Effects of testosterone and estradiol on stress-induced adrenal and hippocampal weight changes in female rats

Anastasia Sfikakis,[1] Pothitos M. Pitychoutis,[2] Aikaterini Tsouma,[1] Ioanna Messari,[1] Zeta Papadopoulou-Daifoti[1]

[1]*Department of Pharmacology, Medical School, National & Kapodistrian University of Athens, Athens, Greece;*
[2]*Department of Biology & Center for Tissue Regeneration and Engineering (TREND), University of Dayton, Dayton, USA*

ABSTRACT

OBJECTIVE: To examine the impact of circulating testosterone (T) and the T/Estradiol (T/Ediol) ratio on chronic stress-induced changes of adrenal and hippocampal weight during proestrus (PE) and estrus (E) in female rats. DESIGN: Stress was composed of repeated vaginal smear screening (VSS) and measured by the emotional reactivity score (ERS). Adrenal and hippocampal weight and the T, Ediol and T/Ediol ratio were assessed in PE and E controls as well as 20 h after sham or left adrenalectomy performed on diestrus-2 (DE-2) and PE, respectively. T was measured in ovariectomized (OVX) rats treated with estradiol benzoate (EB) or vehicle (VEH) and in non-OVX EB-treated rats. RESULTS: In OVX rats EB treatment increased adrenal weight and T levels. After separation of VEH- and EB-treated rats into the low and high T-range (below and above the mean, respectively), it was observed that higher T was accompanied by higher adrenal weight in EB- compared to VEH-treated rats only in the low T-range. Non-OVX EB-treated rats with high T had lower adrenal weight compared to low T. Cycling rats assigned to the high T-range presented higher T/Ediol ratio but similar ERS and Ediol levels compared to rats in the low T-range, and were characterized by reduced adrenal weight, higher hippocampal weight and prevalence of PE *versus* E. CONCLUSIONS: High T and high T/Ediol ratios are prominent in PE compared to E and exert a protective effect on hippocampal neuronal degeneration after similar chronic stress through T-mediated lessening of stress response thus counteracting the stress-promoting effects of Ediol.

Key words: Adrenal hippocampal weight, Emotional stress, Testosterone, Testosterone/estradiol ratio

Address for correspondence:
Anastasia Sfikakis, Associate Professor of Pharmacology,
Department of Pharmacology, Medical School,
University of Athens, Athens, 115 27, Greece;
Tel.: +30210 8040964, Fax: +30210 7258562,
E-mail: psfikakis@med.uoa.gr

INTRODUCTION

It is well established that adrenal weight is a useful bioassay indicative of chronic activation of the hypothalamus-pituitary-adrenal (HPA) axis.[1] Indeed, chronic stress increases adrenal weight in rats of both sexes.[2,3] Similar data are obtained following

chronic ACTH administration.[4] In a previous study from this laboratory conducted in cycling female rats examined in diestrus-2 (DE-2), we reported that chronic stress-induced increase of adrenal weight and a parallel decrease of thymic weight may result from daily handling for vaginal smear screening (VSS).[5]

Male rats show reduced response to stress compared to female rats.[6-8] This appears to be due to the high circulating testosterone (T) and the non-aromatizable dihydrotestosterone (DHT).[9-11] It has been suggested that T affects the corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression in response to stress, thus affecting the HPA axis.[12] On the other hand, the higher HPA axis activation observed in female rats subjected to equal stress has been attributed to a potentiating effect of estradiol (Ediol) on CRH response.[13] Indeed, in the afternoon of proestrus (PE), during which the peak of Ediol levels is observed in rats,[14,15] the expression of CRH mRNA is increased in the parvocellular paraventricular nuclei (PVN).[16] It is of interest that a similar effect takes place in humans, in whom estrogens induce CRH gene expression.[17]

In the present study, we sought to investigate the modifying role of T and of the T/Ediol ratio on the response to chronic stress in female rats. Stress response was assessed by adrenal weight. The levels of T and the modifying effects of exogenous estradiol benzoate (EB) administration were assessed in ovariectomized (OVX) and non-OVX rats following low chronic emotional stress.

In 4-day cycling rats, during PE, estrus (E), diestrus-1 (DE-1) and DE-2, Ediol reaches the highest levels at 14.00-16.00 in PE[14,15] and the lowest between midnight of E and 15.00 h of DE-1.[14] Accordingly, the circulating T/Ediol balance might be differentiated between PE and E, but also during the 3 cycle phases preceding PE compared to the 3 cycle phases preceding E. These hormonal alterations may differently affect stress-response during a period of chronic stress through VSS. Therefore, in the present study we investigated the impact of T, Ediol and T/Ediol ratio on stress-induced adrenal, thymus and hippocampal weight change in PE and E controls and 20 h after surgery in DE-2 and PE, respectively, following similar chronic emotional stress.[18,19]

MATERIALS AND METHODS

Animals and procedures

Female Wistar rats were born and raised in the Department of Pharmacology, School of Medicine, National and Kapodistrian University of Athens, and were kept in groups of 8 rats in large cages under controlled conditions (14 h light: 10 h dark, lights on from 06:00 h to 20:00 h; 21-23°C). Rat chow and water were provided *ad libitum*. Rats were studied at 3-4 months of age. The number of studied rats (n=66) included 24 OVX and 42 non-OVX rats. Circulating T levels were measured by radioimmunoassay (RIA) in the 66 rats (i.e. 24 OVX and 42 non-OVX). Ediol was also measured in PE and E in 24 rats (n=12 per group).

The following experimental protocols were conducted:

Effect of EB administration on stress-induced adrenal weight changes

In this set of experiments, the 24 OVX rats were left undisturbed for 19 days post-OVX. Following this period of rest, the animals were handled for weighing and for a daily subcutaneous (s.c.) injection of EB (25 μg/100g bw; n=12) or olive-oil as vehicle (VEH; n=12) for 3 consecutive days. Two days following the last (third) EB or VEH injection, all OVX rats were sacrificed. The scope of exp. 1 was to evaluate: a) the impact of EB treatment on circulating T and adrenal weight in OVX rats and b) the impact of the low and the high T-range (i.e. circulating T below and above the mean, respectively) on stress-induced adrenal weight in VEH- and EB-treated rats.

