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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)

Greer A. Bennett[1,2], Hannah K. Palliser [1,2], Britt Saxby [1,2], David Walker [3], Jonathan Hirst[1,2].

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[1]*Mothers and Babies Research Centre, Hunter Medical Research Institute, Newcastle, NSW, Australia.* [2]*School of Biomedical Science, University of Newcastle, NSW, Australia.* [3]*Richie Centre for Baby Health, Monash University, Clayton, VIC, Australia.*

Corresponding Author: Greer Bennett.

Mothers and Babies Research Institute,

Hunter medical Research Institute

University of Newcastle.

Callaghan NSW 2308 Australia.

Phone: 61-2-4042 0368.

Fax: 61-24042 0045.

Email: greer.bennett@uon.edu.au

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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].

73 Adverse behavioural outcomes following prenatal stress are supported by data in animal
74 studies which link prenatal stress with perturbations in fetal brain development at particular
75 vulnerable windows of fetal brain growth [17]. Late pregnancy is a time of considerable
76 myelination and glial cell proliferation as well as increased synaptogenesis, neuronal and
77 axonal migration/proliferation and various receptor maturational processes, all of which
78 place high energy demands on the fetal brain [18,19]. Therefore this period has been
79 identified as a vulnerable period for neurodevelopmental delay or damage [20]. Animal
80 studies have linked prenatal stress to alterations in the hippocampus that result in a higher
81 susceptibility to neuropathologies later in life [21-23]. Late gestational social stress has
82 been shown to increase anxiety behaviours in male offspring [24]. High levels of cortisol in
83 fetal circulation following exposure to an acoustic stressor have also been shown to cause
84 disturbed hippocampal development in rhesus monkeys [22]. Prenatal restraint stress in late
85 gestational rats leads to dendritic atrophy in the hippocampus of the offspring as a result of
86 excitotoxicity [25]. Therefore stress-induced increases in glucocorticoid levels and neural
87 excitation may mediate some of these deleterious effects.
88 During gestation there are high levels of endogenous neurosteroids, which act at inhibitory
89 $GABA_A$ receptors to reduce neural excitability. We have previously observed high levels of
90 fetal arousal and neural excitability when neurosteroid synthesis is blocked with finasteride
91 [26] and that a reduction in neurosteroid levels is also associated with reduced levels of
92 REM sleep which in turn, may result in developmental delay [27]. During pregnancy, the
93 placenta has a key role maintaining the endogenously protective neurosteroid levels by
94 providing considerable amounts of precursors for their synthesis. This accounts for the
95 remarkably high levels of the potent neurosteroid allopregnanolone, which is synthesised
96 from progesterone, in the fetus throughout late gestation [26,28-31]. We have chosen to

97 administer allopregnanolone in late gestation (from gestational day 60) to mimic this high
98 endogenous production and to model normal responsiveness to stress exposure at this
99 time. Furthermore, we have previously reported that administration of exogenous
100 glucocorticoids during late pregnancy alters levels of the endogenous allopregnanolone by
101 suppressing synthesising enzymes in the placenta [32]. Our studies also shown a decrease in
102 reactive astrocyte marker expression in the brains of these fetuses, and interestingly, the
103 males and not the female fetuses demonstrated these adverse effects in response to
104 glucocorticoid exposure [32]. Our previous studies have shown that allopregnanolone has
105 potent neuroprotective effects against acute excitotoxicity following hypoxia/ischemia and
106 that reduced concentrations of allopregnanolone conferred increased vulnerability to brain
107 injury in late gestation [33].
108 In the present study we examined the effect of prenatal maternal stress and the associated
109 increase in glucocorticoid exposure on fetal brain development during key growth periods in
110 gestation. We then investigated the effect of prenatal stress when allopregnanolone was
111 administered during the last 8 days of gestation (0.8 of gestation).
112
113 ***Methods:***
114 *Animal stress protocol*
115 Time mated, outbred pregnant guinea pigs were obtained from the University of Newcastle
116 colony. All procedures were approved by the University of Newcastle Animal Care and Ethics
117 Committee and carried out in accordance with the Australian Code of Practice for the Care
118 and Use of Animals for Scientific Purposes. The dams were randomly allocated into either a
119 stress (light) exposure or control (the same handling but no light exposure) group. At 50
120 days of gestational age (GA), dams in the stress exposure group commenced a procedure

121 developed by Matthews et al [20,34,35] in which stress was induced by exposure to strobe
122 light. Briefly, the animals were placed in a ventilated light proof container and exposing the
123 animals to a strobe light for 2hr (9-11am). The high frequency strobe light intensity was 75
124 joules per 10 seconds. This protocol was repeated on GA 55, 60 and 65 (term 69 days).
125 Dams in the control groups were treated in the same way with handling performed but no
126 exposure to the strobe light. Saliva samples were collected from all of the dams by
127 mastication on a cotton bud for approximately 1 minute both immediately (within 30
128 seconds) before and after each of the strobe light exposure or the control events.
129 Food (commercial guinea pig pellets and hay) and/or nutrient intake were not measured in
130 this study, as food and water were available to dams *ad libitum*.
131
132 *Allopregnanolone treatment*
133 Allopregnanolone was obtained from Dr. R. H. Purdy (Department of Psychiatry, Veterans
134 Administration Hospital, San Diego, CA, USA) and administered subcutaneously in a 45% 2-
135 hydroxypropyl-β-cyclodextrin vehicle solution (Sigma Aldrich, Castle Hill, NSW, Australia).
136 Stress and control dams were randomly allocated to either receive allopregnanolone
137 (10mg/kg/day) or vehicle injections twice daily at 9am and 5pm from GA60 to GA68.
138 *Tissue collection*
139 Pregnant dams were euthanased at term (69 days GA) or on the second consecutive day of
140 full pubic symphisis opening (>2cm diameter), which is an established indicator of imminent
141 labour in the guinea pig [36]. Dams were euthanased by inhalation of 100% CO_2. Maternal
142 and fetal blood was collected immediately. Fetal placement, body weight, sex, nose-rump
143 length as well as weights of organs were recorded, including the whole brain, placenta,
144 heart, adrenal glands and liver. Fetal brains were dissected in a sagittal plane with one half

