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Research Article

Aromatase Inhibition Eliminates Sexual Receptivity Without Enhancing Weight Gain in Ovariectomized Marmoset Monkeys

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Abstract

Context: Ovarian estradiol supports female sexual behavior and metabolic function. While ovariectomy (OVX) in rodents abolishes sexual behavior and enables obesity, OVX in nonhuman primates decreases, but does not abolish, sexual behavior, and inconsistently alters weight gain.

Objective: We hypothesize that extra-ovarian estradiol provides key support for both functions, and to test this idea, we employed aromatase inhibition to eliminate extra-ovarian estradiol biosynthesis and diet-induced obesity to enhance weight gain.

Methods: Thirteen adult female marmosets were OVX and received (1) estradiol-containing capsules and daily oral treatments of vehicle (E2; n = 5); empty capsules and daily oral treatments of either (2) vehicle (VEH, 1 mL/kg, n = 4), or (3) letrozole (LET, 1 mg/kg, n = 4).

Results: After 7 months, we observed robust sexual receptivity in E2, intermediate frequencies in VEH, and virtually none in LET females ($P = .04$). By contrast, few rejections of male mounts were observed in E2, intermediate frequencies in VEH, and high frequencies in LET females ($P = .04$). Receptive head turns were consistently observed in E2, but not in VEH and LET females. LET females, alone, exhibited robust aggressive rejection of males. VEH and LET females demonstrated increased % body weight gain ($P = .01$). Relative estradiol levels in peripheral serum were E2 >>> VEH > LET, while those in hypothalamus ranked E2 = VEH > LET, confirming inhibition of local hypothalamic estradiol synthesis by letrozole.

Conclusion: Our findings provide the first evidence for extra-ovarian estradiol contributing to female sexual behavior in a nonhuman primate, and prompt speculation that extra-ovarian estradiol, and in particular neuroestrogens, may similarly regulate sexual motivation in other primates, including humans.

Key Words: estrogen depletion, nonhuman primate model, neuroestrogen, androgen excess, diet-induced obesity, bone density

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; BMC, bone mineral content; BMD, bone mineral density; CG, chorionic gonadotropic; DHEA, dehydroepiandrosterone; DIO, diet-induced obesity; DXA, dual-energy X-ray absorptiometry; E2, estradiol group; EIA, enzyme immunoassay; Erα, estrogen receptor alpha; FFM, fat-free mass; GnRH, gonadotropin-releasing hormone; KO, knockout; LC-MS/MS, liquid chromatography tandem mass spectrometry; LET, letrozole; LH, luteinizing hormone; NHP, nonhuman primate; OGTT, oral glucose tolerance test; OVX, ovariectomy; SERM selective estrogen receptor modifier; SHBG, sex hormone–binding globulin; SME, stalk-median eminence; VEH, vehicle group; 17-OHP$_4$, 17-hydroxyprogesterone.

Aromatase, a cytochrome P450 enzyme, encoded by the *CYP19A1* gene, converts testosterone to estradiol, and androstenedione to estrone. While the ovaries are a major source of estradiol, the same hormone is also produced at aromatase-expressing extra-ovarian sites, including liver, breast, skin, bone, pituitary gland, and various brain regions [1-4]. In particular, neural production of estradiol has been identified in birds [5-7], mice [8], rats [9-12], and in monkeys [13-16]. Brain aromatase is expressed at high levels in the medial basal hypothalamus, preoptic area, amygdala, and hippocampus, and has a higher affinity for androgen substrates than its ovarian counterpart [8, 17]. A role for hypothalamic aromatase in regulating gonadotropin-releasing hormone (GnRH) in female macaques was recently demonstrated to show that (1) estradiol is produced and released at detectable levels within the OVX monkey pituitary stalk-median eminence (SME), (2) estradiol synthesis and release depends upon aromatase activity in the SME, and (3) hypothalamic estradiol can rapidly stimulate GnRH release in the SME [14, 15, 18]. These studies, in addition to previous work in female marmosets [16, 19], provide evidence for extra-ovarian estradiol production in female marmosets with neural action.

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In many mammalian species, including rodents (rats, mice, guinea pigs), sheep, and nonhuman primates (NHPs), estradiol is a major regulator of female sexual behavior and cognition [16, 20-28], body weight regulation, and insulin–glucose homeodynamics [29-31], mediating most of its activity by binding to estrogen receptor alpha (ERα) [32-34]. Not surprisingly, therefore, many female neurological and behavioral symptoms, together with weight gain and metabolic dysfunction, originate from estradiol deprivation [29, 30, 35-39]. Estrogen deficiency is of particular clinical relevance for women in their postreproductive years (menopause) [40], as well as for 80% of girls and young women who survive cancer yet live with premature ovarian insufficiency (premature menopause) as a result of alkylating agent and/or radiation cancer therapy [41, 42]. Women with such estrogen deficiency experience compromised sexual health, impaired psychosocial wellbeing, cardiometabolic dysfunction, and obesity, as well as osteoporosis [43]. Hormonal therapies, including systemically administered estradiol, alleviate clinical conditions to varying degrees [31, 43]. Currently approved estrogen medications, however, increase the risk for harmful systemic side-effects [44, 45], including blood clots and cancer [46, 47]. Estrogen treatments will therefore never realize their full potential until, perhaps, their actions are limited to the brain. In this regard, however, the extent to which estradiol regulates neural control systems in women is largely unknown.

With regard to regulation of female sexual behavior, rodents and sheep have provided invaluable models in which to elucidate molecular mechanisms regulating neural regulatory centers. Expression of female rodent and sheep sexual receptivity, however, is entirely dependent on, and is strictly timed by, a pre-ovulatory surge of estradiol from the ovary and subsequent rapid rise in ovarian progesterone [48-50]. Furthermore, surgical ovariectomy (OVX) abolishes female sexual receptivity, an effect that is rescued by estradiol replacement [25, 51, 52]. Ovarian estradiol mediates female sexual receptivity and other components of female sexual behavior, such as proceptivity, largely through neurons in the ventromedial nucleus of the hypothalamus [25, 53] expressing ERα [54, 55].

Consistent with their rodent counterparts, NHP species such as the Great Apes [21-23], rhesus monkeys [56-59], and the model used in this study, marmoset monkeys [60], females exhibit estradiol-associated increases in sexual receptivity. Female NHP receptivity, nevertheless, is noticeably less restricted and is not strictly limited to peri-ovulatory rises in ovarian estradiol [61, 62]. In addition, captive populations of female NHPs [63], including chimpanzees [64] and rhesus monkeys [65], exhibit a naturally occurring postreproductive period analogous to that of menopause in women, when ovulatory ovarian cycles cease accompanied by dramatic declines in ovarian estradiol release. In contrast to rodents, captive female primate lifespans extend well beyond their reproductive years [66]. Despite minimal postmenopausal circulating levels of estradiol, however, there is conflicting evidence regarding the prevalence and degree of decline in libido in postmenopausal women [67]. Seemingly, there is an unidentified, ovarian estradiol–independent mechanism capable of supporting female sexual behavior in women and NHPs. Consistent with this notion, we have shown that extra-ovarian estradiol in female marmoset monkeys functionally maintains a component of negative feedback regulation on gonadotropin secretion in the absence of ovaries [19]. This raises the possibility that extra-ovarian estradiol in female marmosets may also support sexual receptivity, a notion consistent with OVX diminishing, but not eliminating, female sexual receptivity in marmosets [68, 69], a behavioral pattern more reminiscent of those observed in women than in female rodents.

In parallel consideration, estradiol-regulated metabolic control mechanisms are pronounced in female rodents, exemplified by OVX-mediated estradiol depletion reliably increasing body weight and visceral adiposity, reducing physical activity and energy expenditure, as well as diminishing glucose tolerance and insulin sensitivity [70-74]. Consistent with these findings, aromatase knockout female mice develop obesity with insulin resistance in the absence of endogenous estradiol synthesis [75]. All these effects are prevented or reversed by physiological estradiol replacement [76, 77]. Furthermore, while intact female mice are resistant to high-fat diet-induced obesity (DIO) and its associated sequelae, OVX-mediated estradiol depletion abolishes this protection [78, 79]. Virtually all of these estradiol activities are mediated by ERα, as ERα knockout adult mice (ERαKO) bearing null mutations of ESR1 gene exhibit body weight, adiposity, and energy metabolism phenotypes that largely mimic those observed in long-term OVX adult mice [32-34]. In addition, loss of estradiol bioactivity in bone, particularly that mediated by ERα, reliably results in skeletal bone loss [80].

In menopausal women, declining estradiol concentrations and progressive testosterone predominance are generally associated with increased abdominal fat mass and increased risk for impaired glucose metabolism [38, 81, 82]. Metabolic functions of estradiol in women have been difficult to define, however, partly due to logistical and ethical constraints in designing definitive experiments with rigorous control. OVX-mediated estradiol depletion had small effects on female rhesus macaque body weight in 1 study [83], and no effects on female body weight were observed in 2 studies of cynomolgus macaques [84, 85]. While a putative selective estrogen receptor modifier (SERM) can promote weight loss in OVX rhesus monkeys [86], estradiol replacement therapy has no effect on body weight in OVX cynomolgus macaques [85, 87]. Estradiol and SERM activity, however, both reliably maintain skeletal bone mass in female macaques [88-90].

To address the different contributions of ovarian and extra-ovarian estradiol in a female NHP model, we employ the aromatase inhibitor letrozole© to diminish estradiol production in OVX female marmosets and thereby enable an investigation into whether extra-ovarian estradiol contributes to female sexual receptivity and weight gain in addition to ovarian estradiol. We hypothesize that an extra-ovarian source of estradiol, likely the hypothalamus, will be diminished by aromatase inhibition and subsequently will abolish expression of female receptivity, and enhance weight gain and skeletal bone loss, in female marmoset monkeys compared with both estradiol-replaced females and those experiencing the loss of ovarian estradiol alone.

Materials and Methods

Animals and Estradiol Replacement

Thirteen adult female common marmosets from the Wisconsin National Primate Research Center colony were ovariectomized and randomly assigned to 1 of 3 treatment groups: systemic mid-cycle, peri-ovulatory estradiol replaced (E2; n = 5), systemic estradiol depleted, OVX plus

daily vehicle (VEH; n = 4), or extra-ovarian estradiol depletion, OVX plus daily letrozole administration (LET; n = 4). Treatment groups were balanced by age and body weight at the onset of the study (Table 1). Systemic (mid-cycle, peri-ovulatory) estradiol replacement was achieved through subcutaneous estradiol-filled capsules that maintained a systemic level of estradiol (Table 2) mimicking mid-cycle, peri-ovulatory circulating estradiol levels. The latter were sufficient to maintain negative feedback regulation of circulating pituitary gonadotropin levels within the ovary intact female range (Table 2) [19]. To maintain constant estradiol levels, capsules were replaced every 3 months throughout the study [91]. As an additional biomarker of effective systemic estradiol replacement, uterine dimensions were obtained by transabdominal ultrasonography prior to, and 5 months following, OVX. Using the scanner's calibrated, digitized calipers, uterine width, dorso-ventral uterine depth, and endometrial thickness were measured from transverse views (the last 2 at 5 and 7-8 months only), while fundus–cervix length (7-8 months only) was measured from sagittal views (Table 2). VEH females received a daily oral 200 µL of 1 mL/kg Ensure© as vehicle control, while LET females were given daily oral 1 mg/kg of letrozole dissolved in 200 µL of vehicle, as previously determined [19], and females in both groups received empty capsules when estradiol females received estradiol-filled capsules and replacements.

All females lived with a well-established male cagemate in 0.60 m × 0.91 m × 1.83 m enclosures and were maintained with 12-hour lighting (06:00 hours to 18:00 hours), ambient temperature of ~27°C and humidity of ~50%. This study was reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin, Madison and was performed consistent with the USDA Animal Welfare Act and regulations and the Guide for the Care and Use of Laboratory Animals. The animal care and use program at the University of Wisconsin maintains a Public Health Services Assurance, and is fully accredited by AAALAC.

Ovariectomy

Following baseline assessments, bilateral OVX was performed. Cloprostenol (Estrumate®, 0.75-1.50 µg intramuscular injection for 2 successive days approximately 11-60 days after

Table 1. Age, body weight and uterine characteristics (mean ± SEM) of E2, VEH, and LET ovariectomized adult female marmoset monkeys

Parameter	E2 (n = 5)	VEH (n = 4)	LET (n = 4)	*P* value
Age at baseline (years)	3.1 ± 0.5	3.2 ± 0.3	2.9 ± 0.1	.854
Body weight at baseline (g)	402 ± 26	396 ± 35	382 ± 12	.863
Ultrasonographic imaging				
Baseline (ovary intact)				
Trans-fundus width (mm)	6.7 ± 0.6	6.7 ± 0.2	6.2 ± 0.1	.668
Post-OVX (5 months of study)				
Trans-fundus width (mm)	7.5 ± 0.7	5.2 ± 0.5[a]	4.8 ± 0.1[a]	.010
Dorso-ventral uterine depth (mm)	4.5 ± 0.5	3.3 ± 0.3	2.8 ± 0.2[a]	.020
Endometrial thickness (mm)	1.3 ± 0.1	0.8 ± 0.1[a]	0.7 ± 0.1[b]	.005

[a]$P < .05$ vs E2. [b]$P < .01$ vs E2.

