Implications for Ovarian and Extra-ovarian Estradiol Regulation of Female Marmoset Monkey Sexual Receptivity by Hypothalamic ERα

By

Marissa Kraynak

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The dissertation is approved by the following members of the Final Oral Committee:

David H. Abbott, Professor, Obstetrics and Gynecology
Jon E. Levine, Professor, Neuroscience
Elaine Alarid, Professor, Oncology
Anthony Auger, Professor, Psychology
Avtar Roopra, Professor, Neuroscience

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ABSTRACT

Sexual function is an integral aspect of women's overall health and well-being that is often overlooked. About 14% of women, regardless of reproductive status, suffer from personally-distressing sexual dysfunction, clinical diagnoses as Female Sexual Interest and Arousal Disorder (FSIAD). Despite the prevalence of FSIAD among women, however, there is only one therapeutic option that is only effective for a small number of women affected. One such reason for this is our lack of understanding of the neuroendocrine regulation of sexual function in women. Ovarian estradiol, E_2, supports the expression of female mammalian sexual behavior. In rodents, ovariectomy (OVX) abolishes female sexual behavior. Additionally, estrogen receptor alpha (ERα) in the ventromedial nucleus of the hypothalamus (VMN) has been identified as the major molecular gateway facilitating ovarian E_2-mediated sexual receptivity in rodents. In nonhuman primates, however, OVX decreases, but does not abolish female sexual behavior. The receptor mechanism in the hypothalamus has not been elucidated in any primate models. Additionally, loss of ovarian E_2 in women either due to menopause or surgically does not render total sexual inactivity. Thus, both nonhuman primates (NHPs) and women, in contrast to rodents, display a behavioral emancipation from ovarian E_2. The research presented here hypothesizes that extra-ovarian E_2, likely locally produced in the hypothalamus and acting via ERα, provides key physiological support for female sexual behavior in primates and possibly in women in the absence of ovarian E_2. These hypotheses were tested in a series of experiments manipulating E_2 production and action in a behaviorally-relevant primate species, the female marmoset monkey. In addition to identifying behavioral outcomes in response to the loss of ovarian and extra-

ovarian E_2, these studies also identified key hypothalamic gene targets of E_2 an implicated in the regulation of sexual receptivity. These hypotheses are supported and enhanced through the finding that hypothalamic E_2 can support female sexual receptivity. It is also revealed that while the neuroendocrine facilitator of receptivity is E_2 action through ERα in the VMN, the major facilitator of sexual rejection in the hypothalamus is likely through an androgenic mechanism.

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1.1 Implications for Sexual Dysfunction in Women's Health

Sexual health is an integral aspect of women's overall well-being. Up to 40% of adult women reporting sexual problems have personally distressing sexual dysfunction[1,2]. The current diagnosis according to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) however, is "Female Sexual Interest and Arousal Disorder" or FSIAD[3]. The current diagnostics take into account the complex biopsychosocial nature of female sexual dysfunction, however, until the most recent edition of the DSM-V, the disorder has not been so encompassing. For example, since its original publication in 1952, the first three editions of the DSM described criteria for female sexual dysfunction under 'urogenital disorders,' and those were confined to physiological components[4]. Any additional considerations were labeled as "frigidity" or "vaginismus", in other words, absence of behavioral or vaginal engagements with a male that would permit vaginal intercourse. The initial diagnostic criteria thus omitted inclusion of psychological aspects of female sexual desire or sexual arousal.

Much changed in the 1980's, however, when criteria for sexual dysfunction in women were considered under a new descriptor, "Hypoactive Sexual Dysfunction Disorder" or HSDD, and included psychological domains of female sexual health for the first time. These were considered as quite separate from physiological considerations, such as orgasmic disorder and dyspurenia. The current 2013 diagnostic criteria for FSIAD merge physiological and psychological domains of sexual function into one, requiring three of six signs or symptoms: 1) an absence or decrease of interest in sexual interest; 2) difficulty in initiating sex with a partner; 3) absence or decrease in responsiveness to partner; 4) decrease in sexual excitement or pleasure; 5) decrease in

responsiveness to sexual cues; 6) decreased sensation during sexual activity persisting for at least 6 months, and causing personal distress. One criticism of these diagnostic guidelines is the limitation that sexual dysfunction must be prevalent and personally distressing for at least 6 months. Previous classifications of female sexual dysfunction, such as the common HSDD, identified that clinical sexual dysfunction is a change in sexual function that is personally distressing and that this change occurs for at least 3 months, which may be a more inclusive diagnostic criteria[5]. Regardless, the most common complaint among women who have experienced problems related to sexual health is the decrease in sexual desire or libido[6]. This is true across all age groups of adult women, regardless of reproductive status. There are conflicting data, however, regarding the populations of women affected, as well as the prevalence of FSAID found in different communities and cultures. It is clear, however, that sexual dysfunction affects both pre- and post-menopausal women.

Effective treatment options for women with FSIAD, nevertheless, are lacking. There are effective options to treat physiological aspects of sexual dysfunction such as vaginal dryness, dyspurenia and orgasm disorders. This includes estrogen-based therapies[7], in post-menopausal women. For psychological symptoms of sexual dysfunction such as lowered sexual desire or arousal, there is only one FDA approved pharmacological therapeutic, a mixed serotonergic (5HT) receptor 1A agonist/2A antagonist, flibanserin (® Addyi). Flibanserin entered clinical trials as an antidepressant, but was not incredibly successful. Researchers and physicians however, noticed that the drug improved sexual health in women[8,9]. Thus in 2015, Flibanserin was approved by the FDA to treat women for FSIAD. The caveat for this therapeutic option however, is that it is only

marked for treatment of pre-menopausal women, without concurrent diagnosis of any other psychological disorders, which makes it ineffective for many of the women with FSIAD[10]. Thus, there is still a great need for better therapeutics to treat sexual dysfunction in women.

Sexual function is a universal need for many individuals and can be affected by many psychological, social, and biological factors. Sexual health and its diagnosis varies due to many aspects of a woman's life and health. Studies have shown that the quality of partner relationships and lifestyles factor into sexual satisfaction in women[11,12]. This is often also impacted by cultural norms, community and familial expectations. There are also several medical co-morbidities associated with increased prevalence of FSIAD. One major comorbidity that accounts for about 40% of women diagnosed with FSIAD is clinical depression[2]. Women taking certain antidepressant medications[13] and birth control[14,15] are also more likely to experience issues with sex. Another growing group of women particularly susceptible to FSIAD includes reproductive cancer survivors[16,17], particularly breast cancer survivors. Breast cancer alone affects 1 in 8 women[18]. The early detection of breast cancer has improved[18] and consequently 34% more women are surviving breast cancer than in the last decade[18]. Prevalence of sexual dysfunction in this population of women is increased compared to healthy women[16,17,19]. Additionally, women with endometriosis[20], type 2 diabetes [21,22], and some types of liver disease[14] are more likely to experience personally distressing sexual dysfunction.

Many of these concurrent medical conditions have increasingly become problems within Western societies, thus it's reasonable to believe that FSIAD may also

increasingly become more of a problem in the coming years. Given the lack of treatment options to treat sexual desire and arousal dysfunction in women, and the perceived increase in the prevalence of clinically distressing sexual dysfunction, we need a better understanding of the regulation of female sexual function in order to develop more effective therapeutics. This understanding will come first from controlled studies in behaviorally-relevant animal models.

1.2 Estradiol Production

17β-Estradiol (E_2) is the most common form of bioactive estrogen. E_2 is synthesized from androgen precursors, testosterone (T) and androstenedione (A4), via the CYP19A1 enzyme commonly referred to as aromatase. The major E_2 producing organ in women is the ovary, which highly expresses aromatase in granulosa cells. Aromatase is also expressed in a variety of other E_2 responsive organs, such as adipose, liver, and the brain[23,24].

1.3 Mechanisms of Estradiol Signaling

E_2 exerts physiological action through several mechanisms interacting with different receptors. E_2 binding receptors include the classical nuclear estrogen receptors (ERα and ERβ), membrane bound ERα and ERβ (mERα, mERβ), GPER (a G-protein coupled estrogen receptor formerly known as GPR30), and a novel STX receptor (STXR). These receptors enable E_2 action and elicit several different responses that can be genomic (changes in gene expression of estradiol target genes) or non-genomic (rapid intracellular changes elicited through membrane associated receptors).

