



Hypoestrogenism does not mediate social suppression of cortisol in subordinate female marmosets

Wendy Saltzman^{a,b,c,*}, Brynn K. Hogan^c, Amy J. Allen^c,
Brian M. Horman^c, David H. Abbott^{c,d}

^aDepartment of Biology, University of California, Riverside, CA 92521, USA

^bNeuroscience Graduate Program, University of California, Riverside, CA, USA

^cNational Primate Research Center, University of Wisconsin, Madison, WI, USA

^dDepartment of Obstetrics and Gynecology, University of Wisconsin, Madison, WI, USA

Received 12 August 2005; received in revised form 20 January 2006; accepted 26 January 2006

KEYWORDS

Marmoset;
Reproductive
suppression;
Subordination;
Ovariectomy;
Adrenal cortex;
Cortisol

Summary Behaviorally subordinate female marmosets undergo social suppression of ovulation and hypoestrogenism, as well as chronic reductions in circulating basal cortisol concentrations. Because estrogen elevates hypothalamic-pituitary-adrenal axis activity and circulating glucocorticoid levels in other species, we tested the hypothesis that socially induced hypoestrogenism contributes to cortisol reductions in subordinate female marmosets. We characterized morning basal plasma cortisol levels, as well as cortisol responses to exogenous adrenocorticotrophic hormone (ACTH; 0, 1, or 10 µg/kg), in seven anovulatory subordinate females and six ovariectomized, non-subordinate females under two conditions: during long-term treatment with estradiol (E₂) and control. Circulating E₂ and cortisol levels were compared to those of six dominant females undergoing ovulatory cycles. Basal cortisol concentrations in the control condition were significantly lower in subordinates than in both dominant and ovariectomized females. E₂ treatment elevated circulating E₂ levels of subordinate and ovariectomized females into the range seen in dominant females but did not increase either mean basal or ACTH-stimulated cortisol levels. To the contrary, E₂ treatment caused a decline in basal cortisol levels over time, especially in ovariectomized animals. These results indicate that treatment with exogenous estrogen does not elevate circulating cortisol levels in previously hypoestrogenemic female marmosets and, correspondingly that socially induced hypoestrogenism does not diminish cortisol levels in subordinate females.

© 2006 Elsevier Ltd. All rights reserved.

1. Introduction

Behaviorally subordinate individuals in many species exhibit altered activity of the stress-responsive hypothalamic-pituitary-adrenal (HPA) axis as

* Corresponding author. Tel.: +1 951 827 6356; fax: +1 951 827 4286.

E-mail address: saltzman@ucr.edu (W. Saltzman).

compared to dominants. Traditionally, subordinates have been thought to manifest elevated HPA activity and, consequently, increased circulating glucocorticoid concentrations as a result of psychosocial stress (von Holst, 1998; Blanchard et al., 2002). More recently, however, it has become evident that subordinates may frequently manifest *reduced* circulating or excreted glucocorticoid concentrations as compared to dominant individuals (Saltzman et al., 1994, 1998; Creel, 2001; Goymann and Wingfield, 2004). Several authors have suggested that subordinate individuals in these societies experience less psychosocial stress or lower allostatic load than dominants (Creel et al., 1996; Creel, 2001; Abbott et al., 2003; Goymann and Wingfield, 2004). In addition, we (Saltzman et al., 1994, 1998, 2000) have suggested that low glucocorticoid levels in subordinate animals may be mediated in part by low levels of reproductive hormones, especially in species in which subordinate individuals undergo social suppression of reproduction. A number of reproductive hormones, including estrogen, progesterone, androgens, luteinizing hormone (LH) and chorionic gonadotropin (CG), have been shown to modulate HPA activity (e.g. Phillips and Poolsanguan, 1978; Kime et al., 1980; O'Connell et al., 1994; Young, 1995, 1998). Socially induced suppression of these hormones in subordinate individuals may therefore be expected to contribute to rank-related differences in glucocorticoid levels.

We have been investigating the interactions between reproductive and adrenocortical activity in the common marmoset (*Callithrix jacchus*), a small, New World monkey in which endocrine function is profoundly influenced by social status. Social groups in both the wild and captivity may contain as many as six adult females, but only one or two behaviorally dominant females breed in each group (reviewed by French, 1997; Saltzman, 2003). Subordinate females are often anovulatory and hypoestrogenemic as a result of inadequate secretion of CG (Abbott et al., 1981, 1988; Saltzman et al., 1998), the major luteinizing gonadotropin secreted by the pituitary in this species and possibly other New World primates (Gromoll et al., 2003; Müller et al., 2004). This socially induced infertility can persist for months or even years, but is reversed rapidly following separation of the subordinate female from her dominant female groupmate (Abbott et al., 1988; Abbott and George, 1991).

Anovulatory subordinate female marmosets also undergo chronic suppression of circulating cortisol levels (Saltzman et al., 1994, 1998, 2004; Johnson et al., 1996; Abbott et al., 1997). Morning basal cortisol levels decline dramatically within 6-7 weeks following the onset of social subordination and

anovulation (Saltzman et al., 1994, 1998; Abbott et al., 1997) and can remain suppressed for months to years (Saltzman et al., 1998; unpublished data). Dominant and subordinate females have not been found to differ in morning basal adrenocorticotropin (ACTH) levels or in suppression of ACTH or cortisol by dexamethasone (DEX), a synthetic glucocorticoid (Saltzman et al., 2004). Following suppression of endogenous ACTH and cortisol by DEX, however, subordinates exhibited blunted cortisol responses to exogenous ACTH as compared to dominant females (Saltzman et al., 2000). Moreover, cortisol responses to ACTH were virtually identical in ovary-intact subordinates and ovariectomized, non-subordinate females, indicating that this adrenocortical impairment might be mediated by withdrawal of ovarian hormones rather than by social subordination per se (Saltzman et al., 2000). Consistent with this possibility, basal plasma cortisol concentrations fluctuate reliably across the ovarian cycle in marmosets, showing a midcycle peak during the late follicular, ovulatory, and early luteal phases and a decline across the luteal phase (Saltzman et al., 1998). Thus, circulating cortisol levels may be modulated by reproductive hormones in female marmosets.

These previous findings suggest that cortisol diminution in subordinate female marmosets is effected by two mechanisms: (1) reduced adrenal responsiveness to ACTH, which in turn may be mediated by suppression of ovarian hormones, and (2) an additional inhibitory mechanism, presumably triggered directly by social subordination and acting at the level of the brain or pituitary, which restrains ACTH secretion in spite of the reduced negative-feedback signal from low cortisol levels and reduces basal cortisol levels of subordinates below those of ovariectomized females (Saltzman et al., 1998). In view of the well-known stimulatory effects of estrogen on HPA activity (reviewed by Kime et al., 1980; Young, 1995, 1998; Wilson et al., 2005), we hypothesize that the first of these mechanisms, adrenal hyporesponsiveness, may be mediated by hypoestrogenism. In this study, we tested this hypothesis by determining the effects of long-term estradiol treatment on basal cortisol levels and cortisol responses to exogenous ACTH in both ovariectomized females and anovulatory, socially subordinate females.