Effect of testosterone on stress-induced adrenal weight changes

A set of non-OVX rats (n=12) was exposed only to the mild stress of injections of EB (25 μg/100g bw) during 6 consecutive days. Two days after the last injection, the rats were sacrificed and serum T concentrations were assayed. These rats were not exposed to the chronic emotional stress through handling for VSS. The scope of exp. 2 was to assess the mean levels of circulating T and the impact of the low and the high T-range on the corresponding adrenal weight.

Effect of stress on testosterone-induced changes of adrenal, thymus and hippocampal weight in the DE-2, PE and E

Circulating T levels were measured in DE-2 in 6 cycling rats after low chronic stress, as shown by the low emotional reactivity score (ERS) recorded during VSS, and in 8 rats in PE and 8 in E after similar higher chronic emotional stress. The scope of exp. 3 was to evaluate: a) the mean circulating T levels in DE-2 after low chronic stress and in rats in PE (n=8) and in E (n=8) after similar chronic stress and preceding surgery by 20 h in DE-2 and in PE, respectively, for sham adrenalectomy (ADX) in 4 out of 8 rats in PE and 4 out of 8 rats in E, respectively; b) the impact of stress exposure on the weight of stress-sensitive organs (i.e. adrenals, thymus and hippocampus). Ediol was also measured in parallel with T in PE and E in order to observe differences between PE and E due to the preceding surgery and the interference of high ERS and also the difference in T/Ediol ratio. In the 6 rats examined during DE-2, in order to achieve a low stress through VSS, the protocol was as follows: handling for weighing on 6 consecutive days and handling for weighing and VSS on 6 consecutive days and on another 6 consecutive days with only one day interruption, which resulted in habituation to this kind of VSS and low ERS. This continued until sacrifice on the 5th or 6th day following weighing and VSS. In 24 rats after at least 3 consecutive 4-day estrous cycles, estimation of T and Ediol included 8 controls, 4 in PE and 4 in E, and 16 rats, 8 in PE and 8 in E, that were sacrificed 20 h after surgery either for sham-ADX (n=8) or left-ADX (n=8) on the preceding day of DE-2 or PE, respectively.

Net effect of testosterone and Ediol on stress-induced changes of adrenal, thymus and hippocampal weight

In this set of experiments, rats were examined during PE (n=8) and E (n=8) and were separated into a low and a high T group, irrespective of cycle phase and surgery, in order to assess the effect of circulating T and of T/Ediol ratio on chronic stress-induced changes of adrenal, thymus and hippocampal weight.

Effect of testosterone and the T/Ediol ratio on stress-induced changes of hippocampal weight

This set of experiments included 22 rats (2 out of 24 rats were excluded because of the presence of leucocytes during the PE in 2 preceding cycles). The purpose of this experiment, after grouping the animals into low and high T was: a) to examine the difference in hippocampal weight after exposure to chronic stress; b) to estimate the 24 h body weight difference and the impact of the preceding by 20 hours surgery and cycle phase distribution; and c) to estimate the mean Ediol and the T/Ediol ratio and PE and E distribution in relation to the hippocampal weight.

Vaginal smear screening (VSS) as stress-inducing procedure

VSS was used as a stress-inducing procedure. VSS was carried out between 09:00 and 11:00 h each day. This procedure has been described in detail in a previous study[5] as a means of determining the different reactions that are recorded during VSS.[18,19] The final number of recorded reactions represented the individual emotional reactivity score (ERS) for each rat.

Surgical procedures

Bilateral OVX was performed under ether anesthesia by bilateral dorsal approach. The surgical procedure for sham-ADX (n=8) or left-ADX in cycling rats was performed between 14:00 and 16:00 h either in DE-2 or in PE, as before.[20] In 30 cycling rats, the brain was removed at autopsy and the hippocampus was rapidly dissected on ice and weighed. The boundaries of the whole hippocampus were assessed according to Figure 92 of the Paxinos rat brain atlas.[21] The adrenals and the thymus gland were isolated and weighed in all cycling rats at autopsy.

Assays of testosterone and estradiol

Serum concentrations for T or Ediol were measured upon sacrifice by decapitation between 09:00 and 11:00 h. In order to measure T and Ediol levels, serum samples (0.5mL and 1.0 mL, respectively) were extracted with 3mL of diethyl ether for 2 min with a vortex mixer and centrifuged in a freezing centrifuge. Diethyl ether was used after being freed from peroxides by silver nitrate before distillation in order to reduce solvent blank. The RIA was performed on 2mL and 1.5mL of extract (for T and Ediol, respectively) dried under nitrogen gas at 35-40°C. The standard T curve was prepared in the presence of 2mL of solvent in order to account for solvent blank interference. The

standard Ediol curve was prepared in the presence of 1.5mL of solvent. The sensitivity of the assay was 3.2±1.2pg. T radioimmunoassay was performed using kits supplied by CEA-IRE-SORIN as the Ediol RIA which has been described in detail in a previous study.[15] The antiserum supplied by the kit for T RIA was raised in rabbit against a testosterone-6-conjugate with bovine serum albumin. Final working titre: 1:40,000 following the procedure suggested for the assay. The percent cross-reaction of this antiserum (50% inhibition of the tracer binding) was 75 for dihydro-testosterone, 0.2 for androstenediol and androstenedione, 0.02 for androsterone and epitestosterone, 0.01 for dehydroepiandrosterone, 0.006 for progesterone, 0.001 for Ediol and cortexone and <0.0001 for cortisol and etiocholanolone. The recovery of tritiated testosterone ranged between 97-99%. The intra-assay coefficient of variation (CV) in three different ranges was 9.34% (74.7±6.98 SD), 11.5% (115.3±13.34 SD) and 14% (278.5±39 SD). The sensitivity of the assay was 2.5 pg. The inter-assay coefficient of variation was 15.2%. The interference of cross-reaction of testosterone with dihydrotestosterone (DHT) was considered of minor importance for the purpose of this study, since in male rats after gonadectomy, treatment with DHT beginning at the time of adrenalectomy prevented the significantly elevated levels of hypothalamic CRH compared to intact animals,[9] and since both testosterone and dihydrotestosterone are potent androgens and alter corticotropin-releasing hormone and arginine vasopressin mRNA within forebrain sites known to regulate activity of the HPA axis.[12]

Statistical Analysis

All values are presented as mean ± SEM. Statistical significance by Student's t-test between two independent groups was evaluated at the 2P level. Logarithmic conversion of values was performed when normality assumption was violated. The level of statistical significance was set to 5%. One-way analysis of variance (ANOVA) followed by *Scheffé post hoc* analysis was used to determine significant differences among the DE-2, PE and E phases. Two-way ANOVA, followed by *Tukey's post hoc* analysis was implemented in order to elucidate specific differences among the four groups in EB- and VEH-treated OVX rats.