The first identified mechanism for E_2 action involved classical, genomic signaling from E_2 entering a cell and binding to cytoplasmic estrogen receptor (ER), causing a

conformational change and dimerization to form a molecular complex comprising two ligand bound ERs. The ligand-receptor complex in the nucleus where it accumulates other molecules as a transcription factor by binding to specific genomic DNA sequences termed estrogen response elements (EREs). EREs are located on the genome within or near promoter regions of target genes. Within different estrogen-responsive tissues, different E_2 target genes are available for transcription. Furthermore, EREs found on different promoter regions of target genes differ, which in turn affects the affinity of ER-based transcription complexes for an ERE sequence[25]. Nonetheless, bound estradiol-ER complexes recruit protein regulators, including coactivators and corepressors[26], to the promoter region to subsequently activate or inhibit transcription of estrogen target genes. ERE genomic sites within different tissues types can also act as allosteric regulators of ERs and thus differentially regulate which regulatory proteins can bind to upregulate or diminish gene expression of that target gene[27]. In addition to the classical genomic actions of E_2 at EREs, nuclear receptors do not always bind directly to the DNA, acting directly as a transcription factor. Nuclear ERs can also act as coregulators by interacting with transcription factors on the promoter region of the target genes in order to promote E_2-dependent gene expression regulation. The majority of these signaling pathways include transcription factors such as AP-1 (ERα and ERβ;[28]) or Sp-1 (ERα only;[29]) bound to DNA at or near the promoter region of the target gene and the E_2-bound ER interacts with the DNA-bound transcription factor in order to recruit appropriate regulatory proteins (reviewed in [30,31]).

E_2 has also been found to exert rapid, non-genomic actions through receptors at the cell membrane. Membrane associated ERs (mERs), which are often associated with

membrane G_qPCRs, can elicit intracellular responses in lieu of changes to gene transcription. E_2, through mERs, exerts these rapid intracellular responses by activation of different protein kinase cascades. Some examples of these are mitogen activated protein kinase, MAPK, phospoinositide-3 kinase (PI3K), phospholipase C (PLC/protein kinase C, PKC), and cAMP/protein kinase A (PKA). Signal transduction through these kinase cascades commonly results in increased intracellular Ca^{++} concentrations depolarizing the cell membrane, thus activating voltage-gated channels, as well as enabling exocytosis[32]. These signaling cascades also exert indirect, long-term effects as well as rapid effects, including gene transcription through modification or phosphorylation-mediated activation of transcription factors that bind to estrogen target genes promoters and regulate gene transcription[33].

In addition to mERs rapid, non-genomic estradiol-mediated signaling, a further estrogenic membrane receptor was identified in various tissues, including heart and brain: G-protein coupled estrogen receptor, GPER[34]. GPER has not been as extensively studied as ERα and ERβ, this G-protein coupled receptor however, has been implicated in eliciting intracellular calcium influx and engaging the cAMP/PKA pathway[35].

1.4 Steroid Hormones, Female Reproductive Function and Behavior

E_2 has been identified as a major regulator of female sexual function in mammalian species, including humans. As much of the literature on hormonal control of reproduction and reproductive behavior is understood from animal models, it is important to discern the different ovarian phenotypes between common animal models of reproductive endocrinology. Progesterone is another steroid hormone which has been implicated in the regulation of female reproduction and sex behavior, however,

unlike E_2, the physiological role for progesterone is differs between females of different species.

Sexual behavior in research models include two general classifications of behavior: proceptive sexual behavior, or sex seeking behaviors, and receptive sexual behaviors, or sexually accepting behaviors. The species-typical behaviors that occur in the models described in this section can be found in **Table 1**.

1.4.1 Rodent Models: Ovarian Hormones and Sexual Behavior

Rats, mice, guinea pigs, and hamsters are all rodent models that are used in reproductive physiology research. They also widely contribute to sexual behavior research. In the estrous cycle, there are four phases over 4-5 days[36,37]. Metestrous and diestrus are periods of slow, steady increases of E_2. In the third phase, proestrus, E_2 levels reach a maximal level. An increase in progesterone secretion subsequently, contributes to the positive feedback on GnRH and LH necessary for ovulation to occur 12 hours later. The final day, the estrus phase, is the period of female receptivity. In these rodent models, females are only receptive towards males during the estrous phase after peak levels of E_2 accompanied by the increase in progesterone[38].

In rats, mice, guinea pigs, and hamster there is a common specific behavior elicited preceding ovulation in which the female is receptive. This behavior is termed lordosis, where the female assumes a convex-like posture in which the male can mount and intromit[39-41]. Blocking E_2 production using the transgenic mouse model, the aromatase knockout mouse (ArKO mice), abolishes the expression of lordosis behavior in females[42]. Additionally, ovariectomy (OVX)[40,41] abolishes sexual receptivity, and subsequent E_2 replacement in OVX females restores these behaviors[41,43]. These data

strongly identify E_2 as a major regulator of female sexual receptivity in rodents. In rodents, ovarian produced progesterone also plays an integral role in the specific timing of behavioral expression in female rodents and increasing its intensity.

The dynamics and timing of E_2 and progesterone in the rodent models is crucial to expression of sexual behavior and the reproductive success of females. A variety of experiments have implicated the essential role of not only the rise in E_2, but also the timing of the increase of progesterone in facilitating receptive behaviors[38,44-46]. In the rat, mouse, and guinea pig, it has been shown that E_2 in proestrus 'primes' the hypothalamus for progesterone induction of ovulation and receptivity. In the brain, E_2 functionally upregulates the expression of progesterone receptors (PRs)[47,48]. The brain regions in the rat and mouse in which PRs are upregulated include the amygdala, anterventral periventricular nucleus (AVP), arcuate nucleus (ARC), medial preoptic area (mPOA), and ventromedial nucleus (VMN) and the periaqueductal grey[49]. In guinea pigs, E_2 has also been shown to induce PRs in midbrain and POA[50]. As mentioned, the timing of this effect is crucial in rodent models. If progesterone is administered too early, it exerts an inhibitory effect, rather than a facilitating effect, thus lordosis is not expressed[38]. This is understood to be due to the lack of PR induction by E_2 a 10-12 hour process. This timing is necessary for full expression of receptive behavior in female rodents[51,52]. It has been discerned that progesterone exerts inhibitory effects on different populations of neurons than those induced by E_2 priming. Additionally, the lack of PR activation, both ligand-mediated by progesterone, as well as ligand-independent mechanisms typically through the neurotransmitter dopamine, results in a lack of sexual behaviors in the female mouse[49,53]. The inhibitory PR expressing neurons are located

in midbrain[54]. Following estrous in rats and guinea pigs, progesterone, in the absence of additional E_2 priming, then becomes inhibitory and promotes a refractory period in which, even another bolus of progesterone will not elicit a receptive response in the females[39]. The refractory period is regulated by the sequential inhibitory effects of progesterone in rats[55]. This occurs because progesterone acts a self-regulator by subsequently downregulating PR expression in the hypothalamus[44]. This steroid hormone driven regulation of behavior ensures optimal coordination of fertility and sexual receptivity for reproductive success.

1.4.2 Female Primate Reproductive Cycles

Both nonhuman primates, (NHP) and humans exhibit a variety of female reproductive cycles, and thus there are species specific differences in ovarian hormone dependency of behavior. Like small mammals, there are some NHPs, such as lemurs, lorises and tarsiers that exhibit an estrous cycle in which there is a 4-5 day, peri-ovulatory period of E_2-dependent vaginal opening and female sexual receptivity[56]. Lemurs are also highly olfactory and thus estrous is also associated with olfactory as well as visual cues from the females that increase the likelihood of copulation by males.

Female anthropoid NHPs, however, are emancipated from strict ovarian hormone control of sexual receptivity in females. Some New World Primates (NWPs), such as marmoset monkeys (model used in this thesis) and tamarins, exhibit ovarian cycles. Other NWPs, Old World Primates (OWPs), great apes and women, exhibit a menstrual cycle. Both the ovarian and menstrual cycles share similar endocrine dynamics. Both cycles have two phases: follicular and luteal phases, separated by ovulation. The follicular phase involves ovarian follicle growth phase stimulated by

gonadotropins, FSH and LH (or analogous, chorionic gonadotropin, CG in the marmoset monkey), promote the growth of ovarian follicles, that in turn produce a slow rise in E_2. Toward the end of the follicular phase, estradiol reaches a maximal level, inducing an LH (CG) surge, that ultimately leads to ovulation of the dominant follicle. Following ovulation, the corpus luteum produces progesterone and E_2 and sustains the luteal phase. Key differences between the ovarian and menstrual cycle occur in the luteal phase. In the ovarian cycle, the luteal phase that does not result in pregnancy can last twice as long as anthropoid primates with menstrual cycles[57]. The luteal phase in ovarian cycles do not end with shedding of the uterine endometrium, or menses. The timing of sexual behavior with ovulation in anthropoid primates is not as stringent as many non-primate mammals and NHPs that have estrus cycles, but sexual receptivity can change in these females depending on ovarian hormones.

