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Social suppression of cortisol in female marmosets: Role of luteinizing hormone/chorionic gonadotropin

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Abstract

Behaviorally subordinate female common marmosets (*Callithrix jacchus*) undergo suppression of ovulation and chronic reductions in basal plasma cortisol concentrations. Indirect evidence suggests that hypophyseal chorionic gonadotropin (CG; the major pituitary luteinizing gonadotropin in marmosets) may elevate cortisol concentrations in female marmosets, and therefore that social suppression of CG may contribute to diminution of cortisol in subordinates. To test this hypothesis, we determined whether pharmacological inhibition of pituitary CG release decreases basal and adrenocorticotropin (ACTH)-stimulated cortisol secretion. We characterized cortisol and reproductive hormone concentrations in six ovary-intact and six ovariectomized marmosets during long-term treatment with leuprolide acetate, a gonadotropin-releasing hormone (GnRH) agonist, and vehicle. Leuprolide suppressed basal plasma CG concentrations, abolished the CG response to exogenous GnRH, and, in intact animals, blocked ovarian cyclicity. During treatment with vehicle, plasma cortisol concentrations were elevated during the periovulatory phase in intact females, compared to the follicular phase, the luteal phase, and ovariectomized females. Leuprolide suppressed basal cortisol concentrations of intact females as compared to the periovulatory phase, but did not affect basal cortisol in ovariectomized animals and did not alter responses to exogenous ACTH. These findings suggest that elevations in circulating CG concentrations are associated with elevated cortisol concentrations in female marmosets, and that this relationship requires simultaneous increases in ovarian hormones that occur only during the periovulatory period. Thus, suppression of CG release in anovulatory subordinate females may not play an important role in socially induced diminution of cortisol.

Keywords: Adrenal cortex; *Callithrix jacchus*; Chorionic gonadotropin; Cortisol; Leuprolide acetate; Luteinizing hormone; Marmoset; Ovarian cycle; Ovariectomy; Subordination

1. Introduction

Complex, bidirectional interactions occur between the female reproductive system and the hypothalamic–pituitary–adrenal (HPA) axis. While much attention has been focused on the role of the stress-responsive HPA axis in suppressing female reproductive function, it is also well established that hormones of the hypothalamic–pituitary–ovarian axis can modulate HPA activity (reviewed by Kime et al., 1980; Young, 1998). Estrogen, in particular, is well known to stimulate HPA activity and elevate circulating glucocorticoid concentrations in rodents and primates through actions on the hypothalamus, pituitary, adrenal, and liver. Progesterone may modulate these effects of estrogen and may also interfere with binding of glucocorticoids to their receptors and to corticosteroid-binding globulin, thereby altering glucocorticoid negative-feedback effects. HPA activity may also be modulated by the pituitary gonadotropin luteinizing hormone (LH) and the structurally and functionally homologous placental hormone, chorionic gonadotropin (CG). Although these effects have received much less attention than those

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of the gonadal steroids, evidence from several species suggests that LH/CG can elevate circulating glucocorticoid concentrations through direct actions on the adrenal cortex (Kero et al., 2000; O'Connell et al., 1994; Pabon et al., 1996; Phillips and Poolsanguan, 1978; Vinson and Renfree, 1975; Vinson et al., 1976).

We have been investigating the interactions between reproductive hormones and the HPA axis in the common marmoset (*Callithrix jacchus*), a small New World monkey in which endocrine function is profoundly influenced by social status. Social groups may contain as many as six adult females, but in both the wild and captivity, 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 inhibited hypophyseal secretion of CG (Abbott et al., 1981a,b; Saltzman et al., 1998), which has been shown recently to be the major luteinizing gonadotropin secreted by the pituitary in this species and possibly other New World primates (Gromoll et al., 2003; Müller et al., 2004b). Reproductive suppression can persist for months or even years, but is reversed rapidly following separation of the subordinate female from her dominant female groupmate (Abbott and George, 1991; Abbott et al., 1988).

Anovulatory, subordinate female marmosets also exhibit chronic reductions in circulating cortisol concentrations. Morning basal (i.e., non-stressed) cortisol concentrations decline markedly within 6–7 weeks following the onset of social subordination and anovulation, and can remain low for months to years (Abbott et al., 1997; Johnson et al., 1996; Saltzman et al., 1994, 1998, 2004b, in press, 2006). Diminished cortisol concentrations are associated with reduced adrenal responsiveness to adrenocorticotropic hormone (ACTH): subordinate females have basal plasma ACTH concentrations that are similar to those of dominants (Johnson et al., 1996; Saltzman et al., 2004b, in press), and therefore have reliably lower cortisol-to-ACTH ratios (Saltzman et al., in press). Moreover, subordinates secrete less cortisol than dominants in response to exogenous ACTH (Saltzman et al., 2000). Subordinate females also exhibit elevated ACTH concentrations following treatment with metyrapone, an inhibitor of cortisol biosynthesis, as compared to dominant females, indicative of altered central regulation of the HPA axis (Saltzman et al., in press).

Cortisol diminution in subordinate female marmosets appears to be mediated, in part, by suppression of reproductive hormones. For example, pair-housed females that are anovulatory but not subordinate to other females exhibit low plasma cortisol concentrations and do not show a further reduction in cortisol upon becoming subordinate in a new social group (Saltzman et al., 1994). Moreover, basal cortisol concentrations fluctuate reliably across the ovarian cycle in marmosets, indicating that HPA activity is modulated by reproductive hormones (Saltzman et al., 1998). In a recent study (Saltzman et al., 2006), however, we found that long-term pharmacologic elevation of circulating estradiol concentrations into the range typical of the periovulatory phase of the ovarian cycle did not appreciably elevate basal or ACTH-stimulated cortisol concentrations in either anovulatory subordinates or ovariectomized, non-subordinate females. Hypoestrogenism, therefore, does not appear to contribute to cortisol suppression in subordinates.

