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1 **Female HPA axis displays heightened sensitivity to pre-pubertal stress**

2 **Running title: Pre-pubertal stress and adult HPA axis**

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22 **Abstract**

23 Early life stress (ELS) is a risk factor in the development of psychiatric disorders. The underlying  
24 biological mechanisms governing this phenomenon are not fully understood, but dysregulation of  
25 stress responses is likely to play a key role. Males and females differ in their propensity to develop  
26 psychiatric disorders, with far higher rates of anxiety, major depressive disorder, affective disorders  
27 and post-traumatic stress disorder found in women. We hypothesised that sex differences in response  
28 to ELS may play a crucial role in differential vulnerability between the sexes. To test this, we evaluated  
29 the consequences of pre-pubertal stress (PPS) on the HPA axis in adult female and male Lister Hooded  
30 rats. PPS animals were exposed to swim, restraint and elevated platform stress on postnatal days 25-  
31 27, controls remained in their home cage. Once adult, animals were either a) sacrificed directly and  
32 brains collected or b) sacrificed 20 minutes or 1 week after a social test and trunk blood collected. In  
33 the female hippocampal formation, PPS increased expression of *FKBP5* and *AVPR1a*. In the female  
34 prefrontal cortex, PPS resulted in increased glucocorticoid receptor expression, increased  
35 glucocorticoid:mineralocorticoid (*GR:MR*) receptor expression ratio and decreased *AVPR1a*  
36 expression. Females exposed to PPS did not show the normal rise in blood corticosterone levels  
37 following a social interaction test. In contrast, PPS did not alter the expression of oxytocin or oxytocin  
38 receptors, and no effects of PPS were seen in males. However, striking sex differences were found.  
39 Females had higher oxytocin receptor expression in the prefrontal cortex and *AVPR1a* and oxytocin  
40 expression in the hypothalamus, whereas males demonstrated higher expression of *GR*, *MR*, *GR:MR*,  
41 *FKBP5* and oxytocin receptor in the hypothalamus. These results demonstrate heightened reactivity  
42 of the female HPA axis to PPS and may help explain why in humans females display an increased  
43 susceptibility to certain stress-related psychopathologies.

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47 Lay Summary

48 Women are at greater risk of developing several psychiatric illnesses. Using a rodent model, we show  
49 that the female stress system is more reactive to the lasting effects of early life stress. This heightened  
50 reactivity of the female stress response may help explain why women are at a greater risk of  
51 developing psychiatric disorders.

52 **Keywords:** pre-pubertal stress, HPA axis, sex differences, GR, MR, FKBP5

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68 **Introduction**

69 Adverse experiences early in life are linked with an increased risk of developing psychiatric disorders  
70 later in life(Heim & Nemeroff, 2001; Juruena, Baes, Menezes, & Graeff, 2015; Teicher & Samson, 2016;  
71 Teicher, Samson, Anderson, & Ohashi, 2016). Dysregulation of the stress response is a potential  
72 mechanism through which early life stress (ELS) increases vulnerability to illness. Prolonged or  
73 excessive stress may lead to a maladaptive stress response, and when experienced early in life could  
74 also alter brain development, increasing vulnerability to psychiatric disorders. Stress results in several  
75 adaptive physiological and behavioural responses, a major mediator of this is the hypothalamic-  
76 pituitary-adrenal (HPA) axis.

77 Both psychological and physical stressors result in the release of corticotrophin releasing  
78 hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the  
79 hypothalamus. These neuropeptides act on the pituitary, stimulating the release of  
80 adrenocorticotrophic hormone (ACTH) which in turn causes the release of glucocorticoid stress  
81 hormones (corticosterone in rodents, cortisol in humans (CORT)) from the adrenal cortex(de Kloet,  
82 Joels, & Holsboer, 2005). Glucocorticoids cross the blood brain barrier and bind to corticosteroid  
83 receptors (CR: glucocorticoid (GR) and mineralocorticoid (MR) receptors) distributed throughout the  
84 brain. Feedback mechanisms then ensure the response is terminated in a healthy system. In contrast  
85 to AVP, the closely related neuropeptide oxytocin (OXT) inhibits the activity of the HPA axis(Neumann  
86 & Landgraf, 2019). HPA axis dysfunction is prevalent in psychiatric illness, for example HPA axis  
87 hyperactivity is often found in major depression and bipolar disorder, and increased or decreased HPA  
88 axis activity may be a direct consequence of ELS(Juruena, Cleare, & Young 2018; Murri et al., 2016;  
89 Zorn et al., 2017).