1.4.3 Nonhuman Primates and Female Sexual Behavior

Extensive studies have also examined the role of ovarian hormones has been elucidated in NHP models. Sexual behavioral expression by female great apes and OWPs have demonstrated cyclical control, albeit not to the stringency seen in non-primate mammals. It has been postulated that the larger the brain and more intelligent the mammal is, the more complex sexual relationships are and consequently the expression of female sexual behavior. Gorillas[58], orangutans[59], and chimpanzees[60] exhibit an increase in proceptive and receptive behavior during mid-cycle. These great apes, however, will also display, albiet less so, sexual behaviors outside of mid-cycle. OWPs such as baboons[61] and rhesus monkeys[62] have also showed mid-cycle increases in female sex behaviors. While these phenotypes of behavioral expression

occur, there are also a degrees of ambiguity in these data. In great apes and OWPs, there are hormonally-specific phenotypic changes, such as the reddening and swelling of the anogenital regions of the body, that signal to the male conspecifics when the female is most fertile. This is commonly known as sex skin in the female[63-66]. Male mounting and sexual behavior towards the female also increases when sex skin is visible[58]. Male behavior as well as the sexual maturity of the female and the testing environment have all been shown to yield variations in our understanding for female sexual behavior in NHPs[58].

NWPs, such as common marmosets, *Callithrix jacchus*, the NHP model used in this project, have reproductive patterns of hormones and behavior similar to humans. Marmoset social structure, while different from many other troop living anthropoid primates, have behaviorally-relevant parallels to contemporary humans[67-69]. Marmosets live in stable family groups or thrive well as male-female pairmates. Females typically have one monogamous male partners[67], if they are the dominant female in a family group, or live with just their male partners. This natural relationship-like behavior is not found in rhesus monkeys or Great Apes. Taken with the similar endocrine dynamics in the ovarian cycle, these attributes enable marmosets to be a uniquely translatable NHP model to study reproductive behaviors.

Early work by Kendrick and Dixson[70] identified behavioral changes throughout the ovarian cycle of female marmosets. Similar to other NHPs in laboratory settings, there is increased peri-ovulatory expression of species-typical proceptive and receptive sexual behaviors overlapping with the mid-cycle rise of E_2. Female sexual behavior, however, is not confined to the peri-ovulatory period, thus there is no ‘estrus’.

Frequency of male marmoset mounts increase when there are female-driven solicitation or in response to a change in marmoset genital scent [71] both of which occur during the mid-cycle rise in E_2. This is different from OWPs including Great Apes in that male sexual interest can be additionally increased by the presence of reddened or swollen anogenital regions or chest.

Female marmoset sexual behavior, typical of NHPs nevertheless, diminishes in frequency following OVX-mediated E_2 depletion[72]. Female sexual behavior, however, is not abolished by OVX, in contrast to rodents and many non-primate mammals. Replacing estradiol in an OVX marmoset restores frequent peri-ovulatory levels of sexual behavior [72]. Progesterone also has an effect on female marmoset sexual behavior. Progesterone, in the absence of E_2, has an inhibitory effect on female sexual behavior. Elevated, luteal phase levels of progesterone increase rejection behavior towards male partners, a behavior that is infrequent during the peri-ovulatory phase when E_2 is high and unopposed by progesterone[72]. There has been no facilitative effect of progesterone on female sexual behavior identified in any NHP models.

1.4.4 Women: Ovarian Hormones and Sexual Behavior

Compared with NHPs, correlations between ovarian hormones and sexual behavior in women are less clear. There is evidence for sexual motivation and desire has been shown to increase during the peri-ovulatory period of their cycles[73]. This finding however, can be altered by various aspects of human social and cultural lifestyles. For example, there have been studies identifying differential outcomes between women with and without consistent sexual partners[74]. The role of ovarian hormones is much less clear in partnered women. In women with consistent partners, or spouses, incidence of

sexual activity is not confined to any stage of their menstrual cycles, whereas there is a correlation in single women for increased activity during the peri-ovulatory period[75]. There are also other factors to consider within both of these groups of women. Women that live with partners may have additional constraints, such as children and shared household duties, that dictate the timing of their sexual activity[76]. For single women, social and society pressures can also affect the frequency and timing for their reported sexual encounters. Additionally, sexual activity in women is not solely for reproductive purposes. It is not uncommonly for many women to avoid pregnancy and thus avoid sexual activity during the peri-ovulatory period of their cycles and/or use birth control that inhibits their hormonal cyclicity. Additionally, it has been shown that in women, oral contraceptives can lead to decreased sexual arousal and desire in women[15,77], which leads to another confound in studying sexual desire in women. These are all prevalent confounds win the human population.

Our understanding of how E_2 specifically contributes to sexual behavior in women is lacking. There have been conflicting data about whether[75,78] or not[79] the decline of ovarian E_2 production, or naturally occurring menopause, is associated with a decrease in libido. Utilizing animal models of behavior allows for carefully control environments and other confounding variables in order to get a clearer understanding of how E_2 may regulate different facets of sexual function in women, including an understanding the neural and hormonal contribution to sexual responsiveness and/or sexual desire/libido and the motivation to engage in sexual activity in women. All do these aspects of sexual function in women can contribute either independently or concerted together to their quality of life and satisfaction within their sexuality[80].

1.4.5 Rodents: Mechanisms of Estradiol Regulation of Female Sex Behavior

Predominantly in female rodent models, the receptor mechanisms of E_2-supported sexual behavior have been elucidated. Studies employing mutagenesis as well as pharmacological manipulations have identified which estrogen receptors (ERs) are involved in the expression of female sexual behavior. As mentioned, there are several ERs. Transgenic mouse models in which ERα[81] and ERβ[82] are deleted, have provided evidence that E_2 mediates sex behavior the mouse through ERα, but not ERβ. Studies in rats have also confirmed ERα as the major receptor mediating E_2 action through pharmacological manipulations using ERα agonist PPT, and ERβ agonist DPN[83]. Further studies in rodent models, have also contributed to our understanding of the neural location where ERα mediated effects of E_2 on female sexual behaviors. G-protein coupled estrogen receptor, GPER, has also been implicated in being able to mediate receptivity in female rodents. A GPER specific agonist, G-1, has been shown to be able to elicit lordosis behavior in female rodents similar to the effects seen by stimulating ERα[84]. It is postulated however, that the effect of GPER is additive to that of ERα in order to exert this action [85]. More research is needed to fully understand the direct and indirect effects of GPER on female sexual behavior. Thus, ERα remains as the known major receptor regulating female sexual behavior in rodents.

In the rodent brain, the hypothalamus has implicated as the major brain region responsible for regulating female sex behavior in rodents. There are three different hypothalamic nuclei, in particular, that have been implicated in playing significant roles in regulating female sexual behaviors:

(1) the ventromedial nucleus, VMN, regulates receptive and proceptive sexual

behavior [86-89],

(2) the medial preoptic area, mPOA, predominantly regulates proceptive, butreceptive, behavior[90 91] and

(3) the arcuate nucleus, ARC, plays an accessory role in regulating both proceptive and receptive sexual behaviors[92].

Studies in which the different nuclei of the hypothalamus have been either chemically or electrically lesioned have led to these conclusions in the mPOA and the VMN. To further implicate hypothalamic E_2 facilitated regulation of female sex behavior, targeted gene silencing of ERα in the VMN, abolishes the expression of sex behavior in female rats and mice[89]. Such brain region targeted silencing of ERα has also been utilized to identify behavioral roles of other brain regions. ERα silencing in the mPOA of female mice diminishes proceptive behaviors, but not receptivity[91] and additionally, has been shown to diminish other goal oriented behavior, such as maternal behavior[93].

1.4.6 NHPs: Mechanism of Estradiol Regulation of Female Sex Behavior

Unlike in rodent models, there is considerably less understanding of the mechanism governing E_2-mediated female sexual behavior in NHPs. There is no literature to date examining the E_2 receptor mechanism of sexual behavior expression in female NHPs. There have however, been studies identifying neural regions implicated in behavioral regulation. In the female rhesus monkey, there is evidence that electrical stimulation of neurons in the VMN and mPOA result in the expression of proceptive behavior[94]. In female marmoset monkeys, there have been lesion studies that have confirmed the VMN and the mPOA play roles in regulating female sexual behavior. The ARC, while implicated in rodents to play an accessory role in sex behavior regulation,

has not been examined in a NHP model. Radiofrequency lesions to the mPOA in female marmosets, interestingly, abolishes proceptive behavior, but do not decrease receptive behaviors[95]. Thermal lesions of the VMN in female marmosets, similar to the rodent literature, diminishes sexual receptivity as well as proceptivity[96].

1.5 Neurochemical Regulation of Behavior

Hormonal regulation of female sexual behavior has been established in many mammalian species. It has also been identified that endogenous steroid hormones interact with many different neurochemical systems within the brain. In the scope of sexual behavior, steroid hormones often act upon the hypothalamus and several other limbic and midbrain neurons in order to modulate neurotransmitter and neuropeptide systems that contribute to the expression of female sexual behavior.