2. Methods

2.1. Animals

We used 19 captive-born, adult female common marmosets (*Callithrix jacchus*), including six

socially dominant females undergoing ovulatory cycles, seven anovulatory subordinates, and six long-term ovariectomized (ovx), pair-housed females that were neither dominant nor subordinate to other females. Dominant, subordinate and ovx animals did not differ ($P > 0.05$) in body mass (386 ± 13 vs. 392 ± 12 vs. 384 ± 12 g, respectively; mean \pm SEM) or age (31.0 ± 2.8 vs. 23.6 ± 1.3 vs. 33.0 ± 4.2 months, respectively) at the outset of data collection.

Dominant and subordinate animals were housed in groups containing 2–3 unrelated females and 1–2 gonadally intact adult males. Ovx females had been pair-housed with an intact male for 18.6 ± 11.3 months (mean \pm SEM) prior to data collection and had undergone bilateral removal of the ovaries and fallopian tubes 6 months before the study began. Ovariectomy was performed by midline incision under Saffan anesthesia (8.1 mg alphaxalone:2.7 mg alphadolone acetate, IM; Pitman-Moore, Harefield, Uxbridge, Middlesex, UK).

Social groups were formed as described previously (Saltzman et al., 1998) at least 2 months prior to data collection. Dominance hierarchies in such groups typically are established within 1–2 weeks and may remain stable for several years or more (Abbott, 1986, unpublished data). Behavioral assessments of dominant and subordinate status, based on directionality of submissive behaviors (Saltzman et al., 1994, 1996), were confirmed by the occurrence of ovulatory cycles in dominant females and anovulation in subordinate females, based on plasma progesterone concentrations in blood samples collected twice each week (Saltzman et al., 1994; see below). Subordinates had not ovulated for at least 10 weeks prior to data collection and had not exhibited elevated plasma progesterone concentrations (> 10 ng/ml), characteristic of the luteal phase of the ovarian cycle, for at least 33 days.

Marmosets were housed indoors at the National Primate Research Center at the University of Wisconsin–Madison, with lights on from 0630 to 1830 h, ambient temperature at approx. 27 °C, and humidity at approx. 50%. Most of the animals occupied aluminum and wire mesh cages measuring $61 \times 91 \times 183$ cm, $122 \times 61 \times 183$ cm, or $61 \times 61 \times 183$ cm; however, some were housed in larger rooms measuring $235 \times 370 \times 225$ cm or $310 \times 141 \times 250$ cm. We have not found such variation in cage size to be reflected in circulating cortisol levels in marmosets. Animals were fed Mazuri Callitrichid High Fiber Diet 5M16 (Purina Mills, St Louis, MO) supplemented with fruit, cereal, nuts and miniature marshmallows. Marmosets were fed once daily between 1230 and 1430 h, and water was

available ad lib. Animals were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Additional information on marmoset housing and husbandry is provided by Saltzman et al. (1998).

2.2. Experimental design

Basal and ACTH-stimulated cortisol levels were characterized under two conditions: while marmosets were implanted with two silastic capsules containing 17β -estradiol (E_2 condition) and while they were implanted with two empty silastic capsules (control [C] condition). Each subordinate and ovx female was tested in both the E_2 and C conditions, with the order of conditions balanced across animals within each group. Dominant females were tested in only the C condition.

In each condition, marmosets underwent three ACTH stimulation tests (0, 1 and 10 μ g ACTH/kg body mass; see below) at intervals of at least 4 weeks, beginning at least 25 days after capsule implantation. The order of the three ACTH doses was balanced across females within each group but, for each subordinate and ovx female, was identical in the E_2 and C conditions.

In addition to ACTH stimulation tests, we collected blood samples weekly from each marmoset to characterize morning basal cortisol concentrations. We also measured plasma progesterone in two blood samples collected each week at 3- to 4-day intervals from all ovary-intact (dominant and subordinate) females, to monitor ovulatory activity (see below). E_2 was assayed in blood samples collected from each animal every other week.

2.3. Estradiol implants

Implants comprised 20-mm lengths of silastic tubing (Dow Corning; Sigma-Aldrich, St Louis, MO; inner diameter: 16 mm; outer diameter: 32 mm) that were either filled with crystalline 17β -estradiol (Sigma-Aldrich, St Louis, MO) or left empty. Two implants of this size yielded plasma E_2 concentrations comparable to those in the late follicular/ovulatory phase (approx. 200–500 pg/ml; Kendrick and Dixson, 1985; Saltzman et al., 1998, unpublished data) for at least 17–18 weeks.

For implantation of capsules, marmosets were anesthetized with ketamine hydrochloride (10 mg/kg IV) and lidocaine (2 mg/kg SC). Two capsules were implanted through a small (approx. 1 cm) dorsal midline skin incision between the scapulae, and the incision was closed with surgical

tissue glue or sutures. In some cases, capsules were replaced by identical capsules partway through the E₂ or C condition.

2.4. ACTH stimulation tests and blood sample collection

On the day prior to each ACTH stimulation test, animals were weighed and injected IM with 5 mg/kg dexamethasone sodium phosphate (DEX; American Regent Laboratories, Shirley, NY) at 1550–1630 h to suppress endogenous ACTH secretion. This dose of DEX has been found to suppress plasma cortisol and ACTH levels in female marmosets for 2–3 days (Saltzman et al., 2004; see also Saltzman et al., 2000). At 0900–1000 h on the day of data collection, each animal received an IV injection of either 1 or 10 µg/kg synthetic human ACTH_{1–39} (Sigma-Aldrich, St Louis, MO) in 0.5 ml/kg sterile saline, or an equivalent volume of sterile saline without ACTH. Previous studies have shown that these doses of ACTH produce a robust but submaximal cortisol response in female marmosets (Saltzman et al., 2000, unpublished data). Blood samples (0.2–0.6 ml) were collected at –20 (baseline), 60 and 120 min from the ACTH or saline injection. Between samples, each marmoset remained alone in a stainless steel nest box from its home cage, which also served as a transport cage.

For blood sample collection, animals were briefly restrained in a restraint tube (Hearn, 1977), to which they had been adapted extensively, while blood was collected in a heparinized syringe by femoral venipuncture. Samples were immediately placed on ice, centrifuged at 2000 rpm for 10 min, and the plasma fraction extracted and stored at –20° C. Weekly basal blood samples were collected at 0915–0945 h, several hours after the diurnal cortisol peak (George and Abbott, unpublished data). For these basal samples, latency from initial disturbance of the animal (investigator's entry into the cage) to sample collection was <3 min for 96% of samples and averaged 119 ± 13 s. We found no indication that plasma cortisol levels were elevated as a result of the longer latencies in the remaining 19 samples, which were collected in 185–361 s from cage entry, and therefore we included these values in our analyses.

