<u>Female HPA axis displays heightened sensitivity to pre-pubertal stress</u>

Running title: Pre-pubertal stress and adult HPA axis

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Abstract

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

Lay Summary

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

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

Introduction

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

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

Long-term effects of ELS on the HPA axis differ between the sexes. Early trauma is associated with a more severely blunted cortisol response to social stress in women, and fewer stressful events early in life are required to trigger liability to PTSD in women. Conversely, lower levels of recent stress are capable of provoking major depression in men than women(Bunea, Szentagotai-Tatar, & Miu, 2017; McLaughlin, Conron, Koenen, & Gilman, 2010). Furthermore, women display 2-3 times higher rates of anxiety, affective disorders, major depressive disorder and post-traumatic stress disorder (PTSD)(Christiansen & Hansen, 2015; Kessler et al., 2003; Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Remes, Brayne, van der Linde, & Lafortune, 2016). These differences are not purely attributable to sex-specific life experiences; studies controlling for stressful life events and sex-specific risk factors still find higher prevalence in women(Tiwari & Gonzalez, 2018). Sex differences in HPA axis function may underlie this. Basal secretion of CORT from the adrenal gland is higher in females than males, and this is attributed to sex-differences in gonadal hormones, with estrogen sensitising and testosterone dampening the HPA-axis(Heck & Handa, 2019; Seale et al., 2004).

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

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

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

Methods

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

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

RT-qPCR. Forty rats (male: 12 control, 10 PPS; female: 8 control, 10 PPS) were sacrificed at PND 60-70 using a rising concentration of CO_2. Brains were removed, dissected and stored at -80°C until analysis. Total cell RNA was extracted from hippocampal formation, prefrontal cortex and hypothalamus using the Qiagen RNeasy Kit (Qiagen, Manchester, UK) and DNAse treated in accordance with the supplied protocols. RNA was used to create cDNA for analysis using RNA to cDNA Easy Premix (Clontech Laboratories, France), heated at 42°C for 75 minutes, followed by 80°C for 15 minutes. Sample was then diluted 1:15 in nuclease-free water. 96-well plates were loaded, each well containing a total of 15µl reaction mixture (1.9µl sterile RNAase free water, 0.3µl 10µM forward primer, 0.3µl 10µM reverse primer, 7.5µl SensiMix (Bioline) and 5µl cDNA). *Gapdh* and *Hprt1* primers (Sigma) were used as housekeeping controls and all results were normalised from these values. After loading, plates were spun down at 3,000 rpm for approximately 10-20 seconds before being transferred to Real-Time PCR instrument (Applied Biosystems®) and run for 45 cycles (95°C for 20s, 60°C for 20s, 72°C for 20s). The expression of *GR, MR, AVP, OXT, AVPR1a, OXTR* and *FKBP5* was measured (see Table 1 for primers).

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

Corticosterone ELISA. One animal from each pair was sacrificed 20 minutes after the social test to investigate corticosterone responses to social interaction, the other sacrificed one week later for baseline analysis. Animals were decapitated and trunk blood was collected using EDTA microvette collection tubes (Sarstedt, Germany). Blood was spun at 1500 x g for 10 minutes, plasma was removed and stored at -20^{o}C until analysis. Corticosterone was analysed by ELISA, according to the manufacturer's instructions (Abcam, UK, ab108821). The sensitivity of this ELISA is 0.28ng/ml and the intra-assay coefficient of variation is 5.3%. Samples were run in triplicate on several plates, counterbalancing between groups.

Data analysis

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

Results

mRNA – Hippocampal formation. *FKBP5* (group*sex: $F_{1,29.85}$=4.33, p=0.04, Fig. 1a) and *AVPR1a* (group*sex: $F_{1,26.64}$=4.47, p=0.04, Fig 1b) expression was significantly higher in the female hippocampal 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), *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) and *OXTR* (group: $F_{1,9.84}$=0.02, p=0.9) were unchanged in males and females.

PFC. In the female PFC, PPS resulted in significantly higher *GR* expression (group*sex: $F_{1,24.94}$=7.17, 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* expression (group*sex: $F_{1,23.17}$=6.94, p=0.01, Fig. 2c) when compared to control females. *MR* (group:

$F_{1,8.81}$=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: $F_{1,10.39}$=0.16, p=0.7) and *FKPB5* (group: $F_{1,9.23}$=1.43, p=0.26) were unchanged in male and female PFC following PPS, but *OXTR* expression was higher in females than males (sex: $F_{1,27.9}$=28.65, p<0.0001, Fig. 2d).