90 Long-term effects of ELS on the HPA axis differ between the sexes. Early trauma is associated  
91 with a more severely blunted cortisol response to social stress in women, and fewer stressful events  
92 early in life are required to trigger liability to PTSD in women. Conversely, lower levels of recent stress

93 are capable of provoking major depression in men than women (Bunea, Szentagotai-Tatar, & Miu,  
94 2017; McLaughlin, Conron, Koenen, & Gilman, 2010). Furthermore, women display 2-3 times higher  
95 rates of anxiety, affective disorders, major depressive disorder and post-traumatic stress disorder  
96 (PTSD)(Christiansen & Hansen, 2015; Kessler et al., 2003; Kessler, Chiu, Demler, Merikangas, &  
97 Walters, 2005; Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Remes, Brayne, van der Linde, &  
98 Lafortune, 2016). These differences are not purely attributable to sex-specific life experiences; studies  
99 controlling for stressful life events and sex-specific risk factors still find higher prevalence in  
100 women(Tiwari & Gonzalez, 2018). Sex differences in HPA axis function may underlie this. Basal  
101 secretion of CORT from the adrenal gland is higher in females than males, and this is attributed to sex-  
102 differences in gonadal hormones, with estrogen sensitising and testosterone dampening the HPA-  
103 axis(Heck & Handa, 2019; Seale et al., 2004).

104 Animal studies demonstrate that ELS has profound implications for later HPA axis function.  
105 Prenatal and early post-natal stressors alter basal and stress-induced corticosterone release from the  
106 adrenal glands, brain corticosteroid receptor (*GR* and *MR*) expression, as well as expression of AVP  
107 and OXT in a timing and sometimes sex-specific manner(Llorente et al., 2011; Lupien, McEwen,  
108 Gunnar, & Heim, 2009; Neumann & Landgraf, 2019; Schroeder, Notaras, Du, & Hill, 2018; Tobon,  
109 Newport, & Nemeroff, 2018). However, despite well-established sex differences in the HPA axis,  
110 comparatively few preclinical studies include male and female animals. Compared to the prenatal and  
111 post-natal periods, less is known about the effects of stress experienced in the post-weaning, pre-  
112 pubertal phase (PPS), a time-point suggested as more akin to human childhood(Brydges, 2016). The  
113 limbic system and prefrontal cortex are undergoing maturation during this period, areas which are  
114 crucial for cognition and emotion and are extremely stress reactive due to high densities of CR,  
115 particularly in the hippocampal formation(Herman, 1993).

116 The present study investigated the effects of PPS on long-term neurochemical and molecular  
117 alterations in the adult HPA axis in male and female animals by measuring the brain regional

118 expression of CR (*GR* and *MR*), *AVP*, *OXT* and their receptors (AVP receptor 1a (*AVPR1a*) and oxytocin  
119 receptor (*OXTR*)) and *FKBP5*. *FKBP5* encodes the FK506 binding protein 51 co-chaperone protein of  
120 the GR complex, and is extremely responsive to stress(Wochnik et al., 2005). When *FKBP5* is bound to  
121 the GR complex, *CORT* binds with lower affinity and nuclear translocation of the receptor is less  
122 efficient, decreasing negative feedback regulation of the HPA axis(Wochnik et al., 2005). There is  
123 evidence that genetic modifications in *FKBP5* interact with childhood, but not adulthood stress to  
124 increase risk for several psychiatric disorders(Matossin, Halldorsdottir, & Binder, 2018). We also  
125 measured plasma corticosterone following a social test in adult rats as a behavioural measure of  
126 altered HPA axis function. Altered social function is a core component of several adult psychiatric  
127 illnesses and ELS has been shown to impact on social behaviour and functioning in both animal and  
128 human studies(Nicol, Pope, Romaniuk, & Hall, 2015; Palmier-Claus et al., 2016; Sandi & Haller, 2015).  
129 Furthermore, early life trauma is associated with blunted cortisol responses to social stress in humans,  
130 particularly in women(Bunea et al., 2017). For this reason, we elected to focus on corticosterone  
131 rather than other components of the HPA axis, such as ACTH. Further studies are need to determine  
132 whether ACTH reflects the sex differences we observed in corticosterone.

133 We hypothesised that PPS would alter the expression of *GR*, *MR*, *GR:MR* ratio, *AVPR1a*, *AVP*  
134 and *OXT* in the rodent brain, and the direction of change would be region and receptor/neuropeptide  
135 specific. Given their higher vulnerability to stress-related psychiatric illnesses, we hypothesised  
136 dysregulation resulting from PPS would be more pronounced in females. We also hypothesised that  
137 corticosterone responses to social stress would be blunted in both males and females, but more  
138 exaggerated in females, as early life trauma is often associated with a more pronounced blunting of  
139 the corticosterone response in females(Bunea et al., 2017; McLaughlin et al., 2010).