145 snap frozen at -80°C or and the other half fixed via immersion in a formalin solution (4% w/v
146 Paraformaldehyde in 0.1M Phosphate Buffer (Na_2PO_4; $NaH_2PO_4H_2O$) (Sigma Aldrich).
147
148 *Immunohistochemistry*
149 The fixed brain tissues were embedded in paraffin wax and processed for
150 immunohistochemical staining and analysis using methods we have previously described
151 [37]. Briefly, 8µm brain sections were processed by a method involving dewaxing in xylene,
152 rehydration in a series of ethanol/water washes and finally incubation in a hydrogen
153 peroxide (H_2O_2) and methanol solution to inhibit endogenous peroxidase activity. Antigen
154 retrieval was then performed by incubation in RevealIt Solution (ImmunoSolution Pty Ltd,
155 Everton Park, Qld, Australia). Following blocking with bovine serum albumin in phosphate
156 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
157 sodium azide), sections were incubated with primary antibodies for myelin basic protein
158 (MBP; Sigma Aldrich), glial fibrillary acidic protein (GFAP; Sigma Aldrich) and microtubule
159 associated protein 2 (MAP2; Sigma Aldrich) overnight at concentrations 1:4000, 1:4000 and
160 1:30000 respectively. This was followed by incubation with secondary antibodies at room
161 temperature (MBP, anti-rat IgG biotinylated, Sigma Aldrich; GFAP and MAP2, anti-mouse
162 IgG biotinylated, Amersham, GE Healthcare, Buckinghamshire, UK). Subsequently slides
163 were incubated in Streptavadin-Biotinylated HRP complex (RPN1051, Amersham). Finally,
164 the slides were then incubated in 3,3'-diaminobenzidine (DAB) concentrate with H_2O_2. Slides
165 were fixed with coverslips. Slides were viewed with bright field microscopy on a Nikon
166 Eclipse 90i microscope and images captured on a Nikon DS-Ri1 Digital Sight camera head
167 (Nikon, Australia). All immunoreactivities were analysed by densitometry using ImageJ
168 version 1.46 (National Institutes of Health, Bethesda, MD, USA), made binary by adjusting

169 the threshold manually, with the percentage area of coverage recorded for four fields of
170 view per brain region on two sections per animal. Controls for specificity of primary
171 antibodies were run using the appropriate IgG substituted for each primary antibody.
172
173 *Allopregnanolone radioimmunoassay and cortisol enzyme immunoassay*
174 Allopregnanolone was extracted from fetal and maternal plasma as previously described
175 [32]. Briefly, plasma was treated with 50% methanol with 1% acetic acid in Sep-Pak C_{18}
176 cartridges (Waters, Milford, MA, USA), vacuum dried and then treated with potassium
177 permanganate to reduce cross-reactivity of progesterone [38]. The addition of tritium-
178 labelled allopregnanolone (1000–1500 cpm., 5a-[9, 11, 12, 3H(N)]); PerkinElmer Life and
179 Analytical Sciences, Boston, MA, USA) allowed determination of sample recovery. Each
180 sample was corrected for its extraction loss in the final calculation of allopregnanolone
181 concentrations. Allopregnanolone was quantified by radioimmunoassay using a polyclonal
182 antibody (supplied by Dr. R H Purdy Department of Psychiatry, Veterans Administration
183 Hospital, San Diego, CA, USA) the assay and cross-reactivities of the antisera have previously
184 been described [32]. The limit of detection for allopregnanolone was 35.0 + 2.5pg/tube. The
185 inter and intra-assay coefficients of variation were 12.3% and 8.5% respectively.
186 Cortisol concentrations were determined in maternal saliva obtained before and after each
187 stress or control event using a salivary assay kit (Salimetrics Inc., State College, PA, USA), as
188 per manufacturers instructions. Sensitivity of the assay was 0.012 µg/dL to 3.0 µg/dL and
189 inter- and intra- assay coefficients of variance were 6.89% and 5.52% respectively.
190 Cortisol and progesterone were quantified in fetal and maternal plasma by immunoassay by
191 Hunter Area Pathology Service (HAPS). The assays were conducted on the UniCel Dxl800
192 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:

217 *Effect of prenatal stress on fetal physiological characteristics*

218 Female fetuses showed significantly (ANOVA $p<0.05$, $F=5.172$, $df=1$) larger brain-weight-to
219 body-weight ratios than their male counterparts, irrespective of stress exposure or drug
220 treatment (Table 1). Female fetuses exposed to prenatal stress were also the only group to
221 show a significantly (ANOVA $p<0.05$, $F=3.393$, $df=1$) reduced liver-weight-to-body-weight
222 ratio with no significant changes observed in the male cohort or in the cohort of female
223 fetuses exposed to allopregnanolone treatment. Brain to liver ratio (BLR) in the females, but
224 not males, was significantly higher (ANOVA $p<0.05$, $F=6.472$, $df=1$) in fetuses exposed to
225 stress compared to those in control groups, indicating asymmetric growth and brain sparing.
226 No significant effect of stress, drug treatment or sex was found on body weight or nose-
227 rump length. There were also no significant effects of stress, drug treatment or sex on
228 placental weight, heart weight or adrenal weight when adjusted for individual differences in
229 body weight. It should also be noted that pregnancies exposed to PS showed a modest
230 reduction in GA at the time of post mortem, indicating a shorter gestational length (control
231 68.43+0.47 and stress 67.7+0.35; ANOVA $p<0.05$, $F=4.286$, $df=1$). There was no significant
232 effect of stress exposure or drug treatment on litter size or average litter weight, nor were
233 there any effects on maternal weight gain during pregnancy (data not presented). This
234 suggests that maternal weight gain was not responsible for any difference in fetal growth
235 data.

236

237 *Maternal Salivary Cortisol Concentrations*

238 Maternal salivary cortisol data (presented as the fold change in concentrations from
239 immediately before to after each stress or control handling exposure) is shown in Figure 1.
240 Maternal allopregnanolone treatment did not affect cortisol levels and therefore these data