2.5. Monitoring and control of ovarian function

Progesterone was assayed in blood samples collected twice weekly from each dominant and subordinate female. Ovulation was considered to have occurred on the day before a sustained

(≥2 consecutive samples) elevation of progesterone above 10 ng/ml (Harlow et al., 1983; Saltzman et al., 1994). To prevent term pregnancies and to ensure that all dominant females were in the early to mid-follicular phase of the ovarian cycle during ACTH stimulation tests, we gave each dominant animal an IM injection of 0.75–1.0 µg cloprostenol sodium (Estrumate, Mobay Corp., Shawnee, KS and Cayman Chemicals, Ann Arbor, MI), a prostaglandin F_{2α} analog, 14–45 days after each ovulation and, correspondingly, 3–4 days before each ACTH stimulation test. This treatment causes luteolysis and termination of the luteal phase or early pregnancy (Summers et al., 1985). Ovx and subordinate females were treated with the same dose of cloprostenol 3–4 days before each ACTH stimulation test to control for any potential effects of cloprostenol on HPA activity. This treatment has not been found to alter baseline cortisol levels in marmosets (Saltzman et al., 1998).

2.6. Hormone assays

All hormone assays were fully validated for use with marmoset plasma (Saltzman et al., 1994, 1998). Plasma cortisol levels were determined in duplicate aliquots using an antibody-coated-tube radioimmunoassay (RIA) kit (GammaCoat™, DiaSorin® Corp., Stillwater, MN) as described previously (Saltzman et al., 1994). Assay sensitivity at 90% binding was 0.1 ng/tube (10 µg/dl), and intra- and inter-assay coefficients of variation (CVs) of a plasma pool assayed in quadruplicate in each assay (40% binding) were 5.7 and 10.8%, respectively (*N* = 56 assays).

Plasma progesterone levels were measured in duplicate aliquots using a heterologous enzyme immunoassay (Saltzman et al., 1994). Assay sensitivity at 90% binding was 3.9 pg/tube (2.8 ng/ml), and intra- and inter-assay CV's of a marmoset plasma pool (45% binding) were 5.3 and 21.1%, respectively (*N* = 1725 assays).

Plasma E₂ concentrations were determined by RIA following extraction with 5 ml ethyl ether and celite column chromatography as previously described (Saltzman et al., 1998). Assay sensitivity at 90% binding was 4.6 pg/tube (30.4 pg/ml). Intra- and inter-assay CVs of a marmoset plasma pool (57% binding) were 4.0 and 15.4%, respectively (*N* = 6 assays).

2.7. Analysis

We determined mean plasma E₂ concentrations for each subordinate and ovx marmoset in the E₂ and C

conditions. For dominants, we determined mean plasma E_2 concentrations during the follicular phase ($P < 10$ ng/ml, ≥ 3 days prior to ovulation), ovulatory phase ($P < 10$ ng/ml, 0-2 days prior to ovulation) and luteal phase or early pregnancy ($P \geq 10$ ng/ml; hereafter referred to as the luteal phase). We similarly determined mean basal cortisol levels for each subordinate and ovx female in the E_2 and C conditions. We have previously found that basal cortisol levels of female marmosets are elevated from 4 days before through 4 days after the LH surge, which normally precedes ovulation by 1 day (Harlow et al., 1984; Saltzman et al., 1998). For each dominant female, therefore, we determined mean basal cortisol levels during the early to mid-follicular phase (≥ 6 days prior to ovulation), periovulatory phase (5 days before through 3 days after ovulation) and mid- to late luteal phase/early pregnancy (≥ 4 days after ovulation). For both E_2 and cortisol analyses (except analyses of changes in hormone levels over time), we used only samples collected at least 7 days following implantation of E_2 -containing or empty capsules. For analyses of cortisol responses to ACTH, we determined area under the curve (AUC) using the method of Pruessner et al. (2003) for AUC with respect to increase. We also determined delta scores as each animal's cortisol concentrations at 60 and 120 min after ACTH or saline injection minus her pre-ACTH (DEX-suppressed) baseline cortisol concentration.

E_2 and cortisol data were log-transformed to yield normal distributions and were analyzed by ANOVA, with post hoc analyses performed by Fisher LSD tests. Because hormone concentrations were expected to vary across the ovarian cycle in dominant females, for each hormone (cortisol, E_2) in each treatment condition (E_2 , C) we performed

a series of one-way ANOVAs comparing dominant females in each phase of the cycle to subordinate and ovx females. Analyses were performed using Systat v. 5.2 for the Macintosh (SPSS, Chicago, IL), and results were evaluated at the 0.05 level (2-tailed).

We were unable to obtain E_2 values from two dominant females in the ovulatory phase, and basal cortisol values from two dominants in the early to mid-follicular or periovulatory phase; therefore, these animals were excluded from the relevant comparisons among groups. One subordinate became acutely ill 5.3 weeks into the C condition and had to be euthanized before completing her ACTH stimulation tests; however, there were no clinical, observational or hormonal indications of abnormalities prior to the acute onset of illness. This animal was excluded from analyses of basal cortisol levels over time and cortisol responses to ACTH.

3. Results

3.1. Estradiol

In the control (C) condition, mean plasma E_2 concentrations differed reliably among groups when subordinate and ovx females were compared to dominants in the follicular ($F[2,16]=7.560$, $P=0.005$), ovulatory ($F[2,14]=29.075$, $P<0.0001$) or luteal phase of the ovarian cycle ($F[2,16]=20.260$, $P<0.0001$; Fig. 1). Mean E_2 levels of subordinates were comparable to those of dominant females in the follicular phase and lower than those of dominant females in the ovulatory ($P<0.001$) or