140

141 **Methods**

142 *Animals.* Male and female Lister Hooded rats were bred at Cardiff University from 16 adult pairs  
143 (Charles River). Females were primiparous. Litters ranged between 11 and 18 animals, with an average  
144 of 14.5, and an average sex-ratio of 6.2 males to 8.3 females. All litters were used and weaning from  
145 the birth dams took place on postnatal day (PND) 21, and offspring were housed in groups of 2-4 in  
146 same litter, same sex cages (32cm x 50cm x 21cm) lined with wood shavings. Light was maintained on  
147 12:12 hour light/dark cycle, a wooden stick, nesting material and cardboard tube were provided for  
148 enrichment and food and water provided *ad libitum*. All experiments were approved by Cardiff  
149 University's Animal Welfare and Ethical Review Body and adhered to the UK Home Office Animals  
150 (Scientific Procedures) Act 1986 and European regulations on animal experimentation.

151 *Pre-pubertal stress (PPS).* Half of the offspring (8 litters) were pseudo-randomly allocated to a PPS  
152 protocol (Jacobson-Pick & Richter-Levin, 2010) on PND 25-27 such that litters and sexes were equally  
153 distributed between treatment groups (PPS/control, male/female). PPS took place in a designated  
154 room separate to the holding room, with regular room lighting. On PND 25, animals were placed into  
155 an opaque swim tank (25cm high, 34cm diameter) filled with 6L of 25±1°C water for a 10 minute swim  
156 stress. On PND26 the rats were restrained in plastic restraint tubes (15cm length 5cm diameter) for  
157 3x30 minute sessions (separated by 30 minute breaks in the home cage) and lastly on PND27 they  
158 were exposed to elevated platforms (15x15cm, 115cm high) for 3x30 minute sessions (separated by  
159 60 minute breaks in the home cage). **Animals were observed by the experimenter during all stress**  
160 **procedures, and males and females reacted in a similar manner to each stressor.** Following PPS,  
161 animals were left undisturbed until adulthood aside from weekly cage cleaning. Control animals were  
162 left undisturbed from weaning until adulthood, aside from weekly cage cleaning.

163 *RT-qPCR.* Forty rats (male: 12 control, 10 PPS; female: 8 control, 10 PPS) were sacrificed at PND 60-70  
164 using a rising concentration of CO<sub>2</sub>. Brains were removed, dissected and stored at -80°C until analysis.  
165 Total cell RNA was extracted from hippocampal formation, prefrontal cortex and hypothalamus using  
166 the Qiagen RNeasy Kit (Qiagen, Manchester, UK) and DNase treated in accordance with the supplied

167 protocols. RNA was used to create cDNA for analysis using RNA to cDNA Easy Premix (Clontech  
168 Laboratories, France), heated at 42°C for 75 minutes, followed by 80°C for 15 minutes. Sample was  
169 then diluted 1:15 in nuclease-free water. 96-well plates were loaded, each well containing a total of  
170 15µl reaction mixture (1.9µl sterile RNAase free water, 0.3µl 10µM forward primer, 0.3µl 10µM  
171 reverse primer, 7.5µl SensiMix (Bioline) and 5µl cDNA). *Gapdh* and *Hprt1* primers (Sigma) were used  
172 as housekeeping controls and all results were normalised from these values. After loading, plates were  
173 spun down at 3,000 rpm for approximately 10-20 seconds before being transferred to Real-Time PCR  
174 instrument (Applied Biosystems®) and run for 45 cycles (95°C for 20s, 60°C for 20s, 72°C for 20s). The  
175 expression of *GR*, *MR*, *AVP*, *OXT*, *AVPR1a*, *OXTR* and *FKBP5* was measured (see Table 1 for primers).

176 *Social test.* Sixty-two animals (females: 22 control, 18 PPS; male: 12 control, 10 PPS) were given a social  
177 test in same-sex pairs in adulthood (PND 60-67). Three hours before testing animals were single  
178 housed in the holding room, and one hour before testing transferred to the testing room. All animals  
179 were given an intraperitoneal injection of a vehicle (15%DMSO, 2% Tween 80 in 0.9% saline) 30  
180 minutes before testing as part of a design to measure the effect of PPS on social behaviour directly,  
181 an experiment which included a drug treated group. PPS had a significant effect on social interaction,  
182 and this behavioural data is reported elsewhere (Brydges et al. under review). Animals were weighed  
183 on the day of testing and placed in weight-matched pairs (weight difference did not exceed 20g) into  
184 a clear acrylic arena (65cmx65cmx40cm high) on the floor in the middle of a dimly lit room (45lux) for  
185 15 minutes. Animal pairs were from the same group, control or PPS, but different litters so were  
186 strangers to each other.