Table 2. Circulating levels (mean± SEM) of sex steroid and gonadotropic hormones in ovariectomized female marmosets receiving estradiol replacement (E2), empty capsules (VEH) or the aromatase inhibitor, letrozole (LET) at 6 months following OVX and ~09:00 hours, immediately prior to necropsy

Circulating hormone	E2 (n = 5)	VEH (n = 4)	LET (n = 4)	*P* value
Estradiol (pg/mL)	1174.8 ± 214.0	24.7 ± 11.9[a]	3.0 ± 0.1[a,b]	.001
Estrone (pg/mL)	ND	ND	ND	ND
Progesterone (ng/mL)	1.32 ± 0.40	0.94 ± 0.33	0.47 ± 0.30	.118
17-OHP$_4$ (ng/mL)	3.37 ± 1.89	2.19 ± 0.36	2.00 ± 0.36	.743
DHEA (ng/mL)	0.15 ± 0.02	1.93 ± 0.76 [a]	1.80 ± 0.79[a]	.001
Androstenedione (ng/mL)	3.22 ± 0.37	7.16 ± 3.49	3.23 ± 2.13	.468
Testosterone (ng/mL)	0.18 ± 0.03	1.11 ± 0.62	0.42 ± 0.24	.518
CG (ng/mL)	2.70 ± 0.40	6.31 ± 2.22[c]	8.50 ± 1.52[d]	.014
Hormone ratio				
Estradiol:Testosterone	8.3 ± 1.9	0.3 ± 0.3[a]	0.02 ± 0.01[a]	.001
17OHP$_4$:Progesterone	3.3 ± 1.8	2.8 ± 1.5	6.0 ± 2.1	.482
Androstenedione:DHEA	24.2 ± 4.6	4.1 ± 2.4 [a]	5.2 ± 2.0 [d]	.004
Testosterone:Androstenedione	0.06 ± 0.01	3.87 ± 2.15	3.32 ± 2.19	.225

Abbreviation: DHEA, dehydroepiandrosterone; 17-OHP$_4$, 17-hydroxyprogesterone.
[a]$P < .01$ vs E2. [b]$P < .08$ vs VEH. [c]$P < .08$ vs E2. [d]$P < .05$ vs E2.

ovulation), an analog of prostaglandin-F2-alpha, was administered prior to OVX to facilitate scheduling of OVX during the follicular phase [19]. Treatment onset (0 months) was the day of the OVX procedure.

Hormone Assays

Plasma samples and hypothalami were analyzed for several hormones. Hypothalami were dissected at necropsy and frozen at –80°C. They were subsequently thawed, transected along the midline, and separated into hemi-hypothalami. One hemi-hypothalamus per monkey was divided into 50- to 75-mg aliquots. For steroid hormone analyses, plasma and hemi-hypothalami aliquots underwent extraction [13] and hypothalamic aliquots were recombined after extraction. Extracted samples were subsequently submitted for analysis on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (liquid chromatography tandem mass spectrometry [LC-MS/MS]) [19]. Plasma chorionic gonadotropin (CG) levels (New World primate equivalent of luteinizing hormone, LH) [92] were determined by a validated radioimmunoassay [93], detection limit 0.67 ng/mL (antibody catalogue #518B7, RRID:AB_2756886, https://antibodyregistry.org/search.php?q=AB_2756886). Intra- and interassay coefficients of variation were 17.4% and 8.8%, respectively.

Behavioral Observations

Following treatment onset, pairs were acclimated to a testing arena [94]. At 5 months post-treatment onset, and after animals were acclimated to the testing arena, pairmates were placed into single housing without visual contact for at least 30 days. While in this single housing, pairs were reunited, but only for behavioral testing. The pairs were tested for 3, 30-minute testing sessions per week for 2 weeks. Each test was digitally recorded. At least 2 observers scored frequencies of behavior observed in each test from a well-established ethogram [94]. Inter- and intra-observer reliability was 80% or greater.

Behavioral sequence analysis was used to identify statistically significant sequences of sexual behaviors observed between each of the 13 male–female pairmates and the consequences of estradiol treatment manipulation to disrupt marmoset-typical behavioral transitions during sexual interactions. A behavioral transition included any behavior that followed within 10 seconds of a previous behavior. For example, when "male mount female" is the initiating behavior, well-established male–female marmoset pairmates commonly transition from there to either "female reject mount" or "female receptive posture." Whichever occurred would be scored as a single, "2-act" behavioral transition.

Contingency tables and chi-squared test statistics were used to analyze the probability of each "2-act" transition occurring within a female treatment group. As adapted from those previously described [95, 96], frequencies of initial behaviors and transitions were tabulated from all testing sessions and used to determine the expected frequency occurrence of each behavioral transition derived from 52.5 hours of digitally recorded behavioral observations of all 13 male–female pairs. Five behavioral transition sequences were thus identified; chi-squared statistics were generated for each transition for each female treatment group in the context of the number of observational hours and compared with the expected frequency of the transition generated from the entire cohort of 13 pairs (Table 3). The higher the chi-squared value, the more likely the transition was a statistically significant ($P < .05$) behavioral sequence. In Fig. 1, arrows represent significant transitions present between 2 behaviors.

Gene Expression Analysis

Remaining hemi-hypothalami not processed for hormone analyses were submitted for gene expression analysis. Total RNA was isolated using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen) and cDNA synthesized using the Multiscribe High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative polymerase chain reaction was performed on a StepOnePlus instrument (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression changes are normalized to *TBP* as a reference gene and expressed relative to the estradiol female group, as previously described [94]. Primer sequences were designed using NCBI Primer-Blast [97] and are listed in Table 4.

Diet-induced Obesity From Consumption-driven Weekly Increments in Daily Calories

Animals were fed with Mazuri Callitrichid High Fiber Diet #5MI6 (Purina Mills International, St. Louis, MO), providing 53% carbohydrate, 20% protein, 6% fat, and 10% fiber by weight, with a metabolizable energy of 3.3 kcal/g (~61%, 23%, and 16% kcal from carbohydrate, protein and fat, respectively) [98]. Following OVX at study onset, total daily calorie consumption by each male–female study pair was recorded. To achieve DIO, diet allotment for each male–female study pair was increased weekly by ~66 kcal per day (equivalent to 20 g diet wet weight/day) if the entire daily allotment had been consumed during at least 4 out the 7 previous days. Calorie increments and total calories consumed, however, remained comparable across the pairs between treatment groups, as illustrated in Fig. 2.

Assessment of Daily Calorie Consumption While Maintaining DIO

At 6 to 7 months post-OVX, each female was singly housed within a marmoset housing room, but outside visual contact with her male pairmate, while still maintaining vocal and auditory contact. Each female's daily calorie allotment began at 50% of those provided when housed with their male pairmate. Dietary allotment was increased weekly by ~33 kcal per day (~10 g diet wet weight) only when the entire daily allotment was consumed during at least 4 out the 7 previous days. Calorie increments and total calories consumed, however, remained comparable across females between treatment groups, as illustrated in Fig. 2. The total kilocalories consumed daily were recorded for each female for 8 weeks.

Body Composition and Bone Mass

Animals were weighed weekly. Area under the curve (AUC) assessment of weight parameters over time, calculated by the trapezoidal rule, was employed to better detect recurring differences in weight gain, as previously employed by [99]. At baseline and 5 to 6 months post-OVX, total body composition, as well as bone mineral content (BMC) and bone mineral density (BMD), were assessed by dual-energy X-ray absorptiometry (DXA, iDXA, GE/Lunar Corp., Madison, WI) on sedated animals. Fat, fat-free mass (FFM) (excluding

Table 3. (A) The frequency distribution table illustrates the observed frequencies of subsequent behaviors following initial behaviors (values outside parentheses) compared with expected frequencies of these behavioral transitions for all male–female pair interactions during behavioral tests, and (B) each subtable (B.1-B.3) shows the chi-squared statistic for all behavioral transitions analyzed for each female group

(A)Frequency distribution table

		Subsequent behavior					
		Treatment group	**RP**	**RJ**	**RHT**	**I**	**H**
Initial behavior	M	E2	1.77 (0.78)	0.31 (0.48)	—	—	—
		VEH	0.67 (0.78)	1.22 (0.48)	—	—	—
		LET	0 (0.78)	1.56 (0.48)	—	—	—
	RP	E2	—	—	1.69 (0.52)	1.77 (0.57)	—
		VEH	—	—	0.11 (0.52)	0.67 (0.57)	—
		LET	—	—	0 (0.52)	0 (0.57)	—
	RJ	E2	—	—	—	—	0 (0.20)
		VEH	—	—	—	—	0 (0.20)
		LET	—	—	—	—	1.67 (0.20)

(B)Chi-squared statistic for all behavioral transitions analyzed

B.1. Behavioral transition	**Treatment group: E2**
M→RP	χ^2: 12.55; P = .02
M→RJ	χ^2: 0.06; ns
RP→RHT	χ^2: 30.01; P < .0001
RP→I	χ^2: 25.23; P < .0001
Rj→H	N/A
B.2. Behavioral transition	**Treatment group: VEH**
M→RP	χ^2: 0.16; ns
M→RJ	χ^2: 11.48; P = .005
RP→RHT	χ^2: 0.41; ns
RP→I	χ^2: 0.16; ns
Rj→H	N/A
B.3. Behavioral transition	**Treatment group: LET**
M→RP	N/A
M→RJ	χ^2: 24.10; P < .0001
RP→RHT	N/A
RP→I	N/A
Rj→H	χ^2: 107.5; P < .0001

These data are represented graphically in the behavioral transitions diagram (Fig. 1).
Abbreviations: M, male mount; RP, sexually receptive behavior; RJ, sexual rejection behavior; RHT, sexually receptive head turn; I, penile intromission; H, hitting partner.

bone), BMC, and BMD were determined for total body as well as previously validated body regions of interest, including abdomen, chest, thighs, lower legs, and arms [94].

Locomotor Activity

At baseline and 5 to 6 months post-OVX, a small accelerometer (Actiwatch Mini, CamNtech Ltd, Cambridge UK) was added to each female's standard collar. Activity and intensity of movement were recorded over a ~4-week period after which the accelerometers were removed. For the most part, activity recorded represented whole body movements, and not limb or head movements, alone, as previously validated in marmosets [100]. The accelerometer sampled such activity counts every 30 seconds and these data were averaged for every hour, day (during lights on), night (during lights off), morning (06:00-12:00 hours), afternoon (12:00-18:00 hours), and 24 hours. AUC activity values were also assessed to detect recurring differences in activity over time.

Sucralose Sweet Taste Test

At 5 to 6 months post-OVX, following assessment of individual female daily calorie consumption, each female was tested for the ability to discriminate a highly preferred sweet taste (sucralose) from water, as previously validated in marmosets [101]. Females were separated from their male pairmates within their homecage by a nontranslucent panel between 13:00 hours and 16:00 hours, and were subjected to 3, 30-minute trials, each trial separated by ~3 to 4 days, involving 100 mL of sucralose water bottle solution (2 mM solution) and 100 mL regular water bottle placed ~0.3 m apart [102]. Total volume consumed for each 30-minute period was determined for both sucralose and water solutions.