241 are combined with the vehicle treated animals. As expected, dams in stress exposed groups
242 demonstrated significantly higher (RM_ANOVA $p<0.001$, $F=82.18$, $df=1$) salivary cortisol
243 concentrations after each exposure compared to their control handled counterparts, who
244 showed no change after each event. In addition, there was no difference in the fold change
245 in salivary cortisol concentrations compared to controls, even when adjusting for advancing
246 gestational age.
247
248 Maternal plasma cortisol concentrations taken at the time of post mortem were
249 significantly correlated with cortisol levels in saliva collected at the time of post mortem
250 ($p<0.001$; Spearman $r=0.75$; data not presented) supporting the use of salivary cortisol as a
251 measurement of circulating cortisol concentrations. Furthermore, maternal plasma cortisol
252 negatively correlated with fetal BLR ($p=0.03$; Spearman $r=0.33$; data not presented),
253 indicating that fetal brain growth may be negatively affected as maternal cortisol levels rise.
254
255 *Myelin basic protein (MBP) expression and allopregnanolone response*
256 Figure 2 C and D show representative micrographs of MBP immunostaining in the CA1
257 region of the hippocampus of prenatally stressed, control, vehicle and allopregnanolone
258 treated fetuses. There were significantly lower levels of MBP expression in male prenatally
259 stressed fetuses in the CA1 region of the hippocampus (ANOVA $p<0.001$, $F=13.576$, $df=1$;
260 Figure 2A and C), and in the cerebral cortical white matter (ANOVA $p<0.001$, $F=14.840$, $df=1$;
261 data not shown), compared to controls. This result was not seen in the female cohort
262 (Figure 2B and D). A significant negative correlation was also found between maternal
263 salivary cortisol concentrations at the time of post mortem and MBP expression in the CA1
264 region of the hippocampus when male and female groups were combined ($p=0.02$;

265 Spearman r=-0.53) and between maternal plasma cortisol at the time of post mortem and
266 MBP expression in the cerebral cortical white matter when male and female groups were
267 combined (p=<0.001; Spearman r=-0.62), indicating the relationship between increased
268 cortisol exposure and reduced fetal brain myelination.
269
270 *Glial Fibrillary Acidic Protein (GFAP) and allopregnanolone response*
271 Representative micrographs of GFAP immunostaining in the hippocampus are shown in
272 Figure 3 C and D. Analysis of immunostaining showed there was a marked effect of stress in
273 the male cohort with reduced expression of GFAP in the CA1 region of the hippocampus
274 (ANOVA, p<0.001, F=9.347, df=1; figure 3A and C) and the cerebral cortical white matter
275 (ANOVA p<0.05, F=6.480, df=1; data not shown), which was not seen in the female cohort
276 (Figure 3B and D). Also within the male cohort, there was an interaction between stress and
277 allopregnanolone treatment (ANOVA p<0.05, F=4.541, df=1) indicating that the combination
278 of stress and allopregnanolone treatment caused a differential effect on GFAP expression in
279 the CA1 region, which was not seen in any of the other experimental groups.
280
281 *Microtubule Associated Protein 2 (MAP2) and allopregnanolone response*
282 Figure 4 C and D show representative micrographs showing MAP-2 immunostaining in the
283 hippocampus of prenatally stressed, control, vehicle and allopregnanolone treated fetuses.
284 Analysis showed the significance of stress on MAP2 expression in the CA1 region of the
285 hippocampus revealing stress reduced MAP2 expression in both male and female cohorts
286 (Males ANOVA p<0.01, F=6.443, df=1; Females ANOVA p<0.001, F=11.743, df=1; Figure 4 A
287 and B respectively). There was however, no effect of allopregnanolone treatment on either

288 males or females. There was no effect of stress exposure or allopregnanolone treatment on
289 MAP2 expression within the cerebral cortical white matter (data not shown).
290
291 *Allopregnanolone treatment and fetal plasma concentrations*
292 Maternal plasma allopregnanolone concentrations remained elevated 12 hours after the
293 last maternal allopregnanolone administration in both control and stressed pregnancies
294 compared to vehicle treated controls (RM-ANOVA $p<0.05$, $F=4.859$, $df=1$; Figure 5). In
295 control pregnancies, maternal allopregnanolone treatment resulted in marked fetal plasma
296 allopregnanolone concentrations in both male and female fetuses (ANOVA $p<0.001$,
297 $F=14.598$, $df=1$; Figure 6A and B respectively). In contrast, neither male nor female fetal
298 allopregnanolone concentrations were elevated in pregnancies exposed to stress and
299 allopregnanolone administration (open bars, Figure 6). This observation is consistent with
300 the finding of a significant (ANOVA $p<0.05$, $F=4.090$, $df=1$) interaction between stress and
301 allopregnanolone treatment in fetal plasma indicating that the fetal allopregnanolone levels
302 in response to maternal treatment was altered by stress exposure.
303 There was no difference in plasma progesterone concentrations between any of the
304 experimental groups in fetal plasma at the time of post mortem (average fetal levels were
305 2845.82nmol/L +308.45nmol/L). There was also no significant effect of stress exposure or
306 allopregnanolone treatment on maternal plasma progesterone levels at the time of post
307 mortem (average maternal levels were 10442.83nmol/L +1936.95nmol/L).
308
309
310
311

312 **Discussion**

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

325 There is now increasing evidence for the developmental origins of neuropathologies, such
326 that disturbances in processes such as myelination and neural migration during critical
327 windows of brain development can predispose offspring to abnormalities in efficient
328 synaptic transmission and neural connectivity at birth or later in life. Thus, decreases in
329 MBP, GFAP and MAP2 expression in the male brain indicate decreased myelination,
330 neurogenesis and stability of axons where a lack of sufficient repair processes may confer
331 vulnerability and susceptibility to injury at birth or later in life. The present findings indicate
332 oligodendrocyte maturation and myelination is reduced by late gestation stress. Reactive
333 astrocytes have been shown to have important neuroprotective qualities [42] and are key to
334 supporting development of the CNS [43], such that a reduction in these cells may have a
335 role in the development of detrimental outcomes. The results of this study are consistent

336 with previous studies, which have shown reductions in fetal myelination following brain
337 sparing [44] in both the CA1 region of the hippocampus and the cerebral cortical white
338 matter that are associated with increased incidences of postnatal behavioural pathologies
339 [45]. We assessed white matter tracts within the cerebral cortex, where disturbances during
340 development have been linked to neurodevelopmental damage in other forms of pregnancy
341 compromise such as preterm birth and intrauterine growth restriction [46]. This cerebral
342 cortical white matter deficiency is strongly associated (>90% prevalence) with cognitive,
343 behavioural and attention deficits in children born preterm [46-49]. Previous studies have
344 also found site-specific (CA1 region) disturbances in hippocampal development following
345 exposure to prenatal stressors [50-52]. The CA1 region of the hippocampus contains a high
346 concentration of important efferent projections and pyramidal cells involved in processes
347 such as memory and learning, which are known to be affected by exposure to prenatal
348 stress [53-55]. Furthermore, this region of the hippocampus has also been shown to be
349 selectively vulnerable to the deleterious effects of glucocorticoid exposure [56,57]. This may
350 be attributable in part, to the high levels of expression of glucocorticoid receptors in the
351 CA1 region of the hippocampus [57]. In addition, neurons and glial cells within the CA1
352 region of the hippocampus express high concentration of $GABA_A$ receptors potentially
353 leading to sensitivity to endogenous neurosteroid-dependent neuroprotection. The
354 observation that allopregnanolone metabolism pathways were disrupted by prenatal stress
355 may therefore further contribute to vulnerability.
356 Astrocytes, mature oligodendrocytes and neurons all express steroidogenic enzymes
357 required for neurosteroid synthesis and stress-induced perturbations in the number of
358 these cells could alter endogenous neurosteroid production and therefore contribute to
359 disruption of CNS developmental processes [58]. Allopregnanolone has been shown to have