1.5.1 Rodents: Neurochemical Mechanism of Female Sexual Behaviors

Following E_2 priming in rodent models, there are specific neurochemical changes in the brain that contribute to the expression of female sexual behavior. These studies have been used to identify neural circuits between brain regions in order to understand the complex regulation of behavior within the hypothalamus and other brain structures, in response to E_2. In rodents, the most well characterized female sex behavior is the receptive posture, lordosis. E_2 regulates its expression through a variety of signaling pathways including modulation of neurotransmitters and their receptors. These include opioid signaling and receptor dynamics, oxytocin, dopaminergic, adrenergic, and serotonergic systems[97].

One neurochemical system associated with female sexual behaviors is opioid signaling in the hypothalamus. One major effect of E_2 is on opioid receptor expression

and localization within the hypothalamus. β-endorphin stimulated opioid receptors μ, δ, and κ have all been implicated in playing major roles in the E_2 facilitated regulation of lordosis behavior in rats and mice reviewed in[98,99]. The expression and activation potentials of opioid receptors are dependent upon E_2 followed by progesterone as occurs immediately preceding ovulation. In response to OVX in rodents, opioid receptors, particularly μ and δ in the mPOA are actively recruited to the membrane and activated through E_2-stimulated signaling of β-endorphins that are produced in the ARC nucleus in neurons that project to the mPOA. The activation of opioid receptors in the mPOA exerts inhibitory effects on the VMN which inhibit lordosis behavior in female rats[100-103]. Prolonged E_2 for 48 hours or E_2 followed by progesterone, however, exert the opposite effect. E_2 reduces the transmission of β-endorphins from the ARC to the mPOA, which in turn internalizes μ and δ receptors from the membrane, removing mPOA inhibition on the VMN, subsequently facilitating the expression of lordosis[104]. At the same time, δ and κ opioid receptors in the VMN are upregulated and also play a role in facilitating receptive sex behavior expression in female rodents[100].

In addition to E_2-mediated opioid receptor dynamics, oxytocin (OT) synthesis and its receptor are also modulated via E_2 and play significant roles in both the excitatory pathways and the inhibitory pathways governing sexual behaviors in rodents[105]. The neuropeptide OT, is a prominent excitatory neuroregulator of female sexual behavior that in females is majorly synthesized in the paraventricular nucleus (PVN) . This hormone has also been implicated in having a facilitative effect on female sex behavior expression[106,107]. OT mRNA levels in the (PVN) and the supraoptic nucleus (SON) of the hypothalamus have been shown to increase with E_2 treatment in the female rat

brain[108]. OT infused directly into the VMN or the mPOA also results in receptivity in female rodents[109]. Additionally, OT receptor (OTR) expression is increased[110] and its affinity for OT is increased[106] in response to E_2 in the medial basal hypothalamus (MBH).

Dopaminergic neurons are also modulated by E_2 and implicated in facilitating female sexual behavior. Dopamine producing neuronal cell bodies lie in the striatum, ventral tegmental area (VTA) and the substantial nigra reviewed in[111]. Some of these dopaminergic neurons innervate the hypothalamus, particularly to the mPOA. In female rodents, dopamine is released biphasically in response to E_2[112], and is significantly decreased after OVX, reviewed in[111]. Particular patterns of dopamine release in female rodents are also apparent with appropriately female-paced engagement in sex. Thus, dopamine is released in the anticipation of copulation in female rodents[113].

The final major excitatory neuropeptide involved in the cascade is norepinephrine (NE). NE, a neuropeptide synthesized in the locus coeruleus, is released in the VMN and responds directly to E_2, or indirectly through other signaling pathways in order to engage both sympathetic and parasympathetic nervous systems and facilitate increased heart rate and motor outputs in the expression of female sexual behavior[114]. There are several different adrenergic (NE responsive) receptors expressed in the VMN. The activation of β-adrenergic receptors leads to an inhibition of lordosis behavior, however α_1-adrenergic receptor activation facilitates lordosis in female rodents [115]. The affinity of each of these receptors changes with E_2 priming. Rapid effect of E_2 have been strongly implicated in regulating NE signaling through changes in receptor affinity. E_2 phosphorylates NE receptors via kinase cascades in order to

change protein confirmation and subsequent affinity for NE via kinase cascades[116]. In OVX rodents, β-adrenergic receptors are activated over α_1-adrenergic receptors, leading to a decrease in lordosis behaviors, whereas in E_2 primed rodents, the opposite occurs[117]. E_2 priming also increases α_1-adrenergic receptor density, likely concurrently through classical E_2 signaling through ERα[118].

In order to facilitate the termination of female sexual behavior, there are another set of neurochemical changes that also respond to steroid hormones. These neurochemicals facilitate the satiety response following sex and mostly involve serotonin signaling. Serotonin (5HT) producing neurons are located in the dorsal raphe nucleus of the brainstem. These neurons project to the hypothalamus and the limbic system, facilitating the 'satiety response'. There are also 5HT neuron projections that innervate the spinal cord and the cerebral cortex to engage physiological responses to sex[119]. Depletion of 5HT in the mPOA results in an increase in lordosis behavior in female rodents[120]. E_2, however, has not been particularly implicated in altering 5HT production, but rather modulating both the availability of 5HT in synapses[119] and also increasing receptor density in the mPOA and VMN[119]. There are a variety of 5HT receptors expressed in the hypothalamus that facilitate behavioral inhibition. The two major groups of 5HT receptor (5HTR) subtypes implicated in the regulation of female sex behavior are $5HT_1$ receptors (particularly $5HTR_{1A}$) and $5HT_2$ receptors (particular $5HTR_{2A}$ and $5HTR_{2C}$), which inhibit and facilitate sexual behavior expression, respectively. One inhibitory receptor is $5HTR_{1A}$, has been largely implicated through pharmacological manipulations utilizing the agonist 8OH-DPAT [121,122]. The 5HT receptors have similar dynamics to those observed in the E_2 mediated changes in

adrenergic receptors. In E_2 primed females, there is a decrease in $5HT_{1A}$ receptor subtypes and an increase in $5HT_{2A/C}$ receptors, facilitating receptivity. The opposite is true in OVX-mediated E_2 depletion[123]. Other 5HT receptors have been implicated in more minor roles in inhibiting female sexual receptivity, such as $5HT_3$, $5HT_5$ and the most recently classified $5HT_7$. It is not understood if these subtypes are E_2-dependent.

1.5.2 NHP: Neurochemical Mechanism of Female Sexual Behaviors

The coordination of reproductive steroid hormones and neuropeptides has been much less extensively studied in NHPs than in rodents. However, there is some parallel data to suggest that crosstalk also exist between the steroid hormones and neurotransmitter systems. One such example is the OT system. The neuropeptide OT is highly conserved between smaller mammals, NHPs and through to humans[124]. OT has been implicated in many facets of behavior in female NHPs, including in promoting sexual motivation and also in pro-sociosexual behaviors, such as partner preferences and bonding. Another neural peptide implicated in the facilitation of female sexual behavior in the female marmoset monkey is GnRH II. GnRH II stimulates proceptive sexual behavior in female marmoset monkeys, regardless of peripheral E_2 levels[125]. Additionally in the marmoset monkey, inhibition of sexual behavior by $5HTR_{1A}$ agonist treatment, similar to female rodents has also been shown[126].

1.5.3 Implications for Associated Neurotransmitter Systems and FSIAD

In women, these select neurotransmitters have been identified as playing multiple, and sometimes conflicting roles in the pathology of sexual dysfunction. Intranasal OT has not been shown to improve sexual dysfunction in women [127]. This method of OT administration in women, however, has been shown to increase social behaviors and thus appears to modulate pro-social behaviors rather than overt regulation of sexual

responses and desire in women. As mentioned, depression and associated altered serotonin metabolism are concurrent with FSIAD. Thus, it is not surprising that the only pharmacological treatment available for women with sexual dysfunction is a serotonin receptor modulator. Flibanserin is a $5HT_{1A}$ agonist and a $5HT_{2A}$ antagonist, that leads to improvements in FSIAD. Flibanserin blocks the inhibitory effects of serotonin while simultaneously enhancing the facilitative effects of serotonin, particularly those (through blockade of $5HTR_{2A}$) that facilitate an enhanced dopamine effect in the brain[128].

In women with FSIAD, particularly those with issues relating to the initiation of sex and altered sexual desire, it is possible, but not studied, that dopamine signaling is impaired. Enhancements of dopamine, via dopamine agonist, which can occur in women treated for Parkinson's disease, can lead to abberant hypersexuality, as well as an increase in other reward seeking behaviors, such as increased gambling and drug use[129]. Thus, it can be speculated that, much like observed in female rodents, blunted dopamine signaling be part of the neuronal alterations leading to FSIAD in women.