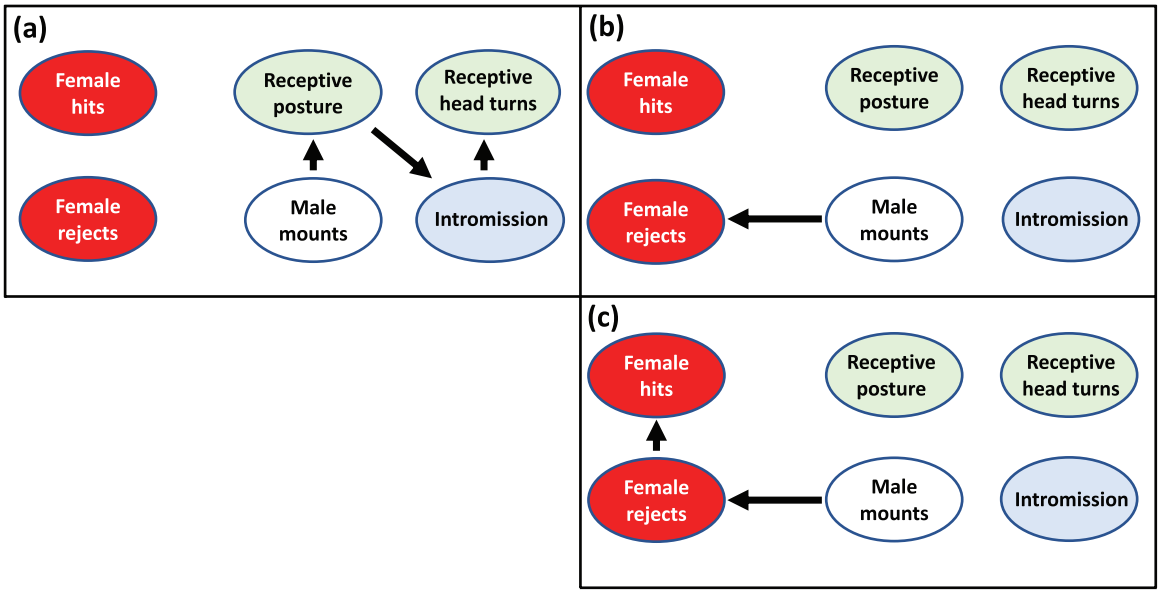

Figure 1. Species typical sexual behavior is observed in the behavioral transitions most likely to occur with (A) estradiol (E_2) replacement. In contrast, (B) loss of ovarian estradiol illustrates the switch in likelihood of behavioral transition from sexual receptivity to sexual rejection, while (C) loss of both ovarian and extra-ovarian estradiol results in not only a high probability of sexual rejection, but also the likelihood that sexual rejection will lead to aggressive hitting behavior. Each black arrow represents a statistically significant ($P < .05$) transition between connected behaviors. Green circles indicate sexually receptive behavior, blue circles indicate male intromission and red circles indicate sexual rejection.

Table 4. Marmoset specific primer sequences employed for the behaviorally related gene expression using NCBI Primer-Blast [97]. TATA-binding protein (TBP) gene expression was used as the housekeeping gene for correction of all other relative gene expression

	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
DRD1	CAGACTTTGCCCTGTGACGA	ACATCGCAGCCCCATTGTTA
DRD2	GCCTCCTTCCTTGACCTTCC	GGCCTTGAAGGGTGTGAACT
DRD4	TTGGCTGGGCTACGTCAACA	CGGCGTTGAAGACCGTGTA
HTR1A	TTAGCAAGGACCACGGCTAC	ATGCGCCCATAGAGAACCAG
HTR1B	TGGGTCTCCTGTGTACGTGA	CCTAGCGGCCATGAGTTTCT
HTR2A	TCAACTCCAGAACGAAGGCA	ATCGTCGGCGAGTAAGCAAC
HTR2B	CAAGCCACCTCAACGCCTAA	CAGAGCCTTGTCCTTCCGAG
HTR2C	CCAAGCAACGCCATCCTTC	TTAGGTGCACAAGGAACGAA
HTR5A	CGTGGTGCTCTTCGTGTACT	TCGGATACGGGTGAGACACT
OXT	CTCGATGTGCGCAAGTGCC	TCCACGCAGCAGATATTCGG
OXTR	ATGCGCCTAAGGAAGCCTCA	GTGACCCGTGAAGAGCATGT
TBP	CCATGACTCCTGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA

Fasting Glucose and Oral Glucose Tolerance Test

Fasting glucose and glucoregulation were assessed in overnight fasted, awake animals. Fasting glucose was determined at baseline and 6 months post-OVX, while glucoregulation was assessed by oral glucose tolerance test (OGTT) at 6 months following OVX. Following a baseline blood sample, animals were given an oral dose (5 mL/kg) of 40% sucrose. Blood samples were then collected at 15, 30, 60, and 120 minutes (2 hours) following sucrose administration and assessed for glucose, as previously validated for marmosets [103]. Glucose was measured by glucometer (Accu-Check Aviva, Roche Diagnostics, Indianapolis, IN). AUC glucose values during the OGTT were also assessed to detect between group differences in accumulating high levels of glucose over time.

Statistical Analysis

Data were analyzed utilizing SPSS software (IBM, Armonk, NY). Hormone measures, behavioral observations, gene expression data and sweet taste data were analyzed with a 1-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison post hoc tests. All other between group analyses were performed using a 2-way ANOVA for repeated measures. Steroid and CG hormone data were log transformed, and behavioral data were transformed to arcsin, prior to ANOVA or correlation tests. Nonparametric Spearman's correlation tests were utilized to identify relationships between gene expression, hormone values, and behavior. Spearman's rho coefficients are expressed as r_s. Statistical significance was determined at $P \leq .05$.

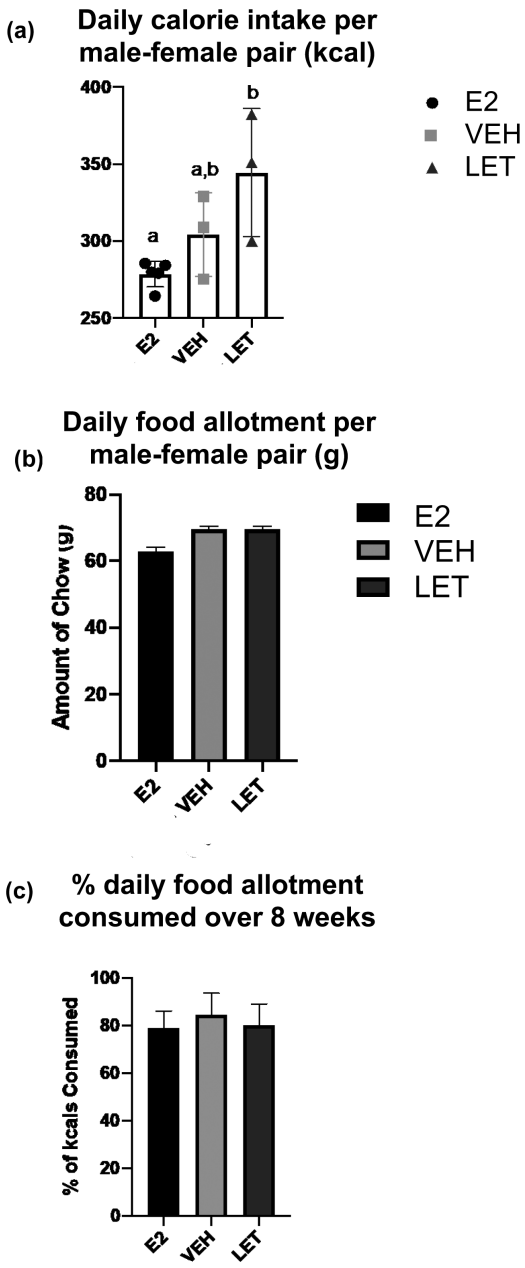

Figure 2. (A) Calories consumed (mean ± SEM) by both males and females combined in the male–female pairs (symbols illustrate individual male–female pair consumption) comprising the treatment groups E2 (black circles), VEH (light gray squares), and LET (dark gray triangles) from ovariectomy (OVX) until 5 months (months) after OVX, (B) amount of chow (mean ± SEM wet weight) provided to singly housed females during months 6 to 7 following OVX (E2 black bar, VEH light gray bar, LET dark gray bar), and (C) % calories consumed (mean ± SEM) by singly housed females during months 6 to 7 following OVX (E2 black bar, VEH light gray bar, LET dark gray bar).

Results

Circulating Estradiol and Pituitary GC Levels, Together With Uterine Dimensions, Confirm Anticipated Estrogen Status

As expected, E2 females alone exhibited circulating estradiol levels approximating those of mid-cycle, ovary intact female marmosets (Table 2). Greatly diminished circulating levels of estradiol were found in both VEH and LET females, with LET females demonstrating the more extreme estradiol depletion (Table 2). Ultrasonographic assessments of uterine dimensions confirmed this systemic estradiol disparity at 5 and 7 months following OVX, respectively, demonstrating maintenance of species typical, estradiol-dependent uterine dimensions in the E2 female group, alone (Table 1). In this context, it was not surprising to find elevated circulating CG levels in VEH ($P < .08$) and LET ($P < .05$) compared with E2 females, reflecting insufficient circulating estradiol for maintenance of negative feedback regulation of pituitary CG release in estradiol-depleted female groups (Table 2). CG levels in LET females, however, were ~35% greater than in VEH females.

Extra-ovarian Estradiol and Androgens Provide Vital Contributions to Female Sexual Responses

Both ovarian and extra-ovarian estradiol contributed to female marmoset sexual behavior. Compared with E2 females, eliminating ovarian (mid-cycle) equivalent estradiol alone (VEH females), diminished ($P = .01$) but did not abolish female sexual receptivity (Fig. 3A). Female sexually receptive behaviors commonly exhibited following acceptance of male mounts, including receptive head turn to nuzzle the mounted male, also greatly diminished in frequency ($P = .04$) following elimination of ovarian estradiol in VEH females (Fig. 3B). These declines in VEH female sexual receptivity were accompanied by an increase ($P = .03$) in female rejection of male sexual advances (Fig. 3A).

Depletion of extra-ovarian estradiol via aromatase inhibition, beyond that in the VEH group, revealed additional detriments in LET female sexual behavior. Female sexual receptivity was eliminated ($P = .003$), except for rare acceptance of male mount attempts (Fig. 3A). Intensity of female sexual rejection increased to include aggressive hitting. These behavioral changes were beyond those observed when eliminating only systemic estradiol, including unanticipated aggressive progression of female sexual rejection.

Behavioral sequence analyses revealed estradiol-manipulated disruption to marmoset-typical behavioral transitions within male–female sexual interactions. Table 3 provides a frequency distribution for each group and analyzed transition. Bracketed frequencies are expected frequency within the entire cohort of 13 male–female pairs observed. Unbracketed frequencies depict the observed frequencies of a specified behavioral transition, such as male mount female—female accepts male mount for each treatment group. Table 3 illustrates chi-squared values for each behavioral transition and their derived *P* values that were used to construct behavioral transition sequences in Fig. 1.

Figure 1 shows a marmoset-typical sexual behavior pattern between male and female pairmates in the E2 group. In this figure, the most likely behavioral sequence following male initiation of a mount is male mount followed by female receptive posture ($P < .001$). Subsequent transitions then follow, resulting in a transition from female receptive posture to male intromission ($P < .0001$), followed by male intromission leading to female receptive head turn ($P < .0001$). Figure 3A subsequently shows that if there is a loss of ovarian estradiol (VEH group), this estradiol decrement is sufficient to increase the likelihood that females will reject their male partners' mounts ($P < .005$). Depletion of both ovarian and extra-ovarian estradiol (LET females), however, as shown in Fig. 3B, not only increases the likelihood of female rejection of male

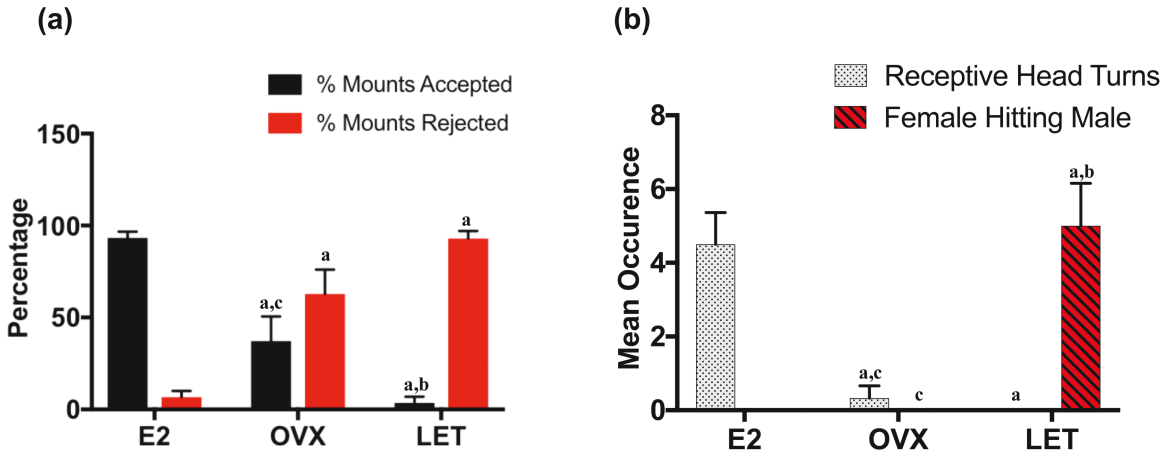

Figure 3. Changes in female sexual behavior (mean ± SEM) in OVX adult female marmosets receiving estradiol replacement (E2), vehicle (VEH), or an aromatase inhibitor, letrozole (LET): (A) percent sexual receptivity (black filled bars) is diminished in OVX (a, $P = .001$) compared to E2 females, and further diminished in LET compared with E2 (a, $P = .001$) and VEH (b, $P = .04$) females, while frequency of sexual rejection (red filled bars) is increased in both OVX (a, $P = .03$) and LET (a, $P = .03$) females compared with the E2 female group, and (B) frequency of receptive female head turns (gray dotted bars) is diminished in the absence of both ovarian (VEH, a, $P = .003$) and extra-ovarian (LET, a, $P = .003$) E2, and LET females exhibit both an increase in sexual rejection of males, and escalation of their rejection into hitting the male partner (black diagonal banded red bars; a,b: $P = .004$). (a = compared with E2 females; b = compared with VEH females; c = compared with LET females).