RESULTS

Effect of EB administration on stress-induced adrenal weight changes

In the OVX rats, EB treatment increased serum T levels and the absolute and relative adrenal weights (Figure 1). After separation of VEH- and EB-treated
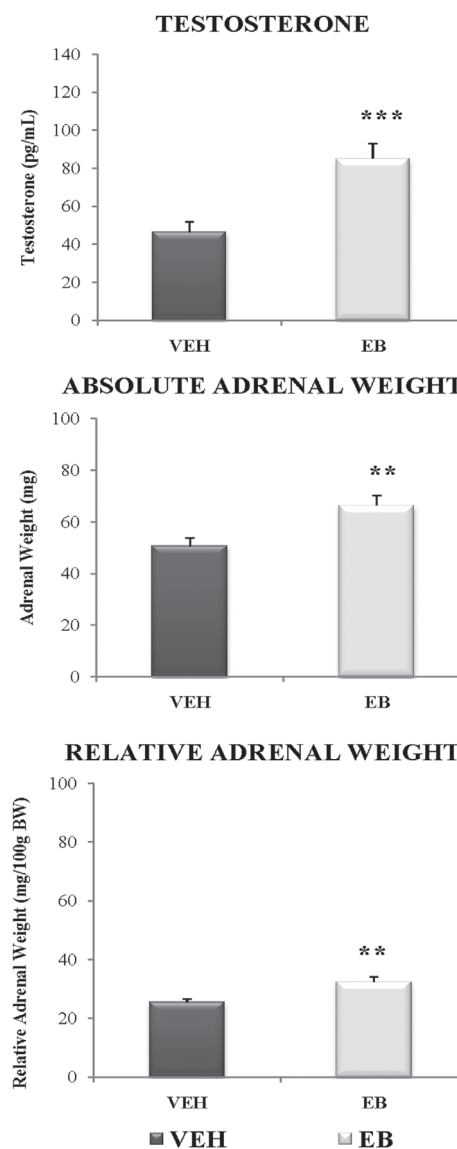
Figure 1. Circulating testosterone (T) and adrenal weight in ovariectomized (OVX) rats estimated 2 days after the mild stress of estradiol benzoate treatment (EB; 25 μg/100 g bw) in 12 rats or the vehicle (VEH) in 12 rats on 3 consecutive days. Body weight did not differ between the two groups. Data are presented as means±SEM **P < 0.01; ***P < 0.001 compared to VEH-treated rats.

rats into the low and high T-range, respectively, a two-way ANOVA followed by *Tukey's post hoc* analysis showed that high T was accompanied by increased adrenal weight in the EB treated group compared to the VEH-treated group only in the low T group (Figure 2).

### Effect of testosterone on stress-induced adrenal weight changes

In the non-OVX rats (n=12) at 4 months of age, exposed only to the mild stress of s.c. EB injections for 6 consecutive days and grouping as per T (low and high T), a significant reduction of adrenal weight was revealed only in the rats assigned to the high T group (Figure 3).

Effect of stress on testosterone-induced changes of adrenal, thymus and hippocampal weight in the DE-2, PE and E

Regarding the rats examined in DE-2 period after low chronic emotional stress through VSS (Table 1) and VEH-treated OVX rats (Figure 1), the circulating mean T levels on DE-2 in 6 rats (265 pg/mL) were higher compared to T of adrenal origin (46.4 pg/mL) found after the mild stress of low duration in 12 VEH-treated OVX rats (Figure 1). Thus, these results point to the ovarian origin of T in DE-2 for the difference of 218.6 pg/mL. One-way ANOVA followed by post hoc analysis revealed no significant difference of T in rats in DE-2, PE and E (Table 1). Comparison of T between E and DE-2 by *Student's t-test* revealed lower T in E. After conversion of ERS values into logarithms, *Student's t-test* revealed higher ERS during PE as compared to DE-2, and in E compared to DE-2 (Table 1). One-way ANOVA also revealed that the relative adrenal weight was higher and the thymus weight was lower in PE and E compared to DE-2. Of note, these differences were accompanied by higher ERS. Moreover, hippocampal weight was also lower in PE compared to DE-2 and in E compared to DE-2. Estimation of Ediol in PE following high chronic stress revealed the cycle phase interference showing higher circulating Ediol in PE compared to E but no differences in T/Ediol ratio (Table 1). Uterine weight, before extrusion of the intralumenal fluid, was significantly higher in PE *versus* E [PE: (8) 770.66±37.24mg; E: (8) 475±20.09mg] at an extremely high significance
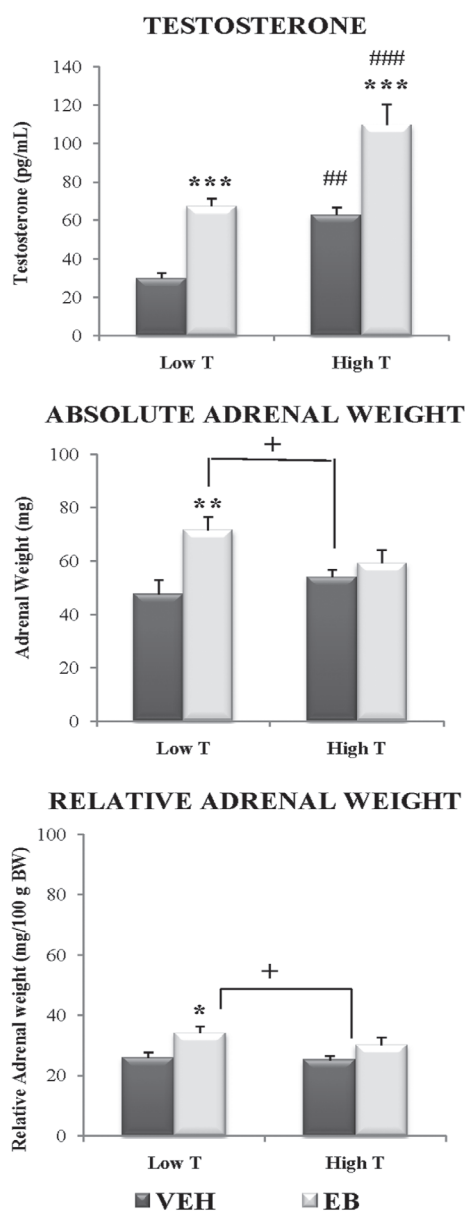
Figure 2. Relation of circulating testosterone (T) levels to adrenal weight after separation of estradiol benzoate (EB; 25 µg/100 g bw) and vehicle (VEH)-treated OVX rats into high T and low T-range groups according to their T levels (below or above the mean). The low T-range group consisted of (6) VEH-treated rats [T: 22-39 pg/mL; below the mean T (12) 46.4 ± 5.46 pg/mL; bw: 182 ± 13.3 g] and (7) EB-treated rats [T: 52-79 pg/mL; below the mean T, EB mean (12) 85.2 ± 7.79 pg/mL; bw: 209±12.5 g]. The high T group consisted of 6 VEH-treated rats [T: 54-79 pg/mL; bw: 218.6±16.55 g] and 5 EB-treated rats [T: 90-150 pg/mL; bw: 200±15.8 g]. Data are presented as means±SEM. *P <0.05; **P <0.01; ***P <0.001, differences between VEH- and EB-treated rats. ##P <0.01; ###P <0.001 differences between high T and low T groups in VEH- and EB-treated rats respectively +P <0.05, differences between EB-treated low T group and VEH-treated high T group.