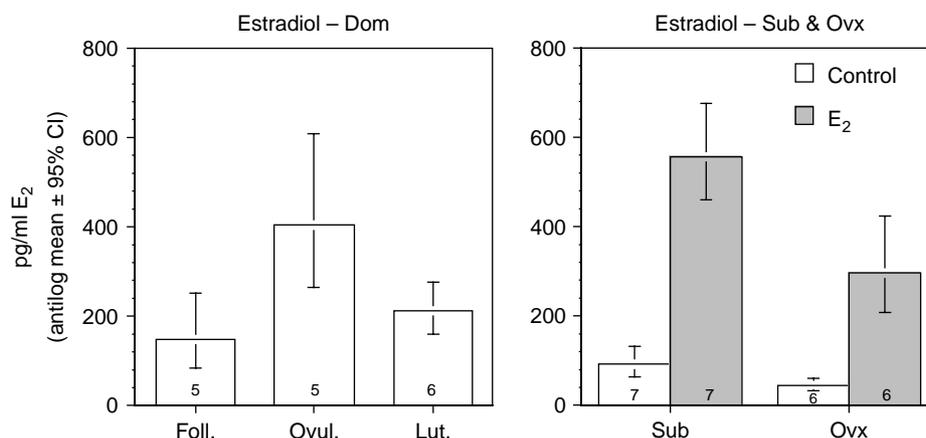


Figure 1 Plasma E_2 concentrations (back-transformed mean \pm 95% confidence intervals) in dominant female marmosets during the follicular, ovulatory and luteal phases of the ovarian cycle, and in subordinate and ovariectomized females in the control and E_2 conditions. Numbers in bars indicate sample sizes. See text for statistical results.

luteal phase ($P=0.003$), whereas mean E_2 concentrations of ovx females were lower than those of dominants in each phase of the cycle ($P<0.005$; Fig. 1). E_2 levels during the C condition were below the assay sensitivity limit in $70.5 \pm 8.1\%$ (mean \pm SEM) of samples from the six ovx females, but in only one sample from a subordinate female. A group (subordinate, ovx) by condition (C, E_2) ANOVA indicated that E_2 levels were higher in subordinate than ovx females across the two treatment conditions ($F[1,11]=16.484$, $P=0.002$).

As expected, E_2 implants dramatically elevated circulating E_2 concentrations ($F[1,11]=138.275$, $P<0.0001$), and this effect did not differ between subordinate and ovx females. In the E_2 condition, as in the C condition, significant differences were found when E_2 levels of subordinate and ovx females were compared to those of dominant females in the follicular ($F[2,16]=12.230$, $P<0.001$), ovulatory ($F[2,14]=4.639$, $P=0.029$) or luteal phase ($F[2,16]=12.706$, $P<0.001$). E_2 levels of subordinate females in the E_2 condition were comparable to those of dominants in the ovulatory phase and higher than those of dominants in the follicular ($P<0.001$) or luteal phase ($P<0.001$). E_2 levels of ovx females in the E_2 condition did not differ from those of dominants in the ovulatory or luteal of the cycle but were higher than those of dominants in the follicular phase ($P=0.024$; Fig. 1). Plasma E_2 concentrations did not change consistently across the course of the E_2 condition in either subordinate (Spearman rank correlation, E_2 vs. days from capsule implantation = -0.643 to 0.314) or ovx animals ($r_s = -0.517$ to 0.252).

3.2. Basal cortisol

Mean basal cortisol concentrations in the C condition differed reliably among groups whether we compared subordinate and ovx females to dominant females in the early to mid-follicular phase ($F[2,15]=3.946$, $P=0.042$), the periovulatory phase ($F[2,15]=5.123$, $P=0.020$) or the luteal phase ($F[2,16]=5.025$, $P=0.020$; Fig. 2). In each of these analyses, subordinates had lower cortisol levels than both dominant ($P<0.05$) and ovx females ($P<0.05$), whereas the latter two groups did not differ from one another. Basal cortisol levels in subordinate and ovx females were not altered by E_2 implants.

Although mean basal cortisol concentrations did not differ between the E_2 and C conditions, visual inspection of the data suggested that cortisol levels declined over time in the E_2 condition. For each subordinate and ovx female, therefore, we calculated the Spearman correlation coefficient between basal cortisol concentrations and days from initial implantation of capsules, and compared these r_s values between the E_2 and C conditions. Both subordinate and ovx females consistently showed a decline in cortisol levels over time in the E_2 condition (ovx: $r_s = -0.297$ to -0.800 ; subordinate: $r_s = -0.042$ to -0.533) but not in the C condition (ovx: $r_s = -0.420$ to 0.807 ; subordinate: $r_s = -0.450$ to 0.691). This difference between conditions was significant for ovx ($P=0.014$, Wilcoxon) but not subordinate females, indicating that in ovx females alone, E_2 treatment caused a decline in basal cortisol concentrations over time.

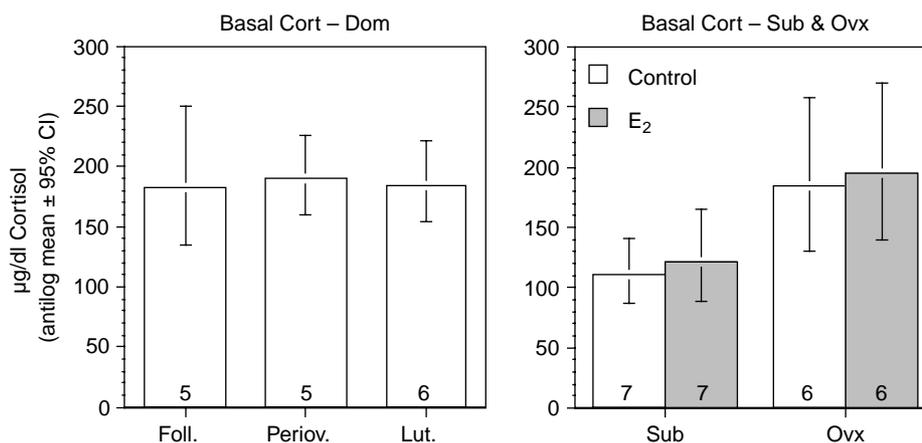


Figure 2 Plasma basal cortisol concentrations (back-transformed mean \pm 95% confidence intervals) in dominant female marmosets during the early to mid-follicular, periovulatory and mid- to late luteal phases of the ovarian cycle, and in subordinate and ovariectomized females in the control and E_2 conditions. Numbers in bars indicate sample sizes. See text for statistical results.

3.3. ACTH-stimulated cortisol

DEX-suppressed cortisol concentrations immediately prior to ACTH treatment, averaged across the three ACTH stimulation tests, did not differ reliably among groups in the C condition and, in subordinate and ovx females, were not altered by

E_2 treatment (Fig. 3). ACTH caused a dose-dependent elevation in cortisol, as determined by net integrated responses, in dominant females in the C condition ($F[2,10]=64.220$, $P<0.001$) as well as in subordinate and ovx females across conditions ($F[2,18]=278.250$, $P<0.001$). In the C condition, the three groups did not differ in their net

