood behavioural problems in infancy and at school age [4–9] with male offspring showing higher rates of learning and memory deficits and hyperactivity disorders [10, 11], particularly when the stress was experienced in late gestation [12, 13]. Prenatal stress has also been associated with disorders in offspring stretching beyond childhood including increased incidences of neuropathologies such as depression and schizophrenia later in life [14–16].

Adverse behavioural outcomes following prenatal stress are supported by data in animal studies which link prenatal stress with perturbations in fetal brain development at particular vulnerable windows of fetal brain growth [17]. Late pregnancy is a time of considerable myelination and glial cell proliferation as well as increased synaptogenesis, neuronal and axonal migration/proliferation and various receptor maturational processes, all of which place high energy demands on the fetal brain [18, 19]. Therefore this period has been identified as a vulnerable period for neurodevelopmental delay or damage [20]. Animal studies have linked prenatal stress to alterations in the hippocampus that result in a higher susceptibility to neuropathologies later in life [21–23]. Late gestational social stress has been shown to increase anxiety behaviours in male offspring [24]. High levels of cortisol in fetal circulation following exposure to an acoustic stressor have also been shown to cause disturbed hippocampal development in rhesus monkeys [22]. Prenatal restraint stress in late gestational rats leads to dendritic atrophy in the hippocampus of the offspring as a result of excitotoxicity [25]. Therefore stress-induced increases in glucocorticoid levels and neural excitation may mediate some of these deleterious effects.

During gestation there are high levels of endogenous neurosteroids, which act at inhibitory $GABA_A$ receptors to reduce neural excitability. We have previously observed high levels of fetal arousal and neural excitability when neurosteroid synthesis is blocked with finasteride [26] and that a reduction in neurosteroid levels is also associated with reduced levels of REM sleep, which in turn may result in developmental delay [27]. During pregnancy, the placenta has a key role maintaining the endogenously protective neurosteroid levels by providing considerable amounts of precursors for their synthesis. This accounts for the remarkably high levels of the potent neurosteroid allopregnanolone, which is synthesised from progesterone, in the fetus throughout late gestation [26, 28–31]. We chose to administer allopregnanolone in late gestation (from gestational day 60) to mimic this high endogenous production and to model normal responsiveness to stress exposure at this time. Furthermore, we have previously reported that administration of exogenous glucocorticoids during late pregnancy alters levels of the endogenous allopregnanolone by suppressing synthesising enzymes in the placenta [32]. Our studies have also shown a decrease in reactive astrocyte marker expression in the brains of these fetuses, and interestingly, the males and not the female fetuses demonstrated these adverse effects in response to glucocorticoid exposure [32]. Our previous studies have shown that allopregnanolone has potent neuroprotective effects against acute excitotoxicity following hypoxia/ischemia and that reduced concentrations of allopregnanolone conferred increased vulnerability to brain injury in late gestation [33].

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

Methods

Animal Stress Protocol

Time-mated, outbred pregnant guinea pigs were obtained from the University of Newcastle colony. All procedures were approved by the University of Newcastle Animal Care and Ethics Committee and carried out in accordance with the Australian Code of Practice

for the Care and Use of Animals for Scientific Purposes. The dams were randomly allocated into either a stress (light) exposure or control (the same handling but no light exposure) group. At 50 days of gestational age (GA), dams in the stress exposure group commenced a procedure developed by Kapoor and Matthews [20, 34] and Kapoor et al. [35] in which stress was induced by exposure to strobe light. Briefly, the animals were placed in a ventilated light-proof container, exposing the animals to a strobe light for 2 h (9–11 a.m.). The high-frequency strobe light intensity was 75 J/10 s. This protocol was repeated on GA 55, 60 and 65 (term 69 days). Dams in the control groups were treated in the same way with handling performed but no exposure to the strobe light. Saliva samples were collected from all of the dams by mastication on a cotton bud for approximately 1 min both immediately (within 30 s) before and after each of the strobe light exposure or the control events.

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

Allopregnanolone Treatment

Allopregnanolone was obtained from Dr. R.H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, Calif., USA) and administered subcutaneously in a 45% 2-hydroxypropyl-β-cyclodextrin vehicle solution (Sigma Aldrich, Castle Hill, NSW, Australia). Stress and control dams were randomly allocated to either receive allopregnanolone (10 mg/kg/day) or vehicle injections twice daily at 9 a.m. and 5 p.m. from GA 60 to GA 68.

Tissue Collection

Pregnant dams were euthanased at term (69 days GA) or on the second consecutive day of full pubic symphysis opening (>2 cm diameter), which is an established indicator of imminent labour in the guinea pig [36]. Dams were euthanased by inhalation of 100% CO_2. Maternal and fetal blood was collected immediately. Fetal placement, body weight, sex, nose-rump length as well as weights of organs were recorded, including the whole brain, placenta, heart, adrenal glands and liver. Fetal brains were dissected in a sagittal plane with one half snap-frozen at –80°C or and the other half fixed via immersion in a formalin solution [4% w/v paraformaldehyde in 0.1 M phosphate buffer (Na_2PO_4; $NaH_2PO_4H_2O$), Sigma Aldrich].

Immunohistochemistry

The fixed brain tissues were embedded in paraffin wax and processed for immunohistochemical staining and analysis using methods we have previously described [37]. Briefly, 8-μm brain sections were processed by a method involving dewaxing in xylene, rehydration in a series of ethanol/water washes and finally incubation in a hydrogen peroxide (H_2O_2) and methanol solution to inhibit endogenous peroxidase activity. Antigen retrieval was then performed by incubation in RevealIt Solution (ImmunoSolution Pty Ltd., Everton Park, Qld., Australia). Following blocking with bovine serum albumin in phosphate-buffered saline (0.1 M phosphate-buffered saline, pH 7.2 with 0.5% w/v bovine serum albumin, 0.05% w/v saponin and 0.05% v/v sodium azide), sections were incubated with primary antibodies for myelin basic protein (MBP; Sigma Aldrich), glial fibrillary acidic protein (GFAP; Sigma Aldrich) and microtubule-associated protein 2 (MAP2; Sigma Aldrich) overnight at concentrations of 1:4,000, 1:4,000 and 1:30,000, respectively. This was followed by incubation with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP and MAP2, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK). Subsequently slides were incubated in streptavidin-biotinylated horseradish peroxidase complex (RPN1051, Amersham). Finally, the slides were then incubated in 3,3′-diaminobenzidine concentrate with H_2O_2. Slides were fixed with coverslips. Slides were viewed with bright field microscopy on a Nikon Eclipse 90i microscope and images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). All immunoreactivities were analysed by densitometry using ImageJ version 1.46 (National Institutes of Health, Bethesda, Md., USA), made binary by adjusting the threshold manually, with the percentage area of coverage recorded for four fields of view per brain region on two sections per animal. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

Allopregnanolone Radioimmunoassay and Cortisol Enzyme Immunoassay

Allopregnanolone was extracted from fetal and maternal plasma as previously described [32]. Briefly, plasma was treated with 50% methanol with 1% acetic acid in Sep-Pak C_{18} cartridges (Waters, Milford, Mass., USA), vacuum-dried and then treated with potassium permanganate to reduce cross-reactivity of progesterone [38]. The addition of tritium-labelled allopregnanolone (1,000–1,500 cpm, 5a-[9,11,12,3H(N)]); PerkinElmer Life and Analytical Sciences, Boston, Mass., USA) allowed determination of sample recovery. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. Allopregnanolone was quantified by radioimmunoassay using a polyclonal antibody (supplied by Dr. R.H. Purdy, Department of Psychiatry, Veterans Administration Hospital, San Diego, Calif., USA); the assay and cross-reactivities of the antisera have previously been described [32]. The limit of detection for allopregnanolone was 35.0 + 2.5 pg/tube. The inter- and intra-assay coefficients of variation were 12.3 and 8.5%, respectively.

Cortisol concentrations were determined in maternal saliva obtained before and after each stress or control event using a salivary assay kit (Salimetrics Inc., State College, Pa., USA), as per manufacturer's instructions. Sensitivity of the assay was 0.012–3.0 μg/dl and inter- and intra-assay coefficients of variance were 6.89 and 5.52%, respectively.

Cortisol and progesterone were quantified in fetal and maternal plasma by immunoassay by Hunter Area Pathology Service. The assays were conducted on the UniCel Dxl800 Access Immunoassay System (Beckman Coulter Inc., Gladesville, NSW, Australia), as per manufacturer's instructions. The inter- and intra-assay coefficients of variance were 5.17 and 4.3%, respectively for cortisol, and 8.2 and 7.9%, respectively for progesterone.

Statistical Analysis

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

A two-way multiple analysis of variance test (ANOVA) was used to further characterise specific relationships within each sex

Table 1. Fetal physical characteristics

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

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. This value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted fetuses. No effect of drug treatment was found. Values are expressed as the mean percentage + SEM and are calculated for animal numbers.

Allo = Allopregnanolone; Cont = control; Veh = vehicle.

[a] Significant effect of stress: $p < 0.05$. [b] Significant effect of sex: $p < 0.05$.

cohort. This same test was also used to assess differences between maternal plasma allopregnanolone data. A repeated measures multiple analyses of variance test (RM-ANOVA) test was used to assess maternal repeated salivary cortisol sampling. All data analysis was performed using the SPSS statistical software package (version 19, SPSS Inc. IBM, Chicago, Ill., USA). In order to prevent pregnancy within litter association and bias, only 1 male and 1 female fetus was used from each pregnancy in the analysis. In a number of pregnancies, there was only 1 male or female fetus and therefore only 1 fetus was available to be used for analysis. All data are presented as mean + SEM with $p < 0.05$ considered significant.