TESTOSTERONE

ADRENAL WEIGHT

RELATIVE ADRENAL WEIGHT
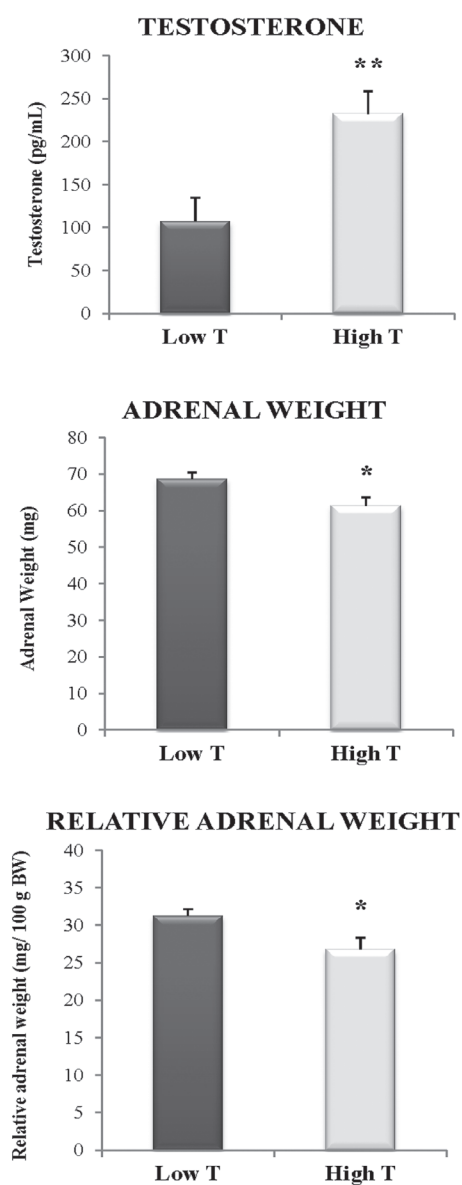
Figure 3. Relation of circulating testosterone (T) levels to adrenal weight after separation of estradiol benzoate (EB; 25 μg/100 g bw) treated non-ovariectomized (non-OVX) rats according to their T levels below or above the mean. Mean T levels (12) 169.6±26.25 pg/mL. In the high T group [range: 200-355 pg/mL; bw: 231.33±11.29 g], as compared to the low T group [range: 60-165 pg/mL; bw: 220.16±6.53 g] (n=6 rats per group). *P <0.05; **P <0.01 differences between low and high T-range groups.

(t=6.12 for the absolute and t=11.5 for the relative weight). In PE, after the extrusion of intralumenal fluid, relative uterine weight was found significantly higher compared to E (p<0.05) [PE: (8) 321.75 ± 14.32 mg/100g bw; E: (8) 274.9 ± 11.82 mg/100g bw]

but no difference in absolute uterine weight was found [PE: (8) 530.38 ± 33.93 mg; E: (8) 475.8 ± 20.9].

Net effect of testosterone and Ediol on stress-induced changes of adrenal, thymus and hippocampal weight

Assignment of the 16 rats (8 in PE and 8 E) into two groups according to serum T concentrations (i.e. low and high T) (Table 2) yielded a 5-fold difference in T levels between these two groups (i.e. 67.5 pg/mL versus 337.5 pg/mL, respectively). Interestingly, the 8 rats assigned to the high T group had reduced adrenal weight and increased hippocampal weight, as compared to the low T group. Moreover, there was prevalence of PE versus E in the high T group with the opposite distribution observed in the low T group. There was no difference in Ediol levels, while the T/Ediol ratio was significantly higher in the high T group (Table 2).

