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Tamoxifen Is an Estrogen Antagonist on Gonadotropin Secretion and Responsiveness of the Hypothalamic–Pituitary–Adrenal Axis in Female Monkeys

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The selective estrogen receptor modulator, tamoxifen, effectively slows the progression of estrogen-positive breast cancer and reduces the possibility of this cancer developing in women at high risk. Despite the widespread acceptance of tamoxifen as a therapeutic agent for this disease, its effects on other estrogen-dependent pathways, particularly on neural circuits regulating brain function and peripheral hormone secretion, are poorly understood. The present study, using previously ovariectomized rhesus monkeys, examined the effects of tamoxifen, in both the presence and absence of estradiol replacement, on the reproductive and hypo-thalamic–pituitary–adrenal (HPA) axes. In Experiment 1, monkeys randomly assigned to three groups (*n* = 8 each) were treated with placebo and either two doses of estradiol, two doses of tamoxifen alone, or two doses of tamoxifen plus high-dose estradiol to assess the effects on negative feedback suppression of luteinizing hormone (LH). Both doses of tamoxifen effectively antag-onized the negative feedback efficacy of estradiol on LH secretion. In contrast, neither the low- or high-dose tamoxifen alone had any effect on LH secretion, as con-centrations during tamoxifen treatments were indistinguishable from those during placebo. In Experiment 2, females were randomly assigned to one of four treatment groups (placebo, *n* = 6; estradiol, *n* = 5; tamoxifen only, *n* = 5; or tamoxifen plus estradiol, *n* = 6) to assess the effects on glucocorticoid negative feedback and pituitary and adrenal responsiveness to exogenous corticotropin-releasing hormone (CRH). Tamoxifen also antagonized the facilitating effects of estradiol on basal and CRH-induced ACTH and cortisol secretion. However, this antagonism produced basal and CRH-stimulated cortisol and ACTH concentrations that were lower than placebo-treated females. Interestingly, tamoxifen in the absence of estradiol produced a similar diminution in ACTH and cortisol response. These data suggest that, in the presence of estradiol, tamoxifen not only antagonized estrogenic facilitation of HPA responsivity but also actually attenuated the response compared with the placebo-treatment condition. Taken together, these data indicate that tamoxifen acts as an estrogen antagonist on the neural circuits controlling the neuroendocrine regulation of the hypothalamic–pituitary–ovarian and adrenal axes in ovariectomized macaque females.

Key Words: Estradiol; tamoxifen; SERM; LH; cortisol; ACTH; dexamethasone; corticotropin-releasing hormone.

Introduction

Tamoxifen is a mixed estrogen agonist—antagonist in the periphery that, through its antagonistic action, effectively slows the progression of estrogen-positive breast cancer and reduces the possibility of this cancer developing in women at high risk *(1,2)*. Despite the widespread acceptance of this selective estrogen receptor modulator (SERM) as a therapeutic agent for the treatment of breast cancer, its effects on other estrogen-dependent pathways, particularly on neural circuits regulating brain function and peripheral hormone secretion, are poorly understood. A better understanding of these nontargeted effects of tamoxifen could not only lead to its use as a tool to elucidate the importance of estradiol in neuroendocrine control of homeostatic systems, but also illustrate how these may impact tamoxifen's therapeutic efficacy.

An appreciation of the specific effect of tamoxifen on the hypothalamic–pituitary regulation of ovarian (HPO) function is critically important for women taking tamoxifen for breast cancer, as factors that perturb estradiol's negative feedback inhibition of gonadotropin secretion *(3)* would alter ovarian estrogen release and bioavailability. This is not only important for premenopausal women whose HPO

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Table 1
Mean ± SEM Serum Concentrations of Estradiol and Tamoxifen for ERT, TAM, and TME Groups During Each Week of Placebo, Low-, and High-Dose Treatment Condition for Experiment 1

		Treatment Condition					
		Estradiol (pg/mL)			Tamoxifen (ng/mL)		
Group	Treatment (d)	Placebo	Low	High	Placebo	Low	High
ERT (n = 8)	3	<5	158 ± 20	214 ± 15	n/a	n/a	n/a
	10	5.9 ± 0.9	73 ± 13	90 ± 9	n/a	n/a	n/a
TAM (n = 8)	3	<5	<5	<5	0	0.84 ± 0.24	2.02 ± 0.66
	10	7.1 ± 2.0	<5	<5	0	0.62 ± 0.11	0.70 ± 0.10
TME (n = 8)	3	7.1 ± 2.1	133 ± 19	188 ± 37	0	0.55 ± 0.14	1.00 ± 0.23
	10	<5	47 ± 12	53 ± 15	0	0.38 ± 0.09	0.75 ± 0.13

axis is intact but also for postmenopausal women who, despite ovarian failure *(4)*, still secrete estrogens, albeit in smaller amounts *(5,6)*, as well as testosterone that is converted to estradiol in target tissues *(7)*. Analysis of gonadotropin secretion in premenopausal women suggests that tamoxifen acts as an estrogen antagonist, increasing serum gonadotropin concentrations and, consequently, serum levels of estradiol *(8–10)*. This antagonistic effect of tamoxifen has lead to its use in ovulation induction in infertile women *(11,12)* and has prompted the recommendation that tamoxifen be combined with a GnRH analog or an aromatase inhibitor in the treatment of breast cancer in order to reduce circulating estrogen concentrations *(13,14)*.

In contrast to these data from premenopausal women, tamoxifen reduces proestrus concentrations of LH in rats suggesting it may facilitate estradiol negative feedback inhibition *(15,16)*, reduce basal and GnRH-stimulated LH release, and, like estradiol, increases pituitary sensitivity to GnRH *(16)*. However, despite this effect on basal LH secretion, tamoxifen blocks the preovulatory LH surge *(15,17)*. The agonist-like action of tamoxifen is also seen in postmenopausal women as gonadotropin secretion is consistently lower in women on tamoxifen therapy compared to nontreated conditions *(8,18–27)*. The discrepancy between the action of tamoxifen in pre- and postmenopausal women is unresolved.

