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17 β -Oestradiol Modulates Glucocorticoid, Neural and Behavioural Adaptations to Repeated Restraint Stress in Female Rats

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Abstract

Sex steroids have a role in modulating responses that extends beyond reproduction. The current study investigated the influence of the sex steroid 17 β -oestradiol on hypothalamic-pituitary-adrenal (HPA) and behavioural responses to acute or repeated restraint stress. Ovariectomized rats treated with 17 β -oestradiol or peanut oil via a subcutaneous silastic capsule were subjected to daily handling (non stressed), acute (single, 1 h) or daily (10 days, 1 h/day) restraint stress. Blood collected at the end of stress revealed that 17 β -oestradiol treatment augmented the corticosterone response to acute restraint. After daily exposure to restraint, the corticosterone response was noticeably diminished in untreated females but 17 β -oestradiol-treated rats still showed an exaggerated response compared to castrated, untreated females. Brain tissue collected 3 h after the end of restraint was probed using isotopic *in situ* hybridization for corticotropin-releasing factor (CRF) and vasopressin gene expression in the paraventricular nucleus (PVN) of the hypothalamus. 17 β -oestradiol treatment at the higher dose (120 μ g/ml) decreased basal CRF mRNA. Stress caused an increase in CRF mRNA expression in 17 β -oestradiol-treated rats but not in the vehicle group. Repeated restraint stress caused an increase in PVN parvocellular vasopressin gene expression, which was more pronounced in 17 β -oestradiol-replaced rats. Animals were exposed to the elevated plus maze for 5 min as a test for anxiety. Non-stressed control rats with or without 17 β -oestradiol replacement spent the same percentage amount of time exploring the open arms of the maze. Previous exposure to acute restraint stress caused a marked reduction in the time spent exploring the open arms, indicating an increase in anxiety levels in these rats; this effect was observed in both vehicle and 17 β -oestradiol-treated rats. After repeated restraint stress, 17 β -oestradiol-replaced rats spent as much time exploring the open arms of the maze as controls, indicating adaptation. By contrast, nonreplaced rats were still showing a significant reduction in open arm exploration after repeated restraint. The present study presents novel data showing that the HPA axis remains reactive to repeated stress in 17 β -oestradiol-treated ovariectomized rats, but stress-induced anxiety behaviour is reduced.

Keywords

stress; oestradiol; adaptation; anxiety; CRF; AVP

The ability of an animal to mount an appropriate response to a stimulus that poses a threat to homeostasis is essential for survival. The hypothalamic-pituitary-adrenal (HPA) axis is a major component of the stress response and sex differences in HPA function have been reported. For example, females exhibit a higher basal rate of adrenocorticotropin (ACTH)

and corticosterone secretion than males (1). It has been shown that female rats respond to various acute stressors, such as ether, restraint or electric shock, with a greater release of corticosterone than males (2, 3).

Several studies have reported the existence of gender differences on behavioural tests that measure anxiety. For example, female rodents exhibit less anxiety on the elevated plus maze by spending more time on the open arms than males (4). Similarly, after acute stress, males show a more pronounced reduction in open arm exploration than females (5). The influence of gender may vary with different stressors. For example, crowding has been shown to be stressful for males whereas isolation is more stressful for females (6). Similarly, group-housing has a positive effect on the ability of females to adapt to repeated foot-shock (7, 8). Male rats show a more pronounced up-regulation of corticotropin-releasing factor (CRF) mRNA in the paraventricular nucleus (PVN) after chronic stress than females (9). Ovarian hormones are implicated in these sex differences. Basal and stress-activated corticosterone levels have been shown to vary across the oestrous cycle, being at their maximal during the proestrous phase when circulating 17β -oestradiol levels are high (10). Anxiety-like behaviour of female rodents has been reported to alter across the oestrous cycle (11), implicating activational effects of gonadal steroids on emotional behaviour. Various stressors, such as foot shock (12), CRF administration (13), restraint (14) and novelty (15), have been shown to result in greater stress-induced increase in ACTH and corticosterone levels ovariectomized 17β -oestradiol-treated compared to nonreplaced rats.

The HPA axis adapts its activation in the face of a repeated stressor. Repeated exposure to restraint causes a reduction in the magnitude of the ACTH and corticosterone response in male rats (16, 17). Coincident changes in CRF and vasopressin mRNA expression are observed in the hypothalamic PVN. The number and transcript density of parvocellular neurones expressing vasopressin is increased after repeated stress (18, 19). Similarly, colocalization of vasopressin with CRF vesicles in the external zone of the median eminence is increased (20). A few studies have looked at sex differences in the adaptation to repeated stress, and demonstrated an effect of gender (21, 22). In addition, it has been shown that 17β -oestradiol modulates neurotransmitter levels after chronic restraint stress (23). Although there is a wealth of literature addressing the nature and manner of the adaptation of the HPA response in males, the role of gonadal steroids in females has been neglected.