In contrast, indirect evidence suggests that hypophyseal CG may elevate circulating cortisol concentrations in female marmosets, and therefore that suppression of CG release in subordinates may contribute to cortisol diminution. First, in females undergoing ovulatory cycles, morning basal plasma cortisol concentrations peak during the periovulatory period, when CG secretion is maximal (Saltzman et al., 1998). Moreover, basal cortisol concentrations of cycling females are reliably higher than those of ovariectomized marmosets only during the periovulatory period, when CG concentrations of cycling females exceed those of ovariectomized females (Saltzman et al., 1998). Subordinates, in contrast, have consistently lower circulating concentrations of both cortisol and CG than ovariectomized females (Saltzman et al., 1998, 2006). Finally, in one study, morning basal plasma cortisol

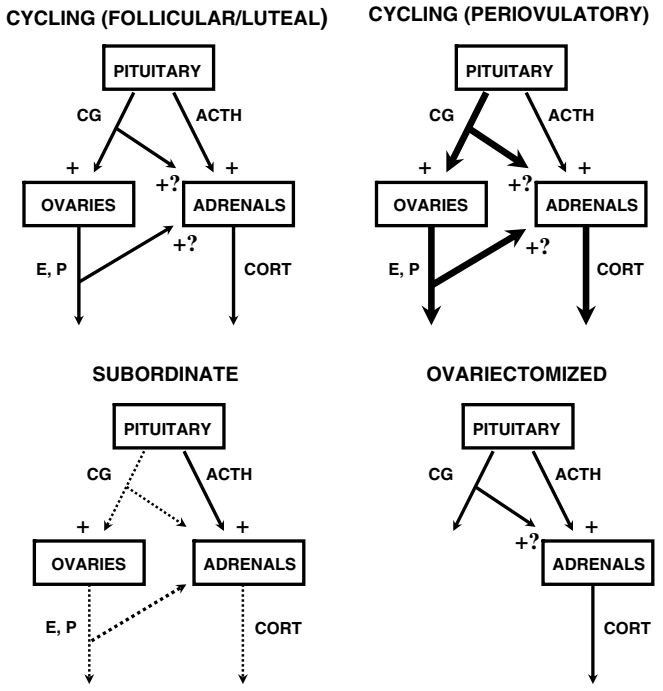

Fig. 1. Hypothesized interactions among hypophyseal chorionic gonadotropin (CG), ovarian steroids (estrogen [E] and progesterone [P]), and cortisol secretion in female marmosets. Anovulatory subordinates have consistently low circulating concentrations of CG and cortisol, but not ACTH, as compared to both cycling and ovx females, as well as low, acyclic concentrations of E and P. Cycling females exhibit elevated CG and cortisol during the periovulatory phase, the only time when both hormones are reliably elevated above those in ovx females. Thus, CG is hypothesized to enhance basal cortisol secretion in cycling and ovx females, as compared to subordinate females, and to further elevate cortisol secretion during the ovulatory phase of the ovarian cycle. This latter effect may be dependent upon the ovarian steroid milieu around the time of ovulation. Thickness of arrow indicates relative hormone concentrations.

concentrations of ovariectomized females were positively correlated with circulating concentrations of CG but not estradiol, estrone, or progesterone (Saltzman et al., 1998). CG may, therefore, modulate cortisol secretion in female marmosets (Fig. 1); however, this possibility has not been tested directly.

In the present study, we used a gonadotropin-releasing hormone (GnRH) agonist to chronically block CG release in both ovary-intact female marmosets undergoing ovulatory cycles and ovariectomized females, to determine whether inhibition of CG release reduced basal or ACTH-stimulated cortisol concentrations and whether these effects might be mediated by ovarian hormones. We predicted that inhibition of CG release would decrease basal plasma cortisol concentrations and cortisol responses to exogenous ACTH in both groups of animals. Such findings would suggest that CG enhances cortisol secretion in female marmosets, possibly through direct actions on the adrenal cortex, and would support our hypothesis that social suppression of CG secretion contributes to diminution of basal cortisol concentrations in subordinate females (Fig. 1).

2. Materials and methods

2.1. Animals

We used 12 captive-born, adult female common marmosets (*C. jacchus*), including six ovary-intact females undergoing regular ovulatory cycles and six long-term ovariectomized (ovx) females. Intact and ovx animals did not differ significantly ($p > 0.05$) in body mass (394 ± 13 g versus 417 ± 19 g, respectively; mean ± SEM) or age (34.2 ± 3.0 months versus 33.9 ± 2.3 months, respectively) at the outset of data collection.

Each intact female was housed with an adult male pairmate and, in four cases, an unrelated, behaviorally subordinate female or up to six offspring. Each ovx female was housed with an adult male pairmate and, in two cases, up to three offspring. Ovx females had undergone bilateral removal of the ovaries and fallopian tubes at least 6 months (8.2 ± 1.3 months, mean ± SEM) 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).

Marmosets were housed indoors at the National Primate Research Center at the University of Wisconsin-Madison, with lights on from 06:30 to 18:30 h, ambient temperature at approximately 27 °C, and humidity at approximately 50%. All marmosets occupied aluminum and wire mesh cages measuring 61 cm × 91 cm × 183 cm, 122 cm × 61 cm × 183 cm, or 61 cm × 61 cm × 183 cm. Animals were fed Mazuri Callitrichid High Fiber Diet 5M16 (Purina Mills, St. Louis, MO, USA), supplemented with fruit, cereal, nuts, and miniature marshmallows, once daily between 12:30 and 14:30 h, and water was available ad lib. Additional information on marmoset housing and husbandry is provided by Saltzman et al. (1998).