In addition to its well-accepted effects on the reproductive axis, estradiol may be important in regulating hypothalamic–pituitary–adrenal (HPA) activity, both under basal conditions and in response to a stressor *(28,29)*. Estradiol increases hypothalamic expression *(30,31)* and content *(32)* of corticotropin-releasing hormone (CRH) in primates, decreases transcription of mineralocorticoid and glucocorticoid receptors in hypothalamic and hippocampal neurons in rodents *(28,33)*, and enhances cortisol and ACTH secretion in a number of species *(33–35)*, including humans *(36, 37)*. Furthermore, the response in ACTH but not cortisol to exogenous CRH is attenuated by ovariectomy in premenopausal women *(38)*. However, other studies in rodents suggest that estradiol decreases hypothalamic CRH gene expres-sion *(28,39,40)* and HPA activity in response to stress *(41)*. The effects of estradiol on arginine vasopressin (AVP), the neuropeptide that synergizes with CRH to regulate pituitary release of ACTH, are cell dependent *(30,42, 43)* but concentrations of AVP are increased by estradiol in portal circulation of sheep *(44)*. Finally, the few studies that have examined the effect of tamoxifen on HPA activity suggest that this SERM increases hypothalamic content of CRH and AVP in sheep *(44)* and increases basal levels of cortisol in women *(45,46)* but blunts the response in ACTH and cortisol to an acute behavioral stressor *(46)*. In contrast, basal levels of corticosterone and ACTH are unaffected by tamoxifen treatment in ovarian intact rats, whereas the ACTH but not the corticosterone response to restraint stress is enhanced by tamoxifen *(41)*. Thus, it is not clear how estradiol or tamoxifen affect the HPA axis, even under basal conditions.

Using ovariectomized, adult rhesus monkeys, the present study examined the effects of tamoxifen, in both the presence and absence of estradiol replacement, on the reproductive and HPA axes. The studies were designed to determine if tamoxifen, in the absence of estradiol, acts as an agonist suppressing LH secretion and, in the presence of estradiol, as an antagonist, blocking the negative feedback inhibition by estradiol. In addition, the effects of estradiol were compared to those of tamoxifen and tamoxifen plus estradiol on the response in cortisol and ACTH to glucocorticoid negative feedback and responsiveness to CRH.

Results

Experiment 1: Role of Tamoxifen in Negative Feedback Suppression of LH Secretion

Table 1 shows that the estradiol and tamoxifen treatments elevated circulating concentrations as expected. Serum estradiol was below or at the sensitivity of the assay during the placebo treatments for all groups and the remainder of the treatments for the group receiving only tamoxifen. For the ERT group, serum estradiol was significantly higher

during high compared to low dose ($F_{1,7} = 12.68$, $p = 0.01$) and levels declined by d 10 compared to d 3 ($F_{1,7} = 135.52$, $p < 0.001$). Because high-dose estradiol was used during the low and high tamoxifen dose conditions for the TME group, it is not surprising serum estradiol did not vary by dose for this group ($F_{1,7} = 1.58$, $p = 0.34$). Like the ERT group, serum estradiol did decrease significantly from d 3 to d 10 ($F_{1,7} = 43.12$, $p < 0.001$). Serum levels of tamoxifen were higher during high- compared to low-dose treatment for both the TAM and TME groups ($F_{1,14} = 13.21$, $p = 0.003$) but, unlike estradiol, serum levels did not decline on d 10 compared to d 3 ($F_{1,14} = 2.93$, $p = 0.11$). Finally, serum levels of tamoxifen were similar between TAM amd TME females across the two doses ($F_{1,14} = 3.25$, $p = 0.93$).

As illustrated in Fig. 1, tamoxifen had no effect on bioLH in the absence of estradiol but significantly blunted estradiol negative feedback in its presence ($F_{4,42} = 6.53$, $p < 0.001$). Since there was no difference between the low and high dose of each of the treatments ($F_{2,21} = 0.10$, $p = 0.90$) data were combined for illustration. During the placebo, serum bioLH was indistinguishable among the three groups. All groups responded to the acute administration of the estradiol bolus during the placebo treatments, with the percentage decrease from time 0 to 6 h similar for ERT (64 ± 10%), TAM (63 ± 4%), and TME groups (65 ± 6%). In addition, serum LH returned to preinjection levels by 12 h for all groups and remained elevated through 96 h, or d 14 of the placebo treatment phase.

For the ERT group, administration of either dose of estradiol significantly inhibited LH secretion compared to the placebo condition ($F_{2,14} = 19.68$, $p < 0.001$). In contrast, serum LH concentrations for the TAM group were not different between the placebo condition and either the low- or high-dose tamoxifen condition ($F_{2,14} = 1.82$, $p = 0.19$). On the other hand, the combination of either the low- or high-dose tamoxifen with high-dose estradiol significantly suppressed serum LH compared with the placebo condition in the TME group ($F_{2,14} = 17.62$, $p < 0.003$).

In terms of between-treatment-group comparisons, serum LH was significantly higher in TAM females compared to both TME and ERT females at both the low and high doses (Fig. 1). The concentration of LH at 6 h following the acute administration of estradiol was similar between ERT and TME females but significantly lower than that observed for TAM females. However, given the preinjection LH concentrations (time 0), the percentage change was greater in TME (68 ± 3%) compared with TAM (57 ± 7%) and both were greater than the ERT females (22 ± 5%; $F_{2,21} = 20.91$). These group differences in response to acute estradiol were similar at both the low and high treatment doses.