Previous exposure to stress has been demonstrated to have an anxiogenic effect on subsequent plus maze behavioural testing. For example, exposure to just a single session of acute restraint stress, followed by exposure to the plus maze, results in a reduction in the amount of time spent in the open arms compared to nonstressed controls (24). This response habituates in male rats after repeated stress (25). The present study examined how 17β -oestradiol treatment affected the adaptation of the HPA (both plasma corticosterone and gene expression in the PVN) and anxiety response to restraint stress under acute and repeated conditions. It shows that 17β -oestradiol appears to facilitate adaptation to stress.

Materials and methods

Animals

Virgin female Lister Hooded rats (Harlan, Bicester, UK) weighing between 190 and 220 g at the start of the experiment were used. They were housed in groups of 4-5 per cage (cage size $36.5 \times 53 \times 20$ cm) in a controlled environment (temperature 21°C and humidity 55%), with *ad libitum* access to food (standard rat chow) and water. Animals were kept on a 12 : 12 h reverse light/dark cycle (lights on 22.00 h), and were left undisturbed for at least 4 days on arrival. They were then handled daily for at least 1 week before experimental manipulations

were carried out. All experiments were carried out in accordance with the Animals Scientific Procedures Act (1986).

Experimental groups

Animals were randomly assigned to one of six groups ($n = 7-8$ per group): (i) ovariectomy + 120 $\mu\text{g/ml}$ 17 β -oestradiol (Sigma-Aldrich Co Ltd, Poole, Dorset, UK): (a) unstressed, (b) acutely stressed and (c) repeatedly stressed; (ii) ovariectomy + peanut oil implant: (a) unstressed, (b) acutely stressed and (c) repeatedly stressed. In experiment 2, an extra three groups were used: (iii) ovariectomy + 40 $\mu\text{g/ml}$ 17 β -oestradiol: (a) unstressed, (b) acutely stressed and (c) repeatedly stressed. Animals were housed in same stress groups.

Surgery

All rats were ovariectomized under isoflurane/N₂O anaesthesia via a dorsal incision. At the time of surgery, rats were implanted with a 25 mm silastic capsule (0.062' inner diameter; 0.03' outer diameter) subcutaneously. The capsule was filled with 40 μl of fluid then the ends were sealed with Type A silastic glue (BDH Laboratory Supplies, VWR, Lutterworth, Leics, UK) so that 20 mm was left for active release of the steroid. Implants were preincubated over night in 0.01 M phosphate-buffered saline (PBS) to activate release. Animals were allowed to recover for at least 7 days before stress procedures. During the recovery period, rats were given a prophylactic dose of aureomycin (Fort Dodge Animal Health, Hedge End, Southampton, UK) in their drinking water.

Stress procedure

Experiment 1: effect of 17 β -oestradiol on adaptation of stress-induced plasma corticosterone levels—Animals in the stress groups were subjected to either a single 60-min restraint session for the acute or repeated daily 60-min restraint sessions for 10 days (repeated stress). Animals were transferred from the holding room to a separate testing room where they were placed in a plexiglass tube (internal diameter 5.5 cm) and placed in the testing cage. The stress sessions were carried out during the dark phase between 11.30 h and 14.30 h. Rats assigned to the repeated stress groups were subjected to daily restraint on days 8-17 post surgery. On day 17, acute stress rats were subjected to a single session of restraint stress after which they were immediately killed. Control rats were handled daily and killed 17 days after surgery. All rats were transferred to another procedure room immediately after the last session of restraint and killed by a lethal dose of sodium pentobarbitone.

Experiment 2: effect of 17 β -oestradiol on changes in PVN CRF and vasopressin mRNA expression—The experiment was conducted as described for experiment 1 except that the rats were returned to their home cage in the holding room at the end of the last session of restraint. Animals were then killed 3 h after the termination of restraint stress and the brains processed as described below.

Experiment 3: behavioural testing on the elevated plus maze—The rats were tested on an elevated plus maze. Behavioural testing was conducted during the dark phase, 4 h after the end of the last stress session. Briefly, the plus maze consisted of two open arms: 50 $\times$ 10 cm, and two closed arms which were 50 $\times$ 10 $\times$ 40 cm. The central platform was 10 $\times$ 10 cm and the entire apparatus was mounted on a metal base 50 cm above the floor. Animals were placed in the central square facing an open arm. The rats were allowed to explore freely for 5 min. During this period, the amount of time spent on the open arms was recorded, as was the number of arm entries. The behavioural testing carried out under red light.