360 potent inhibitory effects, modulating the $GABA_A$ receptor in the late gestation fetus
361 supporting proper neurodevelopment. Thus, fetuses affected by prenatal stress may also be
362 susceptible to damage due to perturbations in neurosteroidogenesis creating an additive
363 environment for damage. These observations have implications for perinatal brain
364 development and psychopathology later in life, particularly for affected male fetuses.
365 We have found a negative effect of stress on the expression of the mature neuronal marker,
366 MAP-2, in both males and females suggesting that females may not be totally protected
367 from maternal stress. This may in part, be attributable to the vulnerability of the
368 hippocampus to prenatal insults including prenatal stress [23] and that some of the first
369 stress episodes, conducted at GA50 and/or 55 (0.7 of gestation) may have damaged these
370 neurons during their peak growth period, before an effective growth adaptation (brain
371 sparing) was employed. Studies have shown the vulnerabilities of the hippocampus to
372 stress-induced reductions in neurogenesis and resultant learning and memory, supporting
373 the idea of a selective effect of stress on certain cell populations and regions within the fetal
374 brain [22,23,59-61]. Male offspring have also shown reduced neurogenesis in the
375 hippocampus following exposure to prenatal restraint stress [62] and experimentally
376 induced hippocampal damage confers increased vulnerability to psychiatric disorders in
377 adulthood [63]. These data suggests that different cell populations within the fetal brain
378 respond differently to prenatal stress.
379 The model of transient stress used in this study was previously developed by Matthews and
380 colleagues, and was used to evaluate the effect of prenatal stress on fetal hypothalamo-
381 pituitary adrenal axis development [20,34]. The maternal salivary cortisol levels observed in
382 this study following prenatal stress exposure were similar to those previously reported [34].
383 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

408 allopregnanolone treatment suggests that the adverse effects of prenatal stress on the fetal
409 brain may be caused by a loss of neurosteroid responses that are normally both trophic and
410 neuroprotective [67]. A stress-induced reduction in allopregnanolone levels could result in
411 exacerbation of brain injury that exceeds normal regenerative processes in the fetal brain,
412 thus resulting in psychopathologies postnatally. A chronic loss of allopregnanolone induced
413 by stress could also directly result in disorders involving excess neural excitation such as
414 increased vulnerability to seizures.
415 In conclusion, this study has shown the pronounced effects of prenatal stress on both fetal
416 brain development and whole body growth adaptations, effects that were sexually
417 dimorphic. The effect on the male brain is consistent with observational studies in humans,
418 which indicate there is an inherent vulnerability of males to prenatal insults and subsequent
419 behavioural abnormalities later in life. Further studies investigating neonatal behaviour
420 following prenatal stress in a guinea pig cohort would be valuable in elucidating the ongoing
421 effects of these changes, the extent to which the placenta impacts fetal adaptation to
422 stress, and the development of treatments and compensatory approaches.
423
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430
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References:

1 King S, Laplante DP: The effects of prenatal maternal stress on children's cognitive development: Project ice storm. Stress 2005;8:35-45.

2 King S, Mancini-Marie A, Brunet A, Walker E, Meaney MJ, Laplante DP: Prenatal maternal stress from a natural disaster predicts dermatoglyphic asymmetry in humans. Dev Psychopathol 2009;21:343-353.

3 Laplante DP, Brunet A, Schmitz N, Ciampi A, King S: Project ice storm: Prenatal maternal stress affects cognitive and linguistic functioning in 5 1/2-year-old children. J Am Acad Child Adolesc Psychiatry 2008;47:1063-1072.

4 Van den Bergh BR, Marcoen A: High antenatal maternal anxiety is related to adhd symptoms, externalizing problems, and anxiety in 8- and 9-year-olds. Child Dev 2004;75:1085-1097.

5 Loomans EM, van der Stelt O, van Eijsden M, Gemke RJ, Vrijkotte T, den Bergh BR: Antenatal maternal anxiety is associated with problem behaviour at age five. Early Hum Dev 2011;87:565-570.

6 O'Connor TG, Heron J, Golding J, Beveridge M, Glover V: Maternal antenatal anxiety and children's behavioural/emotional problems at 4 years. Report from the avon longitudinal study of parents and children. Br J Psychiatry 2002;180:502-508.

7 O'Connor TG, Heron J, Golding J, Glover V: Maternal antenatal anxiety and behavioural/emotional problems in children: A test of a programming hypothesis. J Child Psychol Psychiatry 2003;44:1025-1036.

8 Vedhara K, Metcalfe C, Brant H, Crown A, Northstone K, Dawe K, Lightman S, Smith GD: Maternal mood and neuroendocrine programming: Effects of time of exposure and sex. J Neuroendocrinol 2012;24:999-1011.

9 Buss C, Davis EP, Hobel CJ, Sandman CA: Maternal pregnancy-specific anxiety is associated with child executive function at 6-9 years age. Stress 2011;14:665-676.

10 Li J, Olsen J, Vestergaard M, Obel C: Attention-deficit/hyperactivity disorder in the offspring following prenatal maternal bereavement: A nationwide follow-up study in denmark. European child & adolescent psychiatry 2010;19:747-753.

11 Ronald A, Pennell CE, Whitehouse AJ: Prenatal maternal stress associated with adhd and autistic traits in early childhood. Frontiers in psychology 2010;1:223.

12 Van den Hove DL, Blanco CE, Aendekerk B, Desbonnet L, Bruschettini M, Steinbusch HP, Prickaerts J, Steinbusch HW: Prenatal restraint stress and long-term affective consequences. Dev Neurosci 2005;27:313-320.

13 Clarke AS, Wittwer DJ, Abbott DH, Schneider ML: Long-term effects of prenatal stress on hpa axis activity in juvenile rhesus monkeys. Dev Psychobiol 1994;27:257-269.