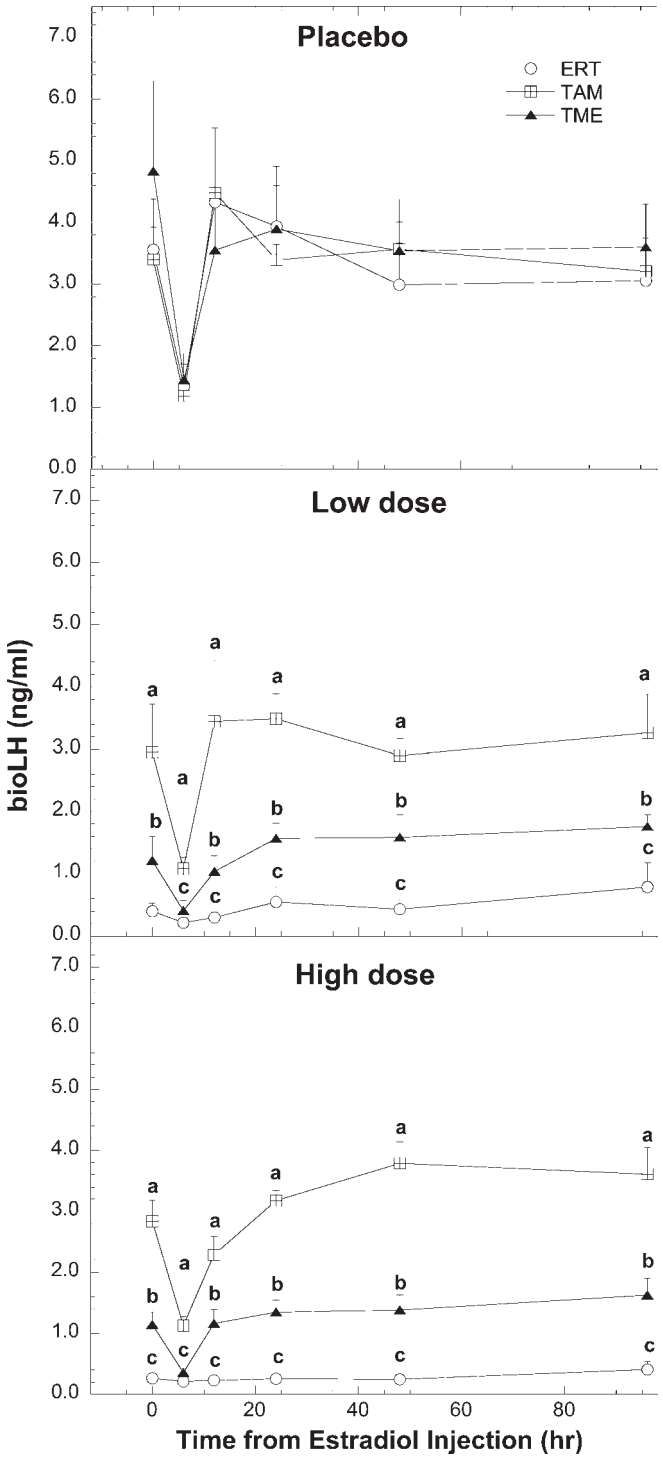

Fig. 1. Mean ± SEM serum concentrations of bioactive LH during wk 2 of placebo (upper panel), low- (middle panel), and high-dose replacement (lower panel) for females receiving estradiol (ERT), tamoxifen (TAM), and tamoxifen plus estradiol (TME) from Experiment 1. Different letters among the three treatment groups at specific time points indicate groups are significantly different ($p \leq 0.05$).

Experiment 2: Role of Tamoxifen and Estradiol on HPA Activity

Table 2 lists the serum estradiol and tamoxifen concentrations for each of the groups from samples collected on d 3 of both the low- and high-dose treatment condition. For those females receiving estradiol (ERT and TME), concentrations were significantly lower during low- compared to

Table 2
Mean ± SEM Serum Concentrations of Estradiol and Tamoxifen for PLC, ERT, TAM, and TME Groups on d 3 of Both the Low- and High-Dose Treatment Conditions for Experiment 2

Treatment Group	Estradiol (pg/mL)		Tamoxifen (ng/mL)	
	Low	High	Low	High
PLC (n = 6)	<5	<5	n/a	n/a
ERT (n = 5)	113 ± 22	188 ± 27	n/a	n/a
TAM (n = 5)	<5	<5	0.40 ± 0.03	0.83 ± 0.08
TME (n = 6)	151 ± 30	210 ± 30	0.33 ± 0.13	0.89 ± 0.25

the high-dose condition ($F_{1, 9} = 5.20$, $p = 0.03$). This pattern was similar between the ERT and TME females ($F_{1, 9} = 1.61$, $p = 0.23$). Serum estradiol was at or near the sensitivity of the assay in the PLC and TAM groups. For the TAM and TME groups, serum tamoxifen also varied significantly as a function of dose ($F_{1, 9} = 15.28$, $p = 0.004$), a pattern which was similar between the two groups ($F_{1, 9} = 0.10$, $p = 0.76$).

Glucocorticoid negative feedback, as assessed by the dexamethasone suppression test, on ACTH and subsequent cortisol secretion was differentially affected by estradiol and tamoxifen. Plasma levels of ACTH varied significantly by treatment both prior to and following dexamethasone (Fig. 2A; $F_{3, 18} = 4.26$, $p = 0.02$). Because the effects of the treatments did not vary significantly by dose ($F_{3, 18} = 1.20$, $p = 0.38$), data were collapsed across doses for analysis. Plasma ACTH was significantly lower in PLC compared to ERT females at 0900, some 12 h prior to DEX, but concentrations were not significantly different thereafter. In contrast, plasma ACTH was significantly lower in both TAM and TME compared to the ERT females except at +15 h from DEX. Importantly, ACTH concentrations escaped from glucocorticoid negative feedback sooner in ERT compared with TAM and TME females, as levels were again significantly higher in the ERT group by +19 h from DEX. With the exception of the pre-DEX samples, plasma ACTH was not different in PLC compared with TAM and TME females. Thus, ACTH was suppressed more by DEX in TAM and TME compared with ERT females, with the PLC group statistically intermediate.