187

188 *Corticosterone ELISA.* One animal from each pair was sacrificed 20 minutes after the social test to  
189 investigate corticosterone responses to social interaction, the other sacrificed one week later for  
190 baseline analysis. Animals were decapitated and trunk blood was collected using EDTA microvette  
191 collection tubes (Sarstedt, Germany). Blood was spun at 1500 x g for 10 minutes, plasma was removed

192 and stored at -20°C until analysis. Corticosterone was analysed by ELISA, according to the  
193 manufacturer's instructions (Abcam, UK, ab108821). The sensitivity of this ELISA is 0.28ng/ml and the  
194 intra-assay coefficient of variation is 5.3%. Samples were run in triplicate on several plates,  
195 counterbalancing between groups.

196

## 197 **Data analysis**

198 JMP statistical software (SAS Institute, Cary, NC, USA) was used to run generalised linear models. For  
199 mRNA analysis, group (control/PPS), sex and group\*sex were fitted as factors and mRNA expression  
200 (normalised to *GAPDH* & *Hprt1*) as response. For corticosterone, group (control/PPS), time of sacrifice  
201 (baseline vs 20 mins post social testing), sex and all two and three way interactions were fitted as  
202 factors, corticosterone level as response. For all models, litter was nested within group and fitted as  
203 a random factor to account for the use of multiple animals per litter. Data were checked for normality  
204 and homogeneity of variance. Post-hoc t-tests were used when significant interactions were found.  
205 The most relevant statistics are reported below, please see Table 2 for a full statistical summary.

206

## 207 **Results**

208 *mRNA* – Hippocampal formation. *FKBP5* (group\*sex:  $F_{1,29.85}=4.33$ ,  $p=0.04$ , Fig. 1a) and *AVPR1a*  
209 (group\*sex:  $F_{1,26.64}=4.47$ ,  $p=0.04$ , Fig 1b) expression was significantly higher in the female hippocampal  
210 formation following PPS, whereas *GR* (group:  $F_{1,7.63}=0.01$ ,  $p=0.92$ ), *MR* (group:  $F_{1,9.44}=0.86$ ,  $p=0.38$ ),  
211 *GR:MR* (group:  $F_{1,8.06}=0.88$ ,  $p=0.38$ ), *AVP* (group:  $F_{1,10.93}=0.62$ ,  $p=0.45$ ), *OXT* (group:  $F_{1,9.56}=0.26$ ,  $p=0.62$ )  
212 and *OXTR* (group:  $F_{1,9.84}=0.02$ ,  $p=0.9$ ) were unchanged in males and females.

213 PFC. In the female PFC, PPS resulted in significantly higher *GR* expression (group\*sex:  $F_{1,24.94}=7.17$ ,  
214  $p=0.01$ , Fig. 2a), a higher *GR:MR* ratio (group\*sex:  $F_{1,25.37}=4.97$ ,  $p=0.03$ , Fig. 2b) and reduced *AVPR1a*  
215 expression (group\*sex:  $F_{1,23.17}=6.94$ ,  $p=0.01$ , Fig. 2c) when compared to control females. *MR* (group:

216  $F_{1,8.81}=0.14, p=0.72$ ), *AVP* (group:  $F_{1,9.77}=0.87, p=0.37$ ), *OXT* (group:  $F_{1,10.34}=0.17, p=0.68$ ), *OXTR* (group:  
217  $F_{1,10.39}=0.16, p=0.7$ ) and *FKBP5* (group:  $F_{1,9.23}=1.43, p=0.26$ ) were unchanged in male and female PFC  
218 following PPS, but *OXTR* expression was higher in females than males (sex:  $F_{1,27.9}=28.65, p<0.0001$ , Fig.  
219 2d).