2.2. Design

Marmosets were tested in two conditions: while undergoing treatment with the GnRH agonist leuprolide acetate and while undergoing identical treatment with vehicle (see below). To avoid carryover effects of leuprolide, we tested each animal first in the vehicle condition and subsequently in the leuprolide condition. In each condition, marmosets underwent two ACTH stimulation tests (1 and 10 μg ACTH/kg body mass) approximately 4 and 8 weeks after the first leuprolide or vehicle injection, to asses adrenocortical responsiveness. The order of ACTH doses was approximately balanced across animals within each group but, for each animal, was identical in the leuprolide and vehicle conditions. Each marmoset also underwent two GnRH (5 μg) stimulation tests in each condition, approximately 2 and 10 weeks after the first leuprolide or vehicle injection, to confirm the efficacy of leuprolide in inhibiting hypophyseal CG release.

In addition to the ACTH and GnRH stimulation tests, we collected weekly blood samples for determination of morning basal cortisol concentrations. Basal CG concentrations were determined in blood samples collected every 1–2 weeks. To monitor ovulatory cycles in intact females, we measured plasma progesterone concentrations in blood samples collected twice each week, at 3- to 4-day intervals (Saltzman et al., 1994). We additionally measured estradiol sulfate (E_2S) in urine samples collected from all females approximately every 2 weeks.

2.3. Leuprolide acetate and vehicle treatment

In the leuprolide condition, each marmoset received a total of four depot injections of leuprolide acetate (Lupron Depot, TAP Pharmaceutical Products, Lake Forest, IL, USA; 198.75 μg, IM) in vehicle (5 mg carboxymethylcellulose sodium + 50 mg D-mannitol + 1 mg polysorbate 80 per 7.5 mg Lupron Depot) at 21-day intervals. This dose and frequency of treatment were based on a published study in another New World primate species (Kuehl et al., 1999) and on pilot tests conducted in marmosets to determine the efficacy of leuprolide in suppressing plasma CG concentrations (unpublished data). In the vehicle condition, marmosets received identical treatment with only the vehicle. Intact females received their first leuprolide and vehicle injections during the follicular phase of the ovarian cycle.

2.4. ACTH stimulation tests

Animals were weighed and given an IM injection of 5 mg/kg dexamethasone sodium phosphate (DEX; American Regent Laboratories, Shirley, NY, USA) at 16:00 h to suppress endogenous cortisol and ACTH secretion. This dose of DEX suppresses plasma cortisol and ACTH concentrations in female marmosets for 2–3 days (Saltzman et al., 2004b). At 09:30–10:00 h the following morning, each animal received an IV injection of either 1 or 10 μg/kg synthetic human $ACTH_{1-39}$ (Sigma–Aldrich, St. Louis, MO, USA) in 0.5 ml/kg sterile saline. These doses of ACTH produce a robust but sub-maximal cortisol response in female marmosets (Saltzman et al., 2000). Blood samples (0.2–0.6 ml) were collected by femoral venipuncture at −20 (baseline), 60, and 120 min from ACTH injection. Between samples, each marmoset remained alone in a stainless steel nestbox from its home cage, which also served as a transport cage.

2.5. GnRH stimulation tests

Animals were injected with human GnRH (Sigma–Aldrich, St. Louis, MO, USA; 5 μg in 0.2 ml sterile saline, IV) at 09:00–09:30 h. Blood samples (0.15–0.2 ml) were collected 0, 30, and 60 min from GnRH injection and subsequently assayed for CG. In the vehicle condition, GnRH stimulation tests were performed when intact females were in the early to mid-luteal phase/early pregnancy (3–8 days post-ovulation).

2.6. Collection of blood and urine samples

Animals were briefly restrained in a marmoset 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 and subsequently centrifuged at 2000 rpm for 10 min. The plasma was extracted and stored at −20 °C until assayed. Blood samples for determination of basal cortisol concentrations were collected between 09:15 and 09:45 h, several hours after the diurnal cortisol peak (L.M. George & D.H. Abbott, unpublished data). For these basal samples, latency from initial disturbance of the animal (investigator's initial entry into the cage) to sample collection was <3 min.

Urine samples were collected using the methods described by Saltzman et al. (2004a). Briefly, marmosets were captured from their nestboxes at

approximately 06:25 h, several minutes prior to lights-on, and immediately placed in a urine-collection chamber within their home cage until they urinated or until 1 h had elapsed, whichever occurred first. Urine samples were placed on ice, centrifuged at 5000 rpm for 5 min, and transferred to a clean vial. Glycerol (0.52 M; 50 μl/ml of urine) was added to each sample to stabilize protein hormones, and samples were stored at −20 °C.

2.7. Monitoring and control of ovarian function

Progesterone was assayed in blood samples collected twice weekly from each intact 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 intact females were in the early to mid-follicular phase of the ovarian cycle during ACTH stimulation tests in the vehicle condition, we gave each intact animal an IM injection of 0.75–1.0 μg cloprostenol sodium (Estrumate, Mobay Corp., Shawnee, KS, USA and Cayman Chemicals, Ann Arbor, MI, USA), a prostaglandin F2α 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). Cloprostenol has not been found to alter baseline cortisol concentrations in marmosets (Saltzman et al., 1998). Nonetheless, to control for any potential effects on HPA activity, intact and ovx females were treated identically with cloprostenol 3–4 days before each ACTH stimulation test, in both the vehicle and leuprolide conditions.

