Effects of prenatal stress on fetal neurodevelopment and responses to maternal neurosteroid treatment in guinea pigs.

Running title: Prenatal stress and neurosteroid treatment (42 characters and spaces)

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Key words: Stress, neurosteroid, cortisol, fetus, myelin basic protein, glial fibrillary acidic protein, microtubule associated protein 2, neonate

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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.

**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<sub>A</sub> receptors to reduce neural excitability. We have previously observed high levels of fetal arousal and neural excitability when neurosteroid synthesis is blocked with finasteride [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 have chosen to
administer allopregnanolone in late gestation (from gestational day 60) to mimic this high endogenous production and to model normal responsiveness to stress exposure at this time. Furthermore, we have previously reported that administration of exogenous glucocorticoids during late pregnancy alters levels of the endogenous allopregnanolone by suppressing synthesising enzymes in the placenta [32]. Our studies 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 Matthews et al [20,34,35] in which stress was induced by exposure to strobe light. Briefly, the animals were placed in a ventilated light proof container and exposing the animals to a strobe light for 2hr (9-11am). The high frequency strobe light intensity was 75 joules per 10 seconds. This protocol was repeated on GA 55, 60 and 65 (term 69 days).

Dams in the control groups were treated in the same way with handling performed but no exposure to the strobe light. Saliva samples were collected from all of the dams by mastication on a cotton bud for approximately 1 minute both immediately (within 30 seconds) before and after each of the strobe light exposure or the control events.

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

Allopregnanolone treatment

Allopregnanolone was obtained from Dr. R. H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA) and administered subcutaneously in a 45% 2-hydroxypropyl-β-cyclodextrin vehicle solution (Sigma Aldrich, Castle Hill, NSW, Australia).

Stress and control dams were randomly allocated to either receive allopregnanolone (10mg/kg/day) or vehicle injections twice daily at 9am and 5pm from GA60 to GA68.

Tissue collection

Pregnant dams were euthanased at term (69 days GA) or on the second consecutive day of full pubic symphisis opening (>2cm diameter), which is an established indicator of imminent labour in the guinea pig [36]. Dams were euthanased by inhalation of 100% CO₂. Maternal and fetal blood was collected immediately. Fetal placement, body weight, sex, nose-rump length as well as weights of organs were recorded, including the whole brain, placenta, heart, adrenal glands and liver. Fetal brains were dissected in a sagittal plane with one half
snap frozen at -80°C or and the other half fixed via immersion in a formalin solution (4% w/v Paraformaldehyde in 0.1M Phosphate Buffer (Na$_2$PO$_4$; NaH$_2$PO$_4$H$_2$O) (Sigma Aldrich).

**Immunohistochemistry**

The fixed brain tissues were embedded in paraffin wax and processed for immunohistochemical staining and analysis using methods we have previously described [37]. Briefly, 8μm 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$_2$O$_2$) and methanol solution to inhibit endogenous peroxidase activity. Antigen retrieval was then performed by incubation in Reveallt Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia). Following blocking with bovine serum albumin in phosphate buffered saline (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide), sections were incubated with primary antibodies for myelin basic protein (MBP; Sigma Aldrich), glial fibrillary acidic protein (GFAP; Sigma Aldrich) and microtubule associated protein 2 (MAP2; Sigma Aldrich) overnight at concentrations 1:4000, 1:4000 and 1:30000 respectively. This was followed by incubation with secondary antibodies at room temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP and MAP2, anti-mouse IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK). Subsequently slides were incubated in Streptavidin-Biotinylated HRP complex (RPN1051, Amersham). Finally, the slides were then incubated in 3,3'-diaminobenzidine (DAB) concentrate with H$_2$O$_2$. Slides were fixed with coverslips. Slides were viewed with bright field microscopy on a Nikon Eclipse 90i microscope and images captured on a Nikon DS-Ri1 Digital Sight camera head (Nikon, Australia). All immunoreactivities were analysed by densitometry using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA), made binary by adjusting
the threshold manually, with the percentage area of coverage recorded for four fields of view per brain region on two sections per animal. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