220 Hypothalamus. In the hypothalamus, *GR* (sex:  $F_{1,30.7}=13.68, p<0.001$ ), *MR* (sex:  $F_{1,25.52}=63.73,$   
221  $p<0.0001$ ), *GR:MR* (sex:  $F_{1,28.33}=8.93, p<0.01$ ), *FKBP5* (sex:  $F_{1,26.23}=43.7, p<0.0001$ ) and *OXTR* (sex:  
222  $F_{1,29.24}=118.81, p<0.0001$ ) were lower in females than males regardless of treatment (Fig 3a-e),  
223 whereas *AVPR1a* (sex:  $F_{1,25.55}=27.06, p<0.0001$ ) and *OXT* (sex:  $F_{1,26.76}=42.06, p<0.0001$ , Fig. 3f-g) were  
224 higher in females. There was no effect of PPS on *GR* (group:  $F_{1,10.79}=0.08, p=0.78$ ), *MR* (group:  $F_{1,9}=0.53,$   
225  $p=0.49$ ), *GR:MR* (group:  $F_{1,11.03}=0.15, p=0.71$ ), *AVP* (group:  $F_{1,7.69}=0.86, p=0.38$ ), *AVPR1a* (group:  
226  $F_{1,10.42}=0.04, p=0.84$ ), *OXT* (group:  $F_{1,10.33}=0.4, p=0.54$ ), *OXTR* (group:  $F_{1,10.84}=0.43, p=0.53$ ) or *FKBP5*  
227 (group:  $F_{1,10.64}=0.63, p=0.44$ ), expression. See Table 3 for summary of regional gene expression  
228 changes.

229 *Corticosterone*. There was no effect of PPS on baseline expression of plasma corticosterone, but 20  
230 minutes after a social interaction test PPS blunted the normal corticosterone rise in females  
231 (group\*sex\*time of sacrifice:  $F_{7,37.77}=3.53, p=0.0052$ , Fig. 4., Table 3).

232

## 233 **Discussion**

234 PPS altered the expression of receptors and neuropeptides involved in HPA axis function in the  
235 hippocampal formation and PFC, but not hypothalamus of adult females, whereas in males the  
236 expression of major HPA axis components were unaffected in all brain regions studied. In the female  
237 prefrontal cortex, PPS increased *GR* and *GR:MR* receptor ratio and reduced *AVPR1a* expression. In the  
238 female hippocampal formation, PPS increased expression of *FKBP5* and *AVPR1a*. In the periphery,  
239 females exposed to PPS did not show the normal rise in blood corticosterone levels following a social

240 interaction test. We also found sex differences in baseline gene expression, particularly in the  
241 hypothalamus.

242 GR and MR are nuclear receptors/transcription factors which mediate the actions of  
243 glucocorticoid stress hormones, playing a key role in the stress response and also regulation of brain  
244 development and neuronal plasticity(Liston & Gan, 2011). These corticosteroid receptors (CR) act  
245 through delayed, long-lasting transcription-dependent mechanisms, but also exert more rapid effects  
246 which dampen the activated HPA axis in a negative feedback, transcription-independent  
247 manner(Gjerstad, Lightman, & Spiga, 2018; Tasker & Herman, 2011). Distributed throughout the brain,  
248 CR expression is highest in the limbic system(Herman, 1993). We found that PPS had no effect on *GR*  
249 or *MR* expression in male or female hippocampal formation. In contrast, stressors applied at earlier  
250 time points (e.g. prenatal stress and maternal separation) generally decrease hippocampal CR  
251 expression in males and females (although precise effects can vary depending on nature of the stress)  
252 (Aisa, Tordera, Lasheras, Del Rio, & Ramirez, 2008; Brunton & Russell, 2010; Kapoor, Dunn, Kostaki,  
253 Andrews, & Matthews, 2006; Levitt, Lindsay, Holmes, & Seckl, 1996; Maccari et al., 1995; Plotsky &  
254 Meaney, 1993; van Bodegom, Homberg, & Henckens, 2017; Welberg, Seckl, & Holmes, 2001).  
255 Stressors at later time points produce different effects, with chronic variable stress in adolescence  
256 decreasing *GR* in the male hippocampus, and increasing/decreasing *MR* in male/female hippocampus  
257 respectively(Isgor, Kabbaj, Akil, & Watson, 2004; Llorente et al., 2011). One study using mice found  
258 that PPS increased *MR* and decreased *GR:MR* ratio in the hippocampus of adult male and female  
259 animals(Brydges et al., 2014). Although PPS did not alter hippocampal CR expression directly in the  
260 present study, we did find evidence of altered CR activity following PPS in females through increased  
261 expression of *FKBP5*. *FKBP5* is a co-chaperone of heat shock protein 90 (hsp90) which regulates GR  
262 sensitivity. Activation of GR leads to increased expression of *FKBP5*, creating an ultrashort negative  
263 feedback loop which inhibits GR signalling(Wochnik et al., 2005; Zannas, Wiechmann, Gassen, &  
264 Binder, 2016). Therefore, increased *FKBP5* likely indicates increased GR activity. Indeed, increased  
265 expression of *FKBP5* in the limbic system (hippocampus and amygdala) is associated with increased

266 stress responsiveness (anxiety) and decreased stress coping behaviours, whereas experimental  
267 reduction of *FKBP5* has opposite effects(Touma et al., 2011; Zannas et al., 2016).