Effect of testosterone and the T/Ediol ratio on stress-induced changes of hippocampal weight

Separation of 22 rats into the low and high T-range (Table 3), after including 6 left-ADX rats (3 in PE and 3 in E) at 20 h following left-ADX in DE-2 and PE, respectively, revealed significantly higher hippocampal weight without alterations in thymus weight in the rats assigned to the high T-range. In these rats T levels were more than 5-fold higher, as compared to the rats assigned to the low T-range. In the low T-range higher 24 h body weight loss was revealed, with E prevailing over PE and prevalence of preceding surgery versus controls. Moreover, PE prevailed over E in rats with a significantly lower body weight loss in the high T group. Given that there was no difference in Ediol levels between the low and high T groups, the T/Ediol ratio was higher in the high T group (i.e. 6.27 versus 1.715; Table 3). In these 22 rats the difference in adrenal weight was not presented in the Table because of the presence of 6 left-ADX rats with only one adrenal and because the difference in adrenal weight in the 16 out of 22 rats has been presented in Table 2. Individual adrenal growth (IAG), defined as the growth seen in the remaining adrenal relative to the corresponding removed adrenal in the same rat, was determined by the weight difference between the remaining right adrenal and the previously excised left adrenal at 20 h following left-ADX in DE-2 or PE.[20] Herein, IAG was found low during

Table 1. Circulating testosterone and estradiol levels, the weight of the hippocampus and the corresponding stress-associated adrenal and thymus weight on diestrus-2 (DE-2) after low chronic emotional stress and on proestrus (PE) and estrus (E) after similar high chronic emotional stress

	Diestrus-2 (DE-2)	Proestrus (PE)	Estrus (E)
Age (days)	(6) 93.0 ± 1.23	(8) 97.0 ± 2.06	(8) 98.0 ± 1.62
Body weight (bw; gr)	(6) 164.6 ± 5.30	(8) 166.81 ± 3.80	(8) 172.92 ± 2.8
Duration of handling (days)	(6) 17.0 ± 1.84	(8) 16.23 ± 2.88	(8) 17.25 ± 1.41
Emotional Reactivity Score (ERS)	(6) 3.0 ± 1.09	(8) 14.0 ± 2.88	(8) 15.37 ± 3.60
ERS (logarithms)	(6) 0.3008 ± 0.155	(8) 1.078±0.109[++]	(8) 1.10±0.106[++]
Adrenal weight (mg/100 g bw)	(6) 24.09 ± 0.51	(8) 32.31 ± 1.80*****	(8) 36.36 ± 0.95*****
Thymus weight (mg/100 g bw)	(6) 256.4 ± 9.42	(8) 204.3 ± 9.89**	(8) 210.83 ± 8.12**
Hippocampal weight (mg/100 g bw)	(6) 70.76 ± 2.66	(8) 55.25 ± 3.48[+++]	(8) 54.49 ± 3.17[+++]
Testosterone (pg/mL)	(6) 265 ± 24.30[+]	(8) 253.75 ± 56.06	(8) 151.0 ± 42.3
Testosterone Range	180-360	60-430	50-350
Estradiol (pg/mL)		(8) 58.47 ± 4.96[+]	(8) 43.18 ± 4.46
Estradiol range		(8) 49-88	(8) 28-64
Testosterone/estradiol ratio		(8) 4.83 ± 1.10	(8) 3.48 ± 0.983

Values represent means±S.E.M. Number of samples is in parenthesis. **P<0.01; ***P<0.001 versus DE-2 assessed by one-way ANOVA; [+] P<0.05 versus E by Student's t-test; [++]P<0.01 versus DE-2 by Student's test after logarithmic conversion of values; [+++] P<0.001 versus DE-2 by Student's t-test; [+] P<0.05 versus E.

Table 2. Relation of circulating testosterone (T) levels Estradiol and T/estradiol ratio to adrenal and hippocampal weight after separation of rats in proestrus (PE) and estrus (E) into low T and high T-range following similar high chronic-stress

	Low testosterone (T) range	High testosterone (T) range
Mean testosterone (pg/mL)	(16) 205 ± 36.08	
Testosterone range (pg/mL)	(8) 50-80	(8) 230-430
Testosterone (pg/mL)	(8) 67.5 ± 3.65	(8) 337.5 ± 21.36
Estradiol range (pg/mL)	(8) 31.5-88	(8) 28.5-56
Estradiol (pg/mL)	(8) 51.31 ± 6.85	(8) 50.75 ± 3.73
Testosterone (T)/estradiol ratio	(8) 1.49 ± 0.218	(8) 6.83 ± 0.363***
Age (days)	(8) 98.5 ± 1.32	(8) 96.5 ± 1.0
Body weight (g)	(8) 169.5 ± 3.21	(8) 167.5 ± 3.86
Duration of handling (days)	(8) 16.87 ± 0.66	(8) 15.87 ± 1.24
Emotional reactivity score	(8) 14.0 ± 2.95	(8) 16.6 ± 3.45
Cycle phase distribution	(8) 3 PE; 5 E	(8) 5 PE; 3 E
Surgery distribution	(8) 5 versus 3 controls	(8) 3 versus 5 controls
Adrenal weight (mg)	(8) 63.16 ± 1.74	(8) 53.91 ± 3.04**
Adrenal weight (mg/100 g bw)	(8) 36.68 ± 0.98	(8) 32.0 ± 1.50*
Thymus weight (mg)	(8) 339.2 ± 17.86	(8) 371.2 ± 13.30
Thymus weight (mg/100 g bw)	(8) 201.67 ± 8.20	(8) 210.78 ± 8.98
Hippocampus (mg)	(8) 83.5 ± 4.23	(8) 101.67 ± 4.07**
Hippocampus (mg/100 g bw)	(8) 48.4 ± 2.43	(8) 60.78 ± 2.48 **

The values are expressed as means ± S.E.M. Number of samples in parenthesis. *P<0.05; **P<0.01; statistically significant differences between the two groups. ***P<0.001, t=12.6.