2.8. Hormone assays

All assays were fully validated for use with marmoset plasma (progesterone, cortisol, CG; Saltzman et al., 1994, 1998) or urine (E_2S; Saltzman et al., 2004a). Plasma progesterone concentrations were measured in duplicate aliquots using a heterologous enzyme immunoassay (Saltzman et al., 1994). Assay sensitivity at 90% binding was 4.85 pg (3.46 ng/ml), and intra- and inter-assay coefficients of variation (CVs) of a marmoset plasma pool assayed in quadruplicate in each assay (46% binding) were 3.6 and 18.7%, respectively.

Plasma cortisol concentrations were determined in duplicate aliquots by radioimmunoassay (RIA) using an antibody-coated-tube kit (Gamma-Coat, DiaSorin Corp., Stillwater, MN, USA) as described previously (Saltzman et al., 1994). Assay sensitivity at 90% binding was 0.054 ng (5.37 μg/dl), and intra- and inter-assay CVs of a plasma pool assayed in quadruplicate in each assay (33% binding) were 6.3 and 8.7%, respectively.

Plasma concentrations of immunoreactive CG were measured by double-antibody RIA using the monoclonal antiserum 518B7 raised in mouse against bovine LH, as previously described (Saltzman et al., 1998). This antibody recognizes both CG and LH; however, we refer to the results exclusively in terms of CG, in view of recent findings that the marmoset pituitary expresses CG but not LH and that the marmoset LH receptor may be activated preferentially by CG (Gromoll et al., 2003; Müller et al., 2004a,b). Assay sensitivity was 0.13 ng (2.67 ng/ml) at 90% binding, and intra- and inter-assay CVs of a quality-control pool assayed in quadruplicate in each assay (26% binding) were 7.5 and 9.8%, respectively.

Urinary E_2S concentrations were measured in duplicate aliquots by RIA as previously described (Saltzman et al., 2004a). Assay sensitivity at 90% binding was 12.01 pg (0.60 μg/ml), and intra- and inter-assay CV's of a quality-control pool assayed in quadruplicate in each assay (48% binding) were 3.0 and 6.8%, respectively. To correct for differences in concentration of urine samples, urinary E_2S concentrations were divided by creatinine concentrations in the same sample. Creatinine was assayed in duplicate as described by Ziegler et al. (1995). Intra- and inter-assay CVs were 1.9 and 6.8%, respectively.

2.9. Data analysis

For each marmoset, we determined mean basal plasma cortisol, mean basal plasma CG, and mean urinary E_2S concentrations during the leuprolide and vehicle conditions, using all samples that had been collected >2 days following the first leuprolide or vehicle injection. For intact females in the vehicle condition, we additionally determined mean basal CG and E_2S concentrations during the follicular phase (plasma progesterone < 10 ng/ml) and luteal phase/early pregnancy (plasma progesterone ⩾ 10 ng/ml). We were unable to characterize periovulatory CG concentrations, because low total blood volumes in these small primates prevented us from collecting blood frequently enough to detect the 1–2-day CG surge (Saltzman et al., 1998). In contrast, morning basal cortisol concentrations in female marmosets are elevated from 4 days before through 4 days after the CG surge, which typically precedes ovulation by 1 day (Harlow et al., 1984; Saltzman et al., 1998); therefore, for each intact female we determined mean basal cortisol concentrations during the early to mid-follicular phase (⩾6 days prior to ovulation), periovulatory phase (5 days before through 3 days after ovulation), and luteal phase/early pregnancy (⩾4 days after ovulation).

For analyses of cortisol responses to ACTH and CG responses to GnRH, we calculated area under the curve (AUC) with respect to increase using the method of Pruessner et al. (2003). For ACTH stimulation tests, we also calculated delta scores as each animal's cortisol concentrations at 60 and 120 min after injection minus her pre-ACTH (DEX-suppressed) baseline cortisol concentrations.

Cortisol and E_2S data were log-transformed to increase normality. Because E_2S, basal CG, and basal cortisol concentrations of intact animals were expected to vary across the ovarian cycle during the vehicle condition, for each of these hormones we initially analyzed all data from intact females (i.e., leuprolide condition + each cycle phase within the vehicle condition) using a repeated-measures ANOVA; pairwise comparisons between treatment conditions/cycle phases were subsequently performed by univariate F tests. Comparisons between ovx females in the leuprolide and vehicle conditions, and between intact and ovx females within either treatment condition, were performed by t tests. Analyses were performed using Systat v. 5.2 for the Macintosh (SPSS, Chicago, IL, USA), and results were evaluated at the 0.05 level (two-tailed).

3. Results

3.1. Plasma progesterone

Progesterone concentrations in twice-weekly blood samples indicated that leuprolide successfully inhibited ovulation in all six intact females (see Fig. 2). One marmoset ovulated 5 days after her first leuprolide injection and underwent a normal luteal phase; this animal subsequently received leuprolide doses twice as high as the other animals for the remainder of the leuprolide condition. Another two females ovulated 7–12 days after their first leuprolide treatment, but had very short luteal phases with low progesterone concentrations (⩽30 ng/ml) and subsequently remained anovulatory. The remaining three marmosets became anovulatory after their first leuprolide injection. Marmosets did not ovulate again until 51–80 days after their final leuprolide treatment.