Blood and tissue collection

Blood was removed at the time of sacrifice via cardiac puncture using heparinized syringes and transferred into centrifuge tubes. Tubes were centrifuged at 3000 r.p.m. for 8 min after which the plasma was collected and stored at -20 °C before assaying for corticosterone. Corticosterone was measured by radioimmunoassay using a standard protocol. The tracer used was corticosterone [1,2,6,7-³H(N)] (Perkin Elmer Life Sciences, Cambridge, UK). Intra- and inter assay coefficient of variation was 3.9% and 5.1%, respectively. 17 β -oestradiol was measured using a commercial ¹²⁵I radioimmunoassay kit (Diagnostic Systems Laboratories, Oxford UK). The assay was carried out on samples obtained from a separate group of rats 17 days after surgery. Intra-assay coefficient of variation was 5.9% and sensitivity of the kit was 0.6 pg/ml.

Brains were collected and frozen on dry ice and stored at -70 °C until sectioning in a cryostat. A 1-cm piece of uterus was collected from each rat postmortem and weighed to assess efficacy of both ovariectomy and 17 β -oestradiol replacement.

In situ hybridization

Brains were sectioned in a cryostat at -20 °C at 12 μ m and thaw-mounted onto poly L-lysine coated microscope slides (BDH Laboratory Supplies). Sections were left to air dry for at least 1 h and then fixed in 4% paraformaldehyde in PBS for 15 min at 4 °C followed by two 5-min washes in PBS. The sections were then dehydrated through a series of 70%, 80%, 90% and 95% ethanol for 3 min each. The slides were then stored at 4 °C in sealed plastic tubs containing 95% ethanol. Before hybridization, slides were removed from the ethanol and air-dried on slide racks for at least 1 h.

Probes for CRF and vasopressin mRNA were 42 and 30 base oligonucleotides complimentary to the exonic mRNA coding for CRF and vasopressin (obtained by 'blast' searches). CRF oligonucleotide was an antisense 42mer sequence, GGC CCG CGG CGC TCC AGA GAC GGA TCC CCT GCT CAG CAG GGC. Vasopressin was a 30mer sequence, GGT GAG GCG GAA AAA ACC CTC TCG ACA CTC. Probes were tail-end labelled with ³⁵S as follows: 4 μ l of purified oligonucleotide (5 ng/ μ l) was added to 4 μ l of 5 $\times$ Buffer (Roche Ltd, Lewes, UK). 1.25 μ l of 1 M cobalt chloride (Roche) was added followed by 8 μ l of DEPC-treated water. 3 μ l of ³⁵S-deoxyadenosine triphosphate (10 mCi/ml) (Amersham Biosciences, Little Chalfont, Bucks, UK) was added followed by 2 μ l of terminal deoxynucleotide transferase enzyme (Roche). The reaction mixture was incubated at 37 °C for 30 min. After the incubation period, the reaction was quenched by adding 12 μ l of DEPC-treated water. Purification of labelled probe from unincorporated nucleotides was carried out by passing the reaction mixture through a G-50 sephadex microspin column (Amersham Biosciences) and centrifuged at 3400 r.p.m. 2 μ l of 1 M dithiothreitol was added to the labelled probe. The labelled probe was then diluted to give a specific activity of 5000 c.p.m. μ l⁻¹ in a hybridization buffer (containing 50% deionized formamide, 20% 20x SSC, 10 $\times$ Denhardt's solution, 0.2% Salmon sperm DNA, 10% dextran sulphate, 0.5 M sodium phosphate and 0.1 M sodium pyrophosphate). 100 μ l of hybridization mixture was added to each slide that was then coverslipped and sealed along the edges with DPX mountant (to prevent the sections from drying out). The slides were then incubated overnight in a fan oven at 43 °C. After incubation, the DPX was peeled off and the coverslips removed in 1 $\times$ SSC solution keeping the slides immersed at all times. The slides were then washed twice for 30 min each wash in 1 $\times$ SSC at 55 °C in a shaking bath, rinsed briefly in water and dehydrated in 300 mM ammonium acetate dissolved in 70% ethanol for 3 min. The sections were then left to air dry for at least 2 h before exposure to X-ray film.