Results

Effect of Prenatal Stress on Fetal Physiological Characteristics

Female fetuses showed significantly (ANOVA, $p < 0.05$, $F = 5.172$, d.f. = 1) larger brain weight-to-body weight ratios than their male counterparts, irrespective of stress exposure or drug treatment (table 1). Female fetuses exposed to prenatal stress were also the only group to show a significantly (ANOVA, $p < 0.05$, $F = 3.393$, d.f. = 1) reduced liver weight-to-body weight ratio with no significant changes observed in the male cohort or in the cohort of female fetuses exposed to allopregnanolone treatment. Brain-to-liver ratio (BLR) in the females, but not males, was significantly higher (ANOVA, $p < 0.05$, $F = 6.472$, d.f. = 1) in fetuses exposed to stress compared to those in control groups, indicating asymmetric growth and brain sparing. No significant effect of stress, drug treatment or sex was found on body weight or nose-rump length. There were also no significant effects of stress, drug treatment or sex on placental weight, heart weight or adrenal weight when adjusted for individual differences in body weight. It should also be noted that pregnancies exposed to PS showed a modest reduction in GA at the time of post-mortem, indicating a shorter gestational length (control 68.43 + 0.47 and stress 67.7 + 0.35; ANOVA, $p < 0.05$, $F = 4.286$, d.f. = 1). There was no significant effect of stress exposure or drug treatment on litter size or average litter weight, nor were there any effects on maternal weight gain during pregnancy (data not presented). This suggests that maternal weight gain was not responsible for any difference in fetal growth data.

Maternal Salivary Cortisol Concentrations

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

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

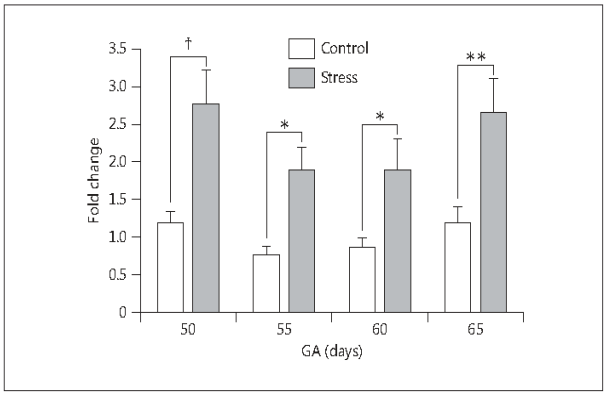

Fig. 1. Effect of strobe light-induced stress episodes on maternal salivary cortisol concentrations. Data are presented as fold change of salivary cortisol between salivary samples taken immediately before and after each stress (grey bars) or control (handling without stress exposure, open bars) episodes that were performed at 50, 55, 60 and 65 days GA. Stress-exposed (n = 14) and control (n = 14) groups contain both vehicle- and allopregnanolone-treated guinea pig dams. * $p = 0.05$, ** $p < 0.01$ and † $p < 0.001$ indicate significance level between changes seen between control and stress groups.

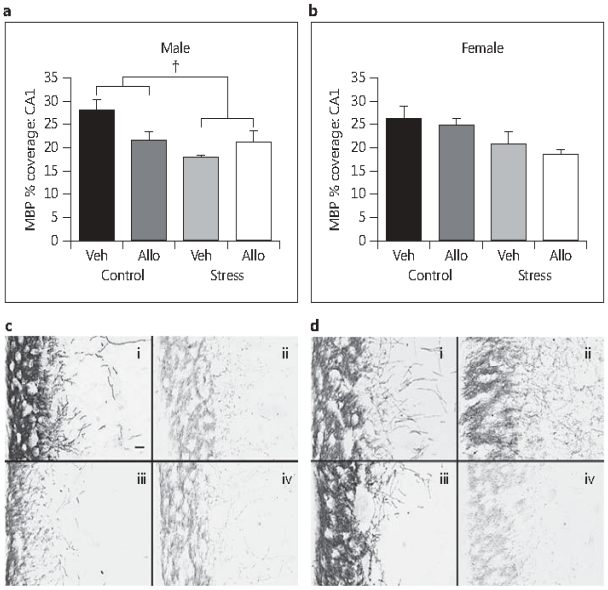

Fig. 2. a, b Effects of prenatal stress on MBP expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. **c, d** Representative images of MBP immunostaining for male and female guinea pig fetuses at term showing staining in a control + vehicle-treated (i), control + allopregnanolone-treated (ii), stress + vehicle-treated (iii) and stress + allopregnanolone-treated fetus (iv). MBP expression calculated coverage areas of immunohistochemical staining (see 'Methods') in male and female guinea pig fetuses at term. MBP staining coverage in: control + vehicle groups in black bars (male n = 7; female n = 5), control + allopregnanolone groups in dark grey bars (male n = 9; female n = 4), stress + vehicle groups in light grey bars (male n = 5; female n = 4) and stress + allopregnanolone groups in open bars (male n = 5; female n = 5). † $p < 0.001$ indicates significance level between control and stress groups of males fetuses. Allo = Allopregnanolone; Veh = vehicle. Scale bar = 50 µm.

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

MBP Expression and Allopregnanolone Response

Figure 2c, d shows representative micrographs of MBP immunostaining in the CA1 region of the hippocampus of prenatally stressed, control, vehicle- and allopregnanolone-treated fetuses. There were significantly lower levels of MBP expression in male prenatally stressed

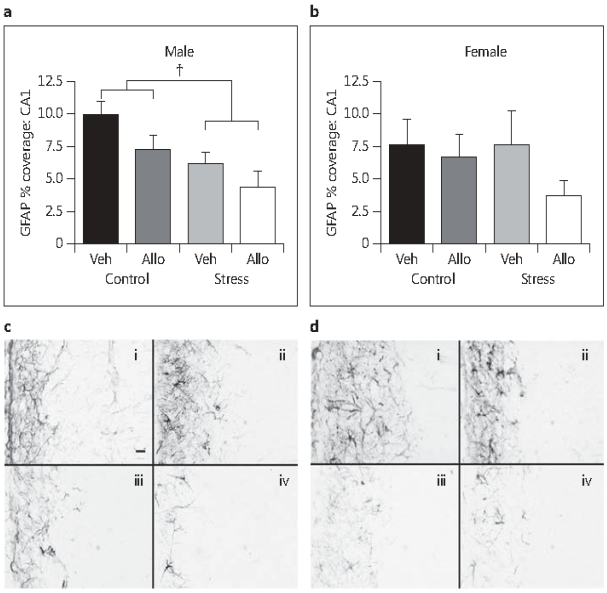

Fig. 3. a, b Effects of prenatal stress on GFAP expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. **c, d** Representative images of GFAP immunostaining for male and female guinea pig fetuses at term showing staining in a control + vehicle-treated (i), control + allopregnanolone-treated (ii), stress + vehicle-treated (iii) and stress + allopregnanolone-treated fetus (iv). GFAP expression calculated coverage areas of immunohistochemical staining (see 'Methods') in male and female guinea pig fetuses at term. GFAP staining coverage in: control + vehicle groups in black bars (male n = 7; female n = 5), control + allopregnanolone groups in dark grey bars (male n = 9; female n = 4), stress + vehicle groups in light grey bars (male n = 5; female n = 4) and stress + allopregnanolone groups in open bars (male n = 5; female n = 5). † $p < 0.001$ between control and stress groups of males fetuses. Allo = Allopregnanolone; Veh = vehicle. Scale bar = 50 μm.

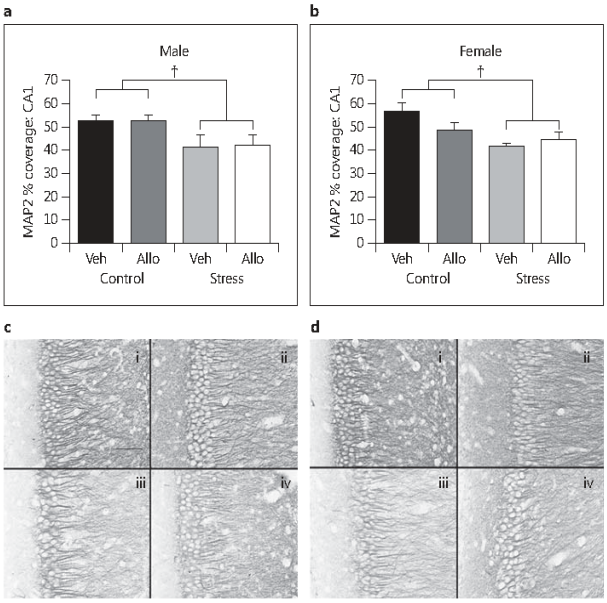

Fig. 4. a, b Effects of prenatal stress on MAP2 expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. **c, d** Representative images of MAP2 immunostaining for male and female guinea pig fetuses at term showing staining in a control + vehicle treated (i), control + allopregnanolone treated (ii), stress + vehicle treated (iii) and stress + allopregnanolone treated fetus (iv). MAP2 expression calculated coverage areas of immunohistochemical staining (see Methods) in male and female guinea pig fetuses at term. MAP2 staining coverage in: control + vehicle groups in black bars (male n = 7; female n = 5), control + allopregnanolone groups in dark grey bars (male n = 9; female n = 4), stress + vehicle groups in light grey bars (male n = 5; female n = 4) and stress + allopregnanolone groups in open bars (male n = 5; female n = 5). † $p < 0.001$ between control and stress groups in male and female fetuses. Allo = Allopregnanolone; Veh = vehicle. Scale bar = 50 μm.