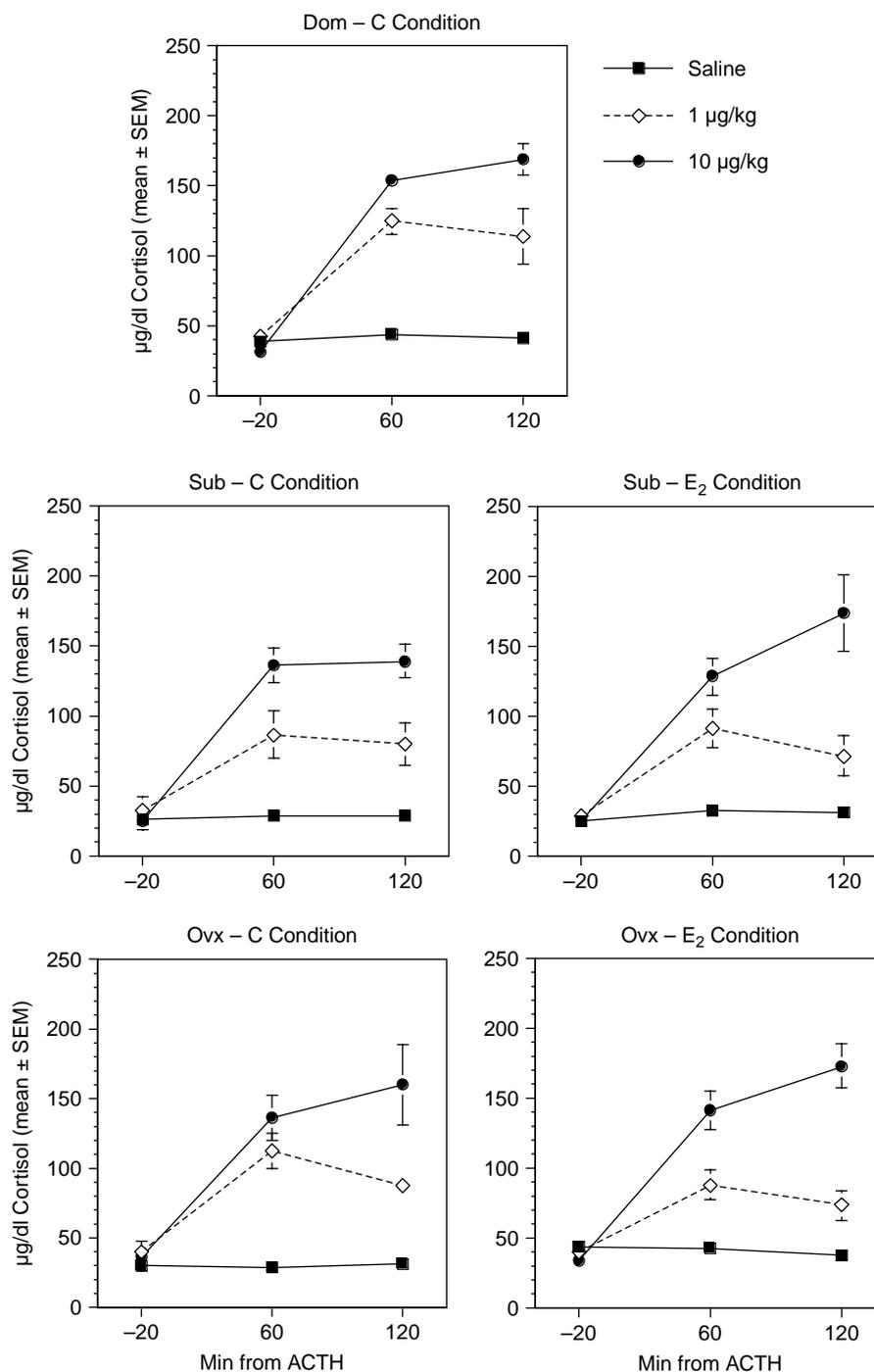


Figure 3 Plasma cortisol concentrations (mean ± SEM) in response to 0, 1 or 10 µg/kg ACTH, following DEX suppression, in six dominant female marmosets in the control condition, and in six subordinate and six ovariectomized females in the control and E_2 conditions. See text for statistical results.

integrated responses to ACTH. E₂ treatment affected the net integrated response to ACTH differently in subordinate and ovx animals (group × condition interaction: $F[1,9]=5.492$, $P=0.044$). When each group was analyzed separately, however, their net integrated responses to ACTH did not differ between the E₂ and C conditions.

To more closely examine the pattern of cortisol responses to ACTH, we analyzed the elevation in cortisol above pre-ACTH, DEX-suppressed baseline levels at both 60 and 120 min after ACTH or saline injection between groups (subordinate, ovx), between conditions (C, E₂), and among ACTH doses using 3-way ANOVAs. The net cortisol elevation at 60 min differed across ACTH doses ($F[2,18]=211.073$, $P<0.0001$; Fig. 3) but not between groups or conditions. The net cortisol elevation at 120 min also differed across ACTH doses ($F[2,18]=205.246$, $P<0.0001$), and this dose effect differed between the C and E₂ conditions ($F[2,18]=3.872$, $P=0.040$). Inspection of the data presented in Fig. 3 suggests that at this timepoint, marmosets showed relatively greater responses to 10 µg/kg ACTH, as compared to 1 µg/kg ACTH, in the E₂ condition than in the C condition. The biological significance of this finding is unclear, but it may indicate a subtle, E₂-mediated increase in adrenocortical steroidogenic capacity.

4. Discussion

Results of this study indicate that diminished circulating basal cortisol levels in anovulatory subordinate female marmosets are not mediated by hypoestrogenism. As in previous studies, anovulatory subordinates in the control condition had markedly lower plasma E₂ and morning basal cortisol concentrations than dominant females undergoing ovulatory cycles (Abbott et al., 1988, 1997; Saltzman et al., 1994, 1998, 2004; see also Johnson et al., 1996). Long-term treatment with physiological doses of E₂, however, did not elevate basal or ACTH-stimulated cortisol levels in either subordinate or ovx females.

We have previously found that subordinate female marmosets have lower basal cortisol levels than dominant females but similar circulating ACTH levels (Saltzman et al., 2004, in press), suggesting that the adrenal cortex is less responsive to ACTH in subordinates than in dominants. Moreover, both subordinate and ovx females showed lower peak and net integrated responses to 10 µg/kg ACTH than follicular-phase dominants (Saltzman et al., 2000). In the present study, in contrast, basal cortisol

levels of subordinates were significantly lower than those of both dominant and ovx females, but cortisol responses to ACTH did not differ among groups. The reason for this disparity is not clear and must await resolution in future studies. Nonetheless, these findings suggest that the principal mechanism underlying reduced basal cortisol levels in subordinate females acts at the level of the brain or pituitary, rather than the adrenal cortex, and is not triggered by suppression of ovarian hormones.

Basal cortisol levels of ovx females in this study did not differ from those of dominant females in any phase of the ovarian cycle. In a previous study, in contrast, basal cortisol levels of ovx females were significantly lower than those of ovary-intact females during the periovulatory (but not the follicular or luteal) phase (Saltzman et al., 1998). This disparity may reflect the small number of dominant females for which we had periovulatory-phase cortisol data ($N=5$), as well as the less systematic collection of blood samples across the cycle, in the present study as compared to the previous one. Importantly, however, the absence of cortisol differences between dominant and ovx females further indicates that estrogen is not an important regulator of cortisol secretion in this species.