1.6 Endocrine Differences in New World Primates

The NHP model used in this thesis is the NWP, the marmoset monkey. While the endocrine dynamics of reproduction are similar to other NHPs and women, it is important to consider the evolution consequence of steroid hormone resistance unique to NWPs. Ovarian production of E_2 in NHPs, like other mammalian species including humans, is centrally regulated by the hypothalamus and pituitary (HP-axis). Gonadotropin-releasing hormone (GnRH), a peptide hormone synthesized in neurons in the ARC nucleus and projecting to the median eminence, is released into the hypophyseal portal veins, and circulates only to the anterior pituitary gland, causing

systemic release of pituitary peptide hormones, gonadotropins, that in turn stimulate the production of E_2 from the ovaries. A notable difference in the female marmoset is that marmosets do not have the same pituitary gonadotropin output as other mammalian species. Possibly due to exaggerated expression of a previously rare gene variant of the 12-exon gene coding for primate luteinizing hormone/chorionic gonadotropin receptor type 1 (*LHCGR type 1)* marmosets, typical of NWPs, only express *LHCGR type 2* (affinity for CG binding, alone) that omits exon 10 of *LHCGR type 1*[130]. In addition, their pituitary gonadotropes, while retaining transcriptional responsiveness to GnRH, only express the β subunit for CG instead of LH[131]. Marmoset gonadotropes utilize a different promoter sequence than the placenta [132], limiting the addition of a novel exon 1 to placental CG, alone.

NWP also exhibit endocrine traits that are distinctly different from other mammalian species, including other NHPs and humans. These traits likely arose during rapid, adaptive selection pressure from undetermined causes, following separation from the OWP lineage, resulting in successive gene changes in a multitude of regulatory systems[133]. One obvious global change includes end-organ resistance to steroid hormones resulting in exaggerated production of NWP steroid hormones driven by increased intracellular concentrations of specific (1) heat shock protein (hsp) chaperones, diminishing steroid hormone binding to intracellular cognate receptors [134,135] [136], and (2) heterogeneous nuclear riboproteins (hnRNPs) that out compete receptor-bound, steroid hormone transcription complexes for DNA response elements, blocking transcription[137-140]. The generic NWP solution to these global endocrine changes has been compensatory increases in endogenous, bioavailable, circulating

steroids [141] sufficient to overwhelm extensive intra-cellular barriers to biological action in target organs in order to engage homeostatic actions[69].

1.7 Objectives

The overall theme of this thesis is to discern and identify the contribution of ovarian and extra-ovarian E_2, likely neurally produced, E_2 on female sexual behavior in a NHP model that shares key translatable features related to sexual behavior in women. Additionally, this thesis aims to identify if the receptor, ERα implicated in regulating sex behavior in female rodents, is conserved in a NHP model. This thesis contains three main objectives to test this. **Objective One (Chapter 2)** serves to test several aspects of ovarian E_2 on female sexual behavior regulation. These experiments aim to test the hypothesis that in well-established male-female marmoset pairs. Additionally, this experiment will test if the loss of ovarian E_2 will lead to altered mRNA expression of ligands and receptors of other neurochemical systems in the MBH that have been implicated in E_2-regulated female sexual behaviors. **Objective Two (Chapter 3 and 5)** focuses on utilizing an aromatase inhibitor to understand the physiological function of extraovarian E_2 on female reproduction. The first part of this objective (**Chapter 3**) aims to identify a functional role of extraovarian E_2 on hypothalamic-pituitary regulation. Subsequently, (**Chapter 5**) examines a role for extraovarian, likely neurally produced, E_2 on female sexual behavior and associated behaviorally-related gene expression in the hypothalamus. Finally, **Objective Three (Chapter 4)** will identify whether hypothalamic ERα, the receptor majorly identified in rodents to regulate female sexual behavior is similarly engaged in a NHP model. **Chapter 6** serves as an additional results chapter that combines previous chapter findings in a qualitative sequence

analysis in order to draw broad conclusions and hypothesize a theoretical model for the components of E_2 mediated patterns of female sexual behavior.

1.8 Tables and Figures

	Female Rodents	*Female Marmoset Monkey*
Proceptive Sexual Behaviors	Ear wiggling, hops and darts	Proceptive stares, postures
Receptive Sexual Behaviors	Lordosis posture	Receptive posture

Table 1: Female rodents and marmoset monkeys both have species specific sex seeking, or proceptive behaviors and sexually accepting, or receptive sexual behaviors.

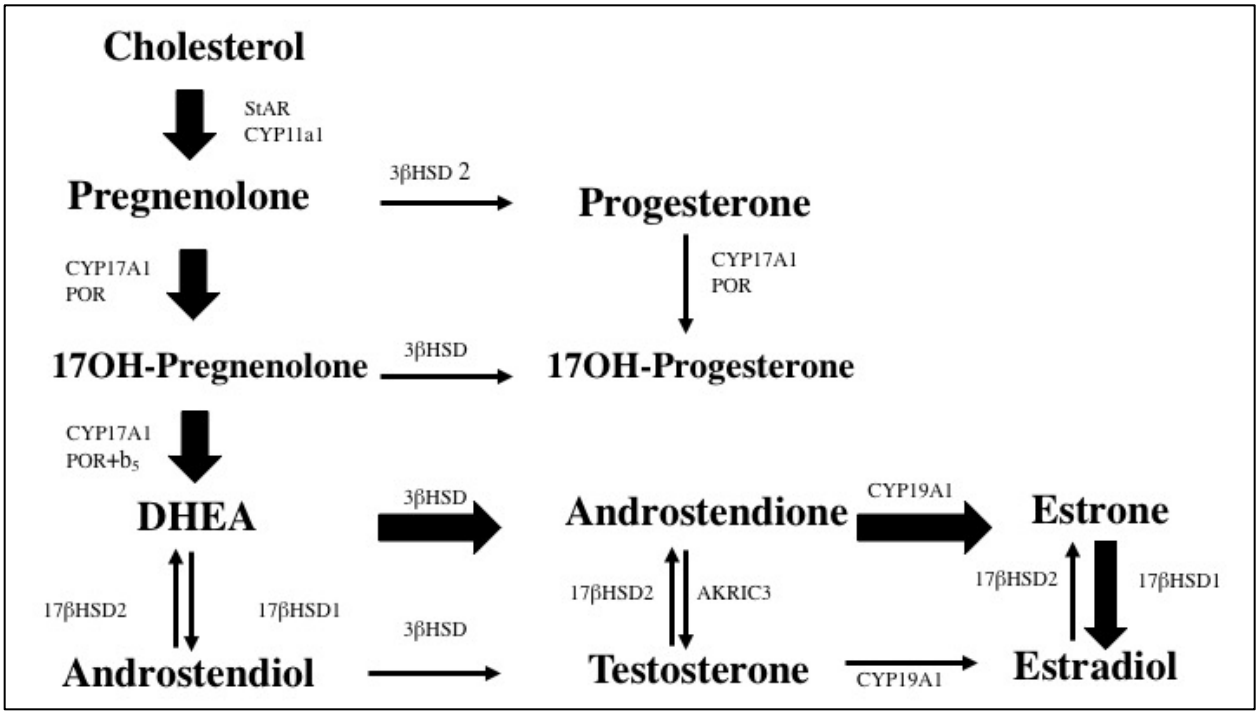

Figure 1: Bolded arrows depict the delta five pathway which is favored in steroidogenic tissues of primate species, including the female marmoset monkey.

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2. CHAPTER TWO: Depletion of ovarian estradiol leads to a decrease in female sex behavior and hypothalamic progesterone receptor mRNA in the female marmoset monkey

Marissa Kraynak, Ricki J. Colman, Matthew T. Flowers, David H. Abbott, Jon E. Levine

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2.1 Abstract

Estradiol (E_2) has been long implicated in the regulation of female sexual receptivity in many mammalian species. In rodents, the neural gene targets of E_2, such as progesterone receptor, dopaminergic receptors, and serotonergic receptors have been identified and functionally linked to the regulation of sexual receptivity. In the marmoset monkey, the mid-cycle rise in ovarian E_2 results in an increase in receptive sexual behaviors, however, the behaviorally related gene targets of ovarian E_2 have not been examined. As these monkeys provide an ethologically relevant model for sexual dysfunction in women, they provide an opportunity to examine the effects of E_2 replacement on sexual support and additionally identify any mRNA gene expression changes of known behaviorally-related genes. In many studies, however, including marmosets, females are placed with males only during behavior testing. This is of concern in marmosets as long-term, male-female pairbonds commonly form the core of social groups. Single housing and intermittent testing prevent species-typical interactions between behavior tests, and limit social and sexual interactions to conspecifics of little familiarity. We thus propose to use pair-housed, well-familiarized male and female marmosets to determine the impact of diet and E_2 on female sexual receptivity and associated neural mRNA gene expression changes. Fifteen female common marmosets (3-6 years) with well-established male pairmates were ovariectomized (OVX) and implanted with capsules that were E_2-filled (E2) or empty (OVX). Behavioral observations were recorded 2 and 5 months post-OVX. A 90-minute (min) separation period preceded each 15-min behavioral test. Observations were validated between and within observers and digitally recorded on JWatcher. Sexual

receptivity decreased and rejection increased in OVX females ($p<0.02$) at 5 months post-treatment. Hypothalamic progesterone receptor mRNA (*PGR*) expression was diminished due to OVX ($p=0.01$), however, no other behaviorally-related gene expression differences were observed. Using well-established, male-female pairs as opposed to singly housed monkeys, appears to slow progression to diminished E_2 support of sexual receptivity (5 months vs 2 weeks). Our results, however, still indicate a supportive role for E_2 in female sexual receptivity and hypothalamic *PGR* expression in this more ethologically relevant paradigm.