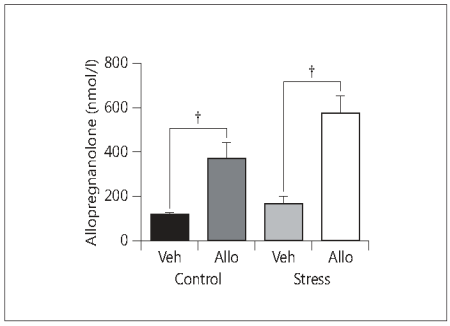

Fig. 5. Maternal plasma allopregnanolone concentrations in stress and control dams 12 h after last vehicle (control + vehicle group, black bars, n = 3; stress + vehicle, light grey bars, n = 4) or allopregnanolone administration (control + allopregnanolone, dark grey bars, n = 4; stress + allopregnanolone, open bars, n = 4). † $p < 0.001$ between vehicle and allopregnanolone treatment. Allo = Allopregnanolone; Veh = vehicle.

fetuses in the CA1 region of the hippocampus (ANOVA, $p < 0.001$, $F = 13.576$, d.f. = 1; fig. 2a, c) and in the cerebral cortical white matter (ANOVA, $p < 0.001$, $F = 14.840$, d.f. = 1; data not shown), compared to controls. This result was not seen in the female cohort (fig. 2b, d). A significant negative correlation was also found between maternal salivary cortisol concentrations at the time of postmortem and MBP expression in the CA1 region of the hippocampus when male and female groups were combined ($p = 0.02$; Spearman's $r = -0.53$) and between maternal plasma cortisol at the time of postmortem and MBP expression in the cerebral cortical white matter when male and female groups were combined ($p = <0.001$; Spearman's $r = -0.62$), indicating the relationship between increased cortisol exposure and reduced fetal brain myelination.

GFAP and Allopregnanolone Response

Representative micrographs of GFAP immunostaining in the hippocampus are shown in figure 3c, d. Analysis of immunostaining showed that there was a marked effect of stress in the male cohort with reduced expression of GFAP in the CA1 region of the hippocampus (ANOVA, $p < 0.001$, $F = 9.347$, d.f. = 1; fig. 3a, c) and the cerebral cortical white matter (ANOVA, $p < 0.05$, $F = 6.480$, d.f. = 1; data not shown), which was not seen in the female cohort (fig. 3b, d). Also within the male cohort, there was an interaction between stress and allopregnanolone treatment (ANOVA, $p < 0.05$, $F = 4.541$, d.f. = 1), indicating that the combination of stress and allopregnanolone treatment caused a differential effect on GFAP expression in the CA1 region, which was not seen in any of the other experimental groups.

MAP2 and Allopregnanolone Response

Figure 4c, d shows representative micrographs showing MAP2 immunostaining in the hippocampus of prenatally stressed, control, vehicle- and allopregnanolone-treated fetuses. Analysis showed the significance of stress on MAP2 expression in the CA1 region of the hippocampus revealing stress-reduced MAP2 expression in both male and female cohorts (males ANOVA, $p < 0.01$, $F = 6.443$, d.f. = 1; females ANOVA, $p < 0.001$, $F = 11.743$, d.f. = 1; fig. 4a, b, respectively). There was, however, no effect of allopregnanolone treatment on either males or females. There was, no effect of stress exposure or allopregnanolone treatment on MAP2 expression within the cerebral cortical white matter (data not shown).

Allopregnanolone Treatment and Fetal Plasma Concentrations

Maternal plasma allopregnanolone concentrations remained elevated 12 h after the last maternal allopregnanolone administration in both control and stressed pregnancies compared to vehicle-treated controls (RM-ANOVA, $p < 0.05$, $F = 4.859$, d.f. = 1; fig. 5). In control pregnancies, maternal allopregnanolone treatment resulted in marked fetal plasma allopregnanolone concentrations in both male and female fetuses (ANOVA, $p < 0.001$, $F = 14.598$, d.f. = 1; fig. 6a, b, respectively). In contrast, neither male nor female fetal allopregnanolone concentrations were elevated in pregnancies exposed to stress and allopregnanolone administration (open bars, fig. 6). This observation is consistent with the finding of a significant (ANOVA, $p < 0.05$, $F = 4.090$, d.f. = 1) interaction between stress and allopregnanolone treatment in fetal plasma, indicating that the fetal allopregnanolone levels in response to maternal treatment were altered by stress exposure.

There was no difference in plasma progesterone concentrations between any of the experimental groups in fetal plasma at the time of post-mortem (average fetal levels were 2,845.82 + 308.45 nmol/l). There was also no significant effect of stress exposure or allopregnanolone treatment on maternal plasma progesterone levels at the time of postmortem (average maternal levels were 10,442.83 + 1,936.95 nmol/l).

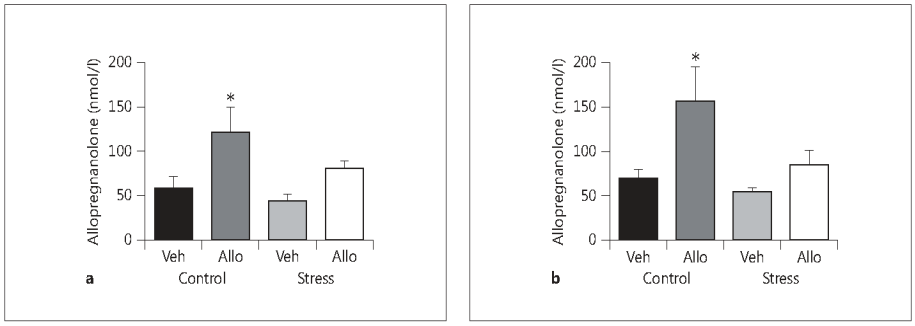

Fig. 6. Fetal plasma allopregnanolone concentrations in stress and control dams 12 h after last vehicle or allopregnanolone administration in male (**a**) and female (**b**) fetuses. Data shown are for: control + vehicle (black bars; males n = 5, females n = 5), control + allopregnanolone (dark grey bars; males n = 6, females n = 5), stress + vehicle (light grey bars; males n = 5, females n = 5) and stress + allopregnanolone treatment (open bars; males n = 6, females n = 6). * $p < 0.05$ difference between levels in the control + allopregnanolone group and all other groups. Allo = Allopregnanolone; Veh = vehicle.

Discussion

The major finding of this study was that prenatal maternal stress had profound, sexually dimorphic effects on the guinea pig fetus. In this study, female brain sparing is seemingly a neuroprotective growth adaptation which may have partially preserved brain growth and development. In contrast, male fetuses demonstrated reduced expression of markers for three major brain cell types (myelinating oligodendrocytes, reactive astrocytes and mature neurons) in both brain regions assessed (CA1 region of the hippocampus and cerebral cortical white matter). These observations suggest an increased vulnerability of males to the effects of prenatal stress on fetal brain growth and development. This study also highlights the differing effects of prenatal stress on each cell type and brain region during fetal neurodevelopment. These findings are consistent with clinical and experimental studies showing an inherent disadvantage of males to prenatal insults such as prenatal stress as well as the vulnerability of the hippocampus to damage [6, 39–41].

There is now increasing evidence for the developmental origins of neuropathologies, such that disturbances in processes such as myelination and neural migration during critical windows of brain development can predispose offspring to abnormalities in efficient synaptic transmission and neural connectivity at birth or later in life. Thus, decreases in MBP, GFAP and MAP2 expression in the male brain indicate decreased myelination, neurogenesis and stability of axons where a lack of sufficient repair processes may confer vulnerability and susceptibility to injury at birth or later in life. The present findings indicate oligodendrocyte maturation and myelination is reduced by late gestation stress. Reactive astrocytes have been shown to have important neuroprotective qualities [42] and are key to supporting development of the central nervous system [43], such that a reduction in these cells may have a role in the development of detrimental outcomes. The results of this study are consistent with previous studies, which have shown reductions in fetal myelination following brain sparing [44] in both the CA1 region of the hippocampus and the cerebral cortical white matter that are associated with increased incidences of postnatal behavioural pathologies [45]. We assessed white matter tracts within the cerebral cortex, where disturbances during development have been linked to neurodevelopmental damage in other forms of pregnancy compromise such as preterm birth and intrauterine growth restriction [46]. This cerebral cortical white matter deficiency is strongly associated (>90% prevalence) with cognitive, behavioural and attention deficits in children born preterm [46–49]. Previous studies have also found site-specific (CA1 region) disturbances in hippocampal development following exposure to prenatal stressors [50–52]. The CA1 region of the hippocampus contains a high concentration of important efferent projections and pyramidal cells involved in processes such as memory and learning, which are known to be affected by exposure to prenatal stress

[53–55]. Furthermore, this region of the hippocampus has also been shown to be selectively vulnerable to the deleterious effects of glucocorticoid exposure [56, 57]. This may be attributable in part, to the high levels of expression of glucocorticoid receptors in the CA1 region of the hippocampus [57]. In addition, neurons and glial cells within the CA1 region of the hippocampus express high concentration of $GABA_A$ receptors potentially leading to sensitivity to endogenous neurosteroid-dependent neuroprotection. The observation that allopregnanolone metabolism pathways were disrupted by prenatal stress may therefore further contribute to vulnerability.

Astrocytes, mature oligodendrocytes and neurons all express steroidogenic enzymes required for neurosteroid synthesis and stress-induced perturbations in the number of these cells could alter endogenous neurosteroid production and therefore contribute to disruption of central nervous system developmental processes [58]. Allopregnanolone has been shown to have potent inhibitory effects, modulating the $GABA_A$ receptor in the late gestation fetus supporting proper neurodevelopment. Thus, fetuses affected by prenatal stress may also be susceptible to damage due to perturbations in neurosteroidogenesis creating an additive environment for damage. These observations have implications for perinatal brain development and psychopathology later in life, particularly for affected male fetuses.

We have found a negative effect of stress on the expression of the mature neuronal marker MAP2 in both males and females suggesting that females may not be totally protected from maternal stress. This may in part be attributable to the vulnerability of the hippocampus to prenatal insults including prenatal stress [23] and that some of the first stress episodes, conducted at GA 50 and/or 55 (0.7 of gestation), may have damaged these neurons during their peak growth period, before an effective growth adaptation (brain sparing) was employed. Studies have shown the vulnerabilities of the hippocampus to stress-induced reductions in neurogenesis and resultant learning and memory, supporting the idea of a selective effect of stress on certain cell populations and regions within the fetal brain [22, 23, 59–61]. Male offspring have also shown reduced neurogenesis in the hippocampus following exposure to prenatal restraint stress [62] and experimentally induced hippocampal damage confers increased vulnerability to psychiatric disorders in adulthood [63]. These data suggest that different cell populations within the fetal brain respond differently to prenatal stress.