Allopregnanolone radioimmunoassay and cortisol enzyme immunoassay

Allopregnanolone was extracted from fetal and maternal plasma as previously described [32]. Briefly, plasma was treated with 50% methanol with 1% acetic acid in Sep-Pak C₁₈ cartridges (Waters, Milford, MA, USA), vacuum dried and then treated with potassium permanganate to reduce cross-reactivity of progesterone [38]. The addition of tritium-labelled allopregnanolone (1000–1500 cpm., 5α-[9, 11, 12, 3H(N)]); PerkinElmer Life and Analytical Sciences, Boston, MA, USA) allowed determination of sample recovery. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. Allopregnanolone was quantified by radioimmunoassay using a polyclonal antibody (supplied by Dr. R H Purdy Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA) the assay and cross-reactivities of the antisera have previously been described [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 manufacturers instructions. Sensitivity of the assay was 0.012 μg/dL to 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 (HAPS). The assays were conducted on the UniCel Dxl800 Access Immunoassay System (Beckman Coulter Inc., Gladesville, NSW, Australia), as per
manufacturers instructions. The inter- and intra- assay coefficients of variance were 5.17% and 4.3% respectively for cortisol and 8.2% and 7.9% respectively for progesterone.

Statistical Analysis

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

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

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

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

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are combined with the vehicle treated animals. As expected, dams in stress exposed groups demonstrated significantly higher (RM_ANOVA p<0.001, F=82.18, df=1) salivary cortisol concentrations after each exposure compared to their control handled counterparts, who showed no change after each event. In addition, there was no difference in the fold change in salivary cortisol concentrations compared to controls, even when adjusting for advancing gestational age.

Maternal plasma cortisol concentrations taken at the time of post mortem were significantly correlated with cortisol levels in saliva collected at the time of post mortem (p<0.001; Spearman r=0.75; data not presented) supporting the use of salivary cortisol as a measurement of circulating cortisol concentrations. Furthermore, maternal plasma cortisol negatively correlated with fetal BLR (p=0.03; Spearman r=0.33; data not presented), indicating that fetal brain growth may be negatively affected as maternal cortisol levels rise.

Myelin basic protein (MBP) expression and allopregnanolone response

Figure 2 C and D show representative micrographs of MBP immunostaining in the CA1 region of the hippocampus of prenatally stressed, control, vehicle and allopregnanolone treated fetuses. There were significantly lower levels of MBP expression in male prenatally stressed fetuses in the CA1 region of the hippocampus (ANOVA p<0.001, F=13.576, df=1; Figure 2A and C), and in the cerebral cortical white matter (ANOVA p<0.001, F=14.840, df=1; data not shown), compared to controls. This result was not seen in the female cohort (Figure 2B and D). A significant negative correlation was also found between maternal salivary cortisol concentrations at the time of post mortem and MBP expression in the CA1 region of the hippocampus when male and female groups were combined (p=0.02;
Spearman $r=-0.53$) and between maternal plasma cortisol at the time of post mortem and
MBP expression in the cerebral cortical white matter when male and female groups were
combined ($p<0.001$; Spearman $r=-0.62$), indicating the relationship between increased
cortisol exposure and reduced fetal brain myelination.

Glial Fibrillary Acidic Protein (GFAP) and allopregnanolone response

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

Microtubule Associated Protein 2 (MAP2) and allopregnanolone response

Figure 4 C and D show representative micrographs showing MAP-2 immunostaining in the
hippocampus of prenatally stressed, control, vehicle and allopregnanolone treated fetuses.
Analysis showed the significance of stress on MAP2 expression in the CA1 region of the
hippocampus revealing stress reduced MAP2 expression in both male and female cohorts
(Males ANOVA $p<0.01$, $F=6.443$, $df=1$; Females ANOVA $p<0.001$, $F=11.743$, $df=1$; Figure 4 A
and B respectively). There was however, no effect of allopregnanolone treatment on either
males or females. There was no effect of stress exposure or allopregnanolone treatment on MAP2 expression within the cerebral cortical white matter (data not shown).

