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Effects of Estrogen Antagonists and Agonists on the ACTH Response to Restraint Stress in Female Rats

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Previous studies have found that female rats are less sensitive than males to hypothalamic-pituitary-adrenal axis feedback inhibition by exogenous glucocorticoid administration. To determine whether estrogen contributes to this sex difference, we examined the effects of the estrogen antagonists tamoxifen and C1628 on the ACTH and corticosterone responses to restraint stress. CI628 increased both the ACTH and corticosterone response to restraint stress, and tamoxifen increased the ACTH response to restraint. Using overiectomized female rats, we also examined the effects of seven days of estradiol and/or progesterone replacement. Low dose estradiol decreased the ACTH but not the corticosterone response to restraint stress while progesterone had no effect on ACTH or corticosterone responses. The combination of estradiol and progesterone also decreased the ACTH response to stress, and the magnitude of the effect did not differ from that found with estradiol treatment alone. These data suggest that in the physiological range estradiol is an important inhibitory factor in the hypothalamic-pituitary-adrenal stress response of females.

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Sex differences in HPA axis regulation in rodents have been demonstrated by a number of investigators, and these differences are generally attributed to activational effects of gonadal steroids on elements of the hypothalamic-pituitary-adrenal axis in females, although there is also evidence that testosterone inhibits HPA axis activity in males (Handa et al. 1994). Female rats have higher levels of total plasma corticosterone (Kitay 1963). Female rats also have higher plasma corticosterone binding globulin levels, which likely contributes to findings of similar free corticosterone levels in male and female rats. Compared with male rats, female rats also have a greater ACTH response to stress, faster onset of corticosterone secretion after stress, and a faster rate of rise of corticosterone (Le Mevel et al. 1979; Jones et al. 1972; Young 1996). Several studies suggest that estradiol plays a role in enhanced stress responses in female

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rats, based on increased HPA axis responses to stress when ovariectomized rats are treated with estradiol (Burgess and Handa 1992; Carey et al. 1995; Viau and Meaney 1991). In addition, female rats have enhanced HPA axis responsivity in proestrus, when estrogen and progesterone levels are relatively high, compared to diestrus, when levels of these hormones are lower (Viau and Meaney 1991).

Our own studies have focused upon sex differences in sensitivity to glucocorticoid feedback. We have found that gonadally intact female rats are insensitive to the feedback effects of 50% corticosterone pellets on stress induced ACTH secretion and that ovariectomy increased the sensitivity to steroid feedback (Young 1996). In another study, a steeper rate of rise of corticosterone was necessary to elicit glucocorticoid fast feedback in female rats than male rats (Jones et al. 1972). Similarly, women are less sensitive to dexamethasone feedback during the luteal phase when estrogen and progesterone levels are high than during the early follicular phase when both estrogen and progesterone are very low (Altemus et al. 1997). There is evidence that both estrogen and progesterone may play a role in the relative resistance to glucocorticoid feedback in females (Burgess and Handa 1992; Ferrini et al. 1995; Patchev et al. 1995; Rousseau et al. 1972; Peiffer et al. 1991).

To further define the underlying mechanisms of exaggerated HPA axis responses to stress in females, we examined the effects of two estrogen antagonists, tamoxifen and CI 628, on stress responses in intact, cycling rats. Use of estrogen antagonists rather than ovariectomy and estrogen replacement allows examination of the effects of estrogen without disruption of other ovarian hormones. In addition, because many prior studies have used supraphysiologal doses of estradiol in replacement studies, in ovariectomized female rats we examined the effects of replacement with low dose estradiol benzoate, progesterone, and the combination of estradiol and progesterone on stress responsiveness and sensitivity to glucocorticoid inhibition.

MATERIALS AND METHODS

All studies used Sprague Dawley female rats maintained on a 14-hr lights on, 10-hr lights off schedule.

Hormone and Drug Treatments

In the following studies, tamoxifen (Sigma, St. Louis, MO) was administered i.p. in a dose of 10 μg/100 g BW dissolved in 10% ethanol 90% normal saline. The dose was based on the studies of Shughrue et al. (1997), who demonstrated that this dose of tamoxifen was able to antagonize estradiol induction of progesterone receptors in the hypothalamus. The vehicle control for tamoxifen was a 10% ethanol 90% normal saline solution. The second estrogen antagonist, CI 628 (Parke-Davis Pharmaceuticals, Ann Arbor, MI) dissolved was administered i.p. in a 2 mg dose dissolved in distilled water (McEwen and Alves 1999), and distilled water was the vehicle control. Corticosterone was administered in a 150 mg pellet containing 50% corticosterone and 50% cholesterol. The control pellet contained 100% cholesterol. Pellets were implanted using metofane anesthesia. Estradiol benzoate (Sigma, St Louis, MO), was administered s.c. as 2 μgms dissolved in sesame oil and sesame oil was the vehicle. Progesterone was administered using silastic implants (Legan et al. 1975). The progesterone implants were constructed from 3.8 cm long silastic tubing with an inner diameter of 0.132 to which progesterone (Sigma, St Louis, MO) was added and then implants are sealed with silastic glue. The implants were washed in ethanol to remove surface progesterone and then primed overnight in tap water prior to implantation. These implants typically produce plasma progesterone concentrations of 50 ng/ml, equivalent to concentrations observed on the morning of proestrous (Legan et al. 1975). An empty silastic implant was used as a conrtol. Following the termination of each experiment, the continued presence of the corticosterone pellet or progesterone implant was verified in all animals.