Autoradiography

Hybridized sections were exposed to Kodak BioMax MR autoradiographic film, in a light-proof X-ray film cassette (Amersham Biosciences) along with a set of ^{14}C labelled standards (range 30–862 mCi/ μg) (Amersham Biosciences). The duration of the exposure varied, according to the strength of the oligonucleotide signal, 1 day for vasopressin and 7 days for CRF. Films were developed under red light in an automated film processor (Fuji Medical Film Processor, FPM-100A) (Fuji Photo Film UK, London, UK). Due to the much higher level of vasopressin expression emanating from the magnocellular cells, it became necessary for cellular resolution, thus emulsion autoradiography was used. Sections processed for vasopressin mRNA were coated with Ilford UK LM-1 emulsion under red light conditions and stored in a light proof box with desiccant at 4 °C for 3–5 days. Slides were developed with 9% Kodak D-19 solution (Kodak Ltd, Hemel Hempstead, Herts, UK) for 5 min at room temperature, rinsed in 1% acetic acid solution for 3 min (stop solution), fixed in 25% Ilford fixer for 5 min and then washed under running water for 30 min. The sections were then stained in 0.5% cresyl violet acetate solution, rinsed in water, dehydrated through a series of ascending alcohol concentration (70%, 80%, 95%, 100% ethanol) and defatted in HistoClear (BDH Laboratory Supplies) before coverslipping with DPX mountant. Adjacent series of sections were stained for reference purposes.

Analysis and quantification

For quantification of CRF mRNA in the PVN, the optical density of the autoradiographic images was measured using a Macintosh-based image analysis system (NIH Image). The optical densities of three to four consecutive sections per rat were analysed bilaterally and the average value for each rat was used to calculate group means. The number of parvocellular cells expressing vasopressin was counted bilaterally for each rat. Three sections were analysed for each rat and an average cell count per section was used to calculate group means.

Statistical analysis

Neuroendocrine data were analysed by two-way univariate analysis of variance (ANOVA) with stress and 17β -oestradiol treatment as factors. Behavioural data were analysed using a multivariate ANOVA with percentage time spent in the open arms, open arm entries and total number of arm entries as variables, and stress and 17β -oestradiol treatment as factors. Subsequent analysis on each variable was carried out using two-way univariate ANOVA with stress and 17β -oestradiol treatment as factors. Post-hoc analysis was carried out (if the ANOVA was significant) using Bonferroni multiple comparisons test. Significance was accepted at the $\alpha = 0.05$ level. Data were log transformed as necessary.

Results

Experiment 1

Corticosterone (Fig. 1)—Blood samples were taken immediately after the end of the stress to ensure that plasma corticosterone levels represent the falling phase of the stress response. There was a significant main effect of both stress [$F(2,36) = 86.62$, $P < 0.001$] and 17β -oestradiol treatment [$F(1,36) = 40.74$, $P < 0.001$] on corticosterone levels. However there was no interaction between the two factors [$F(2,36) = 0.52$, not significant], indicating that 17β -oestradiol increased corticosterone levels independently of stress. Post-hoc analysis showed that levels in the 17β -oestradiol group were more elevated compared to the vehicle group after acute (day 1) stress ($P < 0.01$). After repeated stress (day 10), the corticosterone response had diminished, with both vehicle and 17β -oestradiol-treated groups exhibiting lower levels than corresponding acute stress groups ($P < 0.001$). After repeated stress, mean

corticosterone levels in the 17 β -oestradiol group were still higher than vehicle ($P < 0.05$). Thus, in all groups (non stress, acute and repeated stress), 17 β -oestradiol-replaced rats exhibited higher corticosterone levels compared to corresponding vehicle groups.

Experiment 2

Adrenal and thymus weights—17 β -oestradiol replacement overall significantly increased adrenal weight [$F(2,47) = 4.1$, $P = 0.023$] and stress had no significant effect [$F(2,47) = 0.65$, not significant] on adrenal weight (Table 1). There was no significant interaction between the two factors.

There was a significant main effect of 17 β -oestradiol treatment on thymus weight [$F(2,47) = 15.88$, $P < 0.001$] but no significant effect of stress [$F(2,47) = 0.5$, not significant] (Table 2). There was no significant interaction between the two factors. 17 β -oestradiol replacement significantly reduced thymus weights only in the 120 $\mu\text{g/ml}$ repeatedly stressed group compared to corresponding vehicle group ($P < 0.05$).

CRF mRNA—The expression of CRF mRNA in the PVN is shown in Figs 2_A and 3. There were significant main effects of stress [$F(2,44) = 7.8$, $P < 0.001$] and 17 β -oestradiol treatment [$F(2,44) = 5.44$, $P < 0.005$] on CRF mRNA levels in the PVN. There was also a significant interaction between the two factors [$F(4,44) = 10.5$, $P < 0.001$]. Post-hoc analysis revealed that basal expression of CRF was significantly lower in rats receiving implants containing 120 $\mu\text{g/ml}$ 17 β -oestradiol compared to vehicle and 40 $\mu\text{g/ml}$ 17 β -oestradiol control rats ($P < 0.001$ in both instances). Neither acute nor repeated stress had any effect on CRF expression in vehicle-treated rats, compared to vehicle controls (not significant in both instances). Similarly rats with 40 $\mu\text{g/ml}$ 17 β -oestradiol implants showed no change in CRF expression after acute or repeated restraint stress compared to corresponding controls (not significant). There was no difference in CRF expression between corresponding vehicle and 40 $\mu\text{g/ml}$ 17 β -oestradiol-replaced rats at any condition (not significant in all instances). Animals with 120 $\mu\text{g/ml}$ 17 β -oestradiol implants did show an increase in expression after acute stress compared to corresponding control rats ($P < 0.001$). This increase was still evident after 10 days of restraint ($P < 0.001$).