2.2 Introduction

The prevalence of sexual health issues among adult women has increased over the past decade. About 40%[1,2] of women have clinically diagnosed sexual dysfunction. The clinical diagnosis, Female Sexual Interest and Arousal Disorder, FSIAD, according to the DSM-V[3], is diagnosed from three out of six diagnostic symptoms ranging from physical difficulties with sex, such as vaginal dryness and pain, to low desire and difficulty initiating sex, that are personally distressing about 75% of the time for at least 6 months. FSIAD is concurrently diagnosed with a variety of medical co-morbities that are also becoming more prevalent in western cultures such as depression[2], type 2 diabetes[4], and survivors of reproductive cancers[5,6]. There are few treatment options available for women who suffer from FSIAD. These include psychological therapies and one pharmacological option, flibanserin (Addyi, FDA approved 2015)[7]. The present treatment options are only mildly effective for pre-menopausal women not being treated for depression, which consequently is a small percentage of the women suffering from FSIAD[8]. Thus, the need for better therapeutics is evident.

In many mammalian species, including human and NHPs, ovarian E_2 has been identified as a key regulator of female reproductive behaviors. In women, libido has been reported to fluctuate throughout the natural menstrual cycle, peaking mid-cyle [9]. Increased frequency of sexual behaviors have been shown to occur around midcycle in non-human primates, such as Great Apes [10-12], baboons [13], rhesus monkeys [14], and the marmoset monkey[15]. Additionally, research in the marmoset, the animal model used in this study, has shown marked decrease in sexual behavior expression in response to ovariectomy, OVX[16].

Ovarian E_2 is necessary for any expression of female sexual behavior in rodents[17,18,19]. In the rodent models, the mechanism for how E_2 facilitates the expression of sex behavior has also been elucidated. Estrogen receptor alpha (ERα)[20] [21-25] mediates the majority of E_2 regulation of sexual behavior in female rodents. Whether ERα is the receptor mechanism governing behavior in NHP and/or women has yet to be determined.

The neuropeptide hormone oxytocin, OT, has been implicated in the facilitation of female sex behavior expression [26,27]. OT mRNA levels in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus have been shown to increase with E_2 treatment in the female rat[26]. Additionally, OT receptor, OTR, expression is both increased[28] and the affinity for OT is increased[29], in response to E_2 in the medial basal hypothalamus (MBH).

The dopaminergic neurotransmitter system has also been implicated in facilitating female sexual behaviors. E_2 has been shown to increase dopamine in the dorsal striatum and also has been shown to increase a key dopamine receptor, D2, in

the hypothalamus[30]. Additionally, pharmacological blocking dopamine receptors in the hypothalamus, particularly the medial preoptic area (mPOA), reduces the expression of sexual behavior in the female rat, whereas a dopamine agonist induces receptivity[31].

In addition to excitatory changes in the hypothalamus, there are also neural inhibitors of female sexual behavior. The inhibitory neurochemical modulations include progesterone and the neurotransmitter serotonin (5-HT). In the rodent, E_2 induces the mRNA expression of neural progesterone receptors (PR)[32]. Additionally, in an OWP model, the rhesus monkey, PR protein is upregulated by E_2 in the hypothalamus[33]. The mRNA expression changes in PR have not been examined in a primate brain prior to this study.

Progesterone's role in the regulation of female sexual receptivity differs between rodents and primates. Progesterone, in a female rodent brain, has both stimulatory and then inhibitory action to regulate female sexual behavior[34]. In primates, however, no such excitatory role of neural progesterone action has been elucidated, but rather progesterone has been shown to have inhibitory actions on female receptivity[16]. Despite the many different 5-HT receptors expressed in the brain, the most well-studied 5-HT inhibitory pathway implicated in blocking or reducing female sexual receptivity are located in the MBH, particularly the VMN, is 5-HT_{1A} receptor [35]. In the presence of E_2, the 5-HT_{1A} agonist, 8-OH-DPAT, has been shown to reduce sexual receptivity in female rodents[36,37], and also in the female marmoset monkey[38]. In the rodent however, the effect of 8-OH-DPAT can be attenuated by long-term, large doses of E_2 [39,40]. Other classes of 5-HT receptors have been studied, however, the results on inhibition of female sexual behavior are ambiguous and inconclusive[41].

The female NHP model used in this study has a reproductive system closely paralleling that in women, including ~28-day ovulatory cycles that feature E_2 surges during the peri-ovulatory phase and exhibition of patterns of sexual behaviors that are translatable to those observed in women. Most notable is female engagement in sex with the male partner throughout the ovarian cycle [15]. In the present study, we investigated the role of ovarian E_2 on female sex behavior specifically in females housed with well-establish male partners. Apart from the practical reasons to perform this study in marmosets (i.e., ease of handling, compressed life cycle), we chose to study this New World (NW) monkey because marmosets also exhibit predictable sexual behavior responses to OVX and E_2 treatments. Notably, however, previous studies identified the role of ovarian E_2[15,16] on female sexual behavior in singly-housed females. It is known from other studies in NHPs that the testing environment. including an established relationship with the sexual partner and degree of sexual maturity, can modulate behavioral outcome[42]. Thus, the present study hypothesizes that in female marmoset monkeys, physiological E_2 replacement will be sufficient to maintain the expression of female sexual behaviors. Additionally, we hypothesize that similar to female rodents, E_2 treatment will also elicit transcription related changes in the hypothalamus. As shown in female rodents, E_2 -mediated sexual receptivity in the marmoset monkey is concurrent with an increased expression of mRNA levels for dopamine and for oxytocin receptor, along with a decrease in select serotonin receptors in the MBH.

2.3 Methods

Animals

Sixteen adult female common marmosets (2-6 years of age) from the Wisconsin National Primate Research Center colony were evenly randomized based on age, body weight into two groups (Table 1): E2 replaced (E2; n=7) and E2 depleted (OVX; n=8). Following onset of experiment, one female was excluded from the study. Animals were maintained in these groups for 6 months after which they went to necropsy to permit tissue collection for gene expression analysis.

All animals lived with a cagemate in 0.60m x 0.91m x 1.83m enclosures and were maintained with 12-hour lighting, 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 and estrogen replacement

Following baseline assessments, bilateral OVX was performed in all females to provide gonadal hormone deficiency. Cloprostenol (Estrumate®, 0.75-1.50 μg intramuscular injection for two successive days approximately 11-60 days after ovulation), an analog of prostaglandin-F2-alpha, was administered prior to OVX to facilitate scheduling of OVX during the follicular phase. At the time of OVX, either empty or E_2-filled (to maintain consistent peri-ovulatory E_2 levels, E_2 replaced) silastic capsules were implanted subcutaneously. Silastic capsules were removed and replaced at 3 months post-OVX to maintain consistent E_2 levels. At this time, empty capsules in the OVX groups were replaced as well to maintain consistent conditions among the groups.

As an indicator of functional E_2 depletion, uterine dimensions were obtained monthly by transabdominal ultrasonography. Using the scanner's calibrated, digitized calipers, uterine trans-fundus length (transverse uterine diameter) and dorso-ventral uterine diameter were measured from transverse views, and fundus-cervix length was measured from sagittal views.

Hormone Assay

For steroid hormone analyses, plasma samples underwent extraction and subsequent analysis on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (LC-MS/MS) as described in[43] and Chapter 3.

Behavioral Observations

Following treatment onset, pairs were acclimated to the testing cages, as previously described[44]. Behavior testing took place at two and five months post-treatment onset. There were two, 15-minute tests per week for two weeks. Well-established (paired for at least 6 months) male-female pairmates were used. Pairs were deprived of visual and olfactory contact with one another for 90 minutes immediately before behavioral observations were taken. Following the 90-minute separation period, males were placed in a holding box for 5 minutes before being allowed into the main testing cage with the female. Observations were recorded digitally using JWatcher and via manual scoring of behaviors for 15 minutes. Each test was recorded and inter- and intra-observer reliability was 80% or greater.

Gene Expression Analysis

Medial basal hypothalamus tissues were dissected and frozen at necropsy. 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 PCR 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 E2 treated group. Primer sequences were designed using NCBI Primer-Blast[45] and are listed in Table 2.

Statistical Analysis

Data collected were analyzed utilizing SPSS software. Repeated measures ANOVA were used to analyze data collection that repeated throughout the duration of the study. Behavioral observations and gene expression data were analyzed with an ANOVA test to compare means. All data are expressed as mean+/- SEM. Statistical significance was determined at $p \leq 0.05$.