Allopregnanolone treatment and fetal plasma concentrations

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

There was no difference in plasma progesterone concentrations between any of the experimental groups in fetal plasma at the time of post mortem (average fetal levels were 2845.82nmol/L +308.45nmol/L). There was also no significant effect of stress exposure or allopregnanolone treatment on maternal plasma progesterone levels at the time of post mortem (average maternal levels were 10442.83nmol/L +1936.95nmol/L).
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 types 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 CNS [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 CNS developmental processes [58]. Allopregnanolone has been shown to have
potent inhibitory effects, modulating the GABA<sub>A</sub> receptor in the late gestation fetus supporting proper neurodevelopment. Thus, fetuses affected by prenatal stress may also be susceptible to damage due to perturbations in neurosteroidogenesis creating an additive environment for damage. These observations have implications for perinatal brain development and psychopathology later in life, particularly for affected male fetuses. We have found a negative effect of stress on the expression of the mature neuronal marker, MAP-2, in both males and females suggesting that females may not be totally protected from maternal stress. This may in part, be attributable to the vulnerability of the hippocampus to prenatal insults including prenatal stress [23] and that some of the first stress episodes, conducted at GA50 and/or 55 (0.7 of gestation) may have damaged these neurons during their peak growth period, before an effective growth adaptation (brain sparing) was employed. Studies have shown the vulnerabilities of the hippocampus to stress-induced reductions in neurogenesis and resultant learning and memory, supporting the idea of a selective effect of stress on certain cell populations and regions within the fetal brain [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 suggests that different cell populations within the fetal brain respond differently to prenatal stress.

The model of transient stress used in this study was previously developed by Matthews and colleagues, and was used to evaluate the effect of prenatal stress on fetal hypothalamo-pituitary adrenal axis development [20,34]. 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 et al [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 to 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 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.

Acknowledgements

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## Tables and figure legends:

### Table 1: Fetal Physical Characteristics

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<th>Sex</th>
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<th>Body weight</th>
<th>Brain to Body weight</th>
<th>Nose-Rump Length</th>
<th>Placenta to Body weight</th>
<th>Heart to Body weight</th>
<th>Liver to Body weight</th>
<th>Adrenal Gland to Body weight</th>
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<td></td>
<td>Cont + Veh (n=10)</td>
<td>98.45 + 4.34</td>
<td>2.49 + 0.07</td>
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<td></td>
<td>Stress + Veh (n=11)</td>
<td>88.75 + 4.84</td>
<td>2.84 + 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.25 + 0.38</td>
<td>5.28 + 0.14</td>
<td>0.52 + 0.03</td>
<td>3.88 + 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 + 0.03</td>
<td>0.67 + 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Stress + Allo (n=12)</td>
<td>82.91 + 4.85</td>
<td>2.90 + 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.89 + 0.38</td>
<td>5.14 + 0.29</td>
<td>0.48 + 0.02</td>
<td>4.64 + 0.31</td>
<td>0.05 + 0.02</td>
<td>0.64 + 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are represented as a percentage of body weight at the time of post mortem with the exception of brain to liver weight ratio (BLR), which is a ratio value of brain weight to liver weight. This value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth restricted fetuses. "<sup>a</sup>" indicates significant (p<0.05) effect of stress, and "<sup>b</sup>" indicates significant (p<0.05) effect of sex. No effect of drug treatment was found. Values are expressed as the mean percentage + SEM and are calculated for animal numbers shown in parentheses. Allo = allopregnanolone, BLR = brain to liver ratio, Cont = control, Veh = vehicle.
**Figure 1:**

Figure 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 episode. Stress episodes were performed at 50, 55, 60 and 65 days GA. Stress exposed (n=14) and control (n=14) groups contain both vehicle and allopregnanolone treated guinea pig dams. ‘*’ p=0.05; ‘**’ p<0.01 and ‘†’ p<0.001 indicates significance level between changes seen between control and stress groups.