Vasopressin mRNA—The expression of CRF mRNA in the PVN is shown in Figs 2_B and 4. There were significant main effects of stress [$F(2,35) = 71.6$, $P < 0.001$] and 17 β -oestradiol treatment [$F(2,35) = 30.51$, $P < 0.001$] on the number of vasopressin expressing parvocellular cells in the PVN; the interaction between the two factors was also significant [$F(4,35) = 6.1$, $P < 0.01$]. Post-hoc analysis showed that, after acute stress, there was no change in the number of vasopressin expressing parvocellular cells in any of the groups. After repeated restraint stress, there was a significant increase in the number of parvocellular cells expressing vasopressin in all three treatment groups compared to corresponding controls ($P < 0.05$). This increase was significantly higher in the repeatedly stressed rats receiving 120 $\mu\text{g/ml}$ 17 β -oestradiol implants compared to vehicle-treated rats ($P < 0.001$). The increase in vasopressin after repeated restraint stress was significantly higher in rats replaced with 120 $\mu\text{g/ml}$ 17 β -oestradiol compared to the 40 $\mu\text{g/ml}$ group ($P < 0.001$).

Experiment 3: plus maze behaviour (Fig. 5)

Percentage time spent on open arms—There were significant main effects of stress [$F(2,44) = 15.7$, $P < 0.001$] and 17 β -oestradiol treatment [$F(1,44) = 7.46$, $P < 0.01$] on the percentage time spent on the open arms. The interaction between the two factors was just outside significance [$F(2,44) = 3.1$, $p = 0.055$, significant). Post-hoc analysis showed that not acute restraint stress significantly reduced the percentage time spent out in the open arms in both vehicle and 17 β -oestradiol rats compared to corresponding controls ($P = 0.005$).

There was no significant difference between vehicle and 17 β -oestradiol-treated groups after acute stress (not significant). After repeated stress, vehicle-treated rats still spent less time out on the open arms than corresponding controls ($P = 0.008$). By contrast, repeatedly stressed 17 β -oestradiol-replaced rats had adapted, spending as much time out in the open arms as the corresponding nonstressed controls (not significant). Vehicle-treated rats spent significantly less time on the open arms compared to the 17 β -oestradiol-treated rats ($P = 0.014$). There was no significant difference between vehicle and 17 β -oestradiol control (nonstressed) rats (not significant).

Number of arm entries—There was no effect of stress on the total number of arm entries made [$F(2,44) = 1$, $P = 0.38$, not significant] and no effect of 17 β -oestradiol treatment [$F(1,44) = 0.1$, not significant]. However, there was a significant main effect of stress on the number of open arm entries [$F(2,44) = 7.2$, $P = 0.002$] but no significant effect of 17 β -oestradiol treatment [$F(1,44) = 0.9$, not significant]. There were no effects of either stress or 17 β -oestradiol on closed arm entries ($F < 1$ in both cases; not significant).

17 β -oestradiol increased uterine weight—Uterine weight was used as a bioassay to determine the efficacy of the ovariectomy and 17 β -oestradiol replacement procedure. The results showed a significant effect: vehicle (4.2 ± 0.3) < 40 $\mu\text{g/ml}$ 17 β -oestradiol (9.2 ± 0.5) < 120 $\mu\text{g/ml}$ 17 β -oestradiol (12.3 ± 0.9) mg/100 g body weight.

Determination of plasma 17 β -oestradiol levels—In a separate experiment, plasma 17 β -oestradiol levels were measured 17 days after surgery in ovariectomized rats receiving silastic implants containing 17 β -oestradiol (40 or 120 $\mu\text{g/ml}$). Levels were: 40 $\mu\text{g/ml}$ 17 β -oestradiol implant, 16 ± 1.9 pg/ml; 120 $\mu\text{g/ml}$ 17 β -oestradiol implant, 45 ± 3.7 pg/ml.