3.2. Urinary estradiol sulfate

3.2.1. Vehicle condition

E_2S concentrations of intact females were higher during the luteal phase/early pregnancy than the follicular phase ($F[2,10] = 90.355$, $p < 0.0001$; follicular versus luteal phase: $p < 0.0001$; Fig. 3), consistent with previous findings in this species (Eastman et al., 1984; Kendrick and Dixson, 1983; Saltzman et al., 1998, 2004a; Torii et al., 1987; Ziegler et al., 1996). E_2S concentrations of ovx animals were lower than

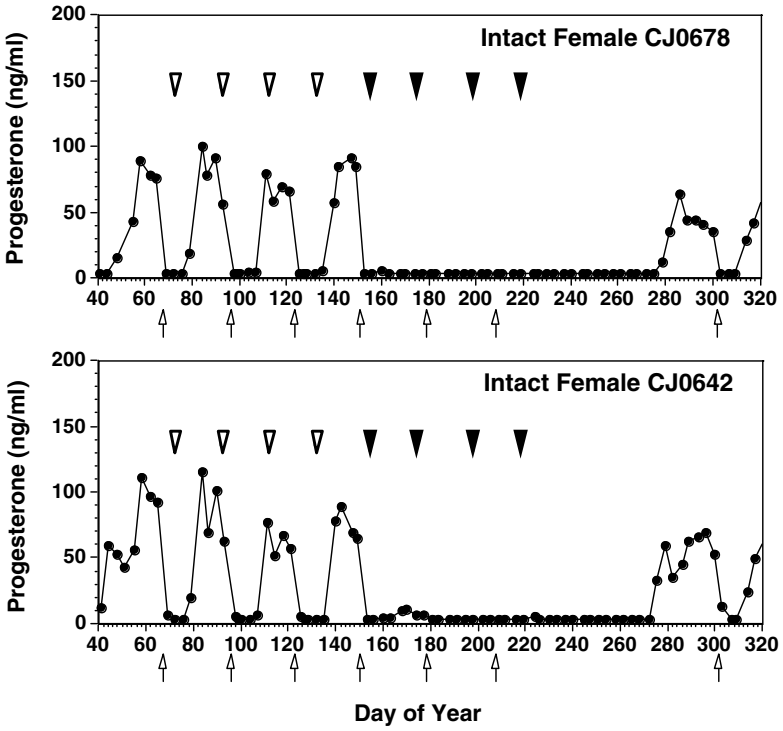

Fig. 2. Plasma progesterone profiles of two representative intact female marmosets. White arrowheads indicate vehicle injections, black arrowheads indicate leuprolide injections, and small arrows indicate cloprostenol ($PGF_{2\alpha}$ analog) injections (to cause luteolysis and terminate ovarian cycles).

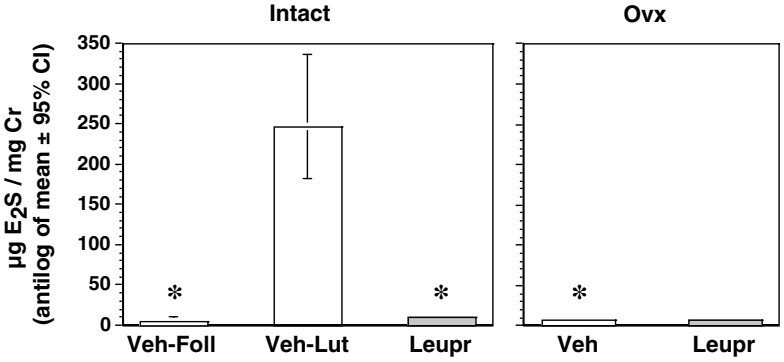

Fig. 3. Urinary E_2S concentrations (backtransformed mean ± 95% confidence intervals) of six intact and six ovariectomized female marmosets during treatment with vehicle (white bars) or leuprolide (gray bars). In the vehicle condition, mean E_2S concentrations of each intact female were determined for the follicular phase and luteal phase/early pregnancy across several ovarian cycles. In the leuprolide condition, all intact females were anovulatory. $^*p < 0.0001$ vs. intact females in the luteal phase/early pregnancy.

those of intact females during the luteal phase/early pregnancy ($T[10] = 12.022$, $p < 0.0001$) but not the follicular phase ($p > 0.3$).

3.2.2. Leuprolide condition

Leuprolide suppressed urinary E_2S concentrations of intact females below their previous luteal-phase concentrations ($p < 0.0001$), to concentrations similar to those in the follicular phase ($p > 0.2$). Leuprolide did not alter E_2S concentrations in ovx females ($p > 0.5$). E_2S concentrations during the leuprolide condition did not differ between intact and ovx animals ($p > 0.2$), implicating a non-ovarian source for the excreted estrogen.

3.3. Plasma chorionic gonadotropin

3.3.1. Vehicle condition

Basal CG concentrations of intact females were higher during the luteal phase/early pregnancy than the follicular phase ($F[2,10] = 12.016$, $p = 0.002$; follicular versus luteal phase: $p = 0.026$; Fig. 4), as previously reported for this species (Abbott et al., 1988; Lunn et al., 1992; Saltzman et al., 1998). Basal CG concentrations of ovx females were significantly higher than those of intact females in the follicular phase ($T[10] = -6.244$, $p = 0.0001$) and marginally higher than those of intact females in the luteal phase/early pregnancy ($T[10] = -2.159$, $p = 0.056$).