Plasma cortisol prior to but not following DEX varied significantly by treatment (Fig. 2B; $F_{12, 72} = 3.14$, $p = 0.001$). The high-dose treatments exacerbated the pre-DEX differences observed during the low dose ($F_{3, 18} = 3.63$, $p = 0.03$). Data were collapsed across doses for illustration. Plasma cortisol prior to DEX was significantly higher in ERT compared with the PLC, TAM, and TME groups, which were not significantly different from one another. Plasma cortisol was suppressed similarly in all groups in response to DEX, as levels were indistinguishable amongst the groups at +10, 15, and 19 h post-DEX.

The response in ACTH to CRH was also significantly affected by treatment (Fig. 3A; $F_{3, 18} = 3.48$, $p = 0.04$). Because the effects of the treatments did not vary significantly by dose ($F_{3, 18} = 1.62$, $p = 0.22$), data were collapsed across doses for analysis. At time 0 (or +19 h following DEX), plasma ACTH was higher but not significantly in ERT compared with PLC females. However, ACTH levels were significantly higher in ERT compared with PLC females at +30 and +120 min following CRH. Although ACTH levels were similar at time 0 in PLC compared with both TAM and TME females, plasma concentrations were significantly higher at every time point following CRH in PLC compared to either tamoxifen-treated group. Importantly, plasma ACTH was significantly higher prior to and following CRH in ERT compared with both TAM and TME females. The tamoxifen-treated females did not differ from one another. Both the PLC and ERT females responded with a significant increase in ACTH within 60 min of the CRH injection, whereas there was no significant increase in ACTH following CRH in either the TAM or TME groups. Indeed, the area under the ACTH response curve was significantly higher in PLC and ERT compared to both TAM and TME females (Fig. 4; $F_{3, 18} = 3.82$, $p = 0.03$).

Plasma concentrations of cortisol following CRH administration were also significantly affected by treatment in a pattern a similar to that of ACTH (Fig. 3B; $F_{9, 54} = 2.78$, $p = 0.03$). Again no dose effect was observed ($F_{3, 18} = 0.34$, $p = 0.79$), so data were combined for illustration. Groups were all similar at time 0 (or +19 h following DEX). Following CRH, plasma cortisol was significantly higher in ERT compared with PLC females at +60 but not 30 and 120 min. Plasma cortisol was significantly lower in TAM females compared to subjects in both PLC and ERT groups at every time point following CRH. Females in the TME group had similar levels of cortisol at +30 min compared to both PLC and ERT subjects, but concentrations were significantly lower at the subsequent time points in the tamoxifen plus estradiol treated group. Finally, plasma cortisol was statistically similar in TAM and TME females following CRH administration. These treatment effects were also reflected in the area under the cortisol response curve to CRH (Fig. 4), with PLC and ERT being significantly greater than both tamoxifen-treated groups ($F_{3, 18} = 4.02$, $p = 0.03$).

Despite these treatment differences in response to dexamethasone and CRH, no differences were observed in CSF concentrations of CRH at either dose tested. CRH concentrations (pg/mL) for PLC (10.0 ± 2.7), ERT (11.4 ± 5.0), TAM (10.1 ± 2.7), and TME (10.2 ± 3.6) were not significantly different from one another ($F_{3, 18} = 0.03$, $p = 0.92$).

Discussion

The results of the present study, using an ovariectomized rhesus monkey model, indicate that the SERM tamoxifen antagonizes the negative feedback effects of estradiol on

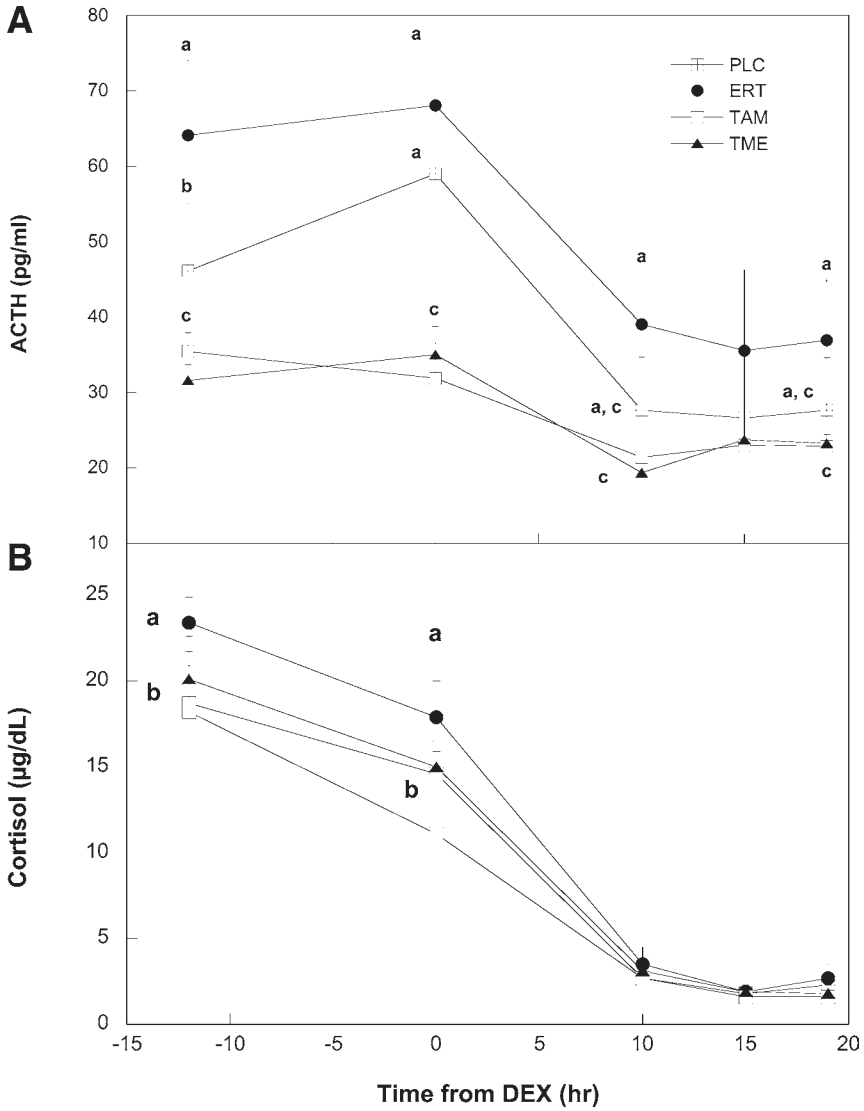

Fig. 2. Mean ± SEM of ACTH (**A**) and cortisol (**B**) prior to and following dexamethasone administration on d 6 and 7 for placebo (PLC), estradiol (ERT), tamoxifen (TAM), and tamoxifen plus estradiol (TME) treatment groups from Experiment 2. Different letters among the four treatment groups at specific time points indicate groups are significantly different ($p \leq 0.05$).