2.4 Results

Reproductive Physiology

Females that received E_2 replacement sustained peri-ovulatory phase levels of E_2 over the course of the study, whereas OVX diminished E_2 to ~10pg/ml, as shown in Figure 1A ($p=0.003$). The achieved level of circulating E_2 in the E_2 replaced females maintained pre-OVX uterine diameter, a key indicator of effective ovarian E_2 replacement, whereas OVX resulted in a diminished uterine diameter over the 6-month experiment ($p=0.01$, Figure 1B). Additionally, E_2 replacement physiologically supported the expression of female sexual behaviors in E_2 replaced females. Figure 2 shows that

by 5 months post OVX, depletion of E_2 leads to an expected decrease (~50%) in sexually receptive behavior (Fig. 2A; p=0.04) and a complementary increase in species-characteristic sexual rejection behavior towards the male partner's mounting behavior (Fig. 2B; p=0.04).

Hypothalamic Gene Expression

Gene expression analysis, via qPCR, of the medial-basal hypothalamus showed that E_2 depletion, regardless of diet, resulted in a decrease in the expression of *PGR,* which encodes progesterone receptor (Figure 3, p=0.01). Other selected, E_2-regulated, behaviorally-related genes did not exhibit any E_2 dependent changes in hypothalamic mRNA expression (Figure 4 A-D). There were also no statistical differences in correlations between mRNA expression of different behaviorally related genes.

2.5 Discussion

Ovarian E_2 has been implicated as a major regulator of female sexual behaviors in several mammalian species. Similar to existing studies[16], the present study also shows that OVX-mediated E_2 depletion decreases sexual receptivity in the female marmoset monkey. Additionally, the decrease in sexual receptivity was observed in females housed full time with well-established male pairmates, a more socially relevant environment. Thus, these results can be more readily translatable to women. Studies in other NHP models have shown that various environments have varying effects on sexual receptivity. Previous to this study, marmoset studies identifying the role of E_2 in regulating female sexual behavior were performed using singly housed females. Additionally, OVX alone did not decrease receptivity compared with E_2 replaced females until 5 months post treatment onset.

In the present study, there was a decrease in hypothalamic progesterone receptor, *PGR*, mRNA expression in the marmoset monkey in response to OVX-mediated E_2 depletion. This has been shown in rodents[32,46], however, E_2 regulation of mRNA or protein expression of PR has not been shown in the marmoset monkey prior to this study. Progesterone regulation of female sexual behavior in rodents is dynamic. Progesterone in rodents can elicit both inhibitory and facilitative functions via hypothalamus and on behavior[34,37]. In female primates, there has been no evidence for a facilitative function of progesterone in relation to female sexual behavior. Progesterone in the marmoset monkey[16] is associated with diminished sexual receptivity and increased sexual rejection behaviors. In marmoset females, this has been examined through progesterone treatment in OVX females [16] as well as the decreased expression of receptivity during the luteal phase of ovarian cycles[15]. On the contrary, in other NHP species such as Great Apes[10] and OWPs[47], hormone induced attractivity, male dominance and behavior towards the female, as well as the testing arena, confound the measures of sexual receptivity throughout the menstrual cycle, effects which are reviewed in[48]. Thus, due to these experimental and species-specific constraints, the role of progesterone in larger NHPs has not been examined under controlled experimental conditions.

In addition to examining E_2 regulation of hypothalamic PR mRNA expression, behaviorally-related genes in the MBH were also analyzed in this study. Despite a decrease in sexual behavior expression, hypothalamic mRNA expression of behaviorally related genes that were implicated from work in rodents, was not altered in response to long-term OVX. There are several explanations for this outcome. While the

ovaries are the major source of E_2 production in female primates, other extraovarian tissues such as adipose, bone, and the brain [49], also express aromatase (CYP19A1), the enzyme required to synthesize E_2 from androgen precursors. Thus, it is possible that locally produced E_2 in the hypothalamus may be sufficient to maintain E_2 primed mRNA expression of the hypothalamic receptors for neural reward systems, even in the absence of the ovaries. This suggests that in order to alter mRNA expression of behaviorally-related genes in a female primate, and possibly women, E_2 produced locally in the brain must also be altered.

While it is possible that the emancipation from ovarian E_2 occurs in many anthropoid primates, and in women, there are marmoset specific considerations. A naturally occurring, and reversible, hypoestrogenic state is observed in socially subordinate females marmosets [50,51]. A key feature of subordinate female marmosets is ovarian suppression. During ovarian suppression in female marmosets, circulating E_2 is low, yet female marmosets do not exhibit typical hyoestrogenic phenotypes, such as loss of bone mass (Colman, personal communication) or altered metabolic function[52](Appendix 2). Thus, it is also possible that in this species, extra-ovarian E_2 or E_2-independent mechanisms are sufficient to maintain physiological function that would otherwise be altered due to hypoestrogenism.

A final consideration is that ovarian E_2 may be modulating mRNA alternately between discrete nuclei of the hypothalamus, thus the analysis of the entire MBH would not be sufficient to detect changes in receptor and peptide expression. Additionally, it is possible that E_2 regulation of hypothalamic reward pathway genes could also occur

post-translationally, possibly through rapid, membrane ER-initiated signaling via kinase cascades.

In summary, ovarian E_2 plays a significant role in the expression of female sexual receptivity in the marmoset monkey, when housed with a well-established pairmate. Additionally, E_2 mediates the hypothalamic mRNA expression of *PGR*, but not other behaviorally related genes. These data suggest that while ovarian-produced E_2 is clearly a major regulator of sexual receptivity in female marmosets, there is another extra-ovarian, possibly hypothalamic E_2-mediated, mechanism that can sustain sexual receptivity in a female marmoset, and possibly in women and other NHPs.

2.6 Tables and Figures

Treatment Group	Age (yrs)	Body Weight (g)
E2	3.53 ±0.40	420.84 ±25.59
OVX	3.44 ±0.43	385.13 ±14.39

Table 1: Females in both treatment groups were of similar ages and body weights prior to study onset.

Gene	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
PGR	CCCTGCATGTCGCCTTAGAA	ATTTGGAATGCCCACTGGCT
TBP	CCATGACTCCTGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA

Table 2: Primers used for qPCR gene expression analysis.

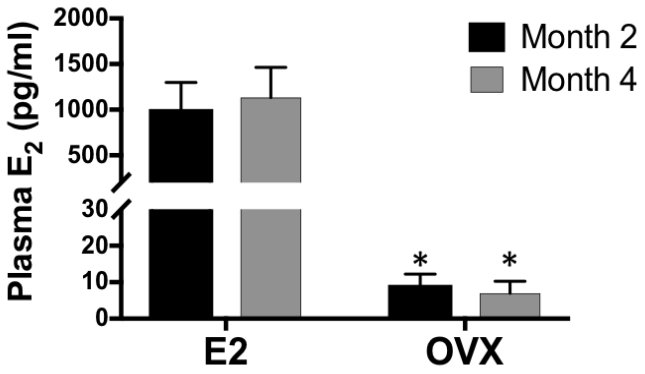

Figure 1: Estradiol, E_2, was diminished in the OVX group. Periovulatory levels of E_2 (1.0 +/- 0.3 ng/ml at 2 months; 1.1 +/- 0.3 ng/ml at 4 months) were obtained with subcutaneous capsules throughout the study.

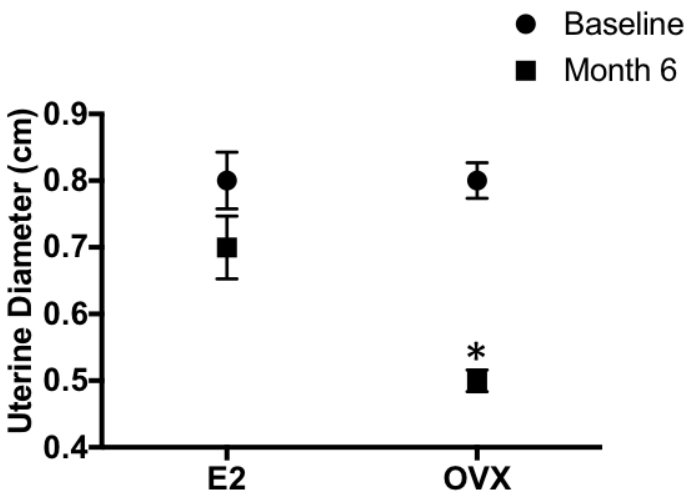

Figure 2: After 6 months of treatment onset, uterine diameter was diminished in OVX females ($p<0.01$). E_2 replacement maintained pre-OVX uterine diameter.