The model of transient stress used in this study was previously developed by Kapoor and Matthews [20, 34] and was used to evaluate the effect of prenatal stress on fetal hypothalamo-pituitary adrenal axis development. The maternal salivary cortisol levels observed in this study following prenatal stress exposure were similar to those previously reported [34]. Maternal plasma cortisol concentrations at the time of post-mortem were positively correlated to maternal salivary cortisol concentrations at this time, suggesting the reliability of this measurement of cortisol whilst minimising the stress of venepuncture which would be difficult to quantify [64, 65]. Furthermore, we have found a significant correlation between maternal cortisol levels and fetal BLR indicating that the higher the maternal cortisol level, the greater the effect on the fetus. The findings of the present study are consistent with the programming effect proposed by Glover and Hill [66]. These investigators suggested that in compromised pregnancies, it may be more advantageous for female fetuses to alter their physical growth in order to maintain optimal brain development, whereas males may grow larger in size to the detriment of their neurodevelopment [66].

This study is the first to demonstrate potential disruption of placental transfer or maternal or fetal metabolism of neurosteroids in pregnancies complicated by prenatal stress. The present observations show that whilst maternal administration of allopregnanolone raised fetal circulating allopregnanolone concentrations in normal pregnancies, this was not achieved in both male and female fetuses of stressed pregnancies. This indicates the marked effects of prenatal stress on the neurosteroid environment. The mechanisms leading to the stress-induced suppression of allopregnanolone levels are unclear. Stress may affect maternal metabolism thereby reducing the effectiveness of exogenous allopregnanolone administration. Alternatively, placental metabolism of allopregnanolone may be increased to result in diminished levels present in the fetal circulation. It is also possible that prenatal stress reduced placental efficiency and therefore reduced the capacity for allopregnanolone to cross the placenta. Investigation of placental function is warranted to further address the mechanisms influencing neurosteroid production and metabolism pathways in the placenta. Regardless of the mechanism involved, the absence of a fetal response to exogenous allopregnanolone treatment suggests that the adverse effects of prenatal stress on the fetal brain may be caused by a loss of neurosteroid responses that are normally both trophic and neuroprotective [67]. A stress-induced reduction in allopregnanolone levels could result in exacerbation of brain injury that exceeds normal regenerative processes

in the fetal brain, thus resulting in psychopathologies postnatally. A chronic loss of allopregnanolone induced by stress could also directly result in disorders involving excess neural excitation such as increased vulnerability to seizures.

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

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Prenatal Stress Alters Hippocampal Neuroglia and Increases Anxiety in Childhood

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Key Words
Prenatal stress · Neurodevelopment · Behaviour · Guinea pigs · Neurosteroids

Abstract
Prenatal stress has been associated with detrimental outcomes of pregnancy, including altered brain development leading to behavioural pathologies. The neurosteroid allopregnanolone has been implicated in mediating some of these adverse outcomes following prenatal stress due to its potent inhibitory and anxiolytic effects on the brain. The aims of the current study were to characterise key markers for brain development as well as behavioural parameters, adrenocortical responses to handling and possible neurosteroid influences towards outcomes in guinea pig offspring in childhood. Pregnant guinea pig dams were exposed to strobe light for 2 h (9–11 a.m.) on gestational days 50, 55, 60, and 65 and were left to deliver spontaneously at term and care for their litter. Behavioural testing (open-field test, object exploration test) of the offspring was performed at postnatal day 18 (with salivary cortisol and DHEA measured), and brains were collected at post-mortem on day 21. Markers of brain development myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) were assessed via immunohistochemistry, and the neurosteroid allopregnanolone and its rate-limiting enzymes 5α-reductase types 1 and 2 (5αR1/2) were measured in neonatal brains by radioimmunoassay, reverse transcriptase polymerase chain reaction (RT-PCR), and Western blot, respectively. Brain-derived neurotrophic factor protein was measured as a marker of synaptic plasticity, and $GABA_A$ receptor subunit expression was also assessed using RT-PCR. Neonates born from mothers stressed during late pregnancy showed a reduction in both MBP ($p < 0.01$) and GFAP ($p < 0.05$) expression in the CA1 region of the hippocampus at 21 days of age. Pups of prenatally stressed pregnancies also showed higher levels of anxiety and neophobic behaviours at the equivalent of childhood ($p < 0.05$). There were no significant changes observed in allopregnanolone levels, 5αR1/2 expression, or $GABA_A$ receptor subunit expression in prenatally stressed neonates compared to controls. This study shows alterations in markers of myelination and reactive astrocytes in the hippocampus of offspring exposed to prenatal stress. These changes are also observed in offspring that show increased anxiety behaviours at the equivalent of childhood, which indicates ongoing structural and functional postnatal changes after prenatal stress exposure.

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Introduction

Maternal experiences during pregnancy can have profound and long-lasting effects on the development of offspring. Indeed, many diseases and pathologies which manifest in later life are now understood to have a prenatal origin [1]. It has been proposed that prenatal maternal stress, particularly if experienced when the developing brain is susceptible to being perturbed, can disrupt normal brain development and thus result in neurobehavioural pathologies that appear later in life [2].

The normal response to stress has the effect of maintaining physiological homeostasis and this usually results in adrenal activation and the release of cortisol. However, repeated or chronic increases in stress, and hence chronically increased plasma cortisol, can entrain responses that have pathological and illness-inducing consequences for adults, including during pregnancy [3]. Indeed, high levels of self-reported maternal stress and salivary cortisol concentrations in pregnant women predict poorer mental and motor development of the neonate and contribute to the determination of infant temperament [4, 5], including an increased risk for the development of attention deficit disorders in childhood [6], schizophrenia [7] and depression in offspring [8]. While these studies show that prenatal stress can have effects on the fetus that persist after the end of pregnancy, the link between prenatal exposure to stress and altered postnatal behaviour is not fully understood. In rats, late gestational prenatal stress has been associated with altered reactive astrocyte expression and reduced synaptic density in the frontal cortex, striatum and hippocampus of adult offspring [9], as well as stress-induced, intensity-dependent alteration of hippocampal growth [10]. Furthermore, prenatal stress was shown to be associated with reduced hippocampal levels of the important marker for neuroplasticity, brain-derived neurotrophic factor (BDNF), as well as social behavioural alterations in adult rat offspring [11], indicating that prenatal stress may act through modifying hippocampal development as a mechanism of programming behaviour in later life. We have previously shown that late gestational prenatal stress in the pregnant guinea pig, a rodent with a relatively long gestation producing mature offspring at birth, results in sexually dimorphic reductions in markers for mature oligodendrocytes, reactive astrocytes and mature neurons, with male fetuses showing profound neurodevelopmental deficits, particularly in the hippocampus [12]. This study also showed a reduction in fetal circulating levels of the neuroactive steroid allopregnanolone following maternal neurosteroid administration in prenatal stress-exposed pregnancies [12].

Neuroactive steroids are steroids that act in the brain with important neuromodulatory actions [13]. The brain possesses the enzymes required for neurosteroid synthesis in astrocytes and oligodendrocytes [14, 15], indicating that it may be able to produce neurosteroids de novo [16]. In pregnancy a considerable source of neuroactive steroids is the placenta [17]. Allopregnanolone, derived from the 5α 3α-reduction of progesterone, is perhaps the most important of these neuromodulators due to its action as a co-agonist with GABA at the $GABA_A$ receptor. $GABA_A$ receptors exist in a hetero-pentameric structure made up of a combination of subunits [18]. The precise combination of subunits, which changes during development and is also influenced by stress, determines the binding affinity of agonists, including allopregnanolone [19]. Social isolation as a stressor has been associated with reductions in tissue and plasma allopregnanolone and the expression of the rate-limiting enzyme in allopregnanolone production, 5α-reductase, indicating that stressful events may have a direct effect on neurosteroidogenesis [20, 21]. Altered neurosteroid levels are also linked with various anxiety-like behaviours in both rodent models and humans [22–24], implicating this neurosteroid pathway in mediating some of the programming effects of prenatal stress exposure.

In the current study, we assessed the effect of prenatal stress on sustained changes in key hippocampal neurodevelopmental markers in guinea pig offspring at 21 days of age (equivalent to childhood in humans), together with its effects on postnatal growth and behaviour. Importantly, we assessed the effects of prenatal stress on neurosteroidogenesis and $GABA_A$ receptor subunit expression in the postnatal brain to determine whether changes in this neuroprotective neurosteroid system are associated with altered brain development and/or behavioural patterns as well as prior prenatal stress exposure.

Methods

Animal Stress Protocol

The University of Newcastle supplied time-mated, outbred tricolour guinea pigs where all procedures were approved under the University of Newcastle Animal Care and Ethics Committee and undertaken in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. We used an established model of prenatal stress [12, 25, 26], in which pregnant dams were randomly assigned to either a stress (strobe light exposure; n = 12) or control (no strobe light exposure; n = 12) group. Briefly, dams allocated to the stress protocol were exposed

to a strobe light for 2 h (9–11 a.m.) on gestational days 50, 55, 60, and 65 (term is ~71 days). The strobe light exposure events were conducted within the normal animal facility and after saliva sampling. The dams were returned to their normal housing cages and placed in a darkened box (dimensions: 1 m wide × 1 m long × 0.7 m tall) for strobe light exposure whereas the control animals were returned in their housing cages to their normal room. This stress container was appropriately ventilated and maintained at a similar temperature to the surrounding housing areas. The strobe light box was cleaned after every use to ensure no adverse reaction of the dams to residual smells within the box. Maternal saliva was collected by allowing the animals to chew on a cotton bud for approximately 30 s immediately before and after the stress protocol. Control animals were handled in the same manner as the stress-exposed dams, including saliva collection, but remained in their normal cage housing during the 2-hour test period until the second saliva sampling. Maternal saliva was analysed by methods previously described [12] to confirm a rise in cortisol concentrations following strobe light exposure, which was previously shown to markedly increase after each exposure [12], ensuring the effectiveness of the stress protocol.