Estrogen elevates circulating glucocorticoid levels in rodents through a number of actions on the brain, pituitary, adrenal cortex and liver (reviewed by Kime et al., 1980; Young, 1995, 1998; Wilson et al., 2005). Estrogenic effects on the HPA axis have also been documented in primates. Ovariectomy decreases mean daily plasma cortisol levels and the amplitude of the circadian change in cortisol in rhesus macaques (Smith and Norman, 1987). Conversely, estrogen treatment elevates plasma cortisol levels in gonadectomized male and female macaques (Norman et al., 1992; Stavisky et al., 2003; Wood et al., 2004) and squirrel monkeys (Coe et al., 1986). In a recent study (Wilson et al., 2005), female rhesus macaques were treated with the gonadotropin-releasing hormone agonist leuprolide acetate to suppress pituitary-ovarian activity. Subsequent E₂ replacement reduced negative-feedback effects of DEX on ACTH and cortisol, and enhanced the cortisol response to corticotropin-releasing factor (CRF; Wilson et al., 2005). Exogenous E₂ has also been shown to elevate plasma ACTH and cortisol levels in baboons undergoing postpartum anovulation (Giussani et al., 2000). In women, the reported effects of estrogen replacement on HPA activity have been more variable, with some authors reporting that estrogen elevated plasma cortisol levels (Burlinson et al., 1998; Gudmundsson et al., 1999)

and others finding no effects of estrogen on basal ACTH or cortisol levels, responses to DEX suppression, or responses to ACTH stimulation (Slayden et al., 1998). Interestingly, in a recent study (Pluchino et al., 2005), long-term E₂ treatment produced a decline in cortisol levels of hysterectomized and ovx postmenopausal women, similar to our findings in ovx marmosets. The mechanisms by which estrogen may stimulate HPA activity in primates have received relatively little attention but include increased expression of CRF in the hypothalamus (Vamvakopoulos and Chrousos, 1993; Roy et al., 1999), alterations in cortisol metabolism (Pepe et al., 1982) and elevation of plasma corticosteroid-binding globulin (CBG) levels (Coe et al., 1986).

Stavisky et al. (2003) recently suggested that New World primates, such as marmosets and squirrel monkeys, show enhanced estrogenic stimulation of HPA activity, as compared to more modest effects in Old World primates such as humans, macaques and baboons. To our knowledge, the present study is only the second to directly characterize effects of estrogen on HPA activity in a New World primate. In the first (Coe et al., 1986), E₂ markedly elevated both CBG and cortisol concentrations in squirrel monkeys; however, it is unclear whether the estrogen treatment used in that study produced circulating E₂ concentrations within or above the physiological range. Thus, we find no support for the suggestion that estrogenic stimulation of HPA activity is more pronounced in New World than in Old World primates.

One possible explanation for the failure of E₂ to increase basal cortisol levels in female marmosets is that marmosets have extremely low circulating levels of CBG, so that virtually all cortisol circulates unbound or weakly bound to albumin (Pugeat et al., 1984; Robinson et al., 1985; Klosterman et al., 1986). Thus, estrogen-mediated increases in circulating CBG levels, which may be one of their key mechanisms by which estrogen increases circulating glucocorticoid concentrations in other species (Sandberg and Slaunwhite, 1959; Coe et al., 1986), are unlikely to occur in marmosets.

We have previously argued (Saltzman et al., 1994, 1998, 2000) that diminished cortisol secretion in subordinate female marmosets may be mediated, in part, by social suppression of reproductive hormones. While our present findings indicate that low basal cortisol levels are not mediated to an appreciable extent by hypoestrogenism, they also suggest that suppression of CG release from the pituitary may play a role. Unexpectedly, E₂ treatment caused a gradual decline in basal cortisol levels in both ovx and

subordinate females; however, this effect differed significantly from the control condition only in ovx females. An intriguing possibility is that this difference between subordinate and ovx animals was mediated by differences in their CG responses to E₂. We were not able to collect sufficient plasma to assay CG, but CG presumably declined in our ovx females in response to E₂ treatment, as previously reported in marmosets (Hodges, 1978). CG levels in anovulatory, ovary-intact subordinates, in contrast, are usually at or near the assay sensitivity limit (Abbott et al., 1981; Abbott, 1988; Saltzman et al., 1998) and, at least in ovx subordinates, are highly sensitive to negative feedback from even low doses of E₂ (Abbott, 1988; Abbott et al., 1990). It is likely, therefore, that CG in our subordinates, in contrast to the ovx females, underwent little additional suppression in response to E₂ treatment.

In summary, results of this study suggest that in the common marmoset, in contrast to several other species, estrogen does not elevate HPA activity: elevation of circulating E₂ levels of anovulatory subordinates and ovx females into the range typical of dominant, cycling females did not increase either mean basal cortisol levels or ACTH-stimulated cortisol levels following DEX suppression. Thus, socially induced hypoestrogenism does not appear to contribute to chronic reductions in circulating cortisol levels in anovulatory subordinate female marmosets.

Acknowledgements

This research was supported by NIH grants MH060728 to W. Saltzman, D.H. Abbott and I.M. Bird, and RR000167 to the National Primate Research Center at the University of Wisconsin–Madison (WPRC), and was conducted at a facility constructed with support from Research Facilities Improvement Program grants RR15459-01 and RR020141-01. We thank A. Drury, R. Froehlich, M. Jacobs, M. Kay, M. Maricek, K. Naruko, K. Raschka and D. Woodward for assistance with data collection; Dr N. Schultz-Darken, S. Brice, R. Kapugi, J. Kemp, S. Scola and M. Sosa for maintenance of the marmoset colony; D. Wittwer, F. Wegner and S. Jacoris for assistance with hormone assays; Dr I. Bolton, Dr K. Brunner, J. Bodden, A. Urbanski and D. Werner-Kelln for veterinary care; D. Mohr for surgical assistance; Dr T. Garland Jr for statistical advice; and Dr I. Bird and three anonymous reviewers for helpful comments on the manuscript. Preparation of the manuscript was facilitated by the staff and resources of the WPRC

Library. Marmosets were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and experimental protocols were reviewed and approved by the Graduate School Animal Care and Use Committee of UW–Madison. WPRC is accredited by AAALAC as part of the UW–Madison Graduate School.