Discussion

There was a robust increase in plasma levels of corticosterone after acute restraint stress, and 17 β -oestradiol-treated rats exhibited a more pronounced response. After repeated stress, the plasma corticosterone response in all treatment groups had diminished, indicating habituation, although levels were still accentuated in 17 β -oestradiol-treated rats. Habituation of the corticosterone response to repeated restraint stress in the rat has been demonstrated on numerous studies on males (18, 26, 27). An interesting feature of this study is that although 17 β -oestradiol-replaced females have enhanced stress-induced corticosterone levels, the rate of adaptation of the corticosterone response was similar across the groups. This implies that the magnitude of initial corticosterone secretion and adaptation of the corticosterone response are differentially controlled by 17 β -oestradiol. This could be due to several reasons. First, it has been shown that 17 β -oestradiol increases glucocorticoid synthesis in the adrenal gland (28, 29). 17 β -oestradiol-replaced rats in the current study tended to have larger adrenal glands (and smaller thymus glands), which may reflect increased release of corticosterone. Alternatively, although ACTH levels were not measured in this study, previous studies have reported augmented acute stress-induced ACTH secretion in 17 β -oestradiol-replaced female rats (2, 10). ACTH secretion is subject to negative feedback control by glucocorticoids (30). 17 β -oestradiol has been strongly implicated in reduced efficacy of glucocorticoid negative feedback inhibition (12, 31). This would result in a greater ACTH response and subsequent corticosterone secretion.

In a separate experiment (data not shown), we compared uterine weights across the oestrous cycle with those of ovariectomized replaced female rats using several doses (40, 120, 240 $\mu\text{g/ml}$ 17 β -oestradiol). We found that 120 $\mu\text{g/ml}$ resulted in uterine weights comparable to rats in proestrous (when 17 β -oestradiol levels are at their maximal). 17 β -oestradiol replacement at the higher dose (120 $\mu\text{g/ml}$) used in the definitive experiments resulted in

significantly lower basal expression of CRF compared to vehicle-treated females. This finding corroborates a previous study (32). Higher basal corticosterone levels may result in increased negative feedback, which may partly contribute to decreased mRNA levels. In support of this, decreases in basal CRF mRNA levels were found to be dependent upon the integrity of the adrenal glands (32). It could be argued that the elevated basal corticosterone levels observed in rats with the higher dose of 17β -oestradiol may be nullified by 17β -oestradiol up-regulation of plasma CBG levels (33). It appears unlikely to be the case in this study because 17β -oestradiol-replaced rats had smaller thymus glands than vehicle-treated rats, suggesting exposure to higher levels of corticosterone (34). It is possible that chronic 17β -oestradiol treatment may decrease CRF mRNA levels directly. A functional ERE has been identified in the human CRF gene (35). One study reported that 17β -oestradiol increased CRF mRNA levels *in vitro* (35). A later report from the same laboratory, using a different cell line, found that 17β -oestradiol decreased CRF transcription (36). In intact female rats, basal CRF mRNA levels have been found to be maximal during prooestrous when 17β -oestradiol levels are high (37). This suggests that, in cycling rats at least, 17β -oestradiol has an excitatory effect on the HPA axis, whereas in the present study chronic 17β -oestradiol (120 μ g/ml) replacement decreased basal CRF mRNA levels. This implies that there is a differential effect of dynamic versus static 17β -oestradiol levels on HPA activity.

After acute and repeated stress, CRF mRNA levels in rats receiving 120 μ g/ml 17β -oestradiol implants had increased. Because we were unable to measure prestress levels at all the times and treatment points, whether or not this represents an acute effect of stress remains uncertain. The important point is that neither vehicle nor lower dose 17β -oestradiol-treated rats exhibited any change in CRF mRNA levels compared to control (unstressed) values. Several studies in male rats have demonstrated an increase in CRF mRNA levels after acute restraint stress (18, 19). Levels in our study were only examined at one time point (i.e. 3 h after stress) and it is possible, although unlikely, that stress-induced changes in CRF mRNA levels could have been observed at other time points. We have data showing that CRF is not increased 1 h after stress in vehicle-treated ovariectomized rats (unpublished data). Previous studies have demonstrated stress-induced increases in CRH hnRNA within 30 min, with a peak in mRNA levels 2-3 h later (18, 38). This suggests that the time point chosen in this study was within the range to detect stress-induced changes in CRF mRNA levels, although this awaits verification with a thorough time course. We carried out the experiment during the dark phase, and this may account for the discrepancy between the current findings and previous studies. This explanation appears unlikely because a previous study using the same stress regimen found an increase in CRF mRNA expression after acute stress (19). The time point of 3 h poststress was chosen to accommodate the different stress-induced responses of both CRF and vasopressin mRNA. Although CRF gene expression occurs within 30 min of exposure to a stressor, that of vasopressin is slower, with detectable changes in vasopressin hnRNA occurring 1-2 h after exposure (39). It is also possible that the already high basal CRF levels in nonreplaced rats may have resulted in a ceiling effect, but levels in adrenalectomized females can rise to even higher levels, which makes this explanation less likely. The relationship between 17β -oestradiol status and the stress induced increases in CRF mRNA is likely to be a complex one with altered glucocorticoid feedback playing a key role. In another study, we found that adrenalectomy caused an increase in CRF mRNA expression in ovariectomized vehicle-treated rats (unpublished data). It is possible that the unstressed vehicle-treated rats were chronically stressed, masking any effects of the restraint stress procedure itself. However, we believe this to be an unlikely explanation because we have found that these rats have larger thymus glands than 17β -oestradiol-treated controls. Chronically stressed rats show thymic involution as a result of chronic exposure to glucocorticoids (34), which is an effect that we did not observe in nonstressed vehicle-treated rats.