Neonatal Protocol

All dams were allowed to deliver spontaneously and on postnatal days (PND) 1 and 21 the pups were weighed, measured for nose-rump length, head length, head circumference, and abdominal circumference. Again, saliva was collected by allowing the animals to chew on a cotton bud for approximately 30 s. All procedures were carried out within visual and auditory contact of both the dam and the remainder of the litter to minimise any stress to the neonate during handling [27]. Dams were kept with their litters throughout the experiment. Data on physical growth parameters was analysed for all offspring used in the analysis in this study (control n = 36: 20 male, 16 female; prenatal stress n = 37: 17 male, 20 female).

Behavioural Testing

On PND 18, the behaviour of offspring (control n = 23: 12 male, 11 female; prenatal stress n = 24: 12 male, 12 female) was assessed using an open-field test and an object exploration test. To achieve this, the neonates were placed in the open-field arena (40 × 40 × 30 cm) for 10 min and their behaviour was recorded using the Stoelting ANY-Maze video tracking and analysis software (Stoelting Co., Wood Dale, Ill., USA). Immediately following this open-field test, the neonates were subjected to an object exploration test for a further 10 min. This object exploration test involved placing two identical objects at fixed locations within the arena and allowing the animal to explore these objects for the full duration of the test. Using ANY-Maze tracking software (Stoelting Co.), the position of the animal's head (object exploration test) and also the whole body (open-field test) were used to determine the distance travelled and time spent in different zones within the arena. The outcome measures from the open-field test include total distance travelled (locomotor activity), time spent and crossings into the inner zone of the arena (inner 9 squares of the field divided into 49 equal squares; anxious behaviour). The object exploration test was used to determine the time spent exploring either object (object + 10% of object diameter as border for total zone) to further determine anxiety and neophobic behaviours as a function of the motivation to explore novelty in the arena or avoid the perceived danger of unknown objects [28]. Saliva was collected by methods previously described immediately before and after the complete testing period. The arena was thoroughly cleaned between each test to ensure the removal of residual olfactory stimuli within the arena.

Tissue Collection

Dams and neonates were euthanised at PND 21 via inhalation of 100% CO_2. Neonatal body weight, crown-rump length, head length, head circumference, and abdominal circumference measurements were recorded at the time of post-mortem, as well as weights of organs, including the whole brain, heart, adrenal glands, and liver. Brains were dissected in a sagittal plane with one half fixed via immersion in a 10% formalin neutral-buffered solution (HT501128; Sigma Aldrich, Castle Hill, N.S.W., Australia) and the other half either further dissected to remove the hippocampus, which was then weighed and snap frozen at –80 °C, or the whole hemisphere was immediately snap frozen still containing the hippocampus. Fixed brains were used for immunohistochemical staining whilst frozen hippocampal samples were used for real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting, with the frozen whole brains used for radioimmunoassay. The animal numbers for each technique are listed in parentheses.

Immunohistochemistry

The fixed brain tissues (control n = 16: 8 male, 8 female; prenatal stress n = 17: 9 male, 8 female) were embedded in paraffin wax and processed for immunohistochemical staining and analysis in methods previously described [12, 29]. Briefly, two 8-μm coronal brain sections per animal underwent a series of chemical washes for dewaxing, rehydration and, finally, inhibition of endogenous peroxidase activity. RevealIt Solution (ImmunoSolution Pty Ltd., Everton Park, Qld., Australia) was used for antigen retrieval followed by blocking with BSA in PBS (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide). The sections were then incubated with primary antibodies for myelin basic protein (MBP; Sigma Aldrich) and glial fibrillary acidic protein (GFAP; Sigma Aldrich) overnight at a concentration of 1:4,000. On the following day, the sections were incubated with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP anti-mouse IgG biotinylated, Amersham, GE Healthcare, Chalfont St Giles, UK) for 2 h. Subsequently, the slides were then incubated in streptavidin-biotinylated HRP complex (RPN1051; Amersham) for 3 h and finally incubated in 3,3′-diaminobenzidine (DAB) concentrate with H_2O_2. Coverslips were fixed and the slides were viewed using bright-field microscopy on a Nikon Eclipse 90i microscope; images were captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). Immunoreactivity was analysed by densitometry using ImageJ version 1.47v (National Institutes of Health, Bethesda, Md., USA) with expression presented as area coverage percentage. The brain regions analysed in the current study include the white matter area of the dorsal CA1 region of the hippocampus as well as the adjacent subcortical white matter [30]. These two regions were imaged using four fields of view on two serial sections per animal (thus, eight fields of view for each animal, each region). Controls for specificity of primary antibodies were run using the appropriate IgG substitute for each primary antibody.

Table 1. Guinea pig-specific primer sequences for quantitative real-time RT-PCR

Gene of interest	Forward primer sequence	Reverse primer sequence	Concentration, nM
5α-reductase type 1	CGA GGA GGG AAG CCA ACA	TAA CCA CAA GGC ACA ACC AGC	400
5α-reductase type 2	TCA GAA AGC CTG GAG AAG TCA TC	CCG AGG AAA CAA AGC GTG AA	800
$GABA_A$ R subunit α5	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA	400
$GABA_A$ R subunit δ	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC	400
β-Actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT	400

Primer sequences designed for guinea pig 5α-reductase types 1 and 2, $GABA_A$ receptor α5 and δ subunits and β-actin gene expression. Primer sequences are displayed from 5′ to 3′ for forward and reverse primer.

Western Blotting

The key neurosteroidogenic enzymes, 5α-reductase types 1 and 2 (5αR-1/2) as well as BDNF were all quantified at a protein level using Western blotting by methods previously described [29].

Briefly, 20 mg of brain tissue was homogenised in RIPA protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with complete protease inhibitor cocktail (Roche Diagnostics Australia Pty Ltd.) and PhosStop phosphatase inhibitor cocktail protein (Roche Diagnostics) with protein concentrations determined using a BCA protein assay kit (Pierce, Rockford, Ill., USA). Subsequently, 70 μg of total protein was separated on a precast NuPAGE Novex 12% Bis/Tris gel (Invitrogen, Carlsbad, Calif., USA) and transferred to a Hybond-P PVDF membrane (GE Healthcare, Sydney, N.S.W., Australia). Membranes were blocked using a BSA blocking solution (5% w/v BSA, 5% skim milk in 1× TBST-T – 25 mM Tris-HCl, 12 mM NaCl, 0.1% v/v Tween-20) and incubated in primary antibody overnight against 5αR1/2 (goat polyclonal antibody to 5αR1 NB100-491; Novus Biologicals, Littleton, Colo., USA; goat polyclonal antibody to 5αR2 Ab27469; Abcam, Cambridge, UK) and BDNF (goat polyclonal antibody to BDNF, P-14, sc-33905; Santa Cruz Biotechnology Inc., Dallas, Tex., USA). Immunoreactive protein was detected using an ECL Western blotting detection kit (GE Healthcare) and the LAS-4000 imaging system (Fuji Photo Film, Tokyo, Japan) following incubation with secondary antibody (anti-goat HRP P0449; DakoCytomation, Glostrup, Denmark). The relative protein levels (5αR1 ~26 kDa; 5αR2 ~29 kDa and BDNF ~32 kDa) were determined using MultiGauge v2.4 software (Fuji Photo Film) against β-actin loading control (ab8227-50; Abcam) and an internal loading control (guinea pig adrenal protein) on each gel. Blocking peptides specific to each primary antibody were used as control membranes in order to confirm the specificity of each respective antibody (5αR1 NB100-1491PEP, Novus Biologicals; 5αR2 ab45681, Abcam; BDNF sc-33905 P, Santa Cruz Biotechnology Inc.). 5αR1/2 and BDNF proteins were quantified in control (n = 8: 4 male, 4 female) and prenatally stressed offspring (n = 8: 4 male, 4 female).

Quantitative Real-Time RT-PCR

Hippocampal homogenates (control n = 15: 7 male, 8 female; prenatal stress n = 16: 9 male, 7 female) dissected from the sagittal frozen brain were extracted for RNA using an RNeasy miniprep kit (Qiagen, Clifton Hill, Vic., Australia) using methods previously described [31] and according to the manufacturer's instructions. The quantity and quality of the RNA was determined using the ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Del., USA) and also by agarose gel electrophoresis. A total amount of RNA (1–3 μg) was reverse transcribed to cDNA using SuperScript III first-strand synthesis system (Invitrogen). Primer sequences and concentrations were selected and sourced from Life Technologies (Mulgrave, Vic., Australia) according to the mRNA sequence available on the guinea pig and are shown in table 1. Real-time quantitative RT-PCR was performed as previously described for $GABA_A$ receptor subunits α5 and δ- and β-actin [32] and 5αR1 and 5αR2 [31]. The abundance of the target sequences was determined relative to β-actin mRNA using the ΔΔCT method with a guinea pig whole brain homogenate as the calibrator sample. There were no differences between the β-actin mRNA levels of control and prenatally stressed neonates (data not shown).

Allopregnanolone Radioimmunoassay

Allopregnanolone was determined by radioimmunoassay by methods previously described [12, 31]. Briefly, 400 mg of whole brain tissue (control n = 16: 8 male, 8 female; prenatal stress n = 16: 8 male, 8 female) was homogenised with 50% methanol and 1% acetic acid, respectively, and after centrifugation the supernatants were applied to Sep-Pak C_{18} cartridges (Waters, Milford, Mass., USA). Tritium-labelled allopregnanolone (1,000–5,000 cpm, 5a-[9,11,12,^{3}H(N)]; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) was used to calculate individual sample recovery concentrations. The average recovery of allopregnanolone was 75.5 ± 1.6% from brain homogenate with recovery values used in the final calculation of allopregnanolone tissue concentration to adjust for extraction losses. Samples were then incubated with allopregnanolone antibody (AS04041; Agrisera AB, Vannas, Sweden) and radioactivity measured using β-counter (LS6500; Beckman Coulter Australia Pty Ltd., Sydney, N.S.W., Australia). The limit of detection was 25 pg/ml and inter- and intra-assay coefficients of variation were 9.39 and 2.75%, respectively.