LH secretion and has no agonistic effects on LH in the absence of estradiol. In addition, tamoxifen also antagonized the facilitating effects of estradiol on basal and CRH-induced ACTH and cortisol secretion. However, this antagonism produced basal and CRH-stimulated cortisol and ACTH concentrations that were lower than placebo-treated females. Interestingly, tamoxifen in the absence of estradiol produced a similar diminution in ACTH and cortisol response. These data suggest that, in the presence of estradiol, tamoxifen not only antagonized estrogenic facilitation of HPA responsivity but actually attenuated the response compared with the placebo-treatment condition.

The data from the present study support previous reports from premenopausal women that tamoxifen antagonizes the effects of estradiol on the negative feedback regulation of LH secretion *(8–10)*. This contrasts with data from rodent models *(15,16)* and postmenopausal women *(18–27)* that suggest tamoxifen is an estrogen agonist in the regulation of LH secretion. It is difficult to reconcile these differences. The monkeys used in this study were "surgically postmenopausal" and differ from postmenopausal women not only in terms of reproductive age but also the presence of a postmenopausal ovary. Evidence from women indicate that gonadotropin concentrations decline during the menopause with advancing chronological age *(54,55)*, likely due to a decrease in pulsatile GnRH *(56)*. However, other data suggest there is no ovarian-independent decline in gonadotropin secretion in aging women *(57)*. Furthermore, older postmenopausal women are thought to be hypersensitive to the negative feedback inhibition of LH compared to younger postmenopausal women *(58)*, suggesting that weak estrogen agonists may effectively suppress LH secretion. How-

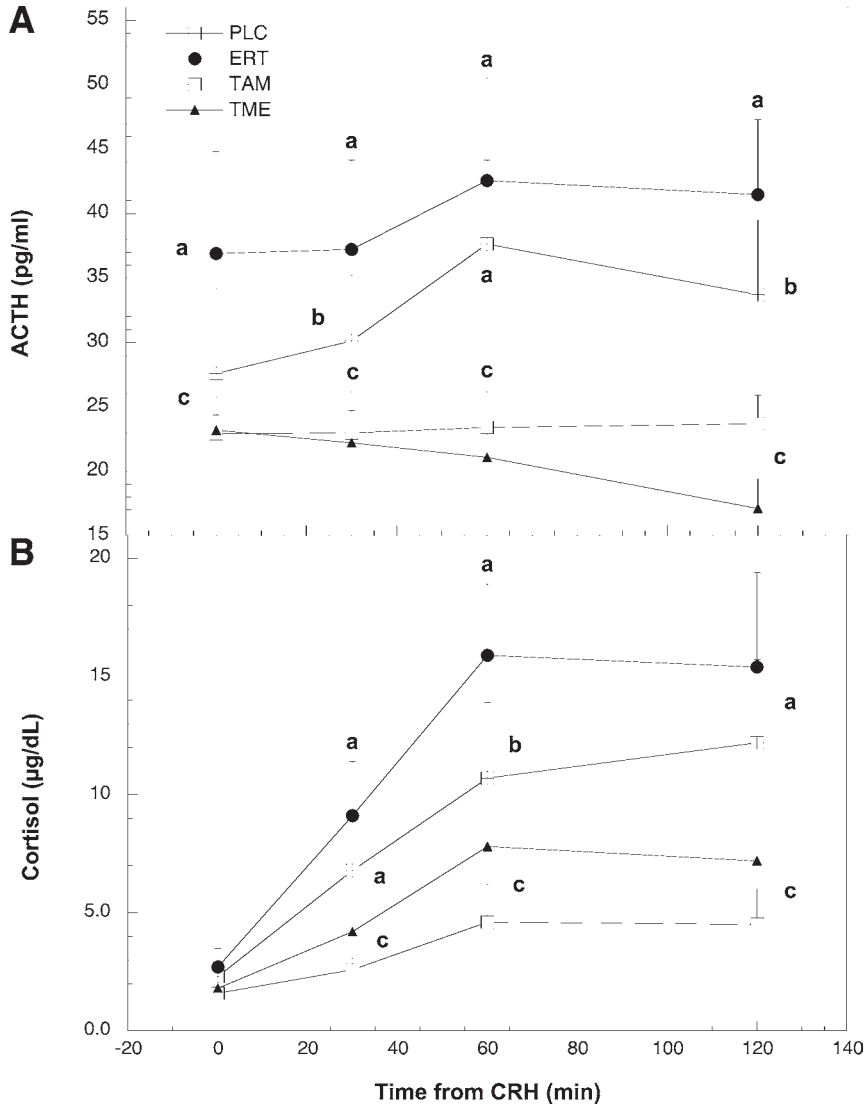

Fig. 3. Mean ± SEM of ACTH (**A**) and cortisol (**B**) prior to and following CRH administration on d 6 and 7 for placebo (PLC), estradiol (ERT), tamoxifen (TAM), and tamoxifen plus estradiol (TME) treatment groups from Experiment 2. Different letters among the four treatment groups at specific time points indicate groups are significantly different ($p \leq 0.05$).

ever, the present study found no evidence of even weak agonist activity by tamoxifen at either the low or high dose on LH secretion. Furthermore, we observed both the low and high doses of tamoxifen equally attenuated estradiol negative feedback, producing serum concentrations of LH intermediate between tamoxifen-only and the estradiol-treated females. It is not known how chronic tamoxifen treatment would affect ovarian cyclicity in nonhuman primates, but it would seem likely that such treatment would produce a persistent elevation in circulating estradiol. Because estradiol negative feedback likely occurs at both hypothalamic *(59–62)* and pituitary sites *(63–65)*, tamoxifen antagonism could be operating at both. Given that fertility is compromised in estrogen receptor (ER) alpha but not ERβ knockout mice *(66)*, it would seem that tamoxifen is disrupting estradiol-dependent transcriptional activity via ER alpha. This hypothesis is further supported by the absence of negative feedback inhibition on LH secretion in ERKO alpha but not β mice *(67,68)*.