Table 5. Hypothalamic levels (mean ± SEM) of sex steroid hormones in ovariectomized female marmosets receiving estradiol replacement (E2), empty capsules (VEH), or the aromatase inhibitor letrozole (LET) at 6 months following ovariectomy

Hypothalamic hormone	E2 (n = 5)	VEH (n = 4)	LET (n = 4)	*P* value
Estradiol (pg/mg)	0.56 ± 0.16	0.77 ± 0.33	0.07 ± 0.04[a,b]	.004
Estrone (pg/mg)	0.26 ± 0.10	0.32 ± 0.15	0.10 ± 0.04	.154
Progesterone (pg/mg)	1.30 ± 0.25	2.55 ± 0.76	1.32 ± 0.40	.266
17-OHP_4 (pg/mg)	8.80 ± 1.46	23.83 ± 5.17[a]	12.75 ± 2.11	.042
DHEA (pg/mg)	0.04 ± 0.01	0.20 ± 0.06[a]	0.09 ± 0.02	.010
Androstenedione (pg/mg)	0.75 ± 0.04	9.34 ± 5.32[a]	4.88 ± 1.75[c]	.008
Testosterone (pg/mg)	0.04 ± 0.01	0.15 ± 0.03[a]	0.08 ± 0.03	.019
Hypothalamic hormone ratio				
Estradiol:Testosterone	127.4 ± 63.2	62.9 ± 0.3[a]	0.02 ± 0.01[a,b]	.002
17OHP_4:Progesterone	6.7 ± 0.8	9.8 ± 1.9	10.9 ± 2.0	.215
Androstenedione:DHEA	17.7 ± 1.2	38.9 ± 11.3	53.3 ± 9.9 [a]	.009
Testosterone:Androstenedione	0.06 ± 0.01	0.03 ± 0.01 [a]	0.02 ± 0.01 [a]	.003

mg = mg wet weight of tissue.
Abbreviation: DHEA, dehydroepiandrosterone; 17-OHP_4, 17-hydroxyprogesterone.
[a]$P < 0.01$ vs E2. [b]$P < .05$ vs VEH. [c]$P < .05$ vs E2.

sexual advances ($P = .001$), instead of female receptive posture, but also changes the most likely subsequent behavior to aggression, namely hitting ($P = .001$).

Circulating Steroid Hormones and Hypothalamic Steroid Content

Eliminating ovarian estradiol (VEH) or both ovarian and extra-ovarian estradiol (LET) produced notable changes in hypothalamic estrogen and androgen content, as well as in circulating steroid hormone levels, when compared with females in the E2 group. Hypothalamic estradiol content was diminished in LET females, alone, when compared with both estradiol ($P = .005$) and VEH ($P = .013$) female groups (Table 5). Notably, comparable hypothalamic estradiol content was maintained between E2 and VEH groups despite the large systemic deficit in circulating estradiol levels ($P = .001$) exhibited by VEH females that greatly increased the ratio of hypothalamic estradiol (pg/g wet weight): systemic circulation estradiol (pg/mL) from approximately 0.5 (E2 females) to 31.2 (VEH). Circulating levels of estradiol, however, tended ($P = .08$) to be lower in LET than in VEH females (Table 2), maintaining a high ratio of hypothalamic estradiol: systemic circulation estradiol of approximately 23.3 (LET).

Hypothalamic androgen content, in contrast to that for estradiol, was generally increased in the absence of ovarian estradiol levels (VEH) and was not further augmented by additional depletion of extra-ovarian estradiol (LET) (Table 5). Hypothalamic androstenedione content was elevated in both VEH ($P = .03$) and LET ($P = .05$) compared with E2 (Table 5) females, resulting in hypothalamic:systemic ratios for androstenedione of 0.2 (E2), 1.3 (VEH), and 1.5 (LET). Hypothalamic dehydroepiandrosterone (DHEA) content,

however, was only elevated in VEH ($P = 0.04$), but not LET, females when compared with those in the E2 group, resulting in hypothalamic:systemic ratios for DHEA of 0.3 (E2), 0.1 (VEH) and 0.05 (LET). Despite demonstrating a similar overall pattern, hypothalamic testosterone values remained comparable across all female groups (Table 5), with relative consistency of hypothalamic vs circulating testosterone ratios between female groups of 0.2 (E2), 0.1 (VEH), and 0.2 (LET). Changes in circulating androgen levels (Table 2), however, did not mirror those found in hypothalamic content (Table 5). Circulating levels of DHEA, alone, increased in VEH ($P < .001$) and LET (Table 2; $P = .001$) groups compared with those in E2 females. Circulating levels of androstenedione and testosterone (Table 2) remained comparable across all female groups.

While circulating levels of progestins, progesterone, and $17OHP_4$, and their ratios, remained comparable across female groups (Table 2), hypothalamic content of $17OHP_4$ more than doubled in VEH compared with E2 and LET female groups (Table 5), thus increasing the hypothalamic to circulating $17OHP_4$ ratio to 10.9 (VEH) in comparison with 2.6 (E2) and 6.4 (LET). Hypothalamic progesterone content, and its hypothalamic to circulating ratio, demonstrated more modest increases following estrogen depletion (E2, 1.0; VEH, 2.7; LET 2.8).

With regard to ratios of hypothalamic steroid hormones, selected to quantify aspects of androgen biosynthesis (Table 5), the ratio of androstenedione:DHEA was higher in both VEH ($P = .05$) and LET ($P = .005$) than in E2 females. There were no effects of eliminating ovarian estradiol (VEH) or both ovarian and extra-ovarian estradiol (LET) on the ratios of hypothalamic testosterone:androstenedione or $17OHP_4$:progesterone (Table 5), respectively. The ratio of hypothalamic estradiol:testosterone, in contrast, was decreased ($P = .002$) in both VEH and LET compared with E2, as well as in LET compared with VEH females ($P = .05$).

Relationships Between Circulating Pituitary CG Levels and Circulating and Hypothalamic Steroid Hormone Values

Circulating CG levels negatively correlated with circulating levels of estradiol and androstenedione, as well as with both circulating and hypothalamic estradiol:testosterone ratios (Table 6). In contrast, a positive correlation was found between circulating CG and DHEA levels. No other correlations were found between circulating levels of CG and circulating or hypothalamic estrogens or androgens (Table 6).

Hypothalamic Gene Expression and Association with Sexual Behavior Frequency

Females groups exhibited only a single difference ($P < .03$) in hypothalamic gene expression relevant to androgen or estrogen biosynthesis (Table 7): CYB5B, an essential allosteric factor enhancing 17,20 lyase activity and thus boosting hypothalamic androgen biosynthesis, demonstrated a strong increasing trend in LET compared with E2 females ($P < .06$). Additionally, there were no hypothalamic gene expression differences between female groups with regard to specific behaviorally related gene targets (Table 8). The ratios of gene expression involving 5HT receptors, however, differed between female groups. Both the ratios for *HTR1A:HTR2A* and *HTR5A:HTR2A* (Table 8) were lower ($P = .05$) in LET than in E2 females, but VEH female 5HTR gene expression ratios were intermediate between E2 or LET female group values.

Correlations between hypothalamic and circulating levels of steroid hormones, behavior, and hypothalamic gene expression were also analyzed for all female groups combined. Table 6 shows the Spearman's correlation coefficient (r_s) and associated P value for each relationship identified with female sexual receptivity and sexual rejection. Overall, there were positive relationships between the frequency of female sexual receptivity and both circulating estradiol levels and hypothalamic estradiol content. Circulating CG concentrations were negatively correlated with female sexual receptivity, likely reflecting the biological impact of estradiol. There was also a positive relationship between female sexual receptivity and the ratio of hypothalamic estradiol:testosterone. Hypothalamic A_4, however, negatively correlated with female sexual receptivity (Table 6). In addition to identified relationships between steroid hormones and female sexual behavior, frequencies of female sexual receptivity were also negatively correlated with hypothalamic mRNA expression ratios for both *HTR1A:HTR2A* ($r_s = 0.645$; $P = .02$) and *HTR5A:HTR2A* ($r_s = 0.682$; $P = .01$), but no other hypothalamic gene expression parameters.

Calorie Consumption, Body Weight, and Body Composition

Only male–female pairs from the LET female group consumed more calories ($P = .02$) per day than male–female pairs from the E2 female group during weekly increments of daily calories from months 1 to 5 following OVX (Fig. 2). When females were separated from their male pairmates at 6 to 7 months following OVX for individual behavioral and calorie intake assessments, total daily calories provided to all 3 female groups were comparable, with female consuming ~80% of calories provided (Fig. 2). Females from both estradiol-depleted, VEH ($P < .001$) and LET ($P < .001$) groups, however, consumed more calories, corrected for FFM, than E2 group females (Fig. 4).

While female body weight increased ~5% to 10% during the DIO study regimen irrespective of treatment group ($P < .016$) (Fig. 4A), the AUC % body weight increase from baseline, with or without correction for FFM (Fig. 4B and 4C), was greater ($P = .02$) in both estradiol-depleted VEH and LET females compared with those in the E2 group. At both baseline and 6 months following OVX, all female marmosets were obese (>14% body fat), with mean fat-to-lean mass ratios in each treatment group exceeding 0.3 (Table 9). While there were no obvious differences in DXA-determined fat mass 6 months following OVX in any female group for total body or previously validated body regions of interest, including abdomen and hips/thighs, in contrast, total body ($P = .014$), abdominal ($P = .002$), and upper leg ($P = .025$) FFM increased ~5% to 10% during the DIO study regimen irrespective of treatment group (Table 9). Increased abdominal FFM, however, was greater ($P = .041$) in VEH than in both E2 and LET female groups. FFM increases in all female groups combined were not correlated with either circulating or hypothalamic steroid hormone levels or ratios, including those for estradiol.

Higher hypothalamic, but not circulating, androstenedione values and the androstenedione to DHEA ratio predicted greater

Table 6. Correlations between female sexual receptivity, sexual rejection, and circulating chorionic gonadotropin (CG) levels with circulating levels and hypothalamic content of estrogens and androgens at ~7 months following ovariectomy, all female groups combined

	Circulating CG (ng/ml)			% female sexual receptivity			% female sexual rejection		
Systemic circulation	**r^2**	**p-value**	**slope**	**r^2**	**p-value**	**slope**	**r^2**	**p-value**	**slope**
E_2 (pg/ml)	**0.60**	**0.003**	**-0.19**	**0.82**	**0.001**	**+0.47**	**0.90**	**0.001**	**-0.54**
T (ng/ml)	0.01	0.712	NS	0.03	0.651	NS	0.02	0.721	NS
A_4 (ng/ml)	**0.36**	**0.038**	**-0.14**	0.28	0.116	NS	0.29	0.110	NS
DHEA (ng/ml)	**0.49**	**0.012**	**+0.35**	**0.75**	**0.001**	**-0.89**	**0.79**	**0.001**	**+0.99**
E_2/T ratio	**0.38**	**0.026**	**-0.12**	**0.69**	**0.002**	**+0.35**	**0.73**	**0.001**	**-0.40**
Hypothalamus	**r^2**	**p-value**	**slope**	**r^2**	**p-value**	**slope**	**r^2**	**p-value**	**slope**
E_2 (pg/mg)	0.25	0.100	NS	0.15	0.272	NS	0.26	0.135	NS
T (pg/mg)	0.07	0.420	NS	**0.44**	**0.036**	**-1.65**	0.36	0.067	NS
A_4 (pg/mg)	0.18	0.175	NS	**0.74**	**0.002**	**-1.47**	**0.65**	**0.005**	**+1.51**
DHEA (pg/mg)	0.03	0.575	NS	0.36	0.067	NS	0.22	0.175	NS
E_2/T ratio	**0.38**	**0.034**	**-0.27**	**0.40**	**0.052**	**+0.62**	**0.52**	**0.019**	**-0.77**

AUC % body weight gain, with (androstenedione: $r^2 = 0.55$, $P = .006$; androstenedione to DHEA ratio: $r^2 = 0.44$, $P = .019$) or without (androstenedione: $r^2 = 0.53$, $P = .007$; androstenedione to DHEA ratio: $r^2 = 0.41$, $P = .025$) correction for FFM, when all female groups were combined. In contrast, circulating but not hypothalamic, androstenedione to DHEA ratio predicted ($r^2 = 0.45$, $P = .011$) greater AUC calories consumed corrected for FFM. No correlations were found between measures of body weight or calories consumed and the remaining hypothalamic or circulating steroid hormone values or ratios, including those for estradiol.