268         The brain undergoes significant development postnatally, and the PFC is one of the last brain  
269 regions to mature, undergoing synaptic remodelling in childhood and adolescence(Barfield & Gourley,  
270 2018). Therefore pre-pubertal and adolescent stress may be particularly detrimental for the PFC, yet  
271 little is known of the effects of **early life stress (ELS)** on CR expression in this region(Patel, Katz, Karssen,  
272 & Lyons, 2008). In agreement with a previous study, we found that PPS did not impact *GR* or *MR*  
273 expression in the male PFC(Fuentes, Carrasco, Armario, & Nadal, 2014). However, PPS did increase *GR*  
274 and *GR:MR* ratio in the female PFC. Stress at an earlier timepoint, between PND 7-14, increased *GR*  
275 expression in the PFC of female and male rats, again highlighting the importance of timing and  
276 sex(Alteba, Korem, & Akirav, 2016). We found no evidence of altered *FKBP5* in the PFC following PPS,  
277 but prenatal stress and maternal separation in rats decreases *FKBP5* expression in the male PFC with  
278 no effects in the hippocampus, whereas chronic unpredictable stress in adolescence increases *FKBP5*  
279 in the male hippocampus, PFC and amygdala(Szymanska et al., 2009; van der Doelen et al., 2014; Xu  
280 et al., 2017; Xu et al., 2019). Overall our results suggest that PPS alters CR function in the adult  
281 hippocampus and PFC, and this effect is specific to females.

282         PPS did not impact baseline corticosterone in males or females in agreement with the majority  
283 of previous rodent research(Fuentes et al., 2014; Grigoryan, Ardi, Albrecht, Richter-Levin, & Segal,  
284 2015; Jacobson-Pick & Richter-Levin, 2010). In humans, studies have found increased, decreased and  
285 no change in basal cortisol following ELS(Agorastos, Pervanidou, Chrousos, & Baker, 2019; Lupien et  
286 al., 2009). Differences are likely attributable to variation in the nature and timing of stress as well as  
287 genetics, factors which are rarely considered in human studies. In the present study, exposure to social  
288 interaction with a stranger resulted in elevated corticosterone in the plasma of control females and  
289 all males, but this response was blunted in females with experience of PPS. Note that the increases in  
290 corticosterone are not an acute response to systemic vehicle administration but are a result of the

291 social interaction, since all animals, both baseline and socially experienced rats, received vehicle  
292 injections. Furthermore, the injection occurred sixty-five minutes before sacrifice, so any acute  
293 corticosterone rise resulting from this would no longer be detectable. In contrast to the results  
294 presented here, mild prenatal stress results in *heightened* corticosterone response to restraint stress  
295 in adult females(Aisa et al., 2008). Interestingly, more prolonged prenatal stress is necessary to induce  
296 the same effects in males(Gobinath, Mahmoud, & Galea, 2015). Maternal separation elevates or  
297 blunts male and female corticosterone responses to restraint stress, depending on the  
298 study(Desbonnet, Garrett, Daly, McDermott, & Dinan, 2008; Lehmann, Russig, Feldon, & Pryce, 2002;  
299 Roman, Gustafsson, Berg, & Nylander, 2006), and chronic variable adolescent stress between PND 45-  
300 58/37-49 blunted corticosterone responses to a stressor in adult females but not males(Bourke &  
301 Neigh, 2011; Wulsin, Wick-Carlson, Packard, Morano, & Herman, 2016). This again suggests that the  
302 female HPA axis is more sensitive to ELS, although specific outcomes are mediated by exact timing of  
303 stress and adult testing paradigm (e.g. social vs restraint). An adaptive stress response is characterised  
304 by a rapid **corticosterone or cortisol** (CORT) increase, followed by a progressive decline. Excessive or  
305 repeated activation of the HPA axis and release of CORT can lead to blunted CORT secretion in  
306 response to acute stress(Kinlein, Wilson, & Karatsoreos, 2015). A healthy CORT response is necessary  
307 for appropriate behaviour and survival, therefore a blunted CORT response to acute stress may be  
308 considered a maladaptive phenotype.