14 van Os J, Selten JP: Prenatal exposure to maternal stress and subsequent schizophrenia. The may 1940 invasion of the netherlands. Br J Psychiatry 1998;172:324-326.

15 Watson JB, Mednick SA, Huttunen M, Wang X: Prenatal teratogens and the development of adult mental illness. Dev Psychopathol 1999;11:457-466.

16 Buitelaar JK, Huizink AC, Mulder EJ, de Medina PG, Visser GH: Prenatal stress and cognitive development and temperament in infants. Neurobiol Aging 2003;24 Suppl 1:S53-60; discussion S67-58.

17 Dobbing J, Sands J: Growth and development of the brain and spinal cord of the guinea pig. Brain Res 1970;17:115-123.

18 Jensen FE: The role of glutamate receptor maturation in perinatal seizures and brain injury. Int J Dev Neurosci 2002;20:339-347.
19 Diamond A: Close interrelation of motor development and cognitive development and of the cerebellum and prefrontal cortex. Child Dev 2000;71:44-56.
20 Kapoor A, Matthews SG: Short periods of prenatal stress affect growth, behaviour and hypothalamo-pituitary-adrenal axis activity in male guinea pig offspring. J Physiol 2005;566:967-977.
21 Hirst JJ, Walker DW, Yawno T, Palliser HK: Stress in pregnancy: A role for neuroactive steroids in protecting the fetal and neonatal brain. Dev Neurosci 2009;31:363-377.
22 Coe CL, Kramer M, Czeh B, Gould E, Reeves AJ, Kirschbaum C, Fuchs E: Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. Biol Psychiatry 2003;54:1025-1034.
23 Lemaire V, Koehl M, Le Moal M, Abrous DN: Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. Proc Natl Acad Sci U S A 2000;97:11032-11037.
24 Brunton PJ, Russell JA: Prenatal social stress in the rat programmes neuroendocrine and behavioural responses to stress in the adult offspring: Sex-specific effects. J Neuroendocrinol 2010;22:258-271.
25 Jia N, Yang K, Sun Q, Cai Q, Li H, Cheng D, Fan X, Zhu Z: Prenatal stress causes dendritic atrophy of pyramidal neurons in hippocampal ca3 region by glutamate in offspring rats. Developmental neurobiology 2010;70:114-125.
26 Hirst JJ, Yawno T, Nguyen P, Walker DW: Stress in pregnancy activates neurosteroid production in the fetal brain. Neuroendocrinology 2006;84:264-274.
27 Mirmiran M: The function of fetal/neonatal rapid eye movement sleep. Behav Brain Res 1995;69:13-22.
28 Bicikova M, Klak J, Hill M, Zizka Z, Hampl R, Calda P: Two neuroactive steroids in midpregnancy as measured in maternal and fetal sera and in amniotic fluid. Steroids 2002;67:399-402.
29 Yawno T, Hirst JJ, Castillo-Melendez M, Walker DW: Role of neurosteroids in regulating cell death and proliferation in the late gestation fetal brain. Neuroscience 2009;163:838-847.
30 Nguyen PN, Billiards SS, Walker DW, Hirst JJ: Changes in 5alpha-pregnane steroids and neurosteroidogenic enzyme expression in the perinatal sheep. Pediatr Res 2003;53:956-964.
31 Bassett JM, Oxborrow TJ, Smith ID, Thorburn GD: The concentration of progesterone in the peripheral plasma of the pregnant ewe. J Endocrinol 1969;45:449-457.
32 McKendry AA, Palliser HK, Yates DM, Walker DW, Hirst JJ: The effect of betamethasone treatment on neuroactive steroid synthesis in a foetal guinea pig model of growth restriction. J Neuroendocrinol;22:166-174.
33 Yawno T, Yan EB, Walker DW, Hirst JJ: Inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in the late gestation fetal sheep. Neuroscience 2007;146:1726-1733.
34 Kapoor A, Matthews SG: Prenatal stress modifies behavior and hypothalamic-pituitary-adrenal function in female guinea pig offspring: Effects of timing of prenatal stress and stage of reproductive cycle. Endocrinology 2008;149:6406-6415.

522 35 Kapoor A, Leen J, Matthews SG: Molecular regulation of the hypothalamic-pituitary-
523 adrenal axis in adult male guinea pigs after prenatal stress at different stages of gestation. J
524 Physiol 2008;586:4317-4326.
525 36 Rodriguez HA, Ortega HH, Ramos JG, Munoz-de-Toro M, Luque EH: Guinea-pig
526 interpubic joint (symphysis pubica) relaxation at parturition: Underlying cellular processes
527 that resemble an inflammatory response. Reprod Biol Endocrinol 2003;1:113.
528 37 Kelleher MA, Palliser HK, Walker DW, Hirst JJ: Sex-dependent effect of a low
529 neurosteroid environment and intrauterine growth restriction on foetal guinea pig brain
530 development. J Endocrinol;208:301-309.
531 38 Bicikova M, Lapcik O, Hampl R, Starka L, Knuppen R, Haupt O, Dibbelt L: A novel
532 radioimmunoassay of allopregnanolone. Steroids 1995;60:210-213.
533 39 Mueller BR, Bale TL: Sex-specific programming of offspring emotionality after stress
534 early in pregnancy. J Neurosci 2008;28:9055-9065.
535 40 Charil A, Laplante DP, Vaillancourt C, King S: Prenatal stress and brain development.
536 Brain Res Rev 2010;65:56-79.
537 41 Bruckner TA, Nobles J: Intrauterine stress and male cohort quality: The case of
538 september 11, 2001. Soc Sci Med 2013;76:107-114.
539 42 Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV: Reactive
540 astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci
541 2004;24:2143-2155.
542 43 Horner PJ, Palmer TD: New roles for astrocytes: The nightlife of an 'astrocyte'. La
543 vida loca! Trends Neurosci 2003;26:597-603.
544 44 Ramenghi LA, Martinelli A, De Carli A, Brusati V, Mandia L, Fumagalli M, Triulzi F,
545 Mosca F, Cetin I: Cerebral maturation in iugr and appropriate for gestational age preterm
546 babies. Reprod Sci 2011;18:469-475.
547 45 Tolsa CB, Zimine S, Warfield SK, Freschi M, Sancho Rossignol A, Lazeyras F,
548 Hanquinet S, Pfizenmaier M, Huppi PS: Early alteration of structural and functional brain
549 development in premature infants born with intrauterine growth restriction. Pediatr Res
550 2004;56:132-138.
551 46 Khwaja O, Volpe JJ: Pathogenesis of cerebral white matter injury of prematurity.
552 Archives of disease in childhood Fetal and neonatal edition 2008;93:F153-161.
553 47 Hack M, Taylor HG, Drotar D, Schluchter M, Cartar L, Wilson-Costello D, Klein N,
554 Friedman H, Mercuri-Minich N, Morrow M: Poor predictive validity of the bayley scales of
555 infant development for cognitive function of extremely low birth weight children at school
556 age. Pediatrics 2005;116:333-341.
557 48 Dyet LE, Kennea N, Counsell SJ, Maalouf EF, Ajayi-Obe M, Duggan PJ, Harrison M,
558 Allsop JM, Hajnal J, Herlihy AH, Edwards B, Laroche S, Cowan FM, Rutherford MA, Edwards
559 AD: Natural history of brain lesions in extremely preterm infants studied with serial
560 magnetic resonance imaging from birth and neurodevelopmental assessment. Pediatrics
561 2006;118:536-548.
562 49 Wilson-Costello D, Friedman H, Minich N, Siner B, Taylor G, Schluchter M, Hack M:
563 Improved neurodevelopmental outcomes for extremely low birth weight infants in 2000-
564 2002. Pediatrics 2007;119:37-45.
565 50 Levitt NS, Lindsay RS, Holmes MC, Seckl JR: Dexamethasone in the last week of
566 pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates
567 blood pressure in the adult offspring in the rat. Neuroendocrinology 1996;64:412-418.