Experiment 1: Effect of 3-day Tamoxifen Treatment on Glucocorticoid Feedback. Four groups of six female rats were used in this study. Weights were 200 ± 11.8 g (SD). Animals were implanted with corticosterone pellets or cholesterol pellets on day 0. Beginning on the morning of day 4, half of the animals in the corticosterone group and half of the animals in the cholesterol group received 3 days of tamoxifen injections and the remaining rats received vehicle injections. On day 6, rats were subjected to a 30 minute restraint stress. Tail blood samples for ACTH and corticosterone were obtained five minutes and thirty minutes into the restraint stress. Immediately after the 30 min restraint stress, rats were placed in ether vapor until unconscious and then decapitated and trunk blood collected for ACTH measurement. Thymus and both adrenals were also collected, dissected and weighed.

Experiment 2: Effect of 6 day Tamoxifen Treatment on Glucocorticoid Feedback. Two groups of seven female rats were used in this study. Weights were 214 ± 11.5 g (SD). All rats were given a vehicle injection 30 minutes before the onset of an initial 30 min restraint stress and tail blood for ACTH and corticosterone was collected at 5 and 30 minutes. Five days later, corticosterone pellets were implanted in all rats. Beginning the day of surgery, the tamoxifen group received 6 daily in-

jections of tamoxifen and the vehicle group received six daily injections of vehicle. After the sixth injection, rats were subjected to a second 30 min restraint stress with blood collected by tail nick at 5 minutes and 30 minutes into the restraint stress.

Experiment 3: Effect of 6 Day CI 628 Treatment on Glucocorticoid Feedback. Two groups of seven female rats were used in this study. Weights were 217 ± 17.7g (SD). All rats were given an initial 30 min restraint stress and blood for ACTH and corticosterone was collected at 5 and 30 minutes. Five days later, corticosterone pellets were implanted in all rats. Beginning the day of surgery, one group received 6 daily injections of CI 628 and the other group received six daily injections of vehicle. After the sixth injection, rats were subjected to a second 30 min restraint stress with blood collected by tail nick at 5 min and 30 min into the restraint stress.

Experiment 4: Effect of Corticosterone Pellets on HPA Axis Stress Response in Male Rats. Because corticosterone pellets had little effect on ACTH responses to restraint stress in Experiments 2, a test in male rats was conducted to assure that the corticosterone pellets were properly functioning. Four male rats were stressed at baseline by restraint stress and blood for ACTH was collected at 5 and 30 minutes. Three days later two pellets from each batch (Experiment 2 and 3) were implanted. Six days later, males were stressed a second time with blood again collected at 5 and 30 min into the restraint stress.

Experiment 5: Effect of 7 Day Estrogen Treatment on Glucocorticoid Feedback. Four groups of seven rats were studied. Rats weighed 204 ± 21 g(SD). Animals were overiectomized under metofane anesthesia, and following a six day recovery period, were then implanted with either cholesterol or corticosterone pellets. Beginning 24 hours later, estradiol benzoate or vehicle was injected for seven days. On day 7, all rats were subjected to 30 min of restraint stress with tail nick sampling of blood for ACTH, corticosterone, cortisol and progesterone at 5 and 30 minutes. Following conclusion of the restraint stress, all animals were anesthetized with ether then decapitated; trunk blood was collected for hormone measures.

Experiment 6: Effect of 7 Day Progesterone Treatment on Glucocorticoid Feedback. Four groups of six rats were to evaluate the effect of progesterone on HPA axis responses to stress. Animals were overiectomized, and following a six day recovery period, were then implanted with progesterone or blank implants and either cholesterol or corticosterone pellets. On the 7th day after implant insertion, all rats were subjected to 30 minutes of restraint stress with tail nick sampling of blood at 5 and 30 minutes for ACTH, corticosterone, estradiol and progesterone.

Experiment 7: Effect of 7 Day Estradiol Alone and Combined with Progesterone on HPA Axis Response to Stress. A final experiment examined the effects of estradiol benzoate injections and progesterone implants in combination on stress responsiveness. There were 3 groups of 8 rats: control (blank implant, sesame oil injected), estradiol alone (blank implant estradiol injection), and estradiol plus progesterone (progesterone implant and estradiol injection). Rats weighed 224 ± 12 g (SD). Animals were overiectomized, and following a six day recovery period were then implanted with progesterone or blank implants. On the same day animals received an estradiol benzoate or vehicle injection. Estradiol or vehicle injections continued daily for seven days. On the 7th day, all rats were subjected to 30 minutes of restraint stress with tail nick sampling of blood at 5 and 30 minutes. Following conclusion of the restraint stress, all animals were anesthetized with ether and then decapitated; trunk blood was collected for ACTH and corticosteron.

Hormone Assays

Following the end of the stress session, all samples for ACTH were centrifuged and the plasma separated and stored at −80°C until assay. The samples were assayed with Allegro HS-ACTH IRMA (Nichols, San Juan Capistrano, CA), using 25 μl of sample with 175 μl of zero standard as diluent. All ACTH data are expressed as pmoles/liter (pM). Progesterone and corticosterone were assayed unextracted using a commercial kit from Diagnostic Products Corporations (DPC-Coat a Count kits, Los Angeles CA) as specified by the manufacturer.

For estradiol, we used a commercial enzyme immunoassay kit for 17β estradiol (Correlate-EIA, Assay Designs, Ann Arbor, MI). Before performing the assay, plasma samples were extracted and concentrated twofold. Five hundred μl of plasma was added to 2 ml of ether and vortexed for 5 min. The organic layer was removed, added to an additional 2 ml ether, and vortexed for 5 minutes. The organic layer was removed again and evaporated to dryness in a Speed Vac (Savant Instruments, Holbrook, NY). Samples were reconstituted in 250 μl of a solution of 20% alcohol and 80% assay buffer from the EIA kit. All samples were run in a single assay. The intra-assay cv was less than 5% for all samples. Recovery of estradiol from the extraction was greater than 90%. Detection limit was 5 pg/ml.