309 PPS increased expression of *AVPR1a* in the female hippocampal formation and decreased it in  
310 the PFC. No changes were observed in the hypothalamus. OXT and AVP are closely related  
311 neuropeptides that exert opposite effects on the HPA axis. Stress results in the release of  
312 hypothalamic AVP, which stimulates the release of adrenocorticotrophic hormone from the pituitary  
313 and eventual production of CORT(de Kloet et al., 2005). In contrast, OXT dampens the HPA  
314 axis(Neumann & Landgraf, 2019). Both AVP and OXT exert effects on behaviour through OXTR and  
315 AVPR1a/AVPR1b situated in the brain(Song & Albers, 2018). Effects of prenatal stress on AVP/OXT  
316 systems are mixed, with some studies finding decreased OXT/AVP expression in the male

317 hypothalamus, others no changes in males or females (Desbonnet et al., 2008; Lee, Brady, Shapiro,  
318 Dorsa, & Koenig, 2007; Schmidt et al., 2018). Poor maternal care in rodents decreases OXT and OXTR  
319 expression centrally (hypothalamus and amygdala) and peripherally (blood plasma) in female  
320 animals (Francis, Young, Meaney, & Insel, 2002; Tobon, Jeffrey, & Nemeroff, 2018), whereas maternal  
321 separation increases hypothalamic AVP and alters OXT expression and OXT/AVP receptor binding in  
322 an age and sex-specific manner (Lukas, Bredewold, Neumann, & Veenema, 2010; Murgatroyd et al.,  
323 2009; Veenema, Bredewold, & Neumann, 2007; Veenema & Neumann, 2009). Our previous work  
324 found PPS increased protein levels of AVP in the supraoptic (but not paraventricular) nucleus of the  
325 hypothalamus and blood plasma in male and female rats (*Brydges et al. in Review*). In the present  
326 study, the hypothalamus was analysed as a whole, it is possible differences may have been found if  
327 the supraoptic and paraventricular nuclei had been analysed separately. Alternatively, PPS may alter  
328 translation rather than transcription of AVP in this region. Considering their opposing effects on  
329 behaviour (AVP exerts anxiogenic and depressive-like effects, whereas OXT is an endogenous  
330 anxiolytic), the balance of AVP and OXT in the brain is thought crucial for appropriate emotional  
331 behaviours (Mak, Broussard, Vacy, & Broadbear, 2012; Neumann & Landgraf, 2012). In the present  
332 study, we find altered *AVPR1a* expression in the female limbic system in the absence of altered  
333 OXT/OXTR expression. This indicates a dysregulated HPA axis which may predispose towards anxiety  
334 or depressive phenotypes following stress (Lesse, Rether, Groger, Braun, & Bock, 2017; Neumann &  
335 Slattery, 2016; Nowacka-Chmielewska, Kasprowska-Liskiewicz, Barski, Obuchowicz, & Malecki, 2017).

336 PPS increased *AVPR1a* expression in the female hippocampal formation, yet decreased  
337 expression in the PFC. Bi-directional projections exist between the hippocampus and hypothalamus  
338 (production site of AVP): the hippocampus is a target of AVP and is capable of decreasing AVP  
339 expression in the hypothalamus (Nettles, Pesold, & Goldman, 2000; Zhang & Hernandez, 2013). PPS  
340 leads to increased AVP (*Brydges et al. under review*), therefore increased *AVPR1a* expression in the  
341 hippocampal formation may be a compensatory mechanism, enhancing the sensitivity to and  
342 subsequent inhibitory effects of the hippocampus on hypothalamic AVP secretion. The PFC is also

343 thought to exert inhibitory effects over the hypothalamus, but direct connections between these two  
344 structures are lacking, and it is hypothesised that the PFC may act via other structures to exert this  
345 influence(Spencer, Buller, & Day, 2005). Whether the decreased *AVPR1a* expression following PPS in  
346 this region is due to adaptation or pathology remains to be elucidated.

347 Striking sex differences were seen regardless of PPS. *GR*, *MR*, *GR:MR*, *FKBP5* and *OXTR*  
348 expression were significantly higher in male than female hypothalamus, whereas *AVPR1a* and *OXT*  
349 showed the opposite pattern. *OXTR* expression was higher in female PFC. These findings are consistent  
350 with previous studies finding *AVPR1a* expression is higher in female vs male rodents, and *GR*, *MR* and  
351 *OXTR* expression higher in the male hypothalamus (although species, age and region studied can all  
352 affect direction of difference)(Albers, 2015; Bale & Dorsa, 1995; Dumais, Bredewold, Mayer, &  
353 Veenema, 2013; Smith et al., 2017; Turner, 1990). One study investigating binding in 35 different  
354 rodent brains regions similarly found sex differences in *OXTR* and *AVPR1a* expression  
355 (increased/decreased depending on region)(Smith et al., 2017), but less is known about *FKBP5*. These  
356 sex differences may confer a natural heightened reactivity to stress in females which may underlie the  
357 greater vulnerability of the female HPA axis to ELS.