Hypothalamus. In the hypothalamus, *GR* (sex: $F_{1,30.7}$=13.68, p<0.001), *MR* (sex: $F_{1,25.52}$=63.73, 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: $F_{1,29.24}$=118.81, p<0.0001) were lower in females than males regardless of treatment (Fig 3a-e), 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 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, 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: $F_{1,10.42}$=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* (group: $F_{1,10.64}$=0.63, p=0.44), expression. See Table 3 for summary of regional gene expression changes.

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

Discussion

PPS altered the expression of receptors and neuropeptides involved in HPA axis function in the hippocampal formation and PFC, but not hypothalamus of adult females, whereas in males the expression of major HPA axis components were unaffected in all brain regions studied. In the female prefrontal cortex, PPS increased *GR* and *GR:MR* receptor ratio and reduced *AVPR1a* expression. In the female hippocampal formation, PPS increased expression of *FKBP5* and *AVPR1a*. In the periphery, females exposed to PPS did not show the normal rise in blood corticosterone levels following a social interaction test. We also found sex differences in baseline gene expression, particularly in the hypothalamus.

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

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

PPS did not impact baseline corticosterone in males or females in agreement with the majority of previous rodent research(Fuentes et al., 2014; Grigoryan, Ardi, Albrecht, Richter-Levin, & Segal, 2015; Jacobson-Pick & Richter-Levin, 2010). In humans, studies have found increased, decreased and no change in basal cortisol following ELS(Agorastos, Pervanidou, Chrousos, & Baker, 2019; Lupien et al., 2009). Differences are likely attributable to variation in the nature and timing of stress as well as genetics, factors which are rarely considered in human studies. In the present study, exposure to social interaction with a stranger resulted in elevated corticosterone in the plasma of control females and all males, but this response was blunted in females with experience of PPS. Note that the increases in corticosterone are not an acute response to systemic vehicle administration but are a result of the social interaction, since all animals, both baseline and socially experienced rats, received vehicle injections. Furthermore, the injection occurred sixty-five minutes before sacrifice, so any acute corticosterone rise resulting from this would no longer be detectable. In contrast to the results presented here, mild prenatal stress results in *heightened* corticosterone response to restraint stress in adult females(Aisa et al., 2008). Interestingly, more prolonged prenatal stress is necessary to induce the same effects in males(Gobinath, Mahmoud, & Galea, 2015). Maternal separation elevates or blunts male and female corticosterone responses to restraint stress, depending on the study(Desbonnet, Garrett, Daly, McDermott, & Dinan, 2008; Lehmann, Russig, Feldon, & Pryce, 2002; Roman, Gustafsson, Berg, & Nylander, 2006), and chronic variable adolescent stress between PND 45-58/37-49 blunted corticosterone responses to a stressor in adult females but not males(Bourke & Neigh, 2011; Wulsin, Wick-Carlson, Packard, Morano, & Herman, 2016). This again suggests that the female HPA axis is more sensitive to ELS, although specific outcomes are mediated by exact timing of stress and adult testing paradigm (e.g. social vs restraint). An adaptive stress response is characterised by a rapid corticosterone or cortisol (CORT) increase, followed by a progressive decline. Excessive or repeated activation of the HPA axis and release of CORT can lead to blunted CORT secretion in response to acute stress(Kinlein, Wilson, & Karatsoreos, 2015). A healthy CORT response is necessary for appropriate behaviour and survival, therefore a blunted CORT response to acute stress may be considered a maladaptive phenotype.