Both total body BMC and BMD, and the same bone parameters in previously validated body regions of interest, were comparable across all 3 female groups (Table 9). No bone parameter was diminished by depletion of either ovarian estradiol or ovarian and extra-ovarian estradiol.

Locomotor Activity

Activity collar assessments of female locomotion were obtained at both baseline (while pair housed with their male cagemate) and 6 months following OVX (when singly housed) in estrogen depleted groups, alone. AUC locomotor activity was greater during the daytime in VEH than in LET females at both baseline ($P = .001$) and 6 months following OVX ($P = .01$) (Fig. 5A and 5B). In contrast, during the resting hours of nighttime, there were no differences in locomotor activity between female groups. When comparing the AUC % change in locomotory activity, however, from baseline to 6 months following OVX, LET females became slightly more active than VEH during both daytime ($P = .057$) and nighttime ($P = .01$) (Fig. 5C). Increased activity in LET females was notable late in the day and throughout much of the night.

Sucralose Sweet Taste Test

There were no between female group differences in the volumes of water or sucralose water consumed during these 15-minute tests. There were also no between group differences for % of sucralose water consumed (Table 10).

Fasting Glucose

By 6 months following OVX, in all female groups combined, DIO induced a trend ($P = .055$) toward increased fasting glucose levels from baseline (Table 11), irrespective of circulating estradiol levels. OGTT 2 hour glucose, similar to fasting glucose, revealed no between group differences. Glycogenic hepatopathy was observed in 20% (1/5) of estradiol females, 50% (2/4) of VEH females, and 75% (3/4) of LET females during postnecropsy histopathological hepatic assessment.

Discussion

In the current study, ovarian and extra-ovarian estradiol depletion of adult female marmoset monkeys, in contrast to depletion of ovarian estradiol, alone, demonstrated a substantial functional contribution provided by extra-ovarian estradiol in support of female NHP sexual engagement and regulation of pituitary gonadotropin release, with little contribution toward metabolic homeostasis. Ovarian estradiol depletion, in addition to ovarian and extra-ovarian estradiol depletion, nevertheless, enhanced DIO calorie consumption and weight gain in comparison to estradiol replete females, illustrating the contribution of estradiol toward diminishing DIO-associated metabolic dysfunction in a female NHP. DIO, in contrast and regardless of estradiol depletion, induced modest glucose intolerance, possibly due to impaired hepatic glucose metabolism related to glycogenic hepatopathy observed in all 3 female groups.

Endogenous estrogenic source(s) beyond the ovaries include a variety of organ systems as well as the brain [7, 8, 11, 12, 17, 104]. This is of considerable importance to clinical management of women's health. For example, the oral, nonsteroidal aromatase inhibitor letrozole, employed in the current NHP study, is widely used in clinical practice, including minimizing

Table 7. Hemi-hypothalamic gene expression[a] and gene expression ratios (mean ± SEM) 7 months after ovariectomy in E2, VEH and LET female groups for selected genes related to estrogen and androgen biosynthesis, reproductive neuroendocrine regulation, and ratios of dopaminergic receptor and serotonergic receptor genes.

Genes	E2	VEH	LET	*P* value
Estrogen and androgen biosynthesis				
Steroidogenic acute regulatory protein (StAR) (*STARD1*)	1.00 ± 0.13	0.82 ± 0.05	0.81 ± 0.02	NS
17-Hydroxylase/17,20 lyase (*CYP17A1*)	1.00 ± 0.19	1.18 ± 0.22	1.15 ± 0.26	NS
Aromatase (*CYP19A1*)	1.00 ± 0.17	1.61 ± 0.46	1.05 ± 0.11	NS
Cytochrome b5a (*CYB5A*)	1.00 ± 0.11	1.21 ± 0.11	1.19 ± 0.08	NS
Cytochrome b5b (*CYB5B*)	1.00 ± 0.17	1.36 ± 0.11	1.35 ± 0.08[b]	.030
Reproductive neuroendocrine regulation				
Gonadotropin releasing-hormone 1 (*GNRH1*)	1.00 ± 0.16	0.71 ± 0.06	0.66 ± 0.09	NS
Kisspeptin (*KISS1*)	1.00 ± 0.57	2.09 ± 1.21	1.50 ± 0.51	NS
Ratios of selected dopaminergic receptor (DR) genes				
DRD1:DRD2	0.72 ± 0.23	0.56 ± 0.12	0.46 ± 0.11	NS
DRD1:DRD4	0.89 ± 0.74	0.73 ± 0.32	0.24 ± 0.03	NS
DRD2:DRD4	0.90 ± 0.60	1.17 ± 0.34	0.57 ± 0.07	NS
Ratios of selected serotonergic receptor (HTR) genes				
HTR1A:HTR1B	1.08 ± 0.15	1.06 ± 0.14	1.01 ± 0.15	NS
HTR1A:HTR2B	1.20 ± 0.38	1.09 ± 0.09	1.61 ± 0.32	NS
HTR1A:HTR2C	1.07 ± 0.14	0.95 ± 0.05	0.91 ± 0.07	NS
HTR1A:HTR5A	1.00 ± 0.08	0.83 ± 0.04	1.38 ± 0.14	NS
HTR1B:HTR2A	1.19 ± 0.26	0.85 ± 0.29	0.65 ± 0.15	NS
HTR1B:HTR2B	1.23 ± 0.48	1.11 ± 0.21	1.59 ± 0.18	NS
HTR1B:HTR2C	1.02 ± 0.09	0.97 ± 0.18	0.91 ± 0.12	NS
HTR1B:HTR5A	0.98 ± 0.26	0.85 ± 0.29	2.70 ± 0.47	NS
HTR2A:HTR2B	1.19 ± 0.40	1.49 ± 0.24	1.38 ± 0.14	NS
HTR2A:HTR2C	0.96 ± 0.14	1.29 ± 0.17	1.48 ± 0.12	NS
HTR5A:HTR2A	1.18 ± 0.16	0.94 ± 0.13	0.67 ± 0.07	NS
HTR5A:HTR2B	1.19 ± 0.37	1.31 ± 0.06	1.73 ± 0.21	NS
HTR5A:HTR2C	1.06 ± 0.07	1.16 ± 0.12	0.97 ± 0.06	NS

[a]Corrected for expression of housekeeping gene TBP and normalized to the E2 group.
[b]$P = 0.058$, E2 vs LET.

recurrence of estrogen receptor positive breast cancer following surgical intervention [105, 106] and enabling menopausal hormone therapy [107, 108]. Not surprisingly, such long-term aromatase inhibition can exert a highly negative effect on female sexuality, engaging personally distressing sexual dysfunction, vaginal atrophy, and dyspareunia [42, 106, 109]. In addition, as found in this NHP study, aromatase inhibition treatment of women with breast cancer enables weight gain (including increased adiposity), perturbing female metabolic homeostasis [110]. In the present NHP study, however, DIO rather than aromatase inhibition, increased glucose intolerance. Women with naturally occurring gene variants in CYP19A1, while exceedingly rare, present with varying degrees of systemic estrogen depletion accompanied by overweight or obesity, impaired glucoregulation, borderline hyperlipidemia, and osteopenia/osteoporosis [111]. Unlike the present study, however, genetically determined CYP19A1 deficiency manifests estradiol depletion through all developmental stages, resulting in widespread organ system abnormalities that are absent from our adult-onset estradiol depletion. Psychosexual information concerning CYP19A1-deficient individuals is lacking [112, 113].

As might be expected, systemically administered estradiol alleviates sexual [114, 115] and metabolic [116] dysfunction in women, but to varying degrees [117], while also increasing the risk for harmful side-effects, including cardiovascular disease and cancer [105, 117]. SERMs, nonsteroidal compounds that interact with estrogen receptors, and display distinct differences in degree of agonism vs antagonism action at estrogen receptors in target tissues, show efficacy for osteoporosis, dyspareunia, and breast cancer [118]. SERMs, however, all carry safety risks, most notably venous thromboembolic events. Treatments that avoid systemic estrogenic activity by delivering bioactive estradiol to the brain, alone, thus have tremendous potential to alleviate female sexual and metabolic dysfunction, as indicated by the findings from our current female NHP study. In this regard a synthetically derived, inactive precursor of estradiol, when administered orally to female rodents, is only metabolized to a bioactive estrogen, in this case estradiol, within the central nervous system following transport across the blood–brain barrier [119], providing amelioration to hypothalamically driven hot flashes without estrogenic effect on systemic estrogen-responsive organs and tissues, such as the uterus.

Estradiol and Female Sexual Receptivity

Although ovarian estradiol has been repeatedly shown to elevate the expression of sexual receptivity in female NHPs

Table 8. Hemi-hypothalamic gene expression[a] and gene expression ratios (mean ± SEM) 7 months after ovariectomy in E2, VEH and LET female groups for selected behaviorally related genes.

Genes	E2	VEH	LET	*P* value
Steroid hormone receptors				
Estrogen receptor α (*ESR1*)	1.00 ± 0.13	0.71 ± 0.06	0.66 ± 0.09	NS
Progesterone receptor (*PRA*)	1.00 ± 0.35	0.49 ± 0.13	0.64 ± 0.11	NS
Dopaminergic receptors (DRs)				
Dopamine receptor 1 (*DRD1*)	1.00 ± 0.85	0.79 ± 0.59	0.26 ± 0.06	NS
Dopamine receptor 2 (*DRD2*)	1.00 ± 0.70	1.02 ± 0.57	0.62 ± 0.11	NS
Dopamine receptor 4 (*DRD4*)	1.00 ± 0.06	0.92 ± 0.32	1.09 ± 0.16	NS
Serotinergic receptors (HTR)				
Serotonin receptor 1A (*HTR1A*)	1.00 ± 0.25	1.00 ± 0.09	1.20 ± 0.15	NS
Serotonin receptor 1B (*HTR1B*)	1.00 ± 0.29	1.04 ± 0.25	1.23 ± 0.13	NS
Serotonin receptor 2A (*HTR2A*)	1.00 ± 0.30	1.35 ± 0.21	2.05 ± 0.31	NS
Serotonin receptor 2B (*HTR2B*)	1.00 ± 0.13	0.92 ± 0.06	0.78 ± 0.07	NS
Serotonin receptor 2C (*HTR2C*)	1.00 ± 0.28	1.07 ± 0.15	1.38 ± 0.14	NS
Serotonin receptor 5A (*HTR5A*)	1.00 ± 0.23	1.20 ± 0.08	1.31 ± 0.06	NS
Ratio of serotonergic receptors				
HTR1A:HTR2A	1.17 ± 0.15	0.78 ± 0.11	0.60 ± 0.05[b]	**.016**
HTR5A:HTR2A	1.18 ± 0.16	0.94 ± 0.13	0.67 ± 0.07[c]	**.037**

[a]Corrected for expression of housekeeping gene TBP and normalized to the E2 group.
[b]$P = 0.02$, E2 vs LET. [c]$P = 0.03$, E2 vs LET.

[21, 28, 59, 60, 114, 120-122], ovarian estradiol depletion fails to completely inhibit female NHP sexual receptivity [27, 122-125], and complicates our understanding of the role of estradiol in regulating sexual function in female NHPs, and likely women. Persistent sexual receptivity across ovarian and menstrual cycles in female NHPs and women, however, stands in contrast to its strictly regulated expression in female rodents and sheep in which mid-cycle, peri-ovulatory ovarian estradiol is necessary to generate the reflexive and receptive posture, lordosis [50, 51] in female rodents, and estrous behavior in female sheep [126]. Such reflexive postures and estrus are not exhibited by female NHPs [121, 122, 124].

In our current NHP study, ovarian estradiol-depleted female monkeys still express receptive postures and gestures toward at least 35% of male mounting behaviors. It is only by removal of both ovarian and extra-ovarian estradiol, including hypothalamic sources, that female marmosets are consistently sexually unreceptive to male sexual advances. Such effective elimination of female marmoset sexual behavior has not been previously achieved, including combined OVX and adrenalectomy [69], as well as bilateral neurolesions of the anterior and medial hypothalamus [68, 125]. While hypothalamic estradiol content maybe necessary for expression of female NHP receptivity, our data also suggest that concurrent with the loss of both ovarian and extra-ovarian estradiol, hypothalamic elevation of androgen content could play a critical role in inhibiting female sexual receptivity and induction of aggressive, female sexual rejection behavior.

Such extreme estradiol depletion-mediated female marmoset sexual rejection of male sexual advances, however, does not appear to implicate a generally diminished engagement in positively rewarding goal-oriented behavior. These same females, when provided with a choice between water and sucralose plus water, clearly maintained their preference for sweet taste despite substantial estradiol depletion. These findings are in contrast to estradiol-associated increases in sucrose preference for women [127] and decreases in glucose preference for female rats [128], but are comparable with the absence of change in saccharin preference for female rats with regard to estradiol levels [128]. The change in female NHP behavioral responses following estradiol depletion may thus be particularly striking with regard to sexual engagement with males.