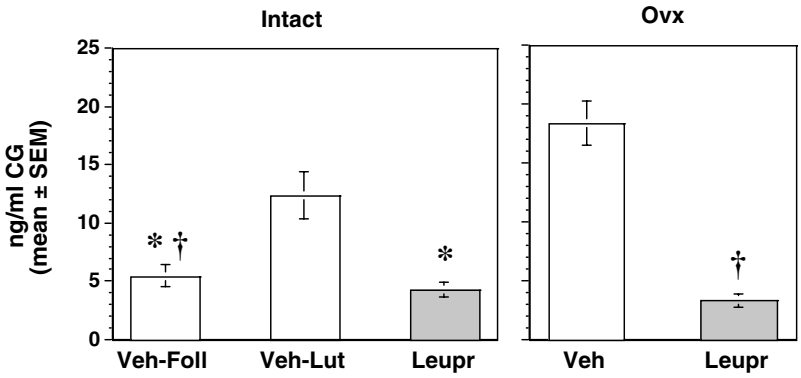

Fig. 4. Plasma CG concentrations (mean ± SEM) of six intact and six ovariectomized female marmosets during treatment with vehicle (white bars) or leuprolide (gray bars). In the vehicle condition, mean CG concentrations of each intact female were determined for the follicular phase and luteal phase/early pregnancy across several ovarian cycles. In the leuprolide condition, all intact females were anovulatory. $^{*}p < 0.05$ vs. intact females in the luteal phase/early pregnancy. $^{\dagger}p < 0.001$ vs. ovariectomized females in the vehicle condition.

3.3.2. Leuprolide condition

Leuprolide sharply decreased plasma CG concentrations in intact females, as compared to the lutealphase/early pregnancy ($p = 0.012$) but not the follicular phase ($p = 0.096$). CG concentrations of intact females were below the sensitivity limit of the assay in $54.4 \pm 14.4\%$ (mean ± SEM) of blood samples in the leuprolide condition, compared to $5.6 \pm 3.5\%$ of samples (two samples, both from the follicular phase) in the vehicle condition. Thus, the assay sensitivity limit may have obscured detection of leuprolide-mediated suppression of CG as compared to follicular-phase values. Leuprolide also lowered CG in ovx females ($T[5] = 10.003$, $p = 0.0002$). CG concentrations of ovx females were below the assay sensitivity limit in $83.3 \pm 16.7\%$ of blood samples in the leuprolide condition but none in the vehicle condition. CG concentrations in the leuprolide condition did not differ reliably between intact and ovx animals ($p > 0.3$).

Leuprolide abolished the CG response to exogenous GnRH in both intact and ovx females, confirming that it effectively blocked pituitary release of CG (Fig. 5). Net integrated responses to GnRH did not differ between the two GnRH stimulation tests in each condition but were higher in the vehicle than the leuprolide condition ($F[1,10] = 37.003$, $p < 0.0001$). Ovx females had markedly greater net integrated responses to GnRH than intact females in the vehicle but not the leuprolide condition, as reflected in a significant main effect of group ($F[1,10] = 5.130$, $p = 0.047$) and group × condition interaction ($F[1,10] = 5.299$, $p = 0.044$).

3.4. Basal plasma cortisol

3.4.1. Vehicle condition

Basal cortisol concentrations of intact females differed across conditions ($F[3,15] = 8.346$, $p = 0.002$; Fig. 6), as they were higher in the periovulatory period than either the early follicular phase ($p = 0.025$) or the mid- to late luteal phase/early pregnancy ($p = 0.001$). Basal cortisol concentrations of ovx females were lower than those of intact females during the periovulatory period ($T[10] = 2.422$, $p = 0.036$) but not the early follicular phase ($p > 0.2$) or the mid- to late luteal phase/early pregnancy ($p > 0.7$).

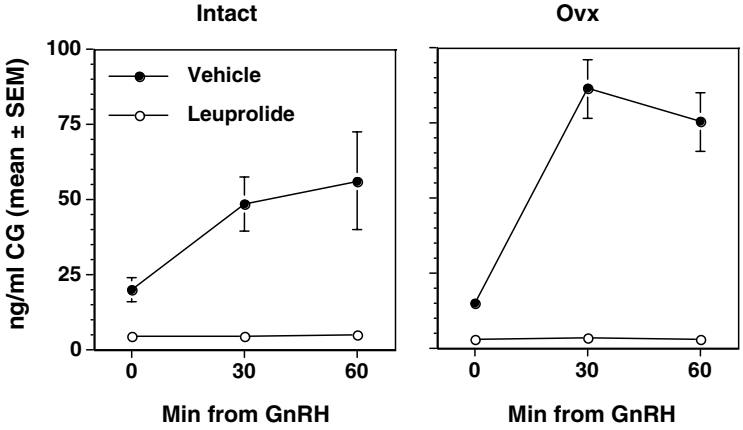

Fig. 5. Plasma CG responses (mean ± SEM) to GnRH (5 μg, IV) in six intact and six ovariectomized female marmosets during treatment with vehicle or leuprolide. Data represent mean values from two GnRH stimulation tests performed on each animal during each treatment condition (2 and 10 weeks after the first leuprolide or vehicle injection). See text for statistical results.