358 The balance between MR and GR functioning is thought crucial for appropriate HPA axis  
359 function, and dysregulation and imbalance between CR is suggested as a candidate mechanism  
360 underlying psychiatric disorders such as major depression, a disorder which has been repeatedly  
361 associated with hyperactive HPA axis function(de Kloet et al., 2005; Juruena et al., 2015; Oitzl,  
362 Champagne, van der Veen, & de Kloet, 2010). Polymorphisms associated with enhanced expression of  
363 *FKBP5* following GR activation are overrepresented in major depression, bipolar and PTSD(Binder,  
364 2009; Matosin et al., 2018). *FKBP5* is implicated in a number psychiatric disorders, particularly in  
365 combination with early life stress(Wang, Shelton, & Dwivedi, 2018). In humans, there is an interaction  
366 between *FKBP5* (FK506 binding protein 5) variability and childhood trauma on psychosis, paranoia,  
367 social stress appraisal and prefrontal cortex function(Harms et al., 2017; Misiak et al., 2018; Wang et

368 al., 2018). Our results suggest that PPS plays a role in altered FKBP5 functioning in females, a key  
369 regulator of the HPA axis. Also in agreement with our findings, the human literature shows early  
370 trauma is associated with blunted CORT responses to social stimuli, particularly in women.

371

## 372 **Conclusions**

373 We found the adult female HPA axis was sensitive to PPS, with changes seen throughout the system.  
374 This highlights the pre-pubertal phase as a particularly sensitive time for re-programming of the  
375 female HPA axis by stress. In contrast, the male HPA axis was unaffected. This sex-specific vulnerability  
376 may underlie the greater propensity for women to develop psychiatric disorders including depression,  
377 anxiety and PTSD, disorders which are frequently associated with HPA axis dysregulation. Although  
378 we found greater effects in females in the present study, males are not immune to the effects of PPS.  
379 For example, in previous studies we found that PPS significantly impaired hippocampal-dependent  
380 behaviour and hippocampal neurogenesis in males but not females, and social behaviour is equally  
381 affected in both sexes(Brydges et al., 2018, *Brydges et al. in review*). Furthermore, others have found  
382 several behavioural and neurobiological effects of PPS in male animals(Albrecht et al., 2017; Brydges,  
383 2016). This suggests males and females differ in their responses to PPS, potentially resulting in sex-  
384 specific vulnerabilities to certain disorders. This strengthens the argument for including both sexes in  
385 preclinical and clinical studies.

386

## 387 **Figure Legends**

388 **Figure 1. Hippocampal formation.** PPS increased expression of a) FKBP5 and b) AVPR1a in the female  
389 hippocampal formation. Con=control, PPS=pre-pubertal stress, F=female, M=male. Male: 12 control,  
390 10 PPS; female: 8 control, 10 PPS. \*=p<0.05. Error bars represent 1 S.E. and bars joined by a line and  
391 asterisk are significantly different to one another.

392 **Figure 2. PFC.** PPS increased a) GR and b) GR:MR ratio and decreased c) AVPR1a in the female PFC.  
393 OXTR expression was higher in female than male PFC. Con=control, PPS=pre-pubertal stress,  
394 F=female, M=male. Male: 12 control, 10 PPS; female: 8 control, 10 PPS. \*=p<0.05, \*\*p<0.01,  
395 \*\*\*p<0.0001. Error bars represent 1 S.E. and bars joined by a line and asterisk are significantly  
396 different to one another.

397 **Figure 3. Hypothalamus.** a) GR, b) MR, c) GR:MR, d) FKBP5 and e) OXTR were higher in male than  
398 female hypothalamus, whereas f) AVPR1a and g) OXT were higher in female hypothalamus.  
399 Con=control, PPS=pre-pubertal stress, F=female, M=male. Male: 12 control, 10 PPS; female: 8 control,  
400 10 PPS. \*\*p<0.01, \*\*\*p<0.0001. Error bars represent 1 S.E. and bars joined by a line and asterisk are  
401 significantly different to one another.

402 **Figure 4. Corticosterone.** Social interaction significantly elevated corticosterone above baseline in  
403 control animals and PPS males. This response was blunted in PPS females. Con=control, PPS=pre-  
404 pubertal stress, F=female, M=male. Females: 22 control, 18 PPS; male: 12 control, 10 PPS \*=p<0.05.  
405 Error bars represent 1 S.E. and bars joined by a line and asterisk are significantly different to one  
406 another.

407

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413

#### 414 **Declaration of interest**

415 The authors declare no competing interest.

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