Assessment of HPA responsiveness to pharmacological challenge indicated that estradiol replacement increases basal circulating concentrations of both cortisol and ACTH. Although glucocorticoid negative feedback was not different between estradiol and placebo-treated groups, it is possible that a lower dose of dexamethasone may have differentiated the effects of estradiol from those of placebo *(69)*. Significant differences in basal cortisol and ACTH would suggest pre-existing differences in feedback sensitivity. Furthermore, the response in both cortisol and ACTH to CRH was significantly higher in estradiol-replaced females. Al-

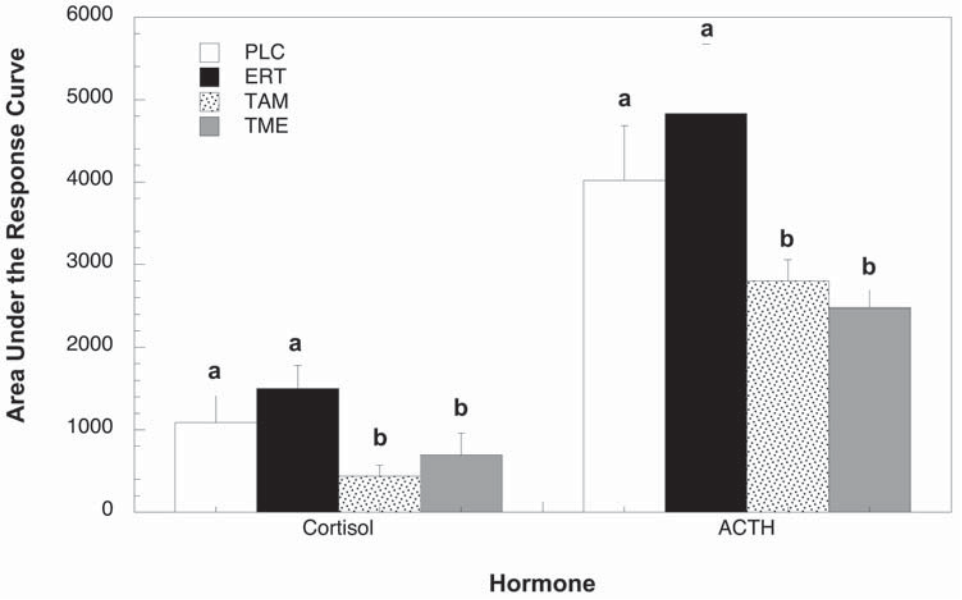

Fig. 4. Mean ± SEM area under the response curve in cortisol and ACTH following CRH administration for placebo (PLC), estradiol (ERT), tamoxifen (TAM), and tamoxifen plus estradiol (TME) treatment groups from Experiment 2. Different letters among the four treatment groups for each hormone indicate groups are significantly different ($p \leq 0.05$).

though no differences in CSF concentrations of CRH were observed between placebo- and estradiol-treated females, these data support the hypothesis that estradiol enhances the activity of HPA axis in female primates and are consistent with data from some *(28–38)* but not all experimental contexts *(28,39–41)*. These activational effects of estradiol may account for the greater reactivity to stress in female compared to males *(70,71)*, although context and the type of stressor is also an important determinant of gender differences in stress responsivity *(72,73)*. Because HPA dysregulation, including elevated cortisol secondary to excess secretion of CRH *(74)* and AVP *(75)*, is associated with depression and anxiety states in women, the possibility exists that the presence or absence of estradiol modulates the ability to terminate the neuroendocrine response in individuals chronically exposed to stressful life events *(76)*.

The effects of tamoxifen on HPA responsiveness were opposite to those of estradiol. Not only did tamoxifen antagonize estradiol action, treatment with tamoxifen alone had opposite effects on HPA regulation relative to the placebo condition. Basal levels of cortisol were similar between placebo- and both tamoxifen-treated groups yet lower than those observed in estradiol replaced females. In contrast, ACTH concentrations were lower in both tamoxifen and tamoxifen plus estradiol groups compared with placebo and estradiol-replaced females. Although the response to dexamethasone was indistinguishable, perhaps given the dose used, the increase in both cortisol and ACTH to CRH was significantly attenuated by the presence of tamoxifen. Previous studies on the effects of tamoxifen have not looked specifically at both agonistic and antagonistic properties but have nonetheless produced equivocal results. Some reports suggest tamoxifen enhances HPA activity *(41,44–46)* while another indicates that, although basal levels of cortisol are elevated in women receiving tamoxifen for breast cancer compared to disease-free controls, the ACTH response to a behavioral stressor in blunted and the decline in cortisol more rapid in the tamoxifen-treated groups *(46)*.