Table 3. Differences between cycling rats with low and high testosterone range after exposure to similar high chronic stress

	Low T-range	High T-range
Testosterone mean (pg/mL)	(22) 196 ± 31.94	
Testosterone range (pg/mL)	(12) 50-80	(10) 230-480
Testosterone (pg/mL)	(12) 65.4 ± 2.98	(10) 348.0 ± 22.64
Estradiol range (pg/mL)	(12) 30.5-88	(10) 28-93
Estradiol (pg/mL)	(12) 48.30 ± 5.66	(10) 56.66 ± 5.42
Testosterone (T)/estradiol ratio	(12) 1.715 ± 0.209	(10) 6.27 ± 0.61
Age (days)	(12) 99 ± 1.53	(10) 96.33 ± 0.915
Body weight at autopsy (g)	(12) 171.8 ± 1.80	(10) 169.04 ± 3.23
24h body weight loss (g)	(12) 4.058 ± 0.86	(10) 1.21 ± 0.64*
Duration of handling (days)	(12) 17.25 ± 0.538	(10) 16.4 ± 1.080
Emotional reactivity score	(12) 14.6 ± 2.17	(10) 16.6 ± 2.72
Cycle phase distribution	(4) PE; (8) E	(7) PE; (3) E
Preceding surgery by 20h	(12) 9 out of 12 rats (5) sham ADX; (4) left ADX	(10) 5 out of 10 rats (3) sham ADX; (2) left ADX
Hippocampal weight (mg)	(12) 81.54 ± 3.30	(10) 96.69 ± 3.61**
Relative hippocampal weight (mg/100g bw)	(12) 47.37 ±1.73	(10) 58.646 ±2.51 **
Thymus weight (mg)	(12) 359.74 ± 13.06	(10) 337 ± 15.18
Relative thymus weight (mg/100g bw)	(12) 212.95 ± 7.61	(10) 199.11 ± 7.689

Values are expressed as mean ± S.E.M. Number of samples in parentheses. *P<0.05; **P<0.01 statistically significant differences between the two groups.

PE in 3 rats (0.933±0.617 mg), with 2 rats being in the high T group (i.e. T: 480 pg/mL and 300 pg/mL) and one in the low T group (i.e. T: 60pg/mL) (Table 3). Conversely, in 3 rats assigned to the low T group IAG was found 6.06±1.91 mg during E, following left-ADX in PE.

DISCUSSION

The purpose of the present study was to investigate the modifying effects of T and Ediol on chronic stress. Stress response was assessed by adrenal, thymus and hippocampal weight changes.

We have found that exogenous Ediol administration augmented the response to stress. Indeed, EB treatment of OVX rats resulted in increased adrenal weight (Exp. 1). Of note, the increase in adrenal weight confirmed previous results in OVX EB-treated rats.[23] Separation of these rats into low and high T revealed that EB treatment in OVX rats induced a robust increase of adrenal weight only in those rats exhibiting low T. The current findings in EB-treated OVX and non-OVX rats (Exp. 2) indicate a negative impact of the high T-range on the corresponding low chronic stress-associated adrenal weight increase. Thus, these results suggest that the reduction of stress response in EB-treated OVX and non-OVX rats assigned to the high T-range may counteract the EB-associated enhancement of stress response by shifting the T/Ediol balance into suppression of the HPA axis.

Concerning androgen involvement in stress-response the non-aromatisable androgen dehydrotestosterone (DHT) and its metabolite 5 alpha-androstane-3 beta, 17 beta diol, inhibit the HPA response to stress by acting through estrogen receptor beta (ER-beta)-expressing neurons in the hypothalamus.[24] Since T can be converted to DHT, an alternative pathway for T and DHT regulation of the reduced response to stress has been revealed.[25] On the other hand, in OVX rats it has been reported that EB-treatment may reduce ER-beta expression in neurons around the PVN of the hypothalamus, shifting the Ediol/T balance towards activation.[26] The latter is in accordance with both the present findings in EB-treated OVX

and with previous results.[23] Taking into account the three important studies[24-26] and our present findings regarding the high and low T-range in EB-treated rats, we may speculate that part of T in the high T-range was converted to DHT and part of DHT to its metabolite. DHT and its metabolite would reduce HPA response to stress, possibly contributing to the overall reduced stress responsiveness manifested in rats assigned to the high T-range.

The low chronic emotional stress (expressed by the lower ERS) in DE-2 compared to PE and compared to E was accompanied by lower adrenal but higher thymus and hippocampal weights. The only difference between PE and E in the present study was the higher Ediol in PE compared to E, as expected.[14]

Separation of cycling rats in PE and E into the low and the high T-range, irrespective of cycle phase and preceding surgery, revealed a reduction of adrenal weight accompanied by increased hippocampal weight in the high T-range without difference in ERS, suggesting that high T levels alleviated stress response. However, no difference in thymus weight was observed. Interestingly, a study in male rats revealed that age-related involution of the thymus from the juvenile period through puberty to post-puberty depends on the rising testosterone levels and represents mainly a decrease of thymic lymphoid cell elements.[27] In another study, immunohistochemical expression of androgen receptors (AR) was detected in a considerable number of thymic epithelial stromal cells in the medulla predominantly and in scattered cells of lymphoid origin in the cortex and beneath the thymic capsule of juvenile, pubertal and post-pubertal male rats.[28] Considering these findings in males, a higher thymus weight, as was found in DE-2 compared to PE and E after low chronic stress that was not accompanied by alterations in T levels, could be expected. However, in the present study the lack of thymus weight change could be attributed to the 5-fold higher T levels. The 5-fold higher T levels in female rats, as compared to rats of similar age subjected to similar high chronic emotional stress, possibly resulted in gradual loss of thymic lymphoid cell elements, thus masking the increase in thymus weight due to high T-associated reduction of stress response. Notably, such a possibility in female rats needs further investigation.

Bearing in mind the far higher T and T/Ediol ratio in rats assigned to the high T-range, we should also consider the putative presence of DHT and its metabolite, derived from conversion of part of the high T, which would inhibit the HPA response to stress through ER-beta expressing neurons in the hypothalamus resulting in reduced ACTH and corticosterone. Theoretically, the decrease in ACTH might have contributed to the reduction of stress-associated adrenal weight increase found in the high T-range. On the other hand, increased hippocampal weight may be attributed to reduced stress-associated hippocampal neuronal degeneration through inhibition of corticosteroid responses.

The prevalence of PE in the high T-range may have resulted from the higher levels of T of adrenal origin in PE from E and the other 2 phases due to the higher response to stress in PE, as was shown by the significantly higher ACTH and corticosterone response to 20 min restraint stress in cycling rats during PE compared to E, DE-1 and DE-2.[29] Recent studies have revealed a T-dependent suppression of CRH-stimulated cortisol secretion in men.[30] Another study exposed a direct androgenic involvement in the expression of CRH in men through colocalization of CRH and AR in PVN neurons.[31] A recent study in healthy young women[32] revealed that exogenous T administration attenuated the integrated stress response, thus highlighting the importance of future investigation regarding the possible role of the HPA axis in disorders that are associated with its dysfunction.