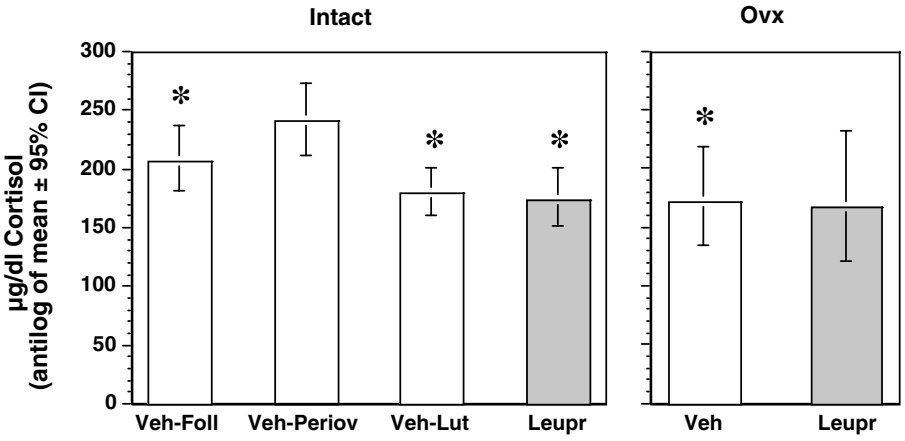

Fig. 6. Basal plasma cortisol concentrations (backtransformed mean ± 95% confidence intervals) of six intact and six ovariectomized female marmosets during treatment with vehicle (white bars) or leuprolide (gray bars). In the vehicle condition, mean basal cortisol concentrations of each intact female were determined for the early follicular phase, periovulatory period, and mid- to late luteal phase/early pregnancy across several ovarian cycles. In the leuprolide condition, all intact females were anovulatory. $^{*}p < 0.05$ vs. intact females in the periovulatory period.

3.4.2. Leuprolide condition

Leuprolide suppressed basal cortisol concentrations of intact females below their periovulatory levels ($p = 0.019$), to levels similar to those in the early follicular phase ($p > 0.1$) and mid- to late luteal phase/early pregnancy ($p > 0.7$). Leuprolide did not, however, alter basal cortisol concentrations in ovx females ($p > 0.6$). Cortisol concentrations during the leuprolide condition did not differ between intact and ovx animals ($p > 0.8$).

3.5. ACTH-stimulated plasma cortisol

Net integrated cortisol values were higher in response to 10 than to 1 µg/kg ACTH ($F[1,9] = 85.050$, $p < 0.0001$; Fig. 7). They did not, however, differ between intact and ovx females ($p > 0.1$) or between the leuprolide and vehicle conditions ($p > 0.3$; group × condition interaction: $p > 0.7$). Similarly, the cortisol elevation above pre-ACTH baseline concentrations at both 60 and 120 min post-ACTH differed

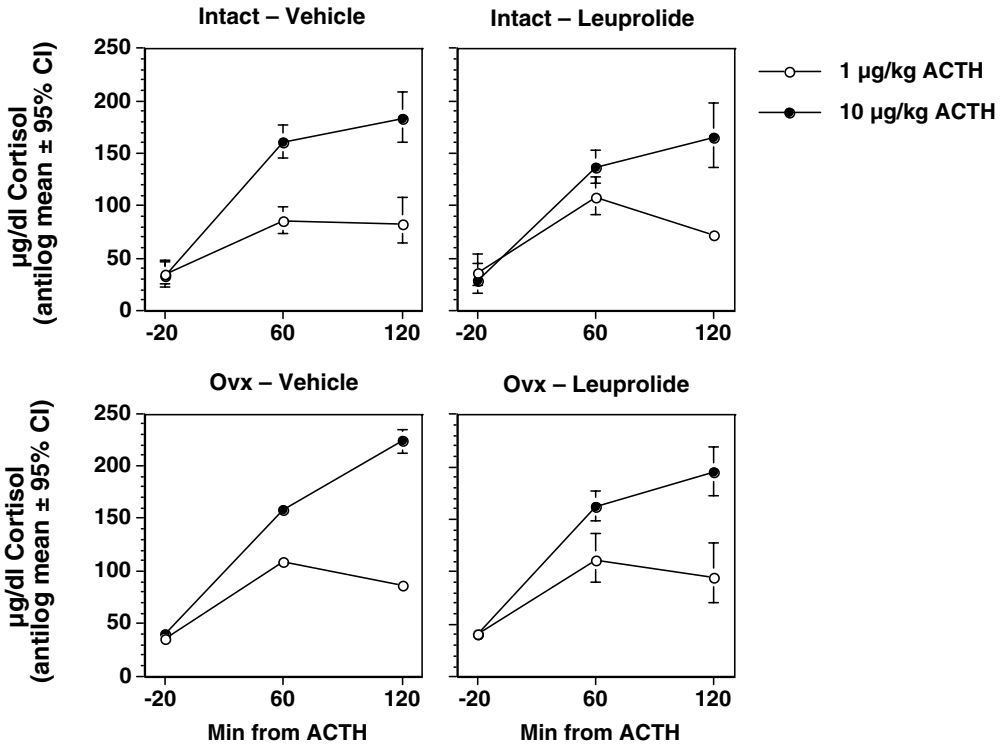

Fig. 7. Plasma cortisol responses (backtransformed mean ± 95% confidence intervals) to ACTH (1 and 10 µg/kg, IV), following dexamethasone (5 mg/kg, IM) treatment the previous day, in six intact and six ovariectomized female marmosets during treatment with vehicle or leuprolide. See text for statistical results.

between the two ACTH doses (60 min: $F[1,10] = 66.314$, $p < 0.001$; 120 min: $F[1,9] = 185.539$, $p < 0.0001$) but not between intact and ovx animals ($p > 0.1$) or between the two treatment conditions ($p > 0.09$; group × condition interaction: $p > 0.4$). Finally, DEX-suppressed cortisol concentrations immediately prior to ACTH injection did not differ between groups ($p > 0.3$) or conditions ($p > 0.9$; group × condition interaction: $p > 0.4$).

4. Discussion

In this study we tested the hypothesis that pharmacological suppression of hypophyseal CG release decreases circulating cortisol concentrations in female marmosets, and, by implication, that socially induced suppression of hypophyseal CG release might therefore contribute to diminished cortisol concentrations in subordinate females (see Fig. 1). The results indicate that CG stimulates cyclical elevations in endogenous cortisol concentrations, but that this effect is small in magnitude and is dependent upon, and may be mediated by, ovarian hormones. Thus, our results suggest that social suppression of CG may not play a major role in mediating chronic reductions of basal cortisol concentrations in anovulatory, subordinate female marmosets, which exhibit low, acyclic concentrations of estrogen and progesterone (Abbott et al., 1981, 1988; Saltzman et al., 1998).