**Figure 2:**

Figure 2: Effects of prenatal stress on myelin basic protein (MBP) expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. A, Representative images of MBP immunostaining for male (A) and female (B) guinea pig fetuses at term showing staining in a control + vehicle treated (i), control + allopregnanolone treated (ii), stress + vehicle treated (iii) and stress + allopregnanolone treated fetus (iv). MBP expression calculated coverage areas of immunohistochemical staining (see methods) in male (A) and female (B) guinea pig fetuses at term. MBP staining coverage in: control + vehicle groups in black bars (male n=7; female n=5), control + allopregnanolone groups in grey bars (male n=9; female n=4), stress + vehicle groups in hatched bars (male n=5; female n=4) and stress + allopregnanolone groups in open bars (male n=5; female n=5). ‘†’ p<0.001 indicates significance level between control and stress groups of males fetuses. Allo = allopregnanolone; veh = vehicle. Scale bar =50µm.

**Figure 3:**

Figure 3: Effects of prenatal stress on glial fibrillary acidic protein (GFAP) expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. A, Representative images of MBP immunostaining for male (A) and female (B) guinea pig fetuses at term showing staining in a control + vehicle treated (i), control + allopregnanolone treated (ii), stress + vehicle treated (iii) and stress + allopregnanolone treated fetus (iv). GFAP expression calculated coverage areas of immunohistochemical staining (see methods) in male (A) and female (B) guinea pig fetuses at term. GFAP staining coverage in: control + vehicle groups in black bars (male n=7; female n=5), control + allopregnanolone groups in grey bars (male n=9; female n=4),
stress + vehicle groups in hatched bars (male n=5; female n=4) and stress + allopregnanolone groups in open bars (male n=5; female n=5). ‘†’ p<0.001 between control and stress groups of males fetuses. Allo = allopregnanolone; veh = vehicle. Scale bar =50µm.

**Figure 4:**

Figure 4: Effects of prenatal stress on myelin basic protein-2 (MAP-2) expression in the CA1 region of the hippocampus with and without allopregnanolone treatment. A, Representative images of MBP immunostaining for male (A) and female (B) guinea pig fetuses at term showing staining in a control + vehicle treated (i), control + allopregnanolone treated (ii), stress + vehicle treated (iii) and stress + allopregnanolone treated fetus (iv). MAP2 expression calculated coverage areas of immunohistochemical staining (see methods) in male (A) and female (B) guinea pig fetuses at term. MAP-2 staining coverage in: control + vehicle groups in black bars (male n=7; female n=5), control + allopregnanolone groups in grey bars (male n=9; female n=4), stress + vehicle groups in hatched bars (male n=5; female n=4) and stress + allopregnanolone groups in open bars (male n=5; female n=5). ‘†’ p<0.001 between control and stress groups in male and female fetuses. Allo = allopregnanolone; veh = vehicle. Scale bar =100µm.

**Figure 5:**

Figure 5: Maternal plasma allopregnanolone concentrations in stress and control dams 12 hours after last vehicle (control + vehicle group, black bars, n=3; stress + vehicle, hatched bars, n=4) or allopregnanolone administration (control + allopregnanolone, grey bars, n=4; stress + allopregnanolone, open bars, n=4). ‘*’ p<0.05, ‘†’ p<0.001 between vehicle and allopregnanolone treatment. Allo= allopregnanolone; Veh = vehicle.

**Figure 6:**

Figure 6: Fetal plasma allopregnanolone concentrations in stress and control dams 12 hours after last vehicle or allopregnanolone administration in male (A) and female (B) fetuses. Data shown are for: control + vehicle (black bars; males n=5, females n=5), control + allopregnanolone (grey bars; males n=6, females n=5), stress +
vehicle (hatched bars; males n=5, females n=5) and stress + allopregnanolone treatment (open bars; males n=6, females n=6). ‘*’ p<0.05 difference between levels in the control + allopregnanolone group and all other groups. Allo = allopregnanolone; Veh = vehicle. Scale bar =10μm.
Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

Figure 6