Statistics

All data was analyzed with a two way or three way analysis of variance except experiments 2 and 3 which used a one way ANOVA. Sheffe F test post-hoc testing was used to test differences between groups at specific time points. Values are presented as mean ± SEM.

RESULTS

Experiment 1: Effect of 3 Day Tamoxifen Treatment on Glucocorticoid Feedback

Restraint stress activated ACTH secretion and ether stress produced a still greater activation [F = 12.78; df = 2, 19; p = .0001]. As expected, implantation of corticosterone pellets resulted in a blunted ACTH response to restraint and ether stress [F = 4.45, df = 1, p = .04] (Figure 1). Tamoxifen produced a significant increase in ACTH secretion [F = 7.2, df = 1, p = .01]. There was no interaction between corticosterone and tamoxifen treatments on the ACTH response to stress.

Baseline corticosterone was not affected by implantation of the corticosterone pellet (vehicle group = 60 ± 13 μg/dl, corticosterone group = 67 ± 3 μg/dl, t = 1.2, df = 1, t = 1.2) or the tamoxifen pellet (vehicle group =60±13 μg/dl; tamoxifen group = 73 ± 5 μg/dl (Table 1). Corticosterone secretion was enhanced in response to restraint stress [F = 33.5, df = 3,48, p <. 001]. As expected, the corticosterone response to stress was blunted in the rats inplanted with corticosterone pellets [F = 4.3; df = 1, 20; p = .04]. In addition, there was significant interaction between corticosterone pellets and tamoxifen on corticosterone secretion [F = 3.96; df = 3, 20; p = .01]. In contrast to the tamoxifen-associated enhancement of the ACTH response to stress seen in this experiment, tamoxifen attenuated the corticosterone response to stress in corticosterone treated rats (Table 1). Despite the lack of change in basal corticosterone level or adrenal weight after corticosterone implants, corticosterone implants did significantly reduce thymus weight [F = 4.5, df = 1, p = .049] (data not shown).

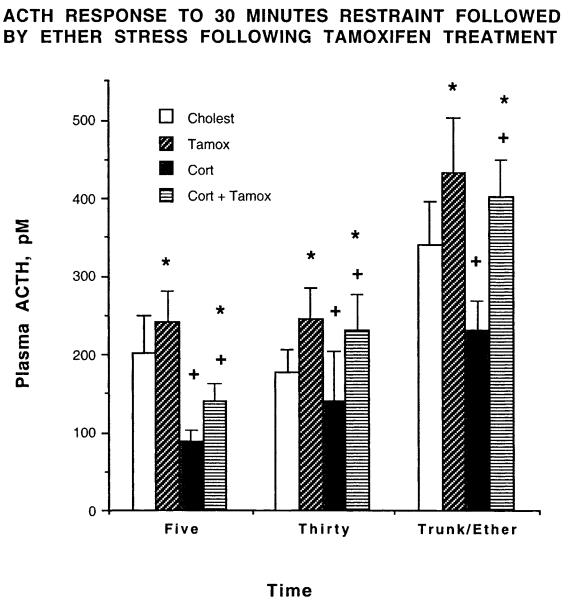

Figure 1. Effect of tamoxifen on ACTH response to stress in animals with either 50% corticosterone pellets or sham implants (cholesterol). Results are shown as mean ± SEM of six rats per group. There was a significant inhibition of the stress response by the corticosterone pellets (+) compared to cholesterol pellets and a significant increase in ACTH response to stress in tamoxifen treated animals (*) compared to vehicle treated animals.

Experiment 2: Effect of 6 Day Tamoxifen Treatment on Glucocorticoid Feedback

There was a significant effect of restraint stress on ACTH (Figure 2) [F = 9.09; df = 1, 14; p = .004] and corticosterone (Table 1) [F = 60.1, df = 2, p = .0001]. In contrast to Experiment 1, neither tamoxifen nor corticosterone produced significant effects on the ACTH response to restraint. Consistent with Experiment 1, there was no difference between baseline corticosterone before and after pellet implantation (basal = 14±3 μg/dl; corticosterone pellet = 13 ±1 μg/dl), but there was a significant effect of corticosterone pellets on the corticosterone response to stress [F = 9.4; df = 1,23; p = .003]. The corticosterone response to stress was blunted in rats with corticosterone implants (Table 1).

Experiment 3: Effect of 6 Day CI 628 Treatment on Glucocorticoid Feedback

Treatment with CI 628 caused an increase in the ACTH response to restraint stress (Figure 3) [F = 4.1; df = 2, 14; p = .02]). In this experiment, corticosterone pellets caused a significant decrease in ACTH response to restraint [F = 4.9, df = 1, 14, p = .03]. There was no difference in basal corticosterone before and after pellet implantation (basal = 15 ± 3 μg/dl; cort pellet = 14 ± 2 μg/dl), but there was the expected significant blunting effect of pellets on the corticosterone response to stress [F = 6.7; df = 1, 13; p = .01]. In addition, there was a significant effect of CI 628 on the corticosterone response to stress [F = 7.8; df = 1,13; p = .0066]. Rats treated with CI 628 had a greater corticosterone response to the stress (Table 1).

Experiment 4: Effect of Corticosterone Pellets on HPA Axis Stress Response in Male Rats

Because only two rats were tested using each corticosterone pellet, statistical analysis was not performed on this data. As shown in Figure 4, both pellets strongly inhibited the ACTH stress response in male rats.