Similar results concerning the increased hippocampal weight and the prevalence of PE were found after including 6 rats following left-ADX, (in 3 rats in DE-2 and in 3 in PE) and separation into the low and high T-range. Concerning the higher hippocampal weight in rats with high T and prevalence of PE *versus* E, we may consider a possible synergism between Ediol and T in this respect. As far as hippocampal weight is concerned, this synergism in PE is expressed by the Ediol-mediated fluctuation of synapse density in the CA1 hippocampal region in PE compared to E[33] during the estrous cycle, with a peak on the day of PE which might increase transiently the hippocampal weight.[34] The lower hippocampal weight in the low T-range with prevalence of E *versus* PE may partly be

attributed to the antagonism of progesterone *versus* Ediol that may result in the rapid regression of CA1 region dendritic spine density in E after ovulation,[35] when Ediol levels are low and progesterone levels are high.[14] Two more recent studies in female rats confirmed that progesterone antagonized the Ediol-dependent neurite outgrowth and neuron viability in the presence of Ediol, as observed *in vivo*, and that progesterone receptor antagonists (i.e. ORG-31710 and RU-486) blocked the antagonism of progesterone on Ediol-dependent sprouting.[36,37] The lower hippocampal weight in rats assigned to the low T-range, with prevalence of E in 8 rats and the prevalence of preceding surgery in 9 out of these 12 rats, implies again the higher response to stress due to higher Ediol in PE preceding E in the hippocampal neuronal degeneration through the rise in corticosterone, which may also exert a negative impact on neurogenesis.[38,39] Hippocampal damage has been associated with preceding glucocorticoid exposure in primates,[40] while MRI-based measurement of hippocampal volume revealed lower volume in patients with combat-related posttraumatic stress disorders.[41] Notably, it would be of great interest to conduct epidemiologic studies in women with polycystic ovary syndrome,[42] and in women with and without hyperandrogenemia and memory dysfunction who were exposed to different chronic emotional stress, in order to elucidate the role of T and T/Ediol balance in this respect.

Rats assigned to the low T-range had significantly higher 24 h body weight loss as compared to their high T-range counterparts. The higher body weight loss in these rats (with prevalence of E in 8 rats) may be attributed to the higher Ediol during PE which would suppress food intake during PE, as shown in an earlier study.[15] Ediol may suppress food intake through increase in CRH secretion which has been shown to acutely stimulate proopiomelanocortin (POMC) neurons of the arcuate nucleus that elicit anorexic signals via a-MSH release and to increase thermogenesis.[43] In the high T-range with prevalence of PE in 7 out of 10 rats, the lower body weight loss may be attributed to the high T levels prevailing over the lower Ediol during the preceding DE-2, hence through lower CRH reduced aMSH, resulting in higher food intake.

CONCLUSIONS

The findings of the present study show the negative impact of the circulating high above mean T-range on stress-induced adrenal weight increase after similar low or high chronic emotional stress. Results in OVX EB- and VEH-treated rats and in the non-OVX EB-treated rats following low stress, and in cycling rats after high chronic emotional stress, indicate the important role of the T/Ediol balance in the modulation of the HPA axis in response to stress. Importantly, there was indication in cycling female rats exposed to high chronic stress and preceding surgery by 20 h that 5-fold higher T levels and 3.65-fold higher T/Ediol ratio antagonized the stress-associated Ediol-dependent activation of the HPA axis. The latter resulted in reduced stress-associated adrenal weight increase in control and rats after sham-ADX and in higher hippocampal weight and reduced 24h bw loss, as compared to rats assigned to low T-range. Notably, chronic elevation of T levels may attenuate the corticosterone-driven hippocampal neuronal degeneration through reduced response to stress. On the other hand, the prevalence of PE associated with the higher hippocampal weight suggests a synergism of PE with T in this respect.[33,34]

REFERENCES

1. Simpson ME, Evans HM, Li CH, 1941 Bioassay of adrenocorticotropic hormone. Endocrinology 33: 261-268.
2. Baron S, Brush FR, 1979 Effects of acute and chronic restraint and estrus cycle on pituitary--adrenal function in the rat. Horm Behav 12: 218-224.
3. Armario A, Restrepo C, Castellanos JM, Balasch J, 1985 Dissociation between adrenocorticotropin and corticosterone responses to restraint after previous chronic exposure to stress. Life Sci 36: 2085-2092.
4. Akana SF, Shinsako J, Dallman MF, 1983 Relationships among adrenal weight, corticosterone, and stimulated adrenocorticotropin levels in rats. Endocrinology 113: 2226-2231.
5. Sfikakis A, Galanopoulou P, Konstandi M, Tsakayannis D, 1996 Stress through handling for vaginal screening, serotonin, and ACTH response to ether. Pharmacol Biochem Behav 53: 965-970.
6. Le Mevel JC, Abitbol S, Beraud G, Maniey J, 1979 Temporal changes in plasma adrenocorticotropin concentration after repeated neurotropic stress in male and female rats. Endocrinology 105: 812-817.

7. Kant GJ, Lenox RH, Bunnell BN, Mougey EH, Pennington LL, Meyerhoff JL, 1983 Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology 8: 421-428.

8. Handa RJ, Burgess LH, Kerr JE, O' Keefe JA, 1994 Gonadal steroid hormone receptors and sex differences in the hyppothalamo-Pituitary-Adrenal Axis. Hormones and Behavior 28: 464-476.

9. Bingaman EW, Magnuson DJ, Gray TS, Handa RJ, 1994 Androgen inhibits the increases in hypothalamic corticotropin-releasing hormone (CRH) and CRH-immunoreactivity following gonadectomy. Neuroendocrinology 59: 228-234.

10. Viau V, Meaney MJ, 1996 The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. J Neurosci 16: 1866-1876.

11. Viau V, Chu A, Soriano L, Dallman MF, 1999 Independent and overlapping effects of corticosterone and testosterone on corticotropin-releasing hormone and arginine vasopressin mRNA expression in the paraventricular nucleus of the hypothalamus and stress-induced adrenocorticotropic hormone release. J Neurosci 19: 6684-6693.