Androgens and Female Sexual Receptivity

The varying effects of androgens on female sexual receptivity have been studied in female rodents, sheep, and NHPs. In female rodents, sheep, and rhesus monkeys, systemic testosterone and androstenedione [58, 114, 129-132], as well as hypothalamic testosterone [133], have been shown to increase or enhance female sexual behavior. In contrast to testosterone, female marmoset hypothalamic androstenedione content, as well as circulating DHEA levels, were elevated in female marmosets depleted of either ovarian estradiol alone, or both ovarian and extra-ovarian estradiol. In addition, both hypothalamic andostenedione and circulating DHEA negatively correlated with female sexual receptivity, while positively correlating with female sexual rejection.

In female rodents and NHPs, androgen effects have been attributed to the aromatization of testosterone to estradiol in the brain as a result of studies showing that nonaromatizable dihydrotestosterone antagonizes estradiol-induced sexual receptivity [58, 114, 129, 134-137]. Potential mechanisms for androgen antagonism of estradiol-mediated female receptivity have been elucidated in female rodent studies. Dihydrotestosterone inhibition of female receptivity in female rats is prevented by the androgen receptor antagonist, flutamide [138]. Additionally, androgens in the hypothalamus have been shown to antagonize estradiol activity by downregulating ERα expression and activity [139].

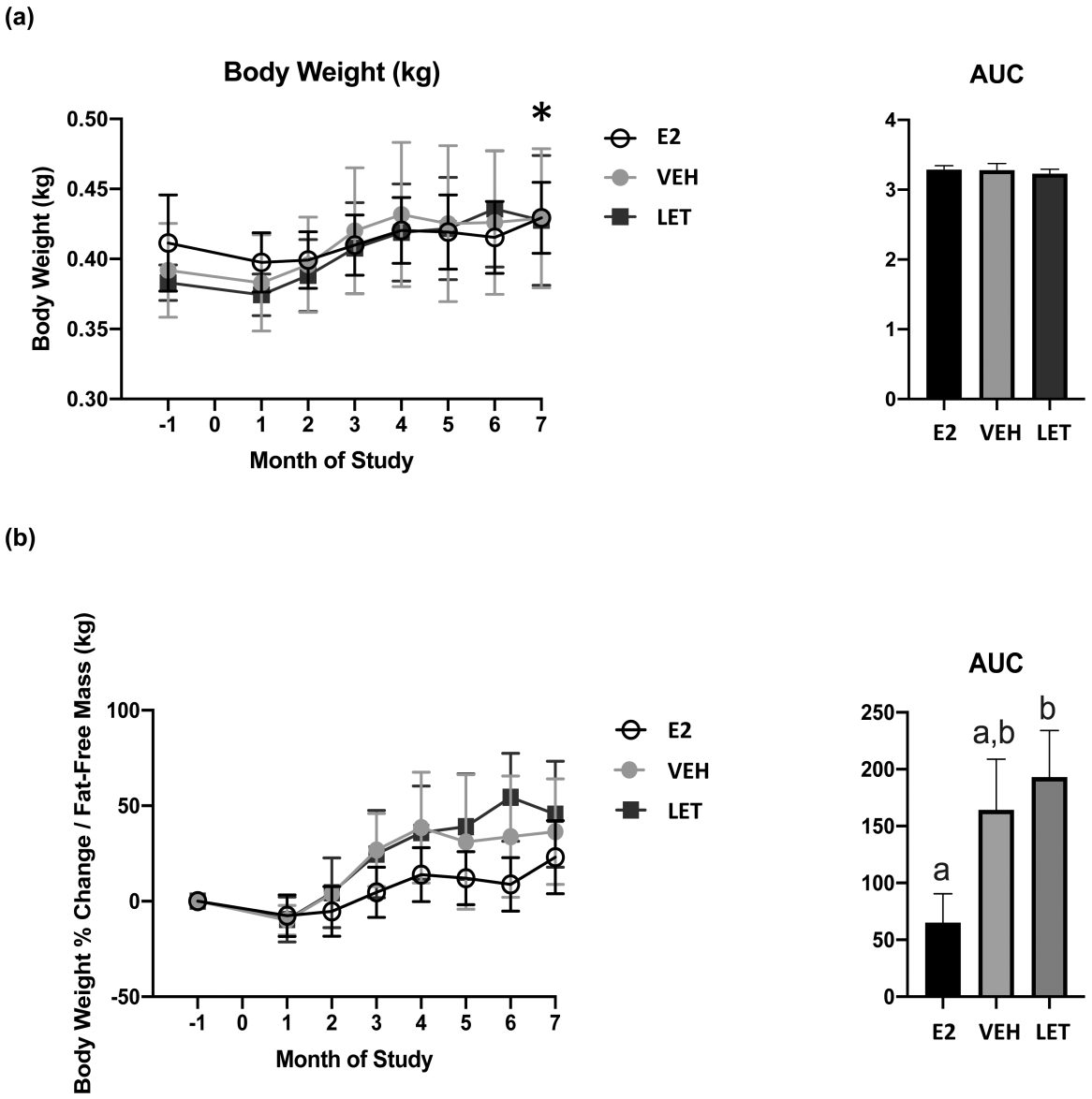

Figure 4. (A) Monthly body weights (mean ± SEM) of adult female marmoset monkeys from baseline until 7 months following ovariectomy, and (B) AUC body weights (mean ± SEM) incorporating all increments in body weight across the entire study, in E2 (open circles and black bars), VEH (light gray circles and bars), and LET (dark gray squares and bars) female groups.

In addition, in the absence of hypothalamic hypoestrogenism accompanying ovarian estradiol depletion in the present study, in contrast to aromatase inhibition-induced ovarian and extra-ovarian estradiol depletion, we suggest that estradiol in the female marmoset hypothalamus, likely locally produced [8, 16], is sufficient to antagonize androgen-facilitated female sexual rejection. Even in the absence of elevated hypothalamic testosterone content, the female marmoset hypothalamic ratio of estradiol:testosterone is only diminished by extreme estradiol depletion engaged by aromatase inhibition accompanying ovariectomy. Estradiol and testosterone in the female marmoset hypothalamus may, therefore, have opposing effects on female sexual behavior. This hypothesis is, in part, supported by studies in female rodents showing that increased estradiol can reduce the receptivity-inhibiting actions of dihydrotestosterone [140], and in female NHPs, in which dihydrotestosterone, in contrast to testosterone or estradiol, diminished female sexual receptivity [58]. Thus, in ovarian and extra-ovarian estradiol–depleted female marmosets, the combination of long-term hypothalamic hypoestrogenism combined with hypothalamic hyperandrogenism may provide 1 mechanistic explanation for robust female rejection of male sexual advances.

Ovarian Estradiol Depletion and Hypothalamic Steroid Hormones

It is important to note that the maintenance of hypothalamic estradiol content in female marmosets depleted of ovarian estradiol provides clear evidence, in a female NHPs, that estradiol concentrations in the hypothalamus are maintained independently of ovarian function. Previous studies investigating estradiol concentrations within the central nervous system of ovary intact adult female NHPs [141, 142] and women [141, 143] have reported predictable relationships between estradiol concentrations in cerebrospinal fluid and circulating concentrations of estradiol, with cerebrospinal fluid

Table 9. Mean (±SEM) regional body composition parameters as determined by dual X-ray absorptiometry (DXA) in E2, VEH, and LET female groups of marmosets at baseline and after 6 months of treatment

Body region of interest	DXA parameter	Treatment group	Baseline	6 months	Change from baseline
Total body	Fat mass (g)	E2	121 ± 17	106 ± 19	–15 ± 18
		VEH	106 ± 22	156 ± 37	38 ± 33
		LET	113 ± 23	126 ± 35	5 ± 45
	Fat-free mass (FFM)[a] (g)	E2	254 ± 15	270 ± 12	16 ± 7
		VEH	231 ± 11	251 ± 14	5 ± 11
		LET	234 ± 16	249 ± 7	22 ± 5
	Fat/FFM ratio	E2	0.5 ± 0.1	0.4 ± 0.1	–0.1 ± 0.1
		VEH	0.5 ± 0.1	0.6 ± 0.1	0.1 ± 0.1
		LET	0.5 ± 0.1	0.5 ± 0.1	–0.1 ± 0.1
	% fat	E2	31.6 ± 2.0	27.6 ± 3.8	–4.1 ± 3.2
		VEH	30.9 ± 4.2	36.6 ± 5.6	5.3 ± 6.3
		LET	32.3 ± 5.8	31.9 ± 6.3	–3.4 ± 7.1
	% FFM	E2	68.4 ± 2.0	72.4 ± 3.8	4.1 ± 3.2
		VEH	69.1 ± 4.2	63.4 ± 5.6	–5.3 ± 6.3
		LET	67.7 ± 5.8	68.1 ± 6.3	3.4 ± 7.1
Abdomen	Fat mass (g)	E2	27 ± 5	22 ± 6	–5 ± 7
		VEH	24 ± 8	43 ± 15	15 ± 14
		LET	23 ± 6	31 ± 13	19 ± 29
	FFM[b] (g)	E2	80 ± 9	85 ± 8	5 ± 3
		VEH	71 ± 8	92 ± 8	16 ± 3[c]
		LET	73 ± 5	81 ± 6	9 ± 5
	Fat/FFM ratio	E2	0.3 ± 0.1	0.3 ± 0.1	–0.1 ± 0.1
		VEH	0.3 ± 0.1	0.4 ± 0.2	0.1 ± 0.2
		LET	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.2
	% Fat	E2	24 ± 2	20 ± 4	–5 ± 3
		VEH	24 ± 5	29 ± 7	4 ± 8
		LET	23 ± 5	24 ± 7	–3 ± 6
	% FFM	E2	76 ± 2	80 ± 4	5 ± 3
		VEH	76 ± 5	71 ± 7	–4 ± 1
		LET	77 ± 5	76 ± 7	2 ± 6
Chest	Fat (g)	E2	37 ± 9	34 ± 7	–4 ± 6
		VEH	32 ± 11	48 ± 14	12 ± 10
		LET	35 ± 10	42 ± 14	1 ± 12
	FFM[c] (g)	E2	57 ± 4	61 ± 4	4 ± 2
		VEH	50 ± 4	44 ± 5	–8 ± 4
		LET	52 ± 8	52 ± 8	5 ± 7
	Fat/FFM ratio	E2	0.7 ± 0.1	0.6 ± 0.1	–0.1 ± 0.1
		VEH	0.7 ± 0.3	1.2 ± 0.4	0.5 ± 0.4
		LET	0.8 ± 0.3	1.0 ± 0.4	0.1 ± 0.5
	% Fat	E2	38 ± 5	35 ± 6	–3 ± 4
		VEH	37 ± 10	48 ± 10	10 ± 7
		LET	40 ± 11	43 ± 12	–4 ± 9
	% FFM	E2	63 ± 5	65 ± 6	3 ± 4
		VEH	63 ± 10	52 ± 11	–10 ± 7
		LET	60 ± 11	57 ± 12	4 ± 9
Upper legs	Fat mass (g)	E2	14 ± 1	12 ± 2	–1 ± 2
		VEH	12 ± 1	16 ± 2	3 ± 3
		LET	13 ± 2	11 ± 3	–1 ± 6
	FFM[d](g)	E2	48 ± 3	50 ± 2	2 ± 1
		VEH	42 ± 3	48 ± 3	2 ± 3
		LET	44 ± 3	45 ± 2	5 ± 4