Experiment 5: Effect of 7 Day Estrogen Treatment on Glucocorticoid Feedback

As before, ACTH increased in response to the restraint stress and showed the greatest response to ether stress [F = 19.8, df = 1, 27; p = .001] (Figure 5). The effect of

Table 1. Effect of Restraint Stress on Plasma Corticosterone Levels (μg/dl)

Drug Treatment	Time 0	+5	+30	trunk blood
Experiment 1 (n = 20)				
Vehicle/vehicle	60 ± 13	112 ± 12	136 ± 13	125 ± 3
Vehicle/tamoxifen	73 ± 5	115 ± 23	155 ± 15	115 ± 12
Corticosterone/vehicle	67 ± 3	96 ± 5	134 ± 9	122 ± 6
Corticosterone/tamoxifen	50 ± 10	102 ± 8	83 ± 9	90 ± 17
Experiment 2 (n = 21)				
Vehicle	14 ± 3	38 ± 4	49 ± 5	
Corticosterone/vehicle	13 ± 1	31 ± 4	39 ± 6	
Corticosterone/tamoxifen	12 ± 2	26 ± 4	33 ± 5	
Experiment 3 (n = 21)				
Vehicle group	15 ± 3	40 ± 3	52 ± 3	
Corticosterone/Vehicle	14 ± 2	32 ± 3	40 ± 3	
Corticosterone/CI628	17 ± 2	38 ± 3	47 ± 3	
Experiement 5 (n = 24)				
Vehicle/Vehicle	7 ± 1	17 ± 1	34 ± 1	30 ± 3
Vehicle/Estradiol	15 ± 5	25 ± 2	29 ± 5	29 ± 4
Corticosterone/Vehicle	8 ± 3	19 ± 1	34 ± 1	35 ± 4
Corticosterone/Estradiol	10 ± 2	32 ± 2	35 ± 4	31 ± 3
Experiment 6 (n = 24)				
Vehicle/vehicle	9 ± 3	28 ± 1	39 ± 2	
Vehicle/progesterone	7 ± 3	25 ± 3	40 ± 2	
Corticosterone/vehicle	8 ± 1	13 ± 2	22 ± 3	
Corticosterone/progesterone	6 ± 1	11 ± 1	21 ± 3	
Experiement 7 (n = 24)				
Vehicle/vehicle	16 ± 12	38 ± 4	48 ± 1	38 ± 1
Vehicle/estradiol	18 ± 1	40 ± 6	48 ± 3	38 ± 1
Progesterone/estradiol	28 ± 5	44 ± 3	48 ± 3	40 ± 4

Mean ± SEM.

corticosterone pellets on the ACTH response was small and demonstrated only a trend towards a decrease in the ACTH response [F = 3.07,df = 1,27; p = .08]. In contrast, estradiol treated rats demonstrated a significant inhibition of the ACTH response to restraint stress [F = 22.6; df = 1,27; p = .001]. There was no significant interaction between corticosterone and estrogen treatment.

As before, baseline corticosterone did not differ between cholesterol and corticosterone treated rats (Table 1; 7 ± 1 μg/dl in cholesterol treated animals; 8 ± 3 μg/dl in corticosterone treated animals). Corticosterone levels increased in response to restraint stress [F = 39.5, df = 1,27; p = .0001]. There was no significant effect of estradiol or corticosterone on corticosterone response to stress and no significant interactions.

There were no significant effects of corticosterone or estrogen on thymus weight (Table 2). Corticosterone cause a significant decrease in adrenal weight (Table 2) [F = 4.6, df = 1, 27; p = .04] while estradiol treatment caused a significant increase in adrenal weight [F = 16.0, df = 1, 27; p = .0005]. Estradiol treatment caused a significant increase in basal plasma estradiol (mean of sesame oil treated animals was 27 ± 5.8 pg/ml; mean of estradiol treated animals was 163 ± 25; [F = 23.5, df = 1, 24, p = .0001]). There was no change in estradiol in response to restraint stress. Using this same assay, we observed estradiol levels during diestrus 1 were 90.4 ± 40.5 pg/ml and proestrus were 215.5 ± 26.1 pg/ml in normal cycling female rats (MA, unpublished data).

Experiment 6: Effect of 7 Day Progesterone Treatment on Glucocorticoid Feedback

As before, stress resulted in activation of ACTH secretion with a significant effect of time [F = 19.9; df = 1, 22; p = .0001]. Implantation of corticosterone pellets produced a blunting of the ACTH response to stress (Figure 6) [F = 30.8, df = 1,22; p = .0001], but progesterone treatment had no effect on ACTH secretion and no significant interaction with the corticosterone pellets. There were no other significant effects or interactions. Again, corticosterone pellets did not significantly increase baseline corticosterone (cholesterol = 9 ± 3 μg/dl; corticosterone pellets = 8 ± 1.0 μg/dl). However there was the expected significant effect of corticosterone pellets to blunt the corticosterone response to stress [F = 75.2; df = 1, 23; p = .0001] (Table 1). There was no interaction between corticosterone and progesterone on the corticosterone response to stress. Mean progesterone levels in rats with progesterone implants was 26.0 ± 8.7 ng/ml, significantly higher than levels in rats with blank implants, 0.9 ± 0.6 ng/ml. There was no