Differences between 17β -oestradiol and vehicle-treated rats in the mRNA expression of vasopressin, the other major ACTH secretagogue, were also evident after repeated stress, although subject to the same reservations of whether these represent altered baseline or acute responses to stress. Due to the high variability in silver grain density as a result of the emulsion protocol, cell counts of vasopressin-positive parvocellular neurones were used to monitor changes in expression. The cells we measured were carefully chosen from the dorsomedial part of the PVN, which are largely, although not entirely, distinct from the magnocellular region. These cells have both autonomic and median eminence connections (40). Several studies have shown that manipulations which alter mRNA expression levels also result in changes in the number of cells expressing vasopressin (41). Under basal conditions, the number of medial PVN parvocellular cells expressing vasopressin was low across all the treatment groups. In the present study, acute stress did not appear to have any effect on vasopressin expression in any of the groups. This agrees with previous investigations that demonstrated either no or a very small increase in parvocellular vasopressin expression after acute stress in males (19, 42). After repeated stress, there was a clear increase in parvocellular vasopressin mRNA expression across the groups, as shown by previous studies in males (18, 19, 43).

The novel finding was that this increase was markedly potentiated in rats replaced with the higher dose of 17β -oestradiol. The site of oestrogen modulation of the HPA axis is not fully elucidated and may occur at several sites, including the adrenal gland, anterior pituitary, hypothalamus and extrahypothalamic regions. Receptors for oestrogen receptors (ER), ER α and/or ER β , are expressed in all of these regions (44, 45). Although ER β -immunoreactivity has been identified in the medial parvocellular part of the PVN, few vasopressin neurones colocalize with the receptor (46). This suggests that the mechanism of 17β -oestradiol action is unlikely to be directly on the parvocellular neurones. However, the results of a recent study suggest that ER β in the PVN may regulate corticosterone responses to stress (47). It is also possible that 17β -oestradiol may modulate afferent inputs into the parvocellular PVN. For example, the PVN receives a substantial excitatory input from brainstem catecholaminergic neurones, and oestrogen has been shown to modulate noradrenaline turnover (48), as well as α -1 β adrenoreceptor expression in the hypothalamus (49). It appears unlikely that reduced corticoid feedback was responsible (50, 51) because the high-dose 17β -oestradiol rats had higher levels of corticosterone compared to vehicle-treated ones.

Another novel finding in this study was the emergence of a difference between 17β -oestradiol and vehicle-treated rats after repeated stress in the elevated plus maze, a recognized test of anxiety-like behaviour (52). Previous exposure to a single session of restraint stress noticeably and significantly reduced open arm time compared to controls in an equivalent manner in vehicle and 17β -oestradiol groups. This anxiogenic effect of acute restraint has been shown in previous studies on male rats (53). However, after 10 daily sessions of restraint, the anxiogenic action of previous stress was no longer observed in 17β -oestradiol-treated rats (i.e. they had completely habituated to the stressor). By contrast, the vehicle-treated group still exhibited pronounced signs of anxiety-like behaviour (decreased percentage time in the open arms). Neither stress nor 17β -oestradiol treatment altered the total number of arms entered or the number of closed arms (i.e. locomotion). Control (unstressed) rats spent a similar amount of percentage time in the open arms (approximately 20%) irrespective of 17β -oestradiol treatment.