As predicted, the GnRH agonist leuprolide acetate reduced basal cortisol concentrations in ovary-intact females; however, this reduction was significant only when compared to the animals' periovulatory cortisol concentrations during the vehicle condition. Basal cortisol concentrations during leuprolide treatment were not reliably lower than those in the early follicular phase or the mid- to late luteal phase/early pregnancy, even though CG concentrations were significantly lower during leuprolide treatment than during the luteal phase/early pregnancy. Furthermore, although leuprolide markedly suppressed both basal and GnRH-stimulated CG concentrations in ovariectomized animals, it did not alter either basal or ACTH-stimulated cortisol concentrations in these females. These results are consistent with findings by Kendrick and Dixson (1985a,b) that plasma cortisol concentrations of ovariectomized, estrogen-treated marmosets were unchanged at 2 and 24 h after GnRH injection, which stimulates release of CG in these monkeys (Abbott et al., 1988; Hodges, 1979). In female marmosets, therefore, CG appears to increase cortisol secretion only during the periovulatory phase of the ovarian cycle.

The mechanism(s) by which CG elevates cortisol during the periovulatory phase is unclear. One possibility is that CG stimulates cortisol secretion indirectly, through actions on other hormones. An obvious candidate would be estrogen, which increases glucocorticoid concentrations in several primate species as well as in rodents (reviewed by Kime et al., 1980; Young, 1998; Wilson et al., 2005). In a recent study, however, we found that long-term estradiol treatment did not appreciably elevate basal or ACTH-stimulated cortisol concentrations in either ovariectomized or anovulatory, subordinate female marmosets (Saltzman et al., 2006). In the present study, moreover, elevated urinary estrogen concentrations in intact females during the luteal phase/early pregnancy, as compared to concentrations in the follicular phase, the leuprolide condition, and ovariectomized females, were not associated with elevated plasma cortisol concentrations. Thus, estrogen does not appear to be an important regulator of cortisol in this species.

Progesterone also seems unlikely to mediate the effects of CG on cortisol, as it has not generally been found to stimulate glucocorticoid secretion in other species (Kime et al., 1980). Moreover, progesterone does not rise appreciably in marmosets until several days after ovulation, whereas cortisol begins to rise several days prior to ovulation (Harlow et al., 1983; Saltzman et al., 1998). Circulating testosterone concentrations, in contrast, are elevated during the late follicular, periovulatory, and early luteal phases in female marmosets (Kendrick and Dixson, 1983), similar to the observed changes in cortisol (Saltzman et al., 1998; this study). Testosterone concentrations also decline after ovariectomy in marmosets (cf. Kendrick and Dixson, 1985a,b). It is unclear, however, what role testosterone plays in regulating cortisol release from the primate adrenal cortex (e.g., Azziz et al., 1991; Hines et al., 2001).

Another possibility is that CG directly stimulates cortisol secretion from the adrenal cortex, but only under hormonal conditions occurring during the periovulatory period (e.g., rising estrogen and low progesterone concentrations). LH/CG has been shown to directly stimulate adrenal glucocorticoid production in several species, at least under pharmacological conditions. For example, LH/CG can stimulate glucocorticoid release from rat, opossum, and guinea-pig adrenal tissue in vitro (O'Connell et al., 1994; Vinson and Renfree, 1975; Vinson et al., 1976). In transgenic mice that express blh β-CTP (a chimeric protein derived from the β-subunits of LH and CG) and hypersecrete LH, CG elevates adrenal release of corticosterone, but only in ovary-intact females (Kero et al., 2000). LH has also been implicated in causing hypertrophy of cellular organelles in the zona fasciculata and zona reticularis of the adrenal cortex, and in increasing adrenal steroidogenic capacity in rats (Chung, 1978). In humans, LH/CG receptor transcripts and receptor protein have been identified in the zona fasciculata (Pabon et al., 1996), and LH/CG-responsive, cortisol-secreting adrenal tumors have been described in adult patients with Cushing's syndrome (Kero et al., 2000; Lacroix et al., 2001).

Nonetheless, it is unclear whether LH/CG plays a physiological role in stimulating glucocorticoid secretion in healthy adults of any species. In one of the few studies to address this question, Piltonen et al. (2002) characterized acute cortisol responses to exogenous CG in pre-menopausal women before and during chronic treatment with a GnRH agonist, and in oophorectomized, postmenopausal women during and after estrogen replacement therapy.

Circulating cortisol concentrations were not altered by either chronic suppression of LH or acute administration of CG. Thus, Piltonen et al. (2002) concluded that LH does not play a major role in regulating cortisol secretion in endocrinologically healthy women, similar to our own conclusions in female marmosets.

In summary, the results of this study indicate that hypophyseal CG may play only a limited, possibly indirect role in stimulating cortisol secretion in female marmosets undergoing ovulatory cycles, and may not stimulate cortisol release in ovariectomized females. Pharmacological suppression of CG in cycling and ovariectomized female marmosets may differ considerably from socially induced suppression of CG in subordinate females; nonetheless, these results suggest that CG suppression in anovulatory subordinates is unlikely to mediate socially induced reductions in basal cortisol concentrations. In conjunction with similar results from women (Piltonen et al., 2002), our findings suggest that although the LH/CG receptor may be expressed in the primate adrenal cortex (Pabon et al., 1996), LH/CG may not be an important regulator of cortisol secretion under physiological conditions in adult female primates.

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