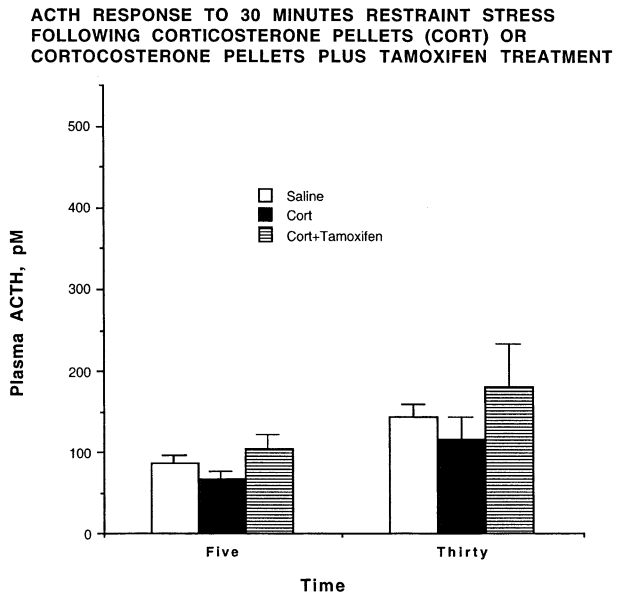

Figure 2. Effect of corticosterone pellets and tamoxifen injection in a repeat experiment. Results are shown as the mean ± SEM of 7 rats per group. Although the direction of the effects are similar to the previous experiment (Figure 1), neither corticosterone pellets nor tamoxifen demonstrated a significant effect.

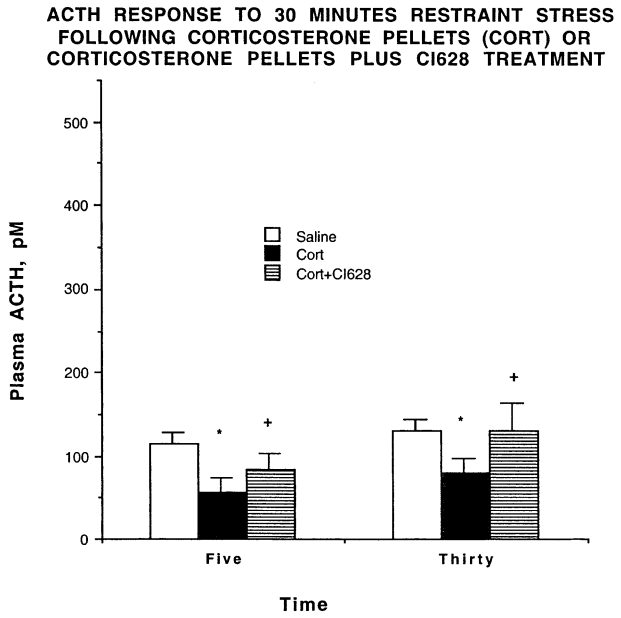

Figure 3. The effects of CI 628 treatment on the response to corticosterone pellets. The results are shown as the mean ± SEM of 7 rats per group. As expected, the corticosterone pellets had a significant inhibition of the ACTH response (*). The corticosterone pellet treated animals given CI628 injections showed a significant reversal (+) of the effect of corticosterone pellets compared to animals given vehicle plus corticosterone pellets.

change in progesterone levels in response to the restraint stress.

Experiment 7: Effect of 7 Day Estradiol Alone and Combined with Progesterone on the HPA Axis Response to Stress

In the final experiment, progesterone was administered in conjunction with estradiol. As expected, ACTH increased in response to the restraint stress [F = 60,4, df = 3,25; $p = .0001$]. As in Experiment 5, there was significant inhibitory effect of estradiol on ACTH response to restraint stress (Figure 7) [F = 19.9, df = 2,25; $p = .0001$]. Post-hoc testing indicated that both the estradiol treated group and the group treated with combined estradiol and progesterone had lower ACTH responses compared to the vehicle treated group ($p < .05$). The estradiol treated group did not differ from the group treated with estradiol plus progesterone.

There was also a significant effect of stress on corticosterone secretion [F = 25.4, df = 2,23; $p = .0001$] but no effect of estradiol or estradiol plus progesterone on corticosterone secretion in response to stress.

A significant effect of estrogen replacement was observed on thymus weight (Table 3) [F = 24,df = 2, 23; $p = .0001$], with no difference between estradiol and estradiol plus progesterone on Scheffe F-test post-hoc testing but a significant difference between control versus estradiol ($p < .05$) and control versus estrogen/progesterone ($p < .05$). A significant increase in adrenal weight (Table 3) was also observed in response to steroid hormone replacement [F = 4.7, df = 2,23; $p = .019$], but only the estradiol plus progesterone group differed from control by Sheffe F-test ($p < .05$).

Basal plasma estradiol in the vehicle treated animals was 27 ± 7.3 pg/ml, while in estradiol treated animals it was 78 ± 36 pg/ml. Progesterone treatment increased baseline progesterone (vehicle treated = 4.66 ± 0.6 ng/ml, estradiol treated = 3.7 ± 0.37 ng/ml, and estradiol + progesterone treated = 12.5 ± 1.1 ng/ml). There was no change in estradiol or progesterone levels in response to the restraint stress.