Cortisol and DHEA Enzyme-Linked Immunoassay

Maternal salivary cortisol (control n = 12; prenatal stress n = 12) and neonatal salivary cortisol (control n = 16: 8 male, 8 female; prenatal stress n = 17: 8 male, 9 female) and DHEA levels were de-

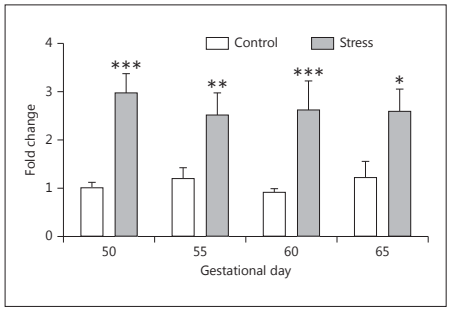

Fig. 1. Maternal salivary cortisol concentrations in prenatal stress-exposed dams (hatched bars n = 12) and control dams (open bars n = 12). Data are expressed as total fold change in concentrations before and after the prenatal stress (or control) event on gestational days 50, 55, 60, and 65 (term 71 days). * p = 0.05, ** p < 0.01, *** p < 0.001: indicate significance level between control and prenatal stress-exposed guinea pig dams.

termined using a Salimetrics cortisol immunoassay kit and a Salimetrics DHEA immunoassay kit, respectively (Salimetrics Inc., State College, Pa., USA) according to the manufacturer's instructions. Sensitivity of the cortisol assay was 0.012–3.0 μg/dl and inter- and intra-assay coefficients of variation were 12.04 and 5.52%, respectively. Sensitivity of the DHEA assay was 10.2–1,000 pg/ml and inter- and intra-assay coefficients of variation were 2.89 and 2.16%, respectively.

Statistical Analysis

For all of the offspring outcome measures, a linear mixed model was used to characterise differences between prenatally stressed and control pups whilst adjusting for the sex of the pups and any correlation between pups from the same dams. In this way, stress exposure (group) and sex were used as independent fixed factors in the linear mixed model; where more than 1 offspring was used from the same pregnancy, this was built into the model as a random factor (familial similarity) to account for any potential correlations between littermates and thus weight the analysis accordingly.

Repeated salivary cortisol concentrations were analysed using a repeated-measures linear mixed model, which again adjusted for the sex of the pups and any correlation between pups from the same dams. Maternal salivary cortisol was analysed using a repeated-measures two-way analysis of variance (ANOVA). All offspring data are expressed as β-coefficient values and 95% confidence intervals (CIs); $p < 0.05$ was considered significant. Unless otherwise specified, all data shown graphically are expressed as means + SEM. Statistical analysis was performed using SPSS software (version 21; SPSS Inc., IBM, Chicago, Ill., USA) and graphs were made using GraphPad Prism Software (version 6; GraphPad Software Inc., La Jolla, Calif., USA).

Results

Maternal Cortisol Responses following Strobe Light Exposure

Maternal salivary cortisol is presented as the fold change in salivary cortisol concentrations from samples collected immediately before and after the stress exposure or control-handling event (fig. 1). Dams that were exposed to prenatal stress in the form of strobe light exposure (n = 12) demonstrated significantly increased salivary cortisol levels compared to controls (n = 12) and thus showed a higher fold change in their cortisol concentrations (fig. 1). This significant rise in cortisol concentrations was observed after all exposure time points (gestational day 50, $p < 0.0001$; gestational day 55, $p < 0.01$; gestational day 60, $p < 0.0001$; gestational day 65, $p < 0.05$; fig. 1).

Effect of Prenatal Stress Exposure on Neonatal Growth Parameters

The offspring of both sexes of prenatally stressed guinea pigs displayed significantly increased head circumference (β = 0.70, 95% CI = –1.21 to –0.20, p = 0.007; fig. 2a), abdominal circumference (β = 1.01, 95% CI = –1.81 to –0.20, p = 0.01; fig. 2b) and greater nose-rump length (β = 0.94, 95% CI = –1.87 to 0.001, p = 0.04; table 2) on PND 21, despite no difference in body weight from birth to 21 days of age, compared to control (non-stressed group). Of note, it is normal for the nose-rump length of females to be smaller than males and this difference was observed in both treatment groups (β = –0.96, 95% CI = 0.05–1.86, p = 0.03). At PND 21, kidney weight was significantly reduced in both male and female offspring after the prenatal stress treatment (β = –0.06, 95% CI = 0.01–0.11, p = 0.01; table 2), as was the adrenal-to-body weight ratio (β = –0.08, 95% CI = 0.01–0.15, p = 0.02; table 2). Gestation length was not affected by the stress paradigm (control = 69.71 ± 0.27; stress = 70.46 ± 0.40), so the changes noted above were not due to preterm birth. Also, average birth weights were not different (control = 83.28 ± 3.20 g; stress = 89.34 ± 2.26 g). In addition, there was no significant overall or sex-related effect of stress exposure on heart weight, brain weight, liver weight, brain-to-liver weight ratio, or hippocampal weight at the time of postmortem at 21 days of age.

Effect of Prenatal Stress Exposure on MBP Expression

Following prenatal stress exposure, offspring at 21 days of age showed a markedly reduced level of MBP expression in the CA1 region of the hippocampus in both

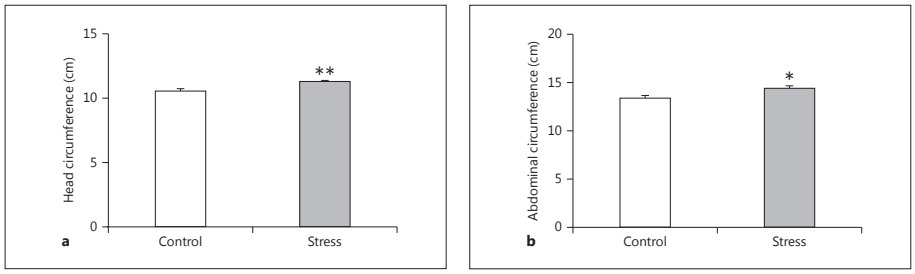

Fig. 2. Head circumference (**a**) and abdominal circumference (**b**) of offspring measured on PND 21 (childhood). Treatment groups: prenatal control guinea pigs (n = 33: 18 male, 15 female); prenatal stress-exposed guinea pigs (n = 36: 16 male, 20 female). * $p < 0.05$, ** $p < 0.01$.

Table 2. Neonatal physical growth parameters at 21 days

Treatment group	BW	Brain to BW	Nose-rump length	Heart to BW	Liver to BW	Kidney to BW	Adrenal gland to BW	Hippo-campus to BW	Brain-to-liver ratio	Birth weight
Male										
control (n = 20)	224.3+10.9	1.3+0.06	21.2+0.4	0.44+0.02	4.06+0.10	1.12+0.03	0.04+0.003	0.09+0.007	0.33+0.02	82.6+4.0
stress (n = 17)	230.7+8.6	1.2+0.04	22.8+0.4[a]	0.43+0.02	4.24+0.18	1.09+0.02[a]	0.04+0.002	0.10+0.009	0.31+0.02	87.7+6.4
Female										
control (n = 16)	213.6+9.5	1.4+0.10	20.8+0.4[b]	0.48+0.05	4.32+0.25	1.20+0.07	0.05+0.003	0.11+0.006	0.33+0.01	86.6+6.8
stress (n = 20)	220.0+7.6	1.3+0.04	21.2+0.4[a, b]	0.41+0.01	3.78+0.07	1.09+0.02[a]	0.04+0.001	0.09+0.018	0.35+0.01	90.7+6.6

Values are expressed as the mean percentage + SEM and are calculated for animal numbers shown in parentheses. All values are represented as a percentage of body weight (BW) at the time of post-mortem with the exception of nose-rump length, which is expressed in centimetres, birth weight which is expressed in grams and brain-to-liver weight ratio, which is a ratio value. This value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted offspring.

[a] $p < 0.05$: significant effect of stress; [b] $p < 0.05$: significant effect of sex.

males and females (β = –6.23, 95% CI = 1.32–11.13, p = 0.015; fig. 3) compared to the control group. There was no effect of prenatal stress exposure on MBP expression in the subcortical white matter, nor was there an effect of the sex of the offspring on MBP expression (area coverage) in this region (male control = 51.4 ± 3.3%, male stress = 44.6 ± 3.1%; female control = 44.7 ± 3.7%, female stress = 42.1 ± 3.7%).

Effect of Prenatal Stress Exposure on GFAP Expression

At 21 days of age, the pups exposed to prenatal stress showed significantly reduced expression of GFAP in the CA1 region of the hippocampus compared to pups from control pregnancies (β = –3.52, 95% CI = 0.07–6.96, p = 0.04; fig. 4). This effect was not present in the subcortical white matter of the cortex, immediately adjacent to the CA1 region of the hippocampus however, nor did the sex of the offspring affect MBP expression (area coverage) in this region (male control = 17.1 ± 1.9%, male stress = 13.1 ± 2.2%; female control = 16.7 ± 1.9%, female stress = 15.5 ± 2.0%).

Neurosteroidogenic Capacity of Neonates following Prenatal Stress

5αR1/2 mRNA (fig. 5a, b) and protein in the hippocampus (fig. 5c, d) were not affected by prenatal stress exposure at 21 days of age. There were no differences in

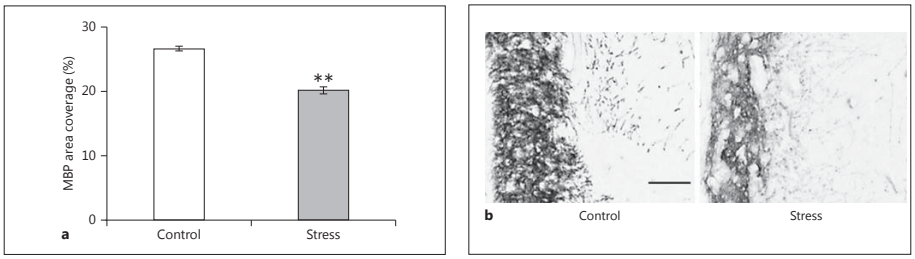

Fig. 3. a Effects of prenatal stress on MBP expression in the CA1 region of the hippocampus in pups at 21 days of age (control n = 16: 8 male, 8 female; prenatal stress = 17: 9 male, 8 female). **b** Representative images of MBP immunostaining for control and prenatally stressed guinea pig offspring, respectively. MBP expression calculated as coverage areas of immunohistochemical staining (see Methods). ** p = 0.01. Scale bar = 100 μm.