568 51 Gilbert ME: Alterations in synaptic transmission and plasticity in area ca1 of adult
569 hippocampus following developmental hypothyroidism. Brain Res Dev Brain Res
570 2004;148:11-18.
571 52 Mallard C, Loeliger M, Copolov D, Rees S: Reduced number of neurons in the
572 hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-
573 restriction. Neuroscience 2000;100:327-333.
574 53 Bartsch T, Dohring J, Rohr A, Jansen O, Deuschl G: Ca1 neurons in the human
575 hippocampus are critical for autobiographical memory, mental time travel, and autonoetic
576 consciousness. Proc Natl Acad Sci U S A 2011;108:17562-17567.
577 54 Uno H, Tarara R, Else JG, Suleman MA, Sapolsky RM: Hippocampal damage
578 associated with prolonged and fatal stress in primates. J Neurosci 1989;9:1705-1711.
579 55 Barros VG, Duhalde-Vega M, Caltana L, Brusco A, Antonelli MC: Astrocyte-neuron
580 vulnerability to prenatal stress in the adult rat brain. J Neurosci Res 2006;83:787-800.
581 56 Kadar T, Dachir S, Shukitt-Hale B, Levy A: Sub-regional hippocampal vulnerability in
582 various animal models leading to cognitive dysfunction. J Neural Transm 1998;105:987-
583 1004.
584 57 Herman JP, Spencer R: Regulation of hippocampal glucocorticoid receptor gene
585 transcription and protein expression in vivo. J Neurosci 1998;18:7462-7473.
586 58 Mellon SH, Griffin LD, Compagnone NA: Biosynthesis and action of neurosteroids.
587 Brain Res Brain Res Rev 2001;37:3-12.
588 59 Dranovsky A, Hen R: Hippocampal neurogenesis: Regulation by stress and
589 antidepressants. Biol Psychiatry 2006;59:1136-1143.
590 60 Mychasiuk R, Gibb R, Kolb B: Prenatal stress produces sexually dimorphic and
591 regionally specific changes in gene expression in hippocampus and frontal cortex of
592 developing rat offspring. Dev Neurosci 2011;33:531-538.
593 61 Mychasiuk R, Gibb R, Kolb B: Prenatal stress alters dendritic morphology and
594 synaptic connectivity in the prefrontal cortex and hippocampus of developing offspring.
595 Synapse 2012;66:308-314.
596 62 Sliwowska JH, Barker JM, Barha CK, Lan N, Weinberg J, Galea LA: Stress-induced
597 suppression of hippocampal neurogenesis in adult male rats is altered by prenatal ethanol
598 exposure. Stress 2010;13:301-313.
599 63 Hayashi F, Takashima N, Murayama A, Inokuchi K: Decreased postnatal neurogenesis
600 in the hippocampus combined with stress experience during adolescence is accompanied by
601 an enhanced incidence of behavioral pathologies in adult mice. Mol Brain 2008;1:22.
602 64 Fenske M: Measurement of salivary cortisol in guinea pigs. J Exp Anim Sci
603 1996;38:13-19.
604 65 Kaliste E, Sachser N, Künzl C, Kaiser S: The welfare of laboratory guinea pigs; The
605 welfare of laboratory animals, Springer Netherlands, 2007, vol 2, pp 181-209.
606 66 Glover V, Hill J: Sex differences in the programming effects of prenatal stress on
607 psychopathology and stress responses: An evolutionary perspective. Physiol Behav
608 2012;106:736-740.
609 67 Nguyen PN, Yan EB, Castillo-Melendez M, Walker DW, Hirst JJ: Increased
610 allopregnanolone levels in the fetal sheep brain following umbilical cord occlusion. J Physiol
611 2004;560:593-602.
612

Tables and figure legends:

Table 1: Fetal Physical Characteristics

Sex	Treatment Group	Body weight	Brain to Body weight	Nose-Rump Length	Placenta to Body weight	Heart to Body weight	Liver to Body weight	Adrenal Gland to Body weight	BLR
Male	Cont + Veh (n=10)	98.45 + 4.34	2.49 + 0.07	14.50 + 0.42	5.84 + 0.30	0.50 + 0.03	5.06 + 0.14	0.03 + 0.002	0.49 + 0.02
	Cont + Allo (n=10)	92.79 + 4.39	2.66 + 0.08	14.20 + 0.22	5.55 + 0.20	0.47 + 0.04	4.49 + 0.22	0.03 + 0.003	0.60 + 0.06
	Stress + Veh (n=10)	86.09 + 4.25	2.71 + 0.12	14.61 + 0.23	5.69 + 0.23	0.59 + 0.05	4.66 + 0.22	0.03 + 0.003	0.56 + 0.03
	Stress + Allo (n=13)	89.61 + 3.36	2.72 + 0.12	14.25 + 0.19	5.51 + 0.22	0.53 + 0.03	4.49 + 0.22	0.04 + 0.006	0.61 + 0.04
Female	Cont + Veh (n=7)	89.39 + 4.72	2.87 + 0.11[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 (n=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 (n=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 (n=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 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. [a] indicates significant ($p<0.05$) effect of stress, and [b] 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 (hatched bars) or control (handling without stress exposure, open bars) episodes that were performed at 50, 55, 60 and 65 days GA. Stress exposed (n=14) and control (n=14) groups contain both vehicle and allopregnanolone treated guinea pig dams. '*' p=0.05; '**' p<0.01 and '†' p<0.001 indicates significance level between changes seen between control and stress groups.