DISCUSSION

These data present a coherent set of findings on the effects of estradiol on glucocorticoid feedback sensitivity and stress responsiveness of the HPA axis. These data suggest that physiological levels of estradiol are inhibitory on stress responsiveness and that blocking estradiol in gonadally intact, normally cycling female rats leads to exaggerated stress responsiveness. In two studies, the second tamoxifen study (Experiment 2) and the estradiol study (Experiment 5), exogenous corticosterone pellets failed to significantly inhibit the ACTH response to stress, which contrasts with the data in male

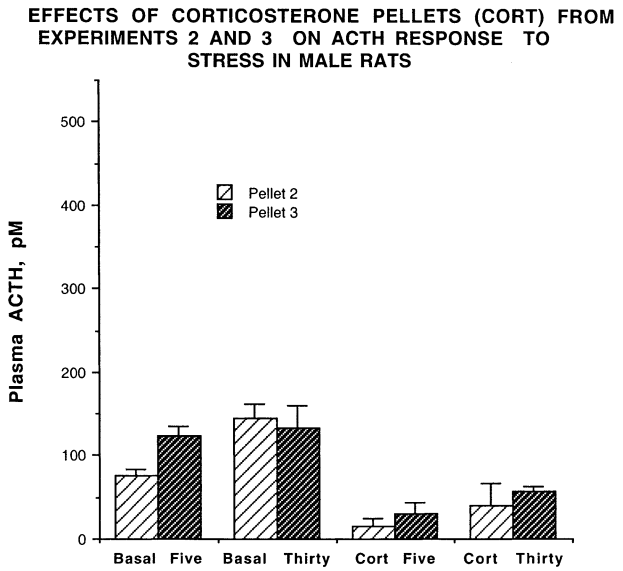

Figure 4. Effect of corticosterone pelllets used in Experiments 2 and 3 in male rats. The data shown are the mean ± SEM of 2 rats per group. The data labeled basal five and thirty are the ACTH response five and thirty minutes into restraint stress prior to pellet implantation, while "cort five" and "cort thirty" are the ACTH response to restraint stress six days following corticosterone pellet implantation. Both sets of pellets significantly inhibited stress responsiveness in male rats.

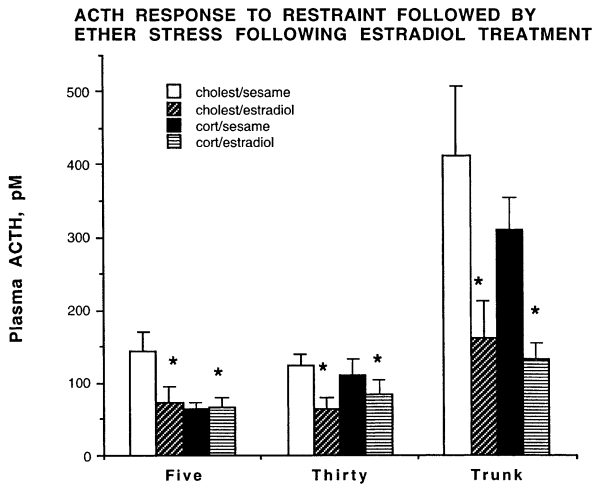

Figure 5. The effect of estradiol replacement and corticosterone pellets on ACTH response to restraint stress in ovariectomized female rats. The results are shown as the mean ± SEM of 7 rats per group. Corticosterone pellets produced only a trend to decreased ACTH response, but estradiol treated rats demonstrated a significant decrease (*) in ACTH response to stress. There was no significant interaction between estradiol and corticosterone.

rats tested both in this study (Experiment 4) and in several other studies (Young et al. 1995; Young 1996; Akana et al. 1992), again underscoring the relative resistance of female rats to the inhibitory effects of corticosterone. Although our original hypothesis was that estrogen played a role in the relative resistance to glucocorticoids in female rats compared to male rats, the results of the estrogen antagonist and estradiol experiments do not support a role for estrogen in this sex difference. Rather, it seems that the overall effect of estrogen is to inhibit activation of the HPA axis.

Although the combined results of the experiments in this study point to an inhibitory effect of estrogen on HPA axis responses to stress, a few of the experimental results are inconsistent. Unlike Experiment 1, in Experiment 2 there was no significant effect of tamoxifen on the ACTH response to restraint stress in corticosterone-treated rats. However, examination of the ACTH responses (Figure 2) shows that responses appeared to increase after tamoxifen treatment, although the difference from the saline treated group was not significant. Another inconsistency is that corticosterone and ACTH levels were elevated in Experiment 1 compared to the other experiments, most likely due to an unidentified environmental stressor which elevated basal levels upon which further restraint-stress induced increases occurred.

The findings in our study contrast with several previous reports of increased HPA axis responses to stress after estradiol treatment (Burgess and Handa 1992; Carey et al. 1995; Viau and Meaney 1991). A major difference between our study and these prior reports is that the dosage of estradiol was much higher in these earlier studies. Viau and Meaney (1991) examined the effects of a single dose of 10 μg of estradiol, which is five-fold higher than our dose. The plasma levels of estradiol achieved with that dose were 535 pg/ml, or five times proestrous levels. They found that estradiol enhanced the ACTH response to stress at 24 hr, but a nor-

Table 2. Effects of Estradiol on Adrenal and Thymus Weight (Experiment 5)

Hormonal Treatment	Adrenal Weight (mg/100 g BW)	Thymus Weight (mg/100 g BW)
Vehicle/vehicle (N = 6)	17.9 ± 1.1	215 ± 42
Vehicle/estradiol (N = 7)	21.4 ± 1.3*	259 ± 22
Corticosterone/vehicle (N = 7)	13.6 ± 1.4*	234 ± 28
Corticosterone/estradiol (N = 8)	20.2 ± 1.2*	273 ± 27

Data are mean ± SEM.

* Significant effect of estradiol in comparison to vehicle by 3 Way ANOVA ($p = .0005$).