PPS increased expression of *AVPR1a* in the female hippocampal formation and decreased it in the PFC. No changes were observed in the hypothalamus. OXT and AVP are closely related neuropeptides that exert opposite effects on the HPA axis. Stress results in the release of hypothalamic AVP, which stimulates the release of adrenocorticotrophic hormone from the pituitary and eventual production of CORT(de Kloet et al., 2005). In contrast, OXT dampens the HPA axis(Neumann & Landgraf, 2019). Both AVP and OXT exert effects on behaviour through OXTR and AVPR1a/AVPR1b situated in the brain(Song & Albers, 2018). Effects of prenatal stress on AVP/OXT systems are mixed, with some studies finding decreased OXT/AVP expression in the male hypothalamus, others no changes in males or females (Desbonnet et al., 2008; Lee, Brady, Shapiro, Dorsa, & Koenig, 2007; Schmidt et al., 2018). Poor maternal care in rodents decreases OXT and OXTR expression centrally (hypothalamus and amygdala) and peripherally (blood plasma) in female animals(Francis, Young, Meaney, & Insel, 2002; Tobon, Jeffrey, & Nemeroff, 2018), whereas maternal separation increases hypothalamic AVP and alters OXT expression and OXT/AVP receptor binding in an age and sex-specific manner(Lukas, Bredewold, Neumann, & Veenema, 2010; Murgatroyd et al., 2009; Veenema, Bredewold, & Neumann, 2007; Veenema & Neumann, 2009). Our previous work found PPS increased protein levels of AVP in the supraoptic (but not paraventricular) nucleus of the hypothalamus and blood plasma in male and female rats (*Brydges et al. in Review*). In the present study, the hypothalamus was analysed as a whole, it is possible differences may have been found if the supraoptic and paraventricular nuclei had been analysed separately. Alternatively, PPS may alter translation rather than transcription of AVP in this region. Considering their opposing effects on behaviour (AVP exerts anxiogenic and depressive-like effects, whereas OXT is an endogenous anxiolytic), the balance of AVP and OXT in the brain is thought crucial for appropriate emotional behaviours(Mak, Broussard, Vacy, & Broadbear, 2012; Neumann & Landgraf, 2012). In the present study, we find altered *AVPR1a* expression in the female limbic system in the absence of altered OXT/OXTR expression. This indicates a dysregulated HPA axis which may predispose towards anxiety or depressive phenotypes following stress(Lesse, Rether, Groger, Braun, & Bock, 2017; Neumann & Slattery, 2016; Nowacka-Chmielewska, Kasprowska-Liskiewicz, Barski, Obuchowicz, & Malecki, 2017).

PPS increased *AVPR1a* expression in the female hippocampal formation, yet decreased expression in the PFC. Bi-directional projections exist between the hippocampus and hypothalamus (production site of AVP): the hippocampus is a target of AVP and is capable of decreasing AVP expression in the hypothalamus(Nettles, Pesold, & Goldman, 2000; Zhang & Hernandez, 2013). PPS leads to increased AVP (*Brydges et al. under review*), therefore increased *AVPR1a* expression in the hippocampal formation may be a compensatory mechanism, enhancing the sensitivity to and subsequent inhibitory effects of the hippocampus on hypothalamic AVP secretion. The PFC is also thought to exert inhibitory effects over the hypothalamus, but direct connections between these two structures are lacking, and it is hypothesised that the PFC may act via other structures to exert this influence(Spencer, Buller, & Day, 2005). Whether the decreased *AVPR1a* expression following PPS in this region is due to adaptation or pathology remains to be elucidated.

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

The balance between MR and GR functioning is thought crucial for appropriate HPA axis function, and dysregulation and imbalance between CR is suggested as a candidate mechanism underlying psychiatric disorders such as major depression, a disorder which has been repeatedly associated with hyperactive HPA axis function(de Kloet et al., 2005; Juruena et al., 2015; Oitzl, Champagne, van der Veen, & de Kloet, 2010). Polymorphisms associated with enhanced expression of FKBP5 following GR activation are overrepresented in major depression, bipolar and PTSD(Binder, 2009; Matosin et al., 2018). FKBP5 is implicated in a number psychiatric disorders, particularly in combination with early life stress(Wang, Shelton, & Dwivedi, 2018). In humans, there is an interaction between FKBP5 (FK506 binding protein 5) variability and childhood trauma on psychosis, paranoia, social stress appraisal and prefrontal cortex function(Harms et al., 2017; Misiak et al., 2018; Wang et al., 2018). Our results suggest that PPS plays a role in altered FKBP5 functioning in females, a key regulator of the HPA axis. Also in agreement with our findings, the human literature shows early trauma is associated with blunted CORT responses to social stimuli, particularly in women.

Conclusions

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

Figure Legends

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

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

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

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

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Declaration of interest

The authors declare no competing interest.

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