12. Viau V, Soriano L, Dallman MF, 2001 Androgens alter corticotropin releasing hormone and arginine vasopressin mRNA within forebrain sites known to regulate activity in the hypothalamic-pituitary-adrenal axis. J Neuroendocrinol 13: 442-452.

13. Haas DA, George SR, 1988 Gonadal regulation of corticotropin-releasing factor immunoreactivity in hypothalamus. Brain Res Bull 20: 361-367.

14. Butcher RL, Collins WE, Fugo NW, 1974 Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 94: 1704-1708.

15. Sfikakis A, Spyraki C, Sitaras N, Varonos D, 1978 Implication of the estrous cycle on conditioned avoidance behavior in the rat. Physiol Behav 21: 441-446.

16. Bohler HC Jr, Zoeller RT, King JC, Rubin BS, Weber R, Merriam GR, 1990 Corticotropin releasing hormone mRNA is elevated on the afternoon of proestrus in the parvocellular paraventricular nuclei of the female rat. Brain Res Mol Brain Res 8: 259-262.

17. Vamvakopoulos NC, Chrousos GP, 1994 Hormonal regulation of human corticotropin-releasing hormone gene expression: implications for the stress response and immune/inflammatory reaction. Endocr Rev 15: 409-420.

18. Sfikakis A, Papadopoulou-Daifotis Z, Sfikaki M, Messari J, 1998 Monoaminergic dysregulation on diestrus-2 and estrus through high emotional reactivity. Pharmacol Biochem Behav 60: 285-291.

19. Sfikakis A, Papadopoulou-Daifotis Z, Bikas N, 2002 Inverse relationship of hippocampal serotonin to avoidance behavior, serotonergic activation by emotional stress differentiated by estrous cycle and surgical stress. Behav Brain Res 128: 41-52.

20. Sfikakis A, Pitychoutis PM, Antoniou K, Bikas N, Papadopoulou-Daifoti Z, 2008 The impact of the hormonal milieu and stress-associated hypothalamic dopaminergic activation on the rapid compensatory adrenal growth in cycling female rats. Hormones (Athens) 7: 303-312.

21. Paxinos R, Watson C, 1998 The rat brain in stereotaxic coordinates. Academic Press California and London.

22. Zarrow MX, Yochim JM, McCarthy JL, 1963 Estrogens in experimental Endocrinology. New York and London Academic Press; pp, 17-64.

23. Sfikakis A, Sfikakis P, Varonos D, 1981 Interaction between adrenals and thymus in the female rat. Acta Edocrinol 244: Suppl: 41-42.

24. Lund TD, Hinds LR, Handa RJ, 2006 The androgen 5 alpha-dihydrotestosterone and its metabolite 5-alpha-androstane-3 beta, 17 beta-diol inhibit the hypothalamo-pituitary-adrenal response to stress by acting through estrogen receptor beta-expressing neurons in the hypothalamus. Neurosci 26: 1448-1456.

25. Handa RJ, Pac TR, Kydwa AE, Lund TD, Hinds L, 2008 An alternate patway for androgen regulation for brain function: Activation of estrogen receptor beta by metabolite of dihydrotestosterone 5-a-androstane-3β, 17β-diol. Horm Behav 53:741-752.

26. Suzuki S, Handa RJ, 2004 Regulation of estrogen receptor beta expression in the female rat hypothalamus: differential effects of dexemethasone and estradiol. Endocrinology 145: 3658-3670.

27. Sfikakis PP, Kostomitsopoulos N, Kittas C, et al, 1998 Tamoxifen exerts testosterone-dependent and independed effects on thymic involution. Intern J Immunofarmacol 20: 305-312.

28. Sfikakis PP, Dellia-Sfikakis A, Karameris A, Varonos D, Katsilambros N, Kittas C, 1995 Immunohistochemical expression of androgen receptors (AR) in the rat thymus during normal thymic involution. Eur J Int Med 6: 62.

29. Viau V, Meaney MJ, 1991 Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129: 2503-2511.

30. Rubinow DR, Roca CA, Schmidt PJ, et al, 2005 Testosterone suppression of CRH-stimulated cortisol in men. Neuropsychopharmacology 30: 1906-1912.

31. Bao AM, Fischer DF, Wu YH, et al, 2006 A direct androgenic involvement in the expression of human corticotropin-releasing hormone. Mol Psychiatry 11: 567-576.

32. Hermans EJ, Putman P, Baas JM, Gecks NM, Kenemans JL, van Honk J, 2007 Exogenous testosterone attenuates the integrated central stress response in healthy young women. Psychoneuroendocrinology 32: 1052-1061.

33. Woolley CS, McEwen BS, 1992 Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12: 2549-2554.

34. McEwen BS, Alves SE, 1999 Estrogen actions in the central nervous system. Endocr Rev 20: 279-307.

35. Woolley CS, McEwen BS, 1993 Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336: 293-306.

36. Rosario ER, Ramsden M, Pike CJ, 2006 Progestins inhibit the neuroprotective effects of estrogen in rat hippocampus. Brain Res 1099: 206-210.

37. Wong AM, Rozovsky I, Arimoto JM, et al, 2009 Progesterone influence on neurite outgrowth involves microglia. Endocrinology 150: 324-332.

38. Schaff MJ, de Jong J, de Kloet ER, Vreungdeuhil E, 1998 Down-regulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. Brain Res 813: 112-120.

39. Woolley CS, Gould E, McEwen BS, 1990 Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res 531: 225-231.

40. Sapolsky RM, Uno H, Rebert CS, Finch CE, 1990 Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 10: 2897-2902.

41. Bremner JD, Randall P, Scott TM, et al, 1995 MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry 152: 973-981.

42. Diamanti-Kandarakis E, Kouli CR, Bergiele AT, et al, 1999 A survey of the polycystic ovary syndrome in the Greek island of Lesbos: hormonal and metabolic profile. J Clin Endocrinol Metab 84: 4006-4011.

43. Kyrou I, Tsigos C, 2008 Chronic stress, visceral obesity and gonadal dysfunction. Hormones (Athens) 7: 287-293.