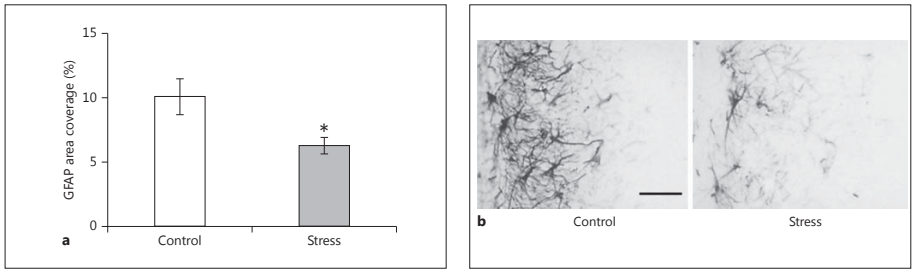

Fig. 4. a Effects of prenatal stress on GFAP expression in the CA1 region of the hippocampus in pups at 21 days of age (control n = 16: 8 male, 8 female; prenatal stress n = 17: 9 male, 8 female). **b** Representative images of MBP immunostaining for control and prenatally stressed guinea pig offspring, respectively. MBP expression calculated as coverage areas of immunohistochemical staining (see Methods). * p = 0.05. Scale bar = 100 μm.

the amount of allopregnanolone extracted from brain homogenates of either sex – or of either treatment group – at 21 days of age (fig. 6).

BDNF Protein Expression in Guinea Pig Hippocampus

Relative expression of BDNF in the hippocampus of offspring exposed to prenatal stress did not differ from that of unaffected controls, in either sex (fig. 7).

$GABA_A$ Receptor Subunit Expression in Guinea Pig Offspring

There was no significant effect of prenatal stress exposure on $GABA_A$ receptor subunit α5 or δ relative mRNA expression in the hippocampus of either sex (fig. 8a, b).

Effect of Prenatal Stress on Behaviour

At 18 days of age, pups from prenatal control and prenatally stressed pregnancies were tested in an open field to determine their innate activity levels, preference for inner/outer zones of the field (fig. 9a) and their propensity to explore objects placed in the field (fig. 9b). In the open-field test, both male and female pups born to a prenatally stressed pregnancy spent significantly less time in the inner zone of the area (β = –32.68, 95% CI = 1.09–64.27, p = 0.04; fig. 9a) compared to controls. Prenatally stressed offspring of both sexes also spent less time exploring the objects (β = –21.97, 95% CI = 1.83–42.10, p = 0.03; fig. 9b) compared to their control counterparts. There were no significant differences between males and

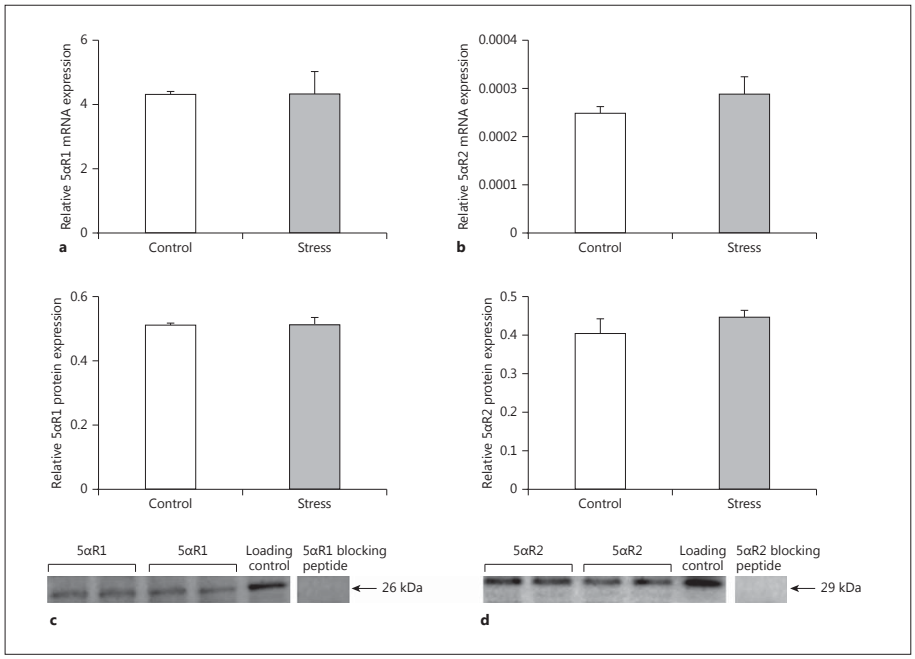

Fig. 5. Relative mRNA and protein expression of 5α-reductase types 1 (**a**, **c**) and 2 (**b**, **d**) in the hippocampus of 21-day-old pups. Prenatal control guinea pigs (n = 15: 7 male, 8 female); prenatal stress-exposed neonates (n = 16: 9 male, 7 female). Representative Western blots show 2 paired guinea pig hippocampal tissue samples and guinea pig adrenal gland internal control (70 μg total protein per lane). Pre-incubation of primary 5αR1 and 5αR2 antibody with respective blocking peptides blocked specific binding at 26 and 29 kDa, respectively, in guinea pig hippocampal sample.

females in any parameter measured during behavioural testing.

Neonatal Salivary Cortisol and DHEA Levels

Serving as an indirect measure of the neonatal stress response, the salivary cortisol concentrations measured immediately before and after behavioural testing did not show a significant difference between prenatally stressed and control animals, despite most neonates exhibiting a modest rise in cortisol following the behavioural testing irrespective of prenatal treatment group (fig. 10a). There was also no effect of sex on the levels of salivary cortisol measured at the time of behavioural testing. There was no effect of prenatal stress exposure or sex on salivary DHEA concentrations either before or after behavioural testing (fig. 9b), nor was there any change in the cortisol/DHEA ratio in offspring (fig. 10c).

Discussion

This study has demonstrated a persistent effect of prenatal stress exposure on the brain development and behavioural outcomes of offspring in postnatal life. We have previously shown a detrimental effect of prenatal stress exposure on fetal brain development [12] and the current

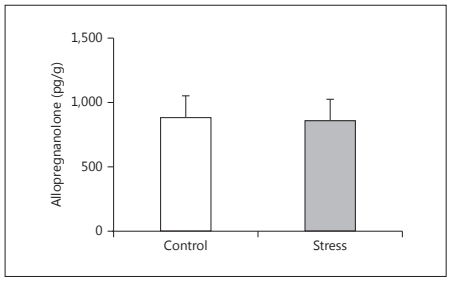

Fig. 6. Allopregnanolone levels as measured by radioimmunoassay in brain homogenate collected at 21 days of age. Prenatal control guinea pigs (n = 16: 8 male, 8 female); prenatal stress-exposed offspring (n = 16: 8 male, 8 female).

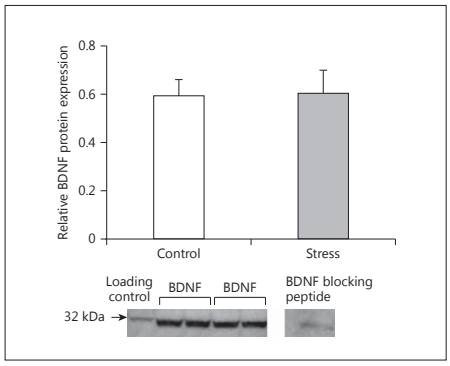

Fig. 7. Relative protein expression of BDNF in the hippocampus of 21-day-old guinea pig pups. Prenatal control guinea pigs (n = 8: 4 male, 4 female); prenatal stress-exposed neonates (n = 8: 4 male, 4 female). Representative Western blots show two paired guinea pig hippocampal tissue samples and guinea pig adrenal gland internal control (70 μg total protein per lane). Pre-incubation of primary BDNF with blocking peptide blocked specific binding at 32 kDa in guinea pig hippocampal sample.