The blunted pituitary, and subsequent adrenal response to CRH, implies a change in sensitivity to CRH. Pituitary corticotropes do express ERβ *(77)*, suggesting that an agonistic effect by estradiol and antagonistic effect by tamoxifen on proopioidmelanocortin (POMC) gene expression and ACTH synthesis could explain group differences *(78)*. On the other hand, CRH down-regulates whereas AVP stimulates the expression of CRH type 1 receptors in the pituitary *(79)*, underscoring the importance of AVP in maintaining pituitary sensitivity to CRH. Because CSF concentrations of CRH did not differ significantly between groups in the present study, it is possible that the difference in the response to exogenous CRH between tamoxifen-treated females and those in the placebo and ERT groups could be explained by differences in AVP release. AVP synergizes with CRH to stimulate ACTH release from the pituitary *(49,80)*, and AVP levels are higher in rats showing a greater response in ACTH to CRH, an effect blocked by a AVP receptor antagonist *(81)*. Although AVP neurons in the rostrocaudal portion of the paraventricular nucleus of the hypothalamus (PVN) coexpress ERβ *(82,83)*, data are mixed on how estradiol regulates AVP synthesis: AVP gene expression or content in the PVN was decreased by estradiol in male mice *(84)*; increased in postpartum *(85)* and ovariectomized rats *(86)*

and adult female sheep *(44)*; and not affected in female monkeys *(30)*. In contrast, estradiol increases AVP expression in extrahypothalamic sites, including the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MA) *(42,43)*. ER alpha is co-localized with AVP cells in the BNST *(87)* and both ER subtypes are found throughout the BNST and MA *(88)*. Although AVP neurons from the parvocellular region of the PVN project to the median eminence to influence ACTH secretion *(89)*, it is clear that other structures, including the MA and BNST, participate in the integration of the stress response *(90–93)*. We were unable to measure CSF concentrations of AVP in the present study due to the availability of adequate volumes of CSF. Additional studies must determine how estradiol and SERMs like tamoxifen simultaneously influence CRH, AVP, and, consequently, pituitary–adrenal function.

The possibility that estradiol and tamoxifen have opposite effects on gene expression and subsequent HPA function relative to placebo condition is supported by observations in female rats that ethinylestradiol down-regulates and tamoxifen up-regulates ERβ expression in the PVN *(94)*. In addition, the phytoestrogen, genistein, has similar effects to tamoxifen on ERβ expression, which, again, are opposite to those of estradiol *(95)*. Finally, estradiol and tamoxifen, through an ERα-mediated mechanism, inhibit the expression of placental CRH in vitro compared with control conditions, while the pure estrogen receptor antagonist, ICI 182780, stimulates CRH synthesis *(96)*. Thus, it is possible that the opposing effects of estradiol and tamoxifen are due to their differential effects on gene expression in the pituitary corticotropes or neurons expressing AVP or CRH, perhaps how they interact with ERα and ERβ. Different ER subtypes may produce different transcriptional effects when bound to respective ligands. Compounds that are normally agonists or antagonists that bind to the estrogen response element site show similar activity through the ERα at AP1 sites on target genes. However, when acting through ERβ at AP1 sites, estradiol is an antagonist and tamoxifen and raloxifene are agonists *(97)*. The binding of a specific ligand–ER subtype with the DNA response element on the target genes produces a specific conformational change in the complex that determines which co-regulating proteins are recruited, either co-activators or co-repressors, and the availability of these co-regulator proteins, in turn, differ between cell types *(98)*. A recent report using a neuronal cell line suggests that AVP transcription could be increased by both estradiol and tamoxifen if cells express ERα, whereas estradiol may inhibit gene transcription if cell express ERβ *(99)*. However, it is difficult to extrapolate these data from in vitro systems that used cells transfected with ER subtypes, as it is not clear how co-regulator proteins in specific cells types in vivo would affect tamoxifen transcriptional effects through ERα or β.

Finally, any possible effects of estradiol and/or tamoxifen on the HPA axis could impact therapeutic efficacy and disease outcome in women being treated for breast cancer. The stress associated with the prognosis, treatment, and uncertainty of disease outcome activates the HPA axis *(100)*. Breast cancer survival is inversely related to nadir concentrations of the cortisol diurnal rhythm *(101)*. Furthermore, cortisol concentrations are significantly higher in breast cancer patients compared to disease-free control women *(46)*, and women at risk for breast cancer show a greater increase in cortisol following an acute psychological stressor *(102)*. Despite the emerging data suggesting dysregulation of the HPA alters immune response and therapeutic outcome *(103, 104)*, it is not known whether hypercortisolemia directly or indirectly affects disease progression, but it certainly could impact quality of life. Data from the present study suggest that tamoxifen may actually dampen HPA activity in response to life stressors, and thus contribute to therapeutic efficacy.

Methods

Subjects were previously ovariectomized rhesus monkeys who were housed in indoor–outdoor runs at the Yerkes National Primate Research Center's Field Station. Females had been ovariectomized from 4 mo to 18 yr and had not received any estrogen replacement for at least 4 mo prior to the start of the study. The average age was 12.1 ± 1.2 yr and none were beyond the age considered to be menopausal for rhesus monkeys *(47)*. Subjects were grouped socially with three to five females and one adult male per run. Animals were fed commercial monkey chow (Ralston-Purina Company, St. Louis, MO) *ad libitum* twice daily and received a daily supplement of fresh fruit and vegetables. The protocol was approved by the Emory University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for Care and Use of Laboratory Animals."

Experiment 1: Role of Tamoxifen in Negative Feedback Suppression of LH Secretion

Females ($n = 24$) were randomly assigned to one of three treatment groups: estradiol (ERT; $n = 8$), tamoxifen (TAM, $n = 8$), and tamoxifen plus estradiol (TME, $n = 8$). The effect of each treatment was assessed separately at three doses: placebo (no treatment), low dose, and high dose. To accomplish this, ERT was administered at 3 and 6 μg/kg/d, producing serum estradiol at mid and late follicular phase levels; TAM was administered at 240 and 480 μg/kg/d, equivalent to the range of doses given to women with breast cancer *(2)*; the combined TME treatment included low-dose TAM plus high-dose ERT and high-dose TAM with high-dose ERT. All treatments were administered subcutaneously by implanting sustained 21-d release pellets of the specific hormone and/or drug (Innovative Research of America, Sarasota, FL), between the scapulas while the animals were anes-

thetized. The study was conducted over a 9-wk period, with each treatment condition lasting 3 wk. The initial condition for all females was placebo followed by the other two doses. The order of low- and high-dose hormone and/or drug treatment was randomized. Samples for estradiol and tamoxifen analyses were collected on d 3 and 10 of the placebo, low-dose, and high-dose treatment conditions. On d 10 of each condition, each female was given an acute intramuscular injection of estradiol (8 μg/kg) and serum samples were obtained just prior to the injection and 6, 12, 24, 48, and 96 h thereafter for the analysis of LH. This design, using animals as their own placebo control, allowed us to assess how each treatment affected LH secretion and whether these augmented or blocked negative feedback suppression of LH in response to the acute administration of estradiol. The study was conducted between October and December 2001.