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.

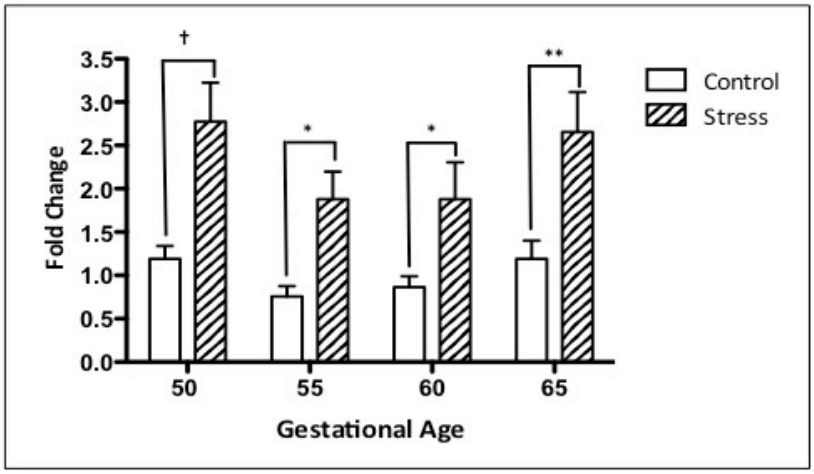

Figure 1

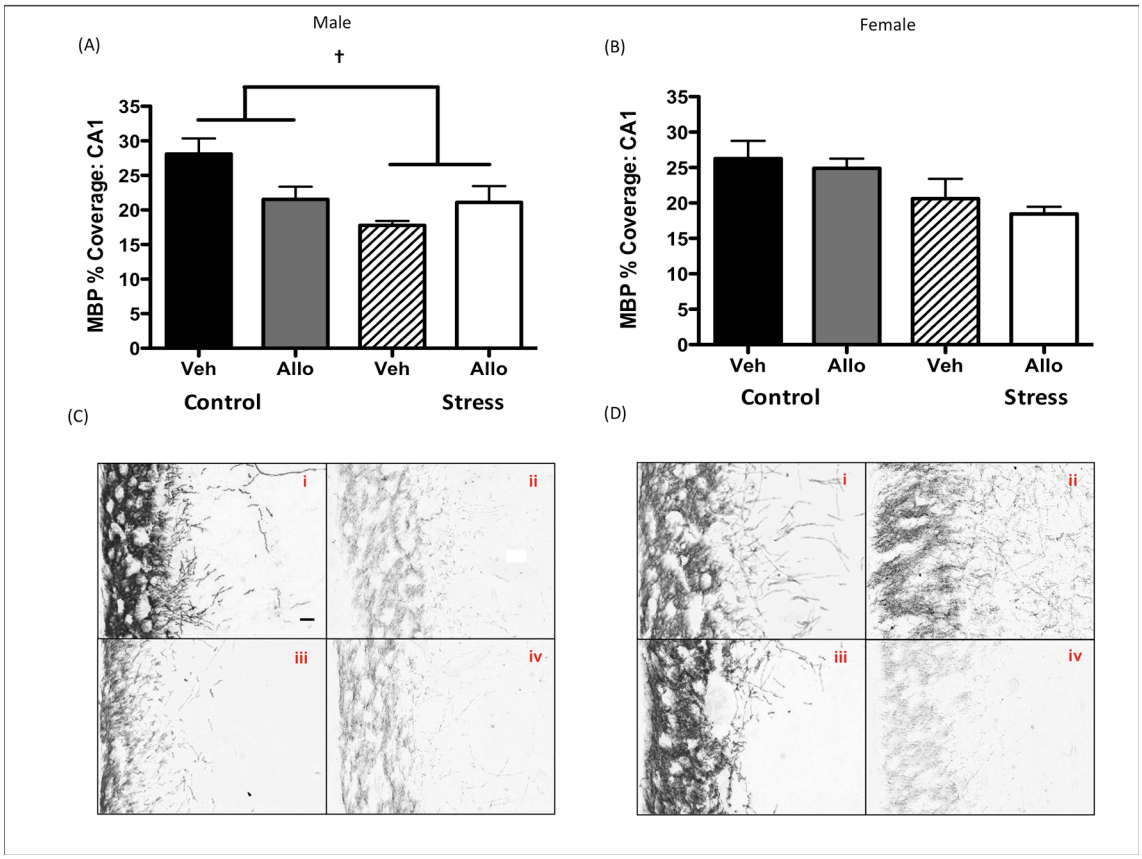

Figure 2

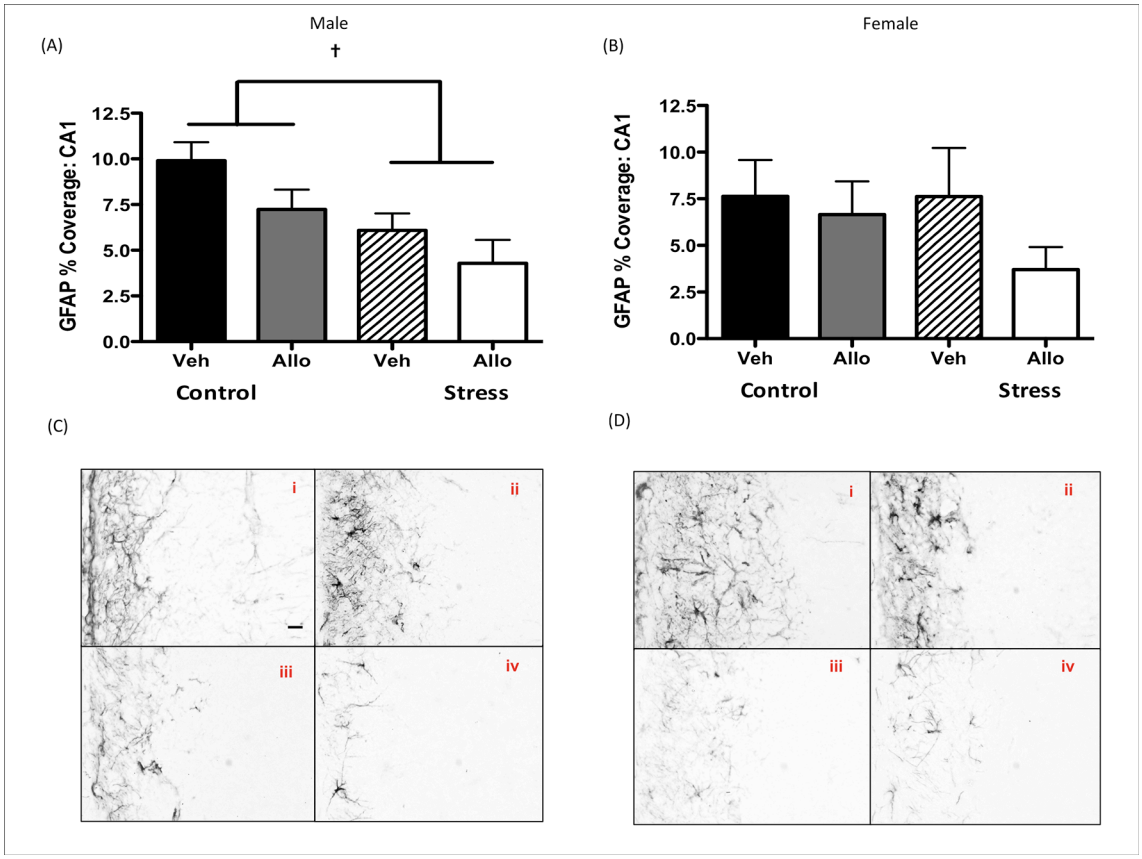