** Significant effect of corticosterone in comparison to vehicle by 3 Way ANOVA ($p = .04$).

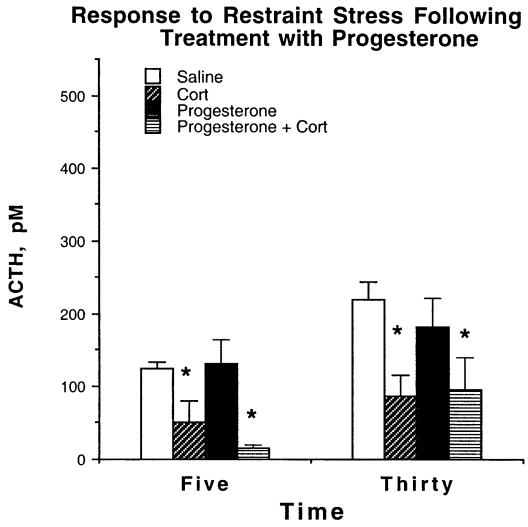

Figure 6. The effect of progesterone implants and corticosterone pellets on the ACTH response to restraint stress in overiectomized female rats. The results are shown as the mean ± SEM of 6 rats per group. There was a significant effect of corticosterone pellet treatment compared to cholesterol pellets, but no effect of progesterone implants compared to blank implants on ACTH response to restraint.

mal response to stress was seen at 48 hr. Carey et al. (1995) used a single dose of approximately 8 μg/rat. Burgess and Handa (1992) used an estradiol implant, which produced constant levels of estradiol of 75 pg/ml, which is a physiological dose, but delivered this dose for 21 days, which is much longer than the duration of estrogen exposure in the normal rat estrus cycle. In contrast, the study by Redei et al. (1994) found a reduced HPA axis response to footshock stress in ovariectomized rats 7 days following implantation of estradiol pellets which produced blood levels in the physiological range (160 pg/ml). The results of the study by Redei et al. (1994) are consistent with the results from our study, which also measured stress responses after 7 days of low dose estradiol replacement. Blunting of the HPA axis response to stress has also been found during physiological dose estrogen replacement for longer periods in species with longer estrus cycles. HPA axis responses were reduced in postmenopausal women treated with estradiol for 6 weeks (Lindheim et al. 1992) and 8 weeks (Komesaroff et al. 1999) and in ovariectomized sheep treated with estradiol for 4 weeks (Komesaroff et al. 1998). In sum, these studies indicate that short-term exposure to low doses of estrogen can suppress the HPA axis response to stress, but higher doses, and more prolonged time periods, enhances HPA axis responses.

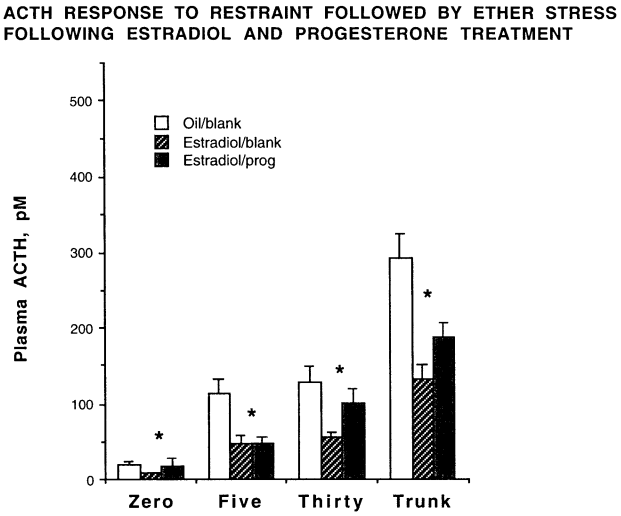

Figure 7. The effects of estradiol and progesterone replacement on ACTH response to restraint stress in ovariectomized female rats. The results are shown as the mean ± SEM of 8 rats per group. As in Figure 4, there was a significant effect of estradiol on ACTH response to restraint, but the estradiol plus progesterone treated rats did not differ from estradiol alone.

Table 3. Effects of Estradiol and Progesterone on Adrenal and Thymus Weight (Experiment 7)

Drug Treatment	Adrenal Weight (mg/100 g BW)	Thymus Weight (mg/100 g BW)
Vehicle/vehicle (N = 9)	16 ± 1	265 ± 28
Estradiol/vehicle (N = 8)	20 ± 2	164 ± 16*
Estradiol/progesterone (N = 9)	23 ± 2*	121 ± 23*

Data are mean ± SEM
*$p < 0.05$ in comparison to vehicle/vehicle group by Sheffe post-hoc.

Because ovariectomy reduces exposure to multiple hormones in addition to estrogen, we initially chose to examine the effects of estrogen antagonists in intact cycling female rats. While this approach holds promise in being less disruptive to the physiology of the animal as well as more specific for estrogen alone, it is complicated by the fact that current estradiol antagonists have agonist effects in some tissues and in some physiological systems. McEwen and Alves et al. (1999) have found that CI628 and tamoxifen both antagonize the effects of estradiol on progesterone receptor induction and lordosis behavior, while CI628 has agonist effects on choline acetyltransferase activity and monamine oxidase A activity. Our finding of opposite effects of estradiol compared to both CI628 and tamoxifen indicates that both of these drugs act as estrogen antagonists at sites that regulate HPA axis responsivity.