Table 9. Continued

Body region of interest	DXA parameter	Treatment group	Baseline	6 months	Change from baseline
	Fat/FFM ratio	E2	0.3 ± 0.1	0.2 ± 0.1	–0.1 ± 0.1
		VEH	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
		LET	0.3 ± 0.1	0.2 ± 0.1	–0.1 ± 0.1
	% Fat	E2	22 ± 1	19 ± 3	–3 ± 3
		VEH	21 ± 2	24 ± 2	2 ± 5
		LET	23 ± 4	19 ± 3	–5 ± 7
	% FFM	E2	78 ± 1	81 ± 3	3 ± 3
		VEH	79 ± 2	76 ± 2	–2 ± 5
		LET	77 ± 4	81 ± 3	5 ± 7
Extremities	Fat mass (g)	E2	27 ± 2	24 ± 3	–3 ± 3
(lower legs + arms)		VEH	24 ± 1	29 ± 4	1 ± 4
		LET	25 ± 3	24 ± 4	–1 ± 6
	FFM (g)	E2	46 ± 2	50 ± 2	4 ± 3
		VEH	47 ± 3	47 ± 3	2 ± 4
		LET	44 ± 3	48 ± 2	1 ± 4
	Fat/FFM ratio	E2	0.6 ± 0.1	0.5 ± 0.1	–0.1 ± 0.1
		VEH	0.5 ± 0.1	0.6 ± 0.1	0.1 ± 0.1
		LET	0.6 ± 0.1	0.5 ± 0.1	– 0.1 ± 0.2
	% Fat	E2	36 ± 1	32 ± 3	–5 ± 3
		VEH	34 ± 1	38 ± 3	–2 ± 2
		LET	36 ± 4	33 ± 3	–2 ± 3
	% FFM	E2	63 ± 1	68 ± 3	4 ± 3
		VEH	66 ± 1	62 ± 3	2 ± 2
		LET	64 ± 4	67 ± 3	2 ± 3
Trunk/Extremities ratio	Fat	E2	2.3 ± 0.4	2.2 ± 0.2	–0.1 ± 0.2
(trunk = abdomen + chest)		VEH	1.6 ± 0.5	2.6 ± 0.7	–0.1 ± 0.2
		LET	2.6 ± 0.6	2.7 ± 0.1	0.1 ± 0.3
Abdomen/Upper legs ratio	Fat	E2	1.9 ± 0.2	1.7 ± 0.1	–0.2 ± 0.2
		VEH	1.5 ± 0.3	2.2 ± 0.6	0.6 ± 0.5
		LET	2.2 ± 0.6	2.4 ± 0.5	0.1 ± 0.1

6 months > baseline, all female groups combined:
[a]P = .01, [b]P = .001; [d]P = .035. Change from baseline, VEH > E2, LET: [c]P = .032.

estradiol values reflecting only 2% to 4% of those in circulation. There is selective, unsaturable entry of estradiol from the circulation into the brain, reflecting not only circulating estradiol unbound by sex hormone–binding globulin (SHBG) [144], 2% to 4% of total circulating estradiol in ovary intact adult female marmosets is not bound to SHBG [145], but also the number of hydrogen bonds estradiol forms with water [146] as well as first pass extraction of estradiol from circulation into the brain likely exceeding 80% [147]. While we did not determine unbound circulating estradiol concentrations in the present study, hypothalamic estradiol concentrations in both estradiol-depleted female groups, representing ~2 to 3×10^3 % of those in circulation, far exceed values consistent with the notion of selective entry of estradiol into the brain from unbound circulating estradiol, clearly indicating that the latter is not the sole contributor to hypothalamic estradiol content. In contrast, hypothalamic estradiol concentrations in estradiol-replete female marmosets, estimated at ~48% of those in circulation, strongly suggest considerable additional factors limiting hypothalamic estradiol content, including elevated estradiol levels stimulating elevations in circulating SHBG concentrations, thus diminishing the amount of unbound estradiol, as well as estradiol inhibition of cytochrome b_5 expression in the hypothalamus, and likely 17,20 lyase activity, of estradiol-replaced female marmosets leading to diminished hypothalamic androgens and estrogens. Selective protection of the brain from circulating concentrations of steroid hormones, likely protects local brain production of estradiol in order to enable its action as a neurosteroid independent of ovarian function [13, 148, 149].

A recent study of brain region–specific concentrations of estradiol in both ovarian, as well as ovarian and extra-ovarian, estradiol–depleted female marmosets demonstrated comparable hypothalamic estradiol concentrations (~0.1 pg/mg) [16] with those reported in this study for ovarian and extra-ovarian estradiol–depleted females, but notably less than those we report in ovarian estradiol–depleted females (~0.8 pg/mg). These study differences may reflect different approaches used to determine estradiol concentrations, LC-MS/MS (present study) compared with enzyme immunoassay (EIA) [16], since the greater specificity of LC-MS/MS determined estradiol values diminishes the likelihood of inaccuracies arising from antibody-based EIA detection [150], including lower LC-MS/MS detection limits (~0.03 pg/mg vs EIA, ~0.1 pg/mg).

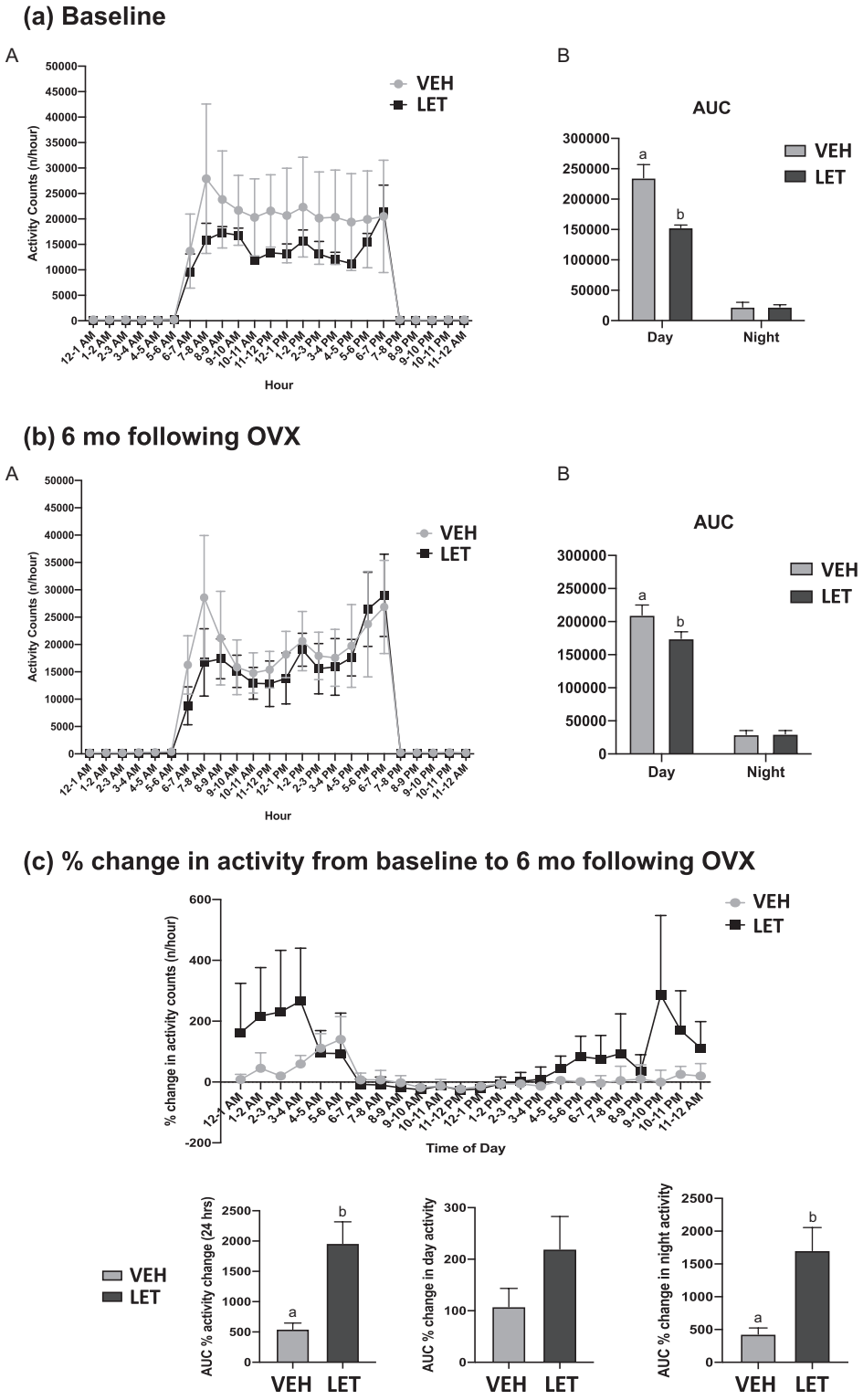

Figure 5. Actical collar determined body motion activity over 21 consecutive days (mean ± SEM) in adult female marmosets in VEH (light gray circles and bars) and LET (dark gray squares and bars) groups (a) at baseline illustrating (A) hourly activity during 12 hour daytime and 12 hour nighttime and (B) AUC activity for daytime and nighttime, a, b: $P = .001$, at (b) 6 months (months) following OVX illustrating (A) hourly activity during 12 hour daytime and 12 hour nighttime and (B) AUC activity for daytime and nighttime, a, b: $P = .01$, and (c) % change in activity from baseline to 6 months following OVX during 12 hour daytime and 12 hour nighttime and AUC % activity change for 24 hours/day, daytime only and nighttime only, a, b: $P = .01$.

Unlike hypothalamic estradiol concentrations, however, the rise in hypothalamic androgen content in females depleted of either ovarian estradiol, or both ovarian and extra-ovarian estradiol, may derive from both increased androgen production within the hypothalamus and a systemic contribution from increased circulating levels of adrenal DHEA in females

depleted of estradiol. Ovarian estradiol depletion in female marmosets has previously been shown to induce protein expression of cytochrome b_5 and a newly differentiated zona reticularis in the adrenal cortex, thus enabling steric enhancement of 17,20 lyase activity within CYP17A1 and de novo adrenal zona reticularis production and release of DHEA, increasing circulating levels [151]. The hypothalamus of female marmosets depleted of estradiol could thus utilize such an increased adrenal source of DHEA to provide increased substrate for hypothalamic synthesis of androgens, including androstenedione and testosterone.

DHEA synthesis also occurs in the brain, where it may be converted to more potent androgens and exert effects via androgen receptor activation. Alternatively, DHEA of either peripheral or central origin may exert nongenomic effects via activation of sigma 1 receptors, or modulation of $GABA_A$ and NMDA receptors in neurons and oligodendrocytes [152]. Previous studies [153] demonstrated that DHEA can enhance brain steroidogenesis as well as sexual motivation in estrogen-primed ovariectomized rats, and DHEA treatments in postemenopausal women have been shown to be effective in increasing sexual arousal and function in some studies (208), but not others [154]. Nevertheless, the inverse relationship between peripheral and central DHEA levels and sexual behavior in the present studies are not consistent with a positive effect of DHEA on sexual motivation in female marmosets. Rather, they suggest that conversion to more potent androgens and enhancement of antagonistic behavior may be the predominate effect of excess DHEA in the hypothalamus of the adult female marmoset.

In addition, the present study suggests an extension of estradiol depletion enhancing androgen biosynthesis in female marmosets to include the hypothalamus, where depletion of both ovarian and extra-ovarian estradiol increases the expression of CYB5B, 1 of 2 genes coding for cytochrome b_5 and enhancing 17,20 lyase androgen biosynthesis [155]. In an analogous mechanisms to estradiol, selective entry of testosterone from the circulation into the brain of ovary intact adult female marmosets represents ~1% to 4% of total circulating testosterone [145], comparable with ~8% in women [156]. While we did not determine unbound circulating testosterone concentrations in the present study, hypothalamic testosterone and androstenedione concentrations represent unexpectedly high 14% to 22% and 23% to 151%, respectively, of circulating values in all female groups, suggesting considerable additional mechanisms contributing to hypothalamic testosterone and andostenedione content beyond circulating androgens not bound to SHBG, likely involving local brain production of androgens involved in neurosteroid action [148]. The proportion of hypothalamic to circulating DHEA values, however, progressively diminished from 27% to 5% following progressive estradiol depletion, suggesting considerable preservation of the neurosteroid action of DHEA within the hypothalamus independent of ovarian and adrenal function.

Previous studies, however, have shown that combined adrenalectomy and OVX in marmosets did not result in the robust lack of female sexual receptivity [69, 130]; in contrast to females in the present study depleted of both ovarian and extra-ovarian estradiol, thus elevated adrenal and hypothalamic androgens may have the potential to contribute to hypothalamic hyperandrogenism and extinguish female sexual receptivity when in tandem with hypothalamic hypoestrogenism. This novel association between elevated androgens and diminished female NHP sexual behavior may not be inconsistent with previous reports demonstrating enhancing androgenic effects on female NHP sexual behavior [114, 130], and on sexual behavior in postmenopausal women [157], since the current study involves an extreme hypo-estrogenic environment in contrast to prior hormonally supplemented conditions. Consistent with this notion, the nonaromatizable androgen, dihydrotestosterone, inhibits female NHP sexual behavior under conditions of ovarian estradiol depletion [58].