References

- Abbott, D.H., 1986. Social suppression of reproduction in subordinate marmoset monkeys (*Callithrix jacchus jacchus*). In: de Mello, M.T. (Ed.), *A Primatologia No Brasil—2*. Anais do 2nd Congresso Brasileiro de Primatologia. Sociedade Brasileira de Primatologia, Brasilia, pp. 15–31.
- Abbott, D.H., 1988. Natural suppression of fertility. In: Hearn, J.P., Smith, G.R. (Eds.), *Reproduction and Disease in Captive and Wild Animals*. Symposia of the Zoological Society of London, vol. 60. Oxford University Press, Oxford, pp. 7–28.
- Abbott, D.H., George, L.M., 1991. Reproductive consequences of changing social status in female common marmosets. In: Box, H.O. (Ed.), *Primate Responses to Environmental Change*. Chapman and Hall, London, pp. 295–309.
- Abbott, D.H., McNeilly, A.S., Lunn, S.F., Hulme, M.J., Burden, F.J., 1981. Inhibition of ovarian function in subordinate female marmoset monkeys (*Callithrix jacchus jacchus*). *J. Reprod. Fertil.* 63, 335–345.
- Abbott, D.H., Hodges, J.K., George, L.M., 1988. Social status controls LH secretion and ovulation in female marmoset monkeys (*Callithrix jacchus*). *J. Endocrinol.* 117, 329–339.
- Abbott, D.H., George, L.M., Barrett, J., Hodges, J.K., O'Byrne, K.T., Sheffield, J.W., Sutherland, I.A., Chambers, G.R., Lunn, S.F., Ruiz de Elvira, M.-C., 1990. Social control of ovulation in marmoset monkeys: a neuroendocrine basis for the study of infertility. In: Ziegler, T.E., Bercovitch, F.B. (Eds.), *Socioendocrinology of Primate Reproduction*. Wiley-Liss, New York, pp. 135–158.
- Abbott, D.H., Saltzman, W., Schultz-Darken, N.J., Smith, T.E., 1997. Specific neuroendocrine mechanisms not involving generalized stress mediate social regulation of female reproduction in cooperatively breeding marmoset monkeys. *Ann. NY Acad. Sci.* 807, 219–238.
- Abbott, D.H., Keverne, E.B., Bercovitch, F.B., Shively, C.A., Mendoza, S.P., Saltzman, W., Snowdon, C.T., Ziegler, T.E., Banjevic, M., Garland Jr., T., Sapolsky, R.M., 2003. Are subordinates always stressed? a comparative analysis of rank differences in cortisol levels among primates. *Horm. Behav.* 43, 67–82.
- Blanchard, D.C., McKittrick, C.R., Hardy, M.P., Blanchard, R.J., 2002. Effects of social stress on hormones, brain, and behavior. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), *Hormones, Brain and Behavior*, vol. 1. Academic Press, San Diego, pp. 735–772.
- Burleson, M.H., Malarkey, W.B., Cacioppo, J.T., Poehlmann, K.M., Kiecolt-Glaser, J.K., Berntson, G.G., Glaser, R., 1998. Postmenopausal hormone replacement: effects on autonomic, neuroendocrine, and immune reactivity to brief psychological stressors. *Psychosom. Med.* 60, 17–25.
- Coe, C.L., Murai, J.T., Wiener, S.G., Levine, S., Siiteri, P.K., 1986. Rapid cortisol and corticosteroid-binding globulin responses during pregnancy and after estrogen administration in the squirrel monkey. *Endocrinology* 118, 435–440.
- Creel, S., 2001. Social dominance and stress hormones. *TREE* 16, 491–497.
- Creel, S., Creel, N.M., Monfort, S.L., 1996. Social stress and dominance. *Nature* 379, 212.
- French, J.A., 1997. Proximate regulation of singular breeding in callitrichid primates. In: Solomon, N.G., French, J.A. (Eds.), *Cooperative Breeding in Mammals*. Cambridge University Press, New York, pp. 34–75.
- Giussani, D.A., Farber, D.M., Jenkins, S.L., Yen, A., Winter, J.A., Tame, J.D., Nathanielsz, P.W., 2000. Opposing effects of androgen and estrogen on pituitary-adrenal function in nonpregnant primates. *Biol. Reprod.* 62, 1445–1451.
- Goymann, W., Wingfield, J.C., 2004. Allostatic load, social status and stress hormones: the costs of social status matter. *Anim. Behav.* 67, 591–602.
- Gromoll, J., Wistuba, J., Terwort, N., Godmann, M., Müller, T., Simoni, M., 2003. A new subclass of the luteinizing hormone/chorionic gonadotropin receptor lacking exon 10 messenger RNA in the New World monkey (*Platyrrhini*) lineage. *Biol. Reprod.* 69, 75–80.
- Gudmundsson, A., Goodman, B., Lent, S., Barczy, S., Grace, A., Boyle, L., Ershler, W.B., Carnes, M., 1999. Effects of estrogen replacement therapy on the circadian rhythms of serum cortisol and body temperature in postmenopausal women. *Exp. Gerontol.* 34, 809–818.
- Harlow, C.R., Gems, S., Hodges, J.K., Hearn, J.P., 1983. The relationship between plasma progesterone and the timing of ovulation and early embryonic development in the marmoset monkey (*Callithrix jacchus*). *J. Zool.* 201, 273–282.
- Harlow, C.R., Hearn, J.P., Hodges, J.K., 1984. Ovulation in the marmoset monkey: endocrinology, prediction and detection. *J. Endocrinol.* 103, 17–24.
- Hearn, J.P., 1977. Restraining device for small monkeys. *Lab. Anim.* 11, 261–262.
- Hodges, J.K., 1978. Effects of gonadectomy and oestradiol treatment on plasma luteinizing hormone concentrations in the marmoset monkey, *Callithrix jacchus*. *J. Endocrinol.* 76, 271–281.
- Johnson, E.O., Kamilaris, T.C., Carter, C.S., Calogero, A.E., Gold, P.W., Chrousos, G.P., 1996. The biobehavioral consequences of psychogenic stress in a small, social primate (*Callithrix jacchus jacchus*). *Biol. Psychiatry* 40, 317–337.
- Kendrick, K.M., Dixson, A.F., 1985. Luteinizing hormone releasing hormone enhances proceptivity in a primate. *Neuroendocrinology* 41, 449–453.
- Kime, D.E., Vinson, G.P., Major, P.W., Kilpatrick, R., 1980. Adrenal-gonad relationships. In: Chester Jones, I., Henderson, I.W. (Eds.), *General, Comparative and Clinical Endocrinology of the Adrenal Cortex*. Academic Press, New York, pp. 183–264.
- Klosterman, L.L., Murai, J.T., Siiteri, P.K., 1986. Cortisol levels, binding, and properties of corticosteroid-binding globulin in the serum of primates. *Endocrinology* 118, 424–434.
- Müller, T., Simoni, M., Pekel, E., Luetjens, C.M., Chandolia, R., Amato, F., Norman, R.J., Gromoll, J., 2004. Chorionic gonadotrophin beta subunit mRNA but not luteinising hormone beta subunit mRNA is expressed in the pituitary of the common marmoset (*Callithrix jacchus*). *J. Mol. Endocrinol.* 32, 115–128.
- Norman, R.L., Smith, C.J., Pappas, J.D., Hall, J., 1992. Exposure to ovarian steroids elicits a female pattern of plasma cortisol levels in castrated male macaques. *Steroids* 57, 37–43.
- O'Connell, Y., McKenna, T.J., Cunningham, S.K., 1994. The effect of prolactin, human chorionic gonadotropin, insulin