Decreased ACTH response to stress following estradiol treatment could either be due to enhanced negative feedback or to decreases in the activational components of the system at either the CRH or ACTH level. A num-

ber of studies have examined the effects of estradiol and progesterone on MR and GR binding, and mRNA expression. There is no clear consensus of the effects; and, all studies examined supra physiological doses. Several studies (Carey et al. 1995; Burgess and Handa 1993; Patchev and Almeida 1996) reported a decrease in MR binding and in MR mRNA in hippocampus, while Ferrini and De Nicola (1991) observed estrogen increased MR binding. A number of reports found that estradiol reduced GR binding and mRNA expression in hippocampus and pituitary (Burgess and Handa 1993; Turner 1990), but no effect was found by others (Peiffer and Barden 1987; Carey et al. 1995). Several studies concluded that estradiol antagonized the ability of glucocorticoids to downregulate GR (Ferrini et al. 1995; Burgess and Handa 1992), while other studies observed no effect (Carey et al. 1995) or that estrogen potentiated glucocorticoid induced GR downregulation in ovariectomized female rats (Patchev and Almeida 1996). While we did not assess GR or MR in our animals, it is of interest to note that weight of the thymus, a GR containing tissue that is a very sensitive index of total glucocorticoid, decreased in response to estrogen in Experiment 7, suggesting increased sensitivity of GR receptors in thymus. However, thymus weight did not change in response to estrogen in Experiment 5 or in response to tamoxifen treatment in Experiment 1.

Since basal total corticosterone levels were not changed by estradiol or estradiol antagonists, and estradiol can raise corticosterone binding globulin (Kitay 1961), it is possible that estrogen treatment reduced free corticosterone levels. However, a reduction in free corticosterone would reduce, not enhance, glucocorticoid feedback, so it is unlikely that changes in corticosterone binding globulin contributed to our findings. Another possibility is that estradiol may enhance corticosterone feedback by stimulating corticosterone production at the adrenal or by reducing corticosterone metabolism (Kitay 1963; Lo et al. 2000). However, we found no evidence of enhanced basal corticosterone levels in estrogen treated rats, or any reduction in basal corticosterone in rats treated with estradiol antagonists.

Another possible mechanism by which estrogen may affect stress responsiveness is by directly influencing CRH. Estrogen increased CRH mRNA in one transvected cell line (Vamvakopoulos and Chrousos 1993) and decreased CRH mRNA expression in another cell line (Makrigiannakis et al 1996). In addition, estrogen may reduce hypothalamic CRH activity by acting on brain neurotransmitter and neuropeptide systems that regulate the HPA axis (Raap et al. 2000). In vivo, some reports have found that estrogen increases CRH mRNA (Patchev and Almeida 1996; Roy et al. 1999), while other reports found no effect (Redei et al. 1994) and others reported a decrease in hypothalamic CRH mRNA or content (Haas and George 1988; Paulmeyer-Lacroix et al. 1996). Again, dose may be critical, since the report of Paulmeyer-Lacroix et al. (1996) demonstrated that reductions in hypothalamic CRH after estrogen treatment were dependent on increases in basal corticosterone secretion, which is likely to vary among different estrogen dosing regimens.

Because corticosterone treatment did reduce adrenal weight, and basal corticosterone levels were not affected by corticosterone implants, it is possible that rats with corticosterone implants had relatively higher corticosterone levels in the dark period compared to rats with cholesterol implants. However, in the current set of studies, corticosterone was only measured during the light period. In addition, the corticosterone pellets may have suppressed the ACTH reactivity to less intense stressors than restraint, and to other stimuli to the HPA axis such as activity, feeding and circadian drive which in turn may have contributed to a reduction in adrenocortical tissue mass.

In the studies described here, estradiol and estradiol antagonists modulated ACTH responses to stress much more consistently than corticosterone responses. Several factors may contribute to the lack of parallel changes in ACTH and corticosterone release in response to stress. First, because corticosterone rises more slowly than ACTH, the 30-min time period is not optimal for detection of differences in corticosterone responses. A longer time course of blood sampling following the stress may have increased our ability to detect effects of estrogen manipulations on corticosterone secretion. Second, it can be difficult to detect changes in corticosterone secretion since even the small rises in ACTH seen in corticosterone treated rats often are able to maximally stimulate adrenal corticosterone release. Finally, estrogen may modulate activity of other systems that control corticosterone release from the adrenal including neural inputs and steroid synthetic enzyme activity (Lo et al. 2000), which may dampen the effects of changes in ACTH stimulation during estrogen manipulations. Support for this possibility comes from the observation the estrogen increased adrenal weight in Experiment 5 and the estradiol plus progesterone treatment increased adrenal weight in Experiment 7, while corticosterone release was unchanged. The increase in adrenal weight following estradiol treatment suggests trophic effects of estrogen on the adrenal, which may enhance the corticosterone response to ACTH after estradiol treatment and possibly reduce corticosterone release after treatment with estradiol receptor antagonists.

In contrast to the effects of estradiol, we observed no effects of progesterone on either stress responsiveness or the feedback effects of glucocorticoids. In the initial progesterone experiment, we did not use estradiol priming, looking instead for an effect of progesterone that might be mediated through other receptors such as

the glucocorticoid receptor (Abou Samra et al. 1984; Rousseau et al. 1972; Svec 1988; Carey et al. 1995) However there was clearly no effect of progesterone in this experiment. Furthermore, when we examined the effect of the combination of estradiol and progesterone the effects of estradiol predominated and the combined steroid hormone treated animals did not differ from estradiol treatment alone on any parameter. These findings are consistent with prior studies using different dosing parameters for estradiol and progesterone (Carey et al. 1995).

In conclusion, our data suggest that low doses of estradiol inhibit the HPA axis response to stress, while physiological doses of progesterone had no effect on HPA axis responses. These effects of estradiol contrast with several prior studies using larger doses of estradiol and point out the importance of estradiol dosing parameters to modulation of HPA axis responses. In addition, these results suggest that the enhanced HPA axis responsivity known to occur during proestrus, is more likely to result from intereactions between estrogen and progesterone and other hormonal changes during proestrus, rather than the increase in estradiol alone.

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