Experiment 2: Role of Estradiol and Tamoxifen on HPA Activity

The ovariectomized females used in Experiment 1 were randomly assigned to four treatment groups: placebo (PLC, $n = 6$) estradiol (ERT, $n = 5$), tamoxifen (TAM, $n = 5$), and tamoxifen plus estradiol (TME, $n = 6$). The effects of each hormone or drug were assessed at two doses, with doses and method of treatment identical to that used in Experiment 1. This study was initiated 6 mo following the completion of Experiment 1. Each treatment condition lasted 3 wk and was separated by a 2-wk wash out period from the assessments described below. Serum samples for estradiol and tamoxifen assay were obtained on d 3 of both the low- and high-dose treatment condition. On d 6 of each treatment, females received a combined dexamethasone (DEX) suppression test–CRH challenge *(48)*. Plasma samples were collected at 0900 and 2100. Following the sample at 2100, DEX was administered (0.50 mg/kg, im) and subsequent samples were collected at 10, 15, and 19 h. Following the 19 h sample, CRH (1.0 μg/kg, iv) was administered and subsequent samples were collected at 30, 60, and 120 min. This approach determined how each treatment affected glu-cocorticoid negative feedback and the pituitary and adrenal response to CRH. Once collected, all blood samples were immediately placed on wet ice until processing for plasma, after which they were stored at –20°C until assay for cortisol and ACTH. On d 13 of each treatment, cerebrospinal fluid (CSF), for the analysis of CRH, was obtained by lumbar puncture *(49)* while the female was anesthetized. All CSF samples were immediately placed on wet ice until storage at –80°C. The study was conducted between June and August 2002.

All subjects were habituated to conscious venipuncture as described previously *(50)*. Rhesus monkeys readily adapt to these procedures showing no adverse effects on behavior or fertility *(50,51)*. Assays for estradiol, cortisol, ACTH, and LH were performed in the Endocrine Core Laboratory at Yerkes. Serum estradiol was determined by a modification of a commercially available radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Prior to assay, samples (250 μL) were extracted twice with 5 mL of anesthesia grade ether. Following evaporation of the solvent, samples were reconstituted with 250 μL of zero calibrator and 100 μL aliquots were assayed in duplicate. The assay has a sensitivity of 5 pg/mL using 100 μL of extracted serum, with an intra- and interassay coefficient of variation (CV) of 5.8%, and 11.7%, respectively. Sample values of estradiol were corrected for extraction efficiency, which exceeded 95%. LH concentrations were determined using the mouse interstitial cell bioassay *(52)*. The standard for the assay was the recombinant monkey LH (purchased from the National Hormone and Pituitary Program, NIDDK). Using 5 μL of serum, the LH bioassay has a sensitivity of 0.20 ng/mL with an intra- and interassay CV of 10.31% and 12.00%, respectively. The LH potency in the standards, controls, and unknown samples was based on the production of testosterone in the incubates, determined using a commercially available radioimmunoassay (Diagnostic System laboratories, Webster, TX). Plasma cortisol was determined by radioimmunoassay of 25 μL duplicates using commercially available reagents (Diagnostic Systems Laboratory). The assay has a sensitivity of 0.02 μg/dL and an intra- and interassay CV of 3.1% and 7.6%, respectively. Plasma ACTH was determined by radioimmunoassay of 100 μL duplicates using commercially available reagents (DiaSorin, Stillwater, MN). The assay has a sensitivity of 4.5 pg/mL and an intra- and interassay CV of 7.1% and 12.4%, respectively. Assays of tamoxifen and CRH were performed by Dr. Robert Bonsall, Dept. of Psychiatry and Behavioral Sciences, Emory University School of Medicine. Tamoxifen concentrations in serum were determined by HPLC as described previously *(53)*. CRH was measured in CSF by radioimmunoassay using reagents from Peninsula Laboratories (San Carlos, CA; part number S-2021). Samples were kept frozen at –80°C until analyzed. Aliquots (50 μL) were pipetted in quadruplicate into 12 × 75 mm culture tubes, dried under vacuum, and reconstituted in 25 μL water. After each of the following reagent additions, sample tubes were briefly centrifuged to keep the contents at the bottom of the tubes. CRF antibody (25 μL) was added and the mixtures were incubated for 24 h at 2°C. Tracer (^{125}I-Tyr-hCRF, 25 μL, approx 3000 cpm) was added and the mixtures incubated for a further 16 h at 2°C. Goat anti-rabbit gamma globulin (25 μL) and normal rabbit serum (25 μL) were added to precipitate the primary antibody and form a pellet, and 1 mL ice cold 5% polyethylene glycol in water was added to accelerate the precipitation. The mixtures were then centrifuged (3000*g* for 15 min) and the supernatants were decanted. Radioactivity was counted in the pellets, and results were computed by a log-logit method using data from hCRF standards (0–32 pg per tube). The sensitivity of the assay (blank + 2 SD) was 0.33 pg and the mean coefficient of variation for samples exceeding 1 pg per tube was 12.3%.

Data were summarized as mean ± SEM and were analyzed by analysis of variance for repeated measures (SPSS, v. 11). Group differences at specific doses and/or times were evaluated with Fisher exact post hoc tests. For Experiment 1, treatment (ERT, TAM, or TME) was a nonrepeated main effect, while dose (placebo, low, high) and time of sample collection repeated main effects. For Experiment 2, treatment group (PLC, ERT, TAM, TME) was the nonrepeated main effect, while dose (low, high) and time of sample collection repeated main effects. Statistical tests having a $p \leq 0.05$ were considered significant.

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