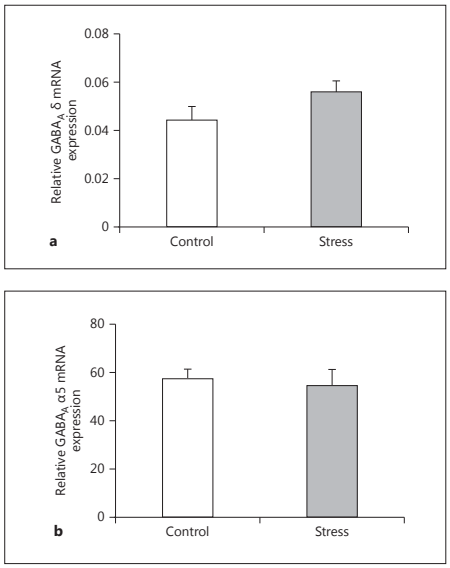

Fig. 8. GABA$_A$ receptor subunit δ (**a**) and α5 (**b**) expression in the hippocampus of prenatal control (n = 14: 6 male, 8 female) and prenatal stress offspring (n = 11: 5 male, 6 female).

study extends these observations by showing that there are ongoing deficits in key markers of mature oligodendrocyte development and reactive astrocyte expression in the postnatal brain, notably the hippocampus. Furthermore, indicators of anxiety were increased in pups born from pregnancies that experienced late gestational prenatal stress and these pups were also less attentive (inquisitive) of objects placed in an open-field environment. However, basal neurosteroidogenic pathways of these offspring as well as the systemic adrenocortical responsiveness (cortisol and DHEA) to handling during the behaviour testing regimens were both not affected by prior in utero stress exposure in childhood. This result potentially implicates persistent structural neural changes – rather than a disturbance in neurosteroid pathways or immature adrenal functioning – in leading changes that result in altered behavioural functioning at this age. In prenatally stressed offspring in this study, the decreased drive to explore new objects within their environment is an indication of heightened avoidance and neophobic actions, which suggests a shift in these offspring towards more anxious behaviour. This study also found that both sexes exposed to prenatal stress spent less time in the inner zone of the open-field arena during behavioural test-

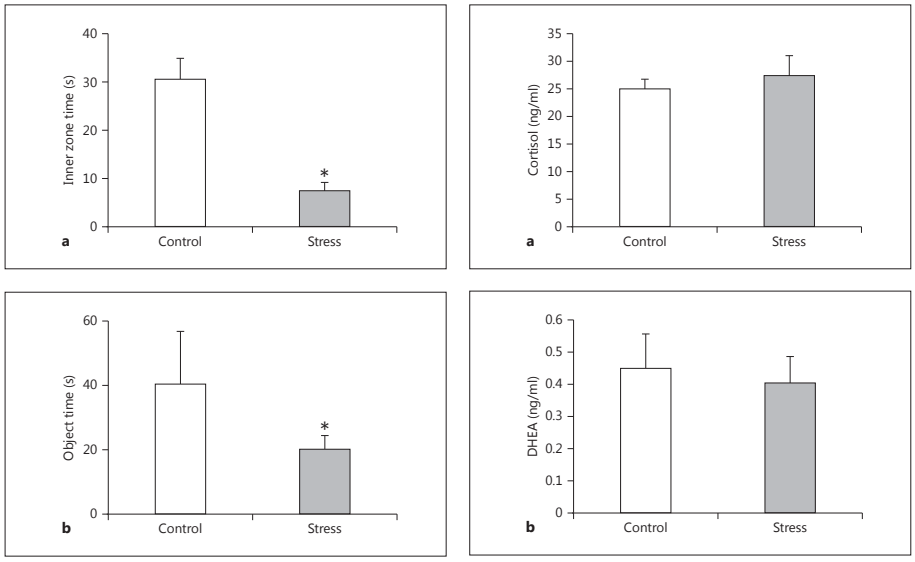

Fig. 9. Time spent in inner zone of open-field arena (**a**) and time spent exploring an object (**b**) during behavioural testing at 18 days of age in prenatal control (n = 23: 12 male, 11 female) and prenatally stressed offspring (n = 24: 12 male, 12 female). * $p < 0.05$.

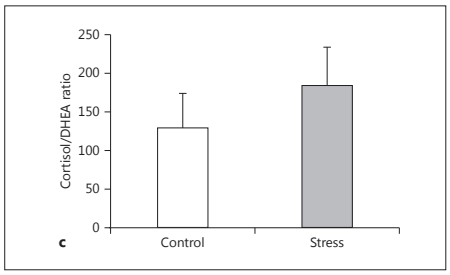

Fig. 10. Salivary cortisol concentrations (**a**), salivary DHEA concentrations (**b**) and cortisol/DHEA ratio (**c**) in prenatal control (n = 16: 8 male, 8 female) and prenatally stressed offspring (n = 17: 8 male, 9 female).

ing, which supports a role for prenatal stress in increasing vulnerability to disorders leading to anxiety-like behaviours [33, 34]. With an increasing number of studies now supporting the striking association between prenatal stress exposure and the development of anxiety-related disorders in offspring [35–37], there appears to be a role for the sex of the offspring in determining the severity of the outcomes associated with prenatal stress exposure [33, 38, 39]. Interestingly, this was not the case in the current study design. Indeed, there is now a growing body of knowledge highlighting the impact of prenatal stress exposure on the outcomes of offspring. However, the particular stressor used and behavioural parameters assessed in offspring are varied between studies and thus the outcomes within the current literature are diverse [40]. Key differences in the species used as well as the type and timing of stressor used are all major variables in determining the outcome of offspring and these factors must be considered when interpreting findings. In the current study, stress was induced in late gestation in a transient manner and was shown to lead to marked rises in maternal cortisol, which has also been previously shown by our group and others using the same model [12, 26]. Prenatal stress was induced in the third trimester of pregnancy in this

study design, which correlates to around 70% of gestation. This is a time of considerable cholesterol accumulation and myelination in the fetal brain [41] as well as glial cell proliferation, synaptogenesis and a number of other neuronal and receptor maturational processes [42, 43], making late gestation a susceptible period for neurodevelopmental damage by the effects of prenatal stress exposure. In human pregnancy, this period of brain growth occurs at around 32 weeks of gestation (0.8), whereas maximal brain growth in rats does not occur until after birth at around PND 7 [44, 45]. These differences support the greater relevance of the guinea pig model in assessing the effects of prenatal perturbations on human behavioural outcomes [25].

The hippocampus has been implicated in many of the behavioural changes observed in offspring associated with prenatal stress exposure and is highly susceptible to adverse adaptations during fetal development and early life [46–50]. It is understood that while the hippocampus has a large population of pyramidal cells and efferent projections to the cortex, thalamus and hypothalamus, which are important for a variety of functions – notably memory and learning – it also has a high level of expression of the glucocorticoid receptor [51], potentially leading to its vulnerability to effects of stress and, thus, increased glucocorticoid exposure. Previously, we have shown a sex-specific effect of prenatal stress on the expression of mature oligodendrocytes and reactive astrocytes in the hippocampus, such that male fetuses exposed to stress in utero had severe reductions in their markers of brain development at term compared to both control fetuses and females [12]. The findings of the current study, however, showed a reduction in these markers in both sexes (in a similar magnitude to that reported previously), indicating that after birth males are unable to recover their levels of oligodendrocyte maturity or reactive astrocytes to the level of unaffected controls but, perhaps more strikingly, that females may develop a delayed reduction in their expression of neurodevelopmental markers that was not present before birth (with no changes observed in hippocampal weight in any group).

The influence of early-life maternal care has also been suggested to be an important factor in determining the developmental outcomes of offspring, with the suggestion that stressed dams may perform worse at early-life care of their pups, potentially exacerbating any deficits present at birth [33, 52, 53]. This effect has even been shown transgenerationally, with prenatal stress being associated with altered maternal care in subsequent generations [54]. However, whilst guinea pig pups demonstrate strong attachments to their mothers [27], maternal care itself is very passive in nature, limited primarily to active licking of the pups over the first postnatal week [55]. In this study, there were no differences observed in neonatal birth weight and weight gain trajectories over the 21 days after birth following prenatal stress exposure, which is indicative of normal feeding and maternal care compared to controls. However, in a model of early-life stress using fragmented (removed) maternal care, the neurosteroid system, which is normally neuroprotective, becomes ineffective due to increased excitatory input in the brains of offspring [56] and, therefore, early-life stress and the quality of maternal care following prenatal stress exposure may challenge vulnerable developing offspring and cause further neurodevelopmental, behavioural and neurosteroid deficits. In this regard, further studies assessing enriched maternal care in early life as a treatment method are warranted to assess whether optimal maternal care at this age and throughout early life may help to counteract detrimental programming effects of prenatal stress before birth. In this context, it is important to consider that previous studies have found increased excitatory input in the brains of offspring, potentially as a programming effect of prenatal stress and elevated glucocorticoid exposure [57, 58], which may be overwhelming any neuroprotective effects of normal basal neurosteroid levels in this cohort. Furthermore, the finding of decreased levels of oligodendrocytes and reactive astrocytes, both of which may be neurosteroid producing [17], in this prenatally stressed population may lead to reduced levels of neuroprotection, both in the face of 'programmed' increased excitatory input and at times of stress. This potentially explains some of the behavioural deficits seen in this population despite the absence of changes in overall neurosteroid levels. This study has also shown no effect of prenatal stress on BDNF expression in the hippocampus, indicating that neurotrophic factors may not be susceptible in the hippocampus in this model of prenatal stress. However, as BDNF is often considered a marker for synaptic plasticity, this may suggest that the changes seen following prenatal stress in this study and previously [12] could be permanent structural deficits.

The $GABA_A$ receptor subunits α5 and δ assessed in this study are dominant within the hippocampus. However, no changes in $GABA_A$ receptor subunit expression were observed following prenatal stress exposure in the current study and whilst we cannot rule out changes to other $GABA_A$ receptor subunits, those measured in this study are important for neurosteroid binding. As such, whilst no changes were observed in the current study with respect

to basal neurosteroidogenesis or receptor expression, any potential deficits in neurosteroid levels during a postnatal stress event cannot be ignored, with the need for further study on the ability of these offspring to cope with stress throughout the lifespan (childhood, sexual maturity, adulthood). Furthermore, in the current study allopregnanolone levels were measured from total brain homogenates and therefore any differences in the hippocampus or other regions could not be detected and may have affected outcomes, although, given the lipid solubility of this steroid, production in one brain region is likely to influence adjacent regions [59]. Considering the involvement of the hippocampus in the adult stress response [60], its population of $GABA_A$ receptors [61] and neuronal and glial cells [50] and its vulnerability to the effects of stress [46], the assessment of allopregnanolone concentrations specifically in this region warrants further investigation.

This study extends a growing body of knowledge highlighting the disruptive role for prenatal stress during important periods of fetal brain development. Although the role of additional brain regions contributing to outcomes in prenatally stressed offspring were not examined in the current study, the finding of marked deficits in mature oligodendrocyte development and reactive astrocyte expression in the hippocampus indicates that the effects of prenatal stress observed in this model are sustained beyond birth and into early life. This suggests that persisting prenatal stress-induced changes are likely to have contributed to the anxious behaviour observed in guinea pigs at the equivalent of childhood in this study. This is a time in postnatal development when many neuropathologies manifest and deficits in brain development may be important for susceptibility to further stress-induced damage and may also be potential targets for amelioration therapies.

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