The apparent discrepancy between the habituation of the corticosterone and behavioural response to repeated stress might imply that the underlying processes are distinct. On the other hand, increased glucocorticoids may enhance memory for adverse effects (54, 55). The latter suggests that 17β -oestradiol may aid habituation of behaviour by increasing

glucocorticoid response to stress. Whether this is so remains to be determined. CRF is thought to play an important role in the coordination of behavioural responses to stress and in anxiety (56). There were no discernible differences in CRF mRNA expression in the PVN between groups after repeated stress in our experiment. This is not surprising because it is thought that the CRF containing neurones involved in this process are distinct from the hypophysiotropic PVN population and, as such, are also differentially regulated by stress; for a review, see (57). Several studies have demonstrated the expression of both or either of the oestrogen receptors in central areas implicated in the expression of anxiety or fear-related behaviours, including the amygdala and the lateral bed nucleus of the stria terminalis (45). 17β -oestradiol administration alters the activity of amygdala neurones (58). The role of the amygdala in anxiety is well-established. The effects of 17β -oestradiol may also be indirect via alteration of neurotransmitter and peptidergic systems. The 5-HT and noradrenergic systems have been implicated in behavioural anxiety-related responses, and 17β -oestradiol modulates the transmitter turnover and the expression of receptors in both these systems. For example, 17β -oestradiol has been shown to down-regulate the 5-HT_{1A} (59), and up-regulate the 5HT_{2A} receptors (60). Furthermore, 17β -oestradiol and vehicle rats exhibit different profiles of central serotonin and noradrenaline metabolites after chronic stress in a region-specific manner (23). Additionally, 17β -oestradiol increases neuropeptide Y expression, a peptide with inhibitory effects on anxiety-related behaviours (61).

In conclusion 17β -oestradiol decreased basal levels of CRF in ovariectomized female rats, but not vasopressin. 17β -oestradiol increased the corticosterone response to stress and, although subsequent adaptation occurs during repeated stress, levels in 17β -oestradiol-treated females remained higher than untreated ovariectomized rats. The process of adaptation or habituation of CRF mRNA is not altered by 17β -oestradiol, in contrast to vasopressin mRNA, which is enhanced in chronically stressed rats by 17β -oestradiol. Levels of anxiety, as measured in the plus maze, adapt in 17β -oestradiol-treated female rats, but not in those without replacement hormone. Taken together, this suggests that 17β -oestradiol plays a significant role in the way that female rats adapt to repeated stress.

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Fig. 1. Effect of 17β-oestradiol treatment on adaptation of the corticosterone response to stress. □, Vehicle; ■, 120 µg/ml 17β-oestradiol *Significantly different from corresponding vehicle group; +significantly different from control group ($P < 0.05$, Bonferroni).

Fig. 2.

Effect of 17β-oestradiol treatment on paraventricular nucleus mRNA expression 3 h after acute or repeated restraint stress. □, vehicle; ▒, 40 μg/ml 17β-oestradiol; ■, 120 μg/ml 17β-oestradiol. (A) basal corticotropin-releasing factor (CRF) mRNA levels were significantly lower in rats receiving 120 μg/ml 17β-oestradiol implants. (B) After repeated restraint, there was an increase in parvocellular cells expressing vasopressin mRNA. Animals with 120 μg/ml 17β-oestradiol implants had significantly higher counts of vasopressin expressing parvocellular cells compared to vehicle. (*P < 0.05 and +P < 0.001, Bonferroni).

Fig. 3. Effect of 17 β -oestradiol treatment on corticotropin-releasing factor (CRF) expression in the paraventricular nucleus after restraint stress. Lightfield photomicrographs of CRF mRNA *in situ* hybridization signal in representative sections. Veh, Vehicle; E₂, 17 β -oestradiol.

Fig. 4. Effect of 17 β -oestradiol treatment on vasopressin expression in the PVN after restraint stress. Darkfield photomicrographs of vasopressin mRNA *in situ* hybridization signal in representative sections. Veh, Vehicle; E₂, 17 β -oestradiol; m, magnocellular cell; p, parvocellular cell.

Fig. 5. Effect of restraint stress and 17β-oestradiol on plus maze behaviour. □, Vehicle; ■, 120 µg/ml 17β-oestradiol. +Significantly different from vehicle control; *significantly different from vehicle repeated restraint group ($P < 0.05$, Bonferroni).

Table 1

Mean Adrenal Weight (mg/100 g Body Weight)

Treatment	Control	Acute stress	Repeated stress
Vehicle	27.8 ± 2.1	27.0 ± 0.91	27.1 ± 1.8
40 µg/ml /17β-oestradiol	31.7 ± 1.5	30.2 ± 1.8	31.0 ± 2.3
120 µg/ml /17β-oestradiol	32.7 ± 0.9	34.4 ± 3	29.7 ± 2

Table 2

Mean Thymus Weight (mg/100 g Body Weight)

Treatment	Control	Acute stress	Repeated stress
Vehicle	209.6 ± 11.5	229.3 ± 12.4	211.7 ± 9.6
40 µg/ml /17β-oestradiol	200.4 ± 8.3	216.1 ± 8	197.2 ± 7.9
120 µg/ml /17β-oestradiol	184.3 ± 10.5	154.6 ± 3.9	165.6 ± 12.3 *

* Significantly different from corresponding vehicle group ($P < 0.05$ Bonferroni).