With regard to estradiol depletion and its obfuscation of reproductive neuroendocrine negative feedback regulation

Table 10. Comparable volumes of sucralose solution and water (mean ± SEM) consumed by E2, VEH, and LET female groups during 3, 30-minute sweet taste tests at 6 months following ovariectomy when females were singly housed

Parameter	E2	VEH	LET	*P* value
Water (mL)	2.1 ± 0.6	1.9 ± 0.7	1.8 ± 0.3	.806
Sucralose solution (mL)	5.5 ± 1.6	2.2 ± 0.4	3.9 ± 0.7	.130
Total volume Consumed (mL)	7.6 ± 1.5	3.8 ± 0.9	6.4 ± 0.7	.099
Ratio of sucralose to Water	3.5 ± 1.5	1.5 ± 0.2	2.6 ± 0.6	.365
Sucralose consumption as % of total volume consumed	66.8 ± 12	58.9 ± 2.9	65.7 ± 4.6	.740

Table 11. Mean (±SEM) basal glucose at baseline and 6 months after ovariectomy, as well as oral glucose tolerance test (OGTT) derived glucose values at 6 months after ovariectomy in E2, VEH and LET females

Parameter	E2	VEH	LET	*P* value
Baseline				
Basal glucose (mg/dL)	114 ± 3	119 ± 3	111 ± 6	—
6 months after ovariectomy				
Basal glucose (mg/dL)[a]	120 ± 11	127 ± 9	137 ± 7	.149
AUC OGTT glucose (mg/dL*120 min)	21251 ± 2386	19380 ± 1548	20843 ± 4829	.555
2 hour OGTT glucose (mg/dL)	130 ± 16	128 ± 13	180 ± 48	.440

Abbreviations: AUC, area under the curve; OGTT, oral glucose tolerance test.
[a]*P* = .055, 6 mo > baseline, all female groups combined. SI unit conversion: glucose × 0.0551 mmol/L.

of pituitary gonadotropin release, ovarian and extra-ovarian estradiol depletion induced extreme hypoestrogenemia after 9 months, resulting in a supra-hypergonadotropic state beyond the hypergonadotropism of ovarian estradiol depletion, alone. Our current findings therefore confirm that gonadotropin-constraining estradiol production is not limited to the ovaries in female primates, but also occurs in other organ systems that include the pituitary [1, 2] and hypothalamus [8, 13]. In addition, we demonstrate for the first time in female NHPs that aromatase inhibition–induced hypoestrogenemia occurs within the hypothalamus, coincident with supra-hypergonadotropism, confirming our earlier findings in marmosets [19]. Such hypothalamically situated estradiol opens the possibility for neuroestrogens contributing toward negative-feedback constraint on release of hypothalamic GnRH, and thus pituitary gonadotropin. Whether extraovarian contribution of estradiol toward negative-feedback control of gonadotropin is another New World primate alternative endocrine and reproductive specialization [92], generated during intense selection pressure following continental separation from a common New World primate - Old World Primate ancestral lineage [92], or is a more generic primate, or mammalian, trait applicable to both sexes, remains to be determined.

In these regards, in the present study ovarian estradiol depletion, with or without accompanying extra-ovarian estradiol depletion, did not diminish expression of progesterone receptor in the female marmoset hypothalamus, as anticipated from earlier marmoset [94] and rhesus macaque studies [158, 159] in concordance with hypergonadotropism, perhaps due to a low numbers of marmosets per female group. Hypothalamic progesterone receptor expression is essential for the homeostatic regulation of hypothalamic GnRH release by ovarian estradiol and progesterone [160]. DIO did not alter expression of either hypothalamic ESR1 or progesterone receptor, as anticipated from previous studies employing female rodents [161].

Estradiol, Body Fat, and Glucoregulation

Attempts have been made in a variety of studies to dissociate effects of normal aging vs declining estradiol levels on adiposity, energy balance, and cardiometabolic health in postmenopausal women [162-167]. In general, these studies support the idea that menopause per se is associated with increasing abdominal obesity and that visceral fat accumulation may, in part, be secondary to an acceleration of aging-related decline in fat oxidation and metabolic energy expenditure [81]. While these changes parallel those observed in OVX rodents [168], a causal relationship between declining ovarian estradiol in menopause and altered body composition and energy balance has been difficult to confirm [169, 170]. Some randomized controlled studies have demonstrated that both oral and transdermal estradiol therapy in postmenopausal women are associated with a reduction in central adiposity and an increase in lean body mass [171, 172], as well as reductions in insulin resistance and fasting glucose, new-onset type 2 diabetes, blood lipids, blood pressure, adhesion molecules, and procoagulant factors [173]. Of the few studies focusing on energy expenditure during menopausal hormone replacement therapy comprising a variety of estrogenic formulations, some demonstrate increases in lipid oxidation and energy expenditure [174, 175], while others reveal acute decreases in lipid oxidation and energy expenditure [176]. There are similarly conflicting data on the effects of hormone replacement therapy on insulin sensitivity, with some suggesting beneficial effects [173], while others find no consistent improvement [177-179].

In the current female marmoset monkey study, mean baseline body weights in each female group (~400 g) were typical for this colony [98, 180, 181], while baseline total body fat exceeded 14% of body mass in all females. Body fat in excess of 14% body mass is considered obese for this laboratory NHP [182], but is typical for this colony [94]. By 9 months following OVX, however, ovarian as well as ovarian and extra-ovarian estradiol depletion resulted in DIO relative weight gain, with and without correction for FFM, as well as increased calorie consumption, in excess of the gains made by estradiol females. Estradiol, therefore, diminishes increased calorie consumption and weight gain in female marmosets in the context of DIO, as has previously been reported in mice [29, 30, 161]. There were, nevertheless, no changes in DXA-determined total, or depot-specific, body fat associated with either estradiol depletion. Such relatively modest fat accumulation in female marmosets may thus occur across a variety of specific depots.

Increased DXA-determined FFM, particularly in the abdomen and upper legs, unexpectedly contributed to combined estradiol depletion and DIO-induced weight gain in female marmosets. While neither treatment has previously been associated with gain in FFM, estradiol depletion in female marmosets also induced hyperandrogenism. Induction of hyperandrogenism in female NHPs [183] and female-to-male transexuals [184] is associated with increased FFM, particularly in upper legs in female-to-male transexuals [184], and with androgen-receptor activity in NHPs [183], but in the present study no correlations between circulating or hypothalamic androgen concentrations or ratios were associated with increases in FFM.

The actions of estradiol via ERα on adiposity may occur directly in white adipose tissue, liver, muscle, and/or pancreas [70, 74], as well as in hypothalamic neurons expressing ERα [29, 185]. The latter exert descending control over systemic organ systems via autonomic innervation [186-188], including estradiol-induced alterations in food intake and energy expenditure, producing secondary metabolic states, or by a combination of these. Stimulatory effects of estradiol on energy expenditure are transduced in ERα expressing neurons of the ventromedial nucleus of the hypothalamus [185] by nonclassical ERα signaling [29] coupled to activation of PI3-kinase [9]. Estradiol also regulates gene expression associated with regulation of food intake and energy expenditure in the hypothalamus, largely through ERα activation [30]. Furthermore, a study by Musatov et al [185] demonstrated that viral vector–mediated ERα gene silencing in the ventromedial nucleus of both female mice and rats largely recapitulates a metabolic phenotype observed in whole-body ERαKO mice, including obesity, hyperphagia, impaired glucose tolerance, and reduced energy expenditure [32-34]. In the current female NHP study, however, and in contrast to female rodents, we found no evidence of impaired glucoregulation at 6 months following estradiol depletion. We nevertheless identified a trend toward DIO-associated glucose intolerance in all female groups combined, likely the result of insufficient increase in compensatory pancreatic

beta cell insulin release to accommodate DIO-induced systemic insulin resistance. Such DIO-associated impairments of glucoregulatory function in the present study are reminiscent of glucoregulatory dysfunction reported in a previous female marmoset study employing a glucose-enriched DIO [189]. Therefore, while estradiol ameliorated DIO increased energy intake and relative weight gain, estradiol may have failed to ameliorate pancreatic beta cell insulin decompensation due to weight gain–mediated systemic insulin resistance [189]. It is unclear whether the 50% to 75% incidence of glycogenic hepatopathy (excessive accumulation of glycogen in hepatocytes) found in estradiol-depleted female marmosets in the present study represents an increase above a previously reported incidence of 34% in laboratory housed female marmosets [190]. Glycogenic hepatopathy can indicate chronic recurrence of hyperglycemic episodes [191].

Since estradiol depletion enables maturation of the female marmoset's androgenic zona reticularis in the adrenal cortex [151], and aromatase inhibition commonly results in androgenic precursor excess [19, 192], the positive correlations between hypothalamic androstenedione and androstenedione to DHEA ratio with weight gain may simply represent biomarkers for the degree and duration of estradiol depletion rather than androgenic effects per se. Long-term testosterone treatment, however, increases body weight in ovary intact prepubertal [193] and adolescent–young adult [194] as well as ovariectomized adult [183] female rhesus macaques, without glucoregulatory impairment. Furthermore, addition of DIO to testosterone treatment of adolescent female rhesus macaques exaggerates weight gain and induces glucoregulatory impairments accompanying insulin resistance [194]. The absence of glucoregulatory impairments in our estradiol-depleted and hyperandrogenic ovariectomized female marmosets would be consistent with the requirement for estradiol activity, likely in the liver, to complete androgen-mediated glucoregulatory dysfunction, as found in organ-selective androgen receptor knockout female mouse models [195].

Skeletal Bone Mass Maintained Independently of Estradiol in Female Marmosets

In almost all female mammals with regular, frequent ovarian cycles, a reduction in circulating estrogen concentrations, either spontaneous or experimentally induced, leads to a reduction in bone mass. This has been demonstrated in numerous primate species and occurs in as little as 3 months in rhesus macaques [196-200]. Therefore, it is surprising that we found no deficit in bone mass or bone density of either the total body or lumbar spine associated with systemic or systemic and hypothalamic estradiol deficiency, though, importantly, estrogen and aromatase activity within the bone microenvironment [4] were not assessed. There are several potential reasons for this finding.

Evidence for estrogen control of bone mass in common marmosets is controversial. In several experiments, we were unable to find any evidence of bone loss following estrogen depletion due to either social subordination or OVX in marmosets [180]. Similar to the conclusions of Kraynak and colleagues [94] that primates evolved metabolic control systems regulated by extra-ovarian estradiol or that are generally less subject to estradiol regulation, a conclusion arising from this study was that brain-derived estrogen may be enough to maintain bone mass even in the context of circulating estradiol deficiency. There have been some limited reports of bone degeneration in marmosets. For example, Seidlová-Wuttke and colleagues [201] found some evidence of bone loss in orchidectomized male marmosets though the level of bone loss only reached WHO criteria for osteoporosis in 2 older animals (10 and 11 years of age). It is challenging, however, to separate potential hormonal causes from overall gastrointestinal health issues, nutritional deficits, and vitamin D malabsorption [202, 203]. While marmosets may represent an intriguing model of estrogen deficiency bone loss, some caution must be taken particularly given their high circulating vitamin D levels and associated end organ resistance related to the overexpression of vitamin D response element binding proteins [92, 204]. In addition, such end organ resistance may be evidence of a broader resistance to select actions of steroid hormones. Bone in marmosets may therefore have evolved to be less subject to estrogen regulation.

Applications to Understanding Sexual Dysfunction in Women

The lack of effective treatment options for women with FSIAD or with obesity is likely directly related to the dual lack of understanding regarding NHP- and human-specific biology of female sexuality, and regulation of body weight and metabolism. With regard to sexual behavior, the present study suggests that in the absence of ovarian estradiol, extra-ovarian hypothalamic estradiol, or neuroestrogen, plays a pivotal role in maintaining sexual receptivity in female NHPs. The implications of these findings for women open up a wealth of opportunities to explore novel, central nervous system–targeted approaches to treating sexual dysfunction in women. In an analogous fashion, the lack of effective weight management for captive NHPs [205, 206] and women [207] has led to successive gains in body weight over recent decades among immature and adult female NHPs and humans alike. The findings presented in this female NHP study suggests that future treatments designed to deliver bioactive estradiol to only the central nervous system, including the hypothalamus [119], may have potential to alleviate both sexual and metabolic dysfunction.

In conclusion, this female marmoset monkey study identifies key relationships between steroid hormones in circulation and the hypothalamus, with behavior and behaviorally related hypothalamic gene expression, with neuroendocrine regulation of ovarian function, as well as with weight gain and calorie consumption that suggest possible neural mechanisms for estradiol regulation of female sexual behavior, ovarian function, and metabolic homeostasis in NHPs and women. This study also identifies a specific role for extra-ovarian estradiol, possibly neuroestrogens locally produced in the hypothalamus and other brain regions, regulating NHP, and likely human, female sexual behavior and pituitary gonadotropin release beyond ovarian influence.

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Author Contributions

M.K., M.T.F., R.A.S., J.E.L., and D.H.A. conceived and designed research; M.K., M.M.W., and M.T.F. performed experiments; M.K., M.M.W., A.L.K., A.K., M.T.F. and R.J.C. analyzed data; M.K., M.M.W., M.T.F., A.K., R.J.C., J.E.L., and D.H.A. interpreted results of experiments; M.K. and M.M.W. prepared figures; M.K. and D.H.A. drafted manuscript; M.K., J.E.L., and D.H.A. edited and revised manuscript; M.K., M.M.W., A.L.K., A.K., M.T.F., R.J.C., J.E.L., and D.H.A. approved final version of manuscript.

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Disclosure Summary

No conflicts of interest, financial or otherwise, are declared by the authors.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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