- and insulin-like growth factor 1 on adrenal steroidogenesis in isolated guinea-pig adrenal cells. *J. Steroid Biochem. Mol. Biol.* 48, 235-240.
- Pepe, G.J., Johnson, D.K., Albrecht, E.D., 1982. The effects of estrogen on cortisol metabolism in female baboons. *Steroids* 39, 471-477.
- Phillips, J.G., Poolsanguan, W., 1978. A method to study temporal changes in adrenal activity in relation to sexual status in the female laboratory rat. *J. Endocrinol.* 77, 283-291.
- Pluchino, N., Genazzani, A.D., Bernardi, F., Casarosa, E., Pieri, M., Palumbo, M., Picciarelli, G., Gabbanini, M., Luisi, M., Genazzani, A.R., 2005. Tibolone, transdermal estradiol or oral estrogen-progestin therapies: effects on circulating allopregnanolone, cortisol and dehydroepiandrosterone levels. *Gynecol. Endocrinol.* 20, 144-149.
- Pruessner, J.C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003. Two formulas for computation of the area under the curve represent measures of total concentration versus time-dependent change. *Psychoneuroendocrinology* 28, 916-931.
- Pugeat, M.M., Chrousos, G.P., Nisula, B.C., Loriaux, D.L., Brandon, D., Lipsett, M.B., 1984. Plasma cortisol transport and primate evolution. *Endocrinology* 115, 357-361.
- Robinson, P.A., Hawkey, C., Hammond, G.L., 1985. A phylogenetic study of the structural and functional characteristics of corticosteroid binding globulin in primates. *J. Endocrinol.* 104, 251-257.
- Roy, B.N., Reid, R.L., Van Vugt, D.A., 1999. The effects of estrogen and progesterone on corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid levels in the paraventricular nucleus and supraoptic nucleus of the rhesus monkey. *Endocrinology* 140, 2191-2198.
- Saltzman, W., 2003. Reproductive competition among female common marmosets (*Callithrix jacchus*): proximate and ultimate causes. In: Jones, C.B. (Ed.), *Sexual Selection and Reproductive Competition in Primates: New Perspectives and Directions*. American Society of Primatologists, Norman, OK, pp. 197-229.
- Saltzman, W., Schultz-Darken, N.J., Scheffler, G., Wegner, F.H., Abbott, D.H., 1994. Social and reproductive influences on plasma cortisol in female marmoset monkeys. *Physiol. Behav.* 56, 801-810.
- Saltzman, W., Schultz-Darken, N.J., Abbott, D.H., 1996. Behavioural and endocrine predictors of dominance and tolerance in female common marmosets, *Callithrix jacchus*. *Anim. Behav.* 51, 657-674.
- Saltzman, W., Schultz-Darken, N.J., Wegner, F.H., Wittwer, D.J., Abbott, D.H., 1998. Suppression of cortisol levels in subordinate female marmosets: reproductive and social contributions. *Horm. Behav.* 33, 58-74.
- Saltzman, W., Prudom, S.L., Schultz-Darken, N.J., Abbott, D.H., 2000. Reduced adrenocortical responsiveness to adrenocorticotrophic hormone (ACTH) in socially subordinate female marmoset monkeys. *Psychoneuroendocrinology* 25, 463-477.
- Saltzman, W., Prudom, S.L., Schultz-Darken, N.J., Wittwer, D.J., Abbott, D.H., 2004. Social suppression of cortisol in female marmoset monkeys: role of circulating ACTH levels and glucocorticoid negative feedback. *Psychoneuroendocrinology* 29, 141-161.
- Saltzman, W., Hogan, B.K. Abbott, D.H., in press. Diminished cortisol levels in subordinate female marmosets are associated with altered central drive to the HPA axis. *Biol. Psychiatry*.
- Sandberg, A.A., Slaunwhite Jr., W.R., 1959. Transcortin: a corticosteroid-binding protein of plasma. II. Levels in various conditions and the effects of estrogens. *J. Clin. Invest.* 38, 1290-1297.
- Slayden, S.M., Crabbe, L., Bae, S., Potter, H.D., Azziz, R., Parker Jr., C.R., 1998. The effect of 17 β -estradiol on adrenocortical sensitivity, responsiveness, and steroidogenesis in postmenopausal women. *J. Clin. Endocrinol. Metab.* 83, 519-524.
- Smith, C.J., Norman, R.L., 1987. Influence of the gonads on cortisol secretion in female rhesus macaques. *Endocrinology* 121, 2192-2198.
- Stavisky, R.C., Watson, S.L., Anthony, M.S., Manuck, S.B., Adams, M.R., Kaplan, J.R., 2003. Influence of estradiol on cortisol secretion in ovariectomized cynomolgus macaques (*Macaca fascicularis*). *Am. J. Primatol.* 60, 17-22.
- Summers, P.M., Wennink, C.J., Hodges, J.K., 1985. Cloprostenol-induced luteolysis in the marmoset monkey (*Callithrix jacchus*). *J. Reprod. Fertil.* 73, 133-138.
- Vamvakopoulos, N.C., Chrousos, G.P., 1993. Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression: potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. *J. Clin. Invest.* 92, 1896-1902.
- von Holst, D., 1998. The concept of stress and its relevance for animal behavior. *Adv. Stud. Behav.* 27, 1-131.
- Wilson, M.E., Legendre, A., Pazol, K., Fisher, J., Chikazawa, K., 2005. Gonadal steroid modulation of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis is influenced by social status in female rhesus monkeys. *Endocrine* 26, 89-97.
- Wood, C.E., Cline, J.M., Anthony, M.S., Register, T.C., Kaplan, J.R., 2004. Adrenocortical effects of oral estrogens and soy isoflavones in female monkeys. *J. Clin. Endocrinol. Metab.* 89, 2319-2325.
- Young, E.A., 1995. The role of gonadal steroids in hypothalamic-pituitary-adrenal axis regulation. *Crit. Rev. Neurobiol.* 9, 371-381.
- Young, E.A., 1998. Sex differences and the HPA axis: implications for psychiatric disease. *J. Gen. Specif. Med.* 1, 21-27.

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®