Figure 3

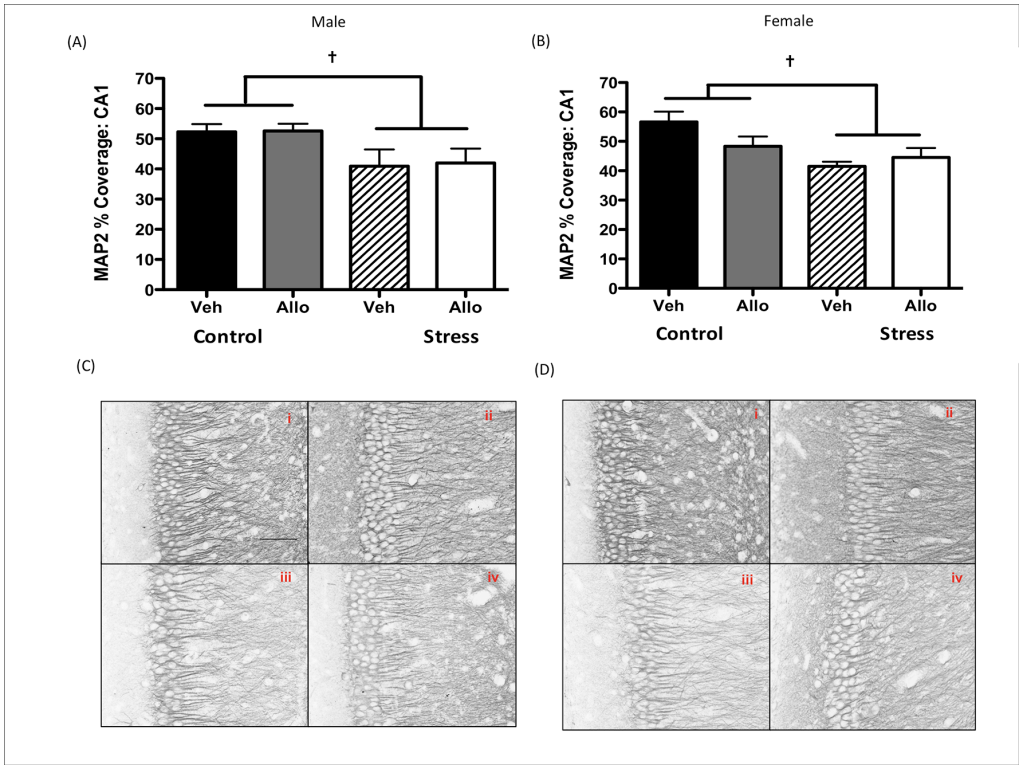

Figure 4

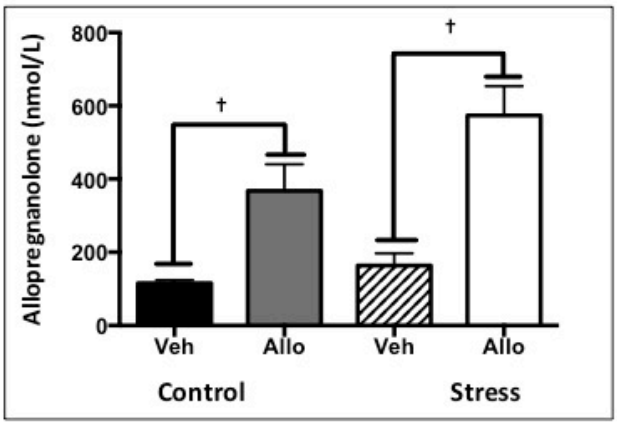

Figure 5

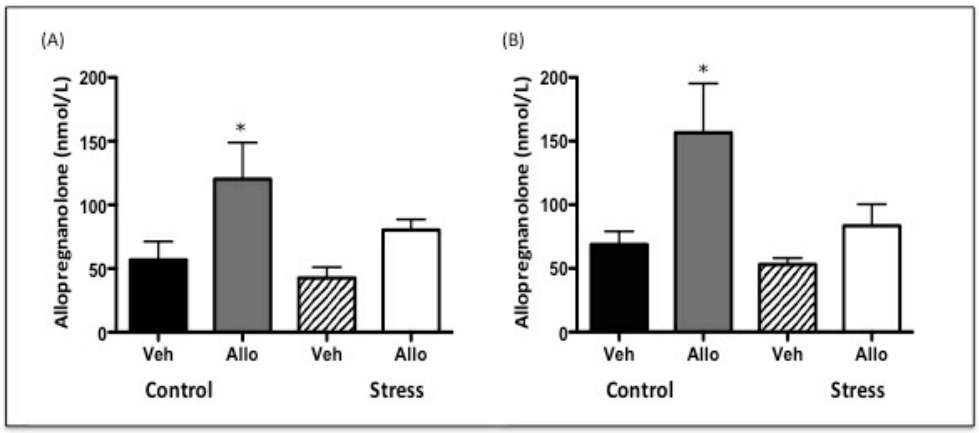

Figure 6