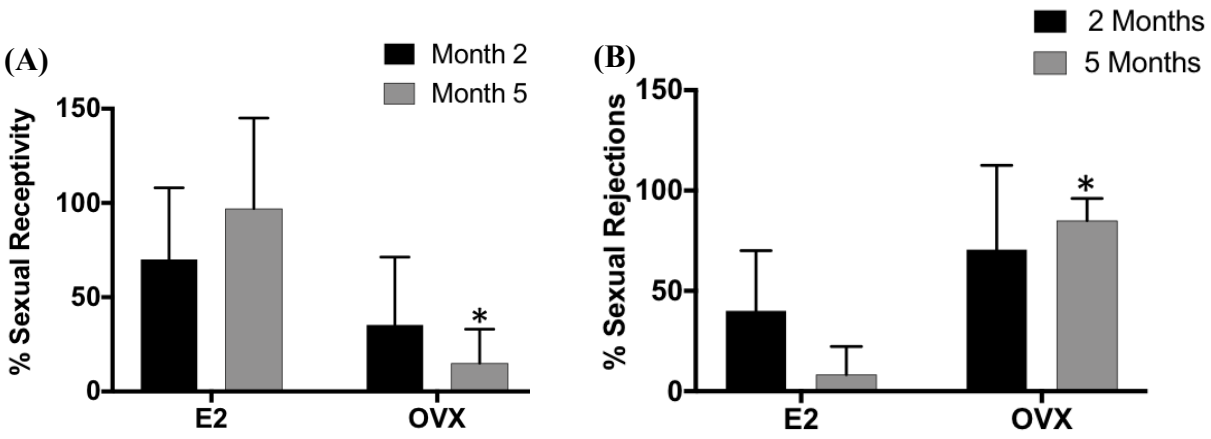

Figure 3: (**A**) Only after 5 months of treatment onset, OVX-mediated E_2 depletion induced diminished expression of sexually receptive behaviors ($p=0.04$) compared to E_2 treated females. (**B**) OVX females, complimentary to the receptivity decline at 5 months post treatment onset, exhibited an increase in rejection behavior towards their male partners ($p=0.04$).

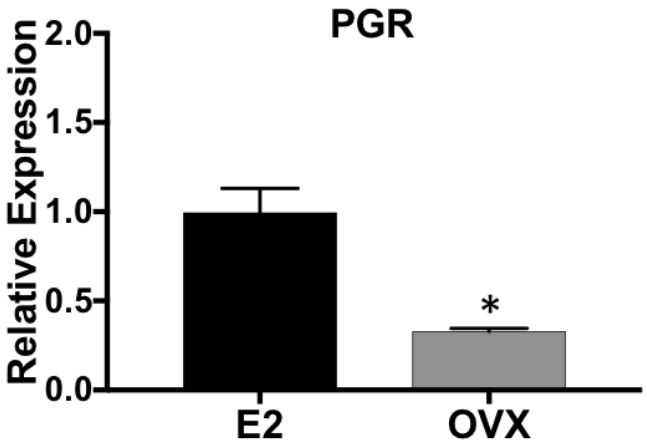

Figure 4: qPCR-determined mRNA expression of progesterone receptor (PGR) was diminished in OVX compared to E_2 replaced females ($p<0.01$).

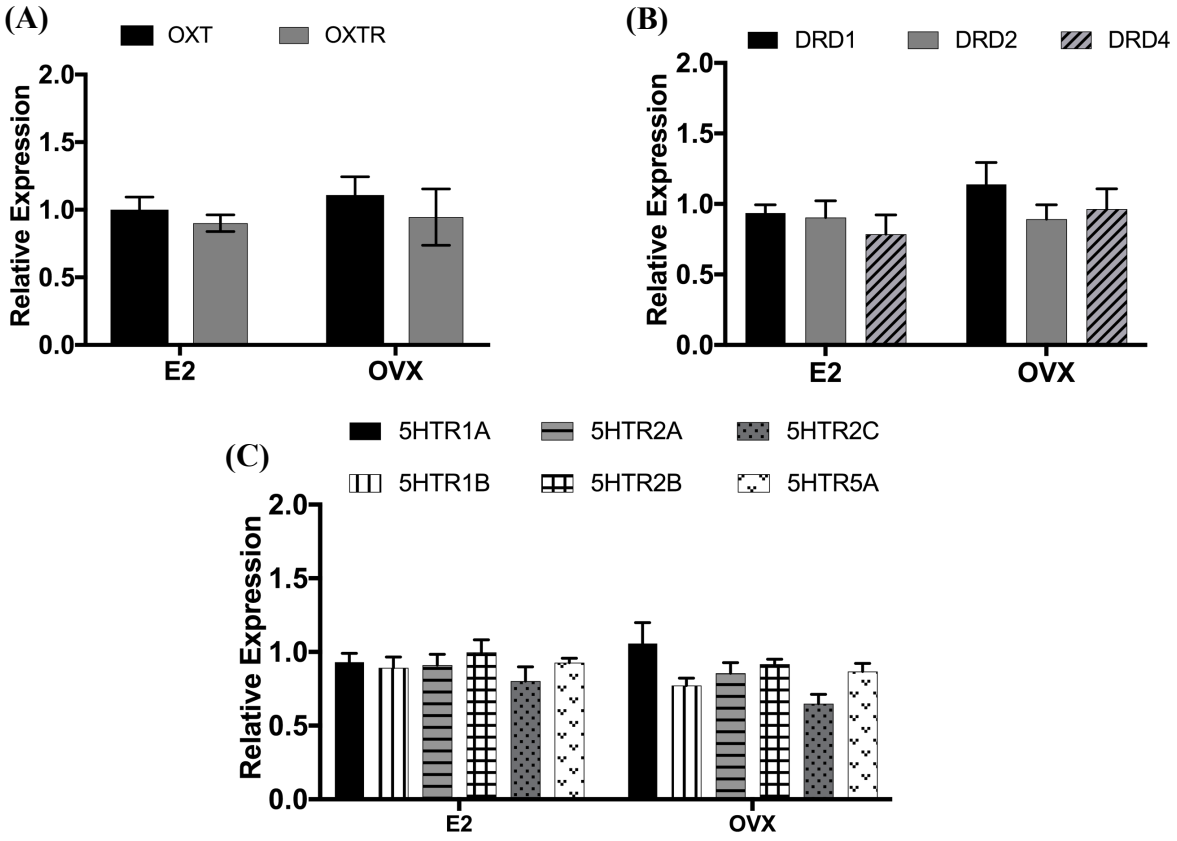

Figure 5: qPCR-determined mRNA expression of other behaviorally related genes (OT and OTR (**5A**), Dopamine receptors DRD1, DRD2, and DRD4 (**5B**), and a variety of serotonin receptors (**5C**) were not significantly altered by OVX.

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3. CHAPTER THREE: Extra-Ovarian Gonadotropin Negative Feedback Revealed by Aromatase Inhibition in Female Marmoset Monkeys

Marissa Kraynak, Matthew T Flowers, Robert A Shapiro, Amita Kapoor, Jon E Levine, and David H Abbott

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3.1 Abstract

While the ovary produces the majority of estradiol (E_2) in mature female primates, other organs including the brain also contribute to E_2 synthesis and action, including regulation of hypothalamic gonadotropin-releasing hormone (GnRH). Aromatase inhibition (AI) has been shown in ovary-intact female rodent models to induce a PCOS-like hypergonadotropic hyperandrogenism due to the absence of E_2-mediated negative feedback. The present study employs letrozole to elicit AI in female marmoset monkeys in order to examine the role of extra-ovarian E_2 on nonhuman primate gonadotropin regulation. Sixteen adult female marmoset monkeys (*Callithrix jacchus*) (>2yrs) were randomly assigned to ovary intact or ovariectomy (OVX) conditions and subsequently placed on a daily oral regimen of either 1 ml/kg/day vehicle (ovary intact Control, n=3, OVX, n=3) or 1 mg/kg/day Letrozole (ovary intact AI, n=4; OVX+AI, n=6). Blood samples were collected every 10 days and plasma chorionic gonadotropin (CG) and steroid hormone levels were determined by validated RIA and LC-MS/MS, respectively. Ovary intact AI-treated and OVX females exhibited elevated CG ($p<0.01$, $p=0.004$, respectively) compared with controls, and after 30 days, OVX+AI females exhibited a suprahypergonadotropic phenotype ($p=0.004$) above ovary intact+AI and OVX females. Androstenedione, A_4 ($p=0.03$) and testosterone, T ($p=0.05$) were also elevated in ovary intact AI-treated females above all other groups. The current study thus confirms in a nonhuman primate that E_2 depletion and reduction of negative feedback in ovary-intact females engages hypergonadotropic hyperandrogenism. Additionally, we have shown evidence that extra-ovarian estrogens, possibly neuroestrogens, may contribute to the negative feedback regulation of gonadotropin release.

3.2 Introduction

The mature, premenopausal ovary of female primates contributes the majority of circulating estradiol (E_2) in support of many aspects of female physiology, including reproductive function. Classic studies in Old World primates (OWPs), such as rhesus monkeys, show that bilateral ovariectomy (OVX) and thus abrogation of ovarian-mediated negative feedback at the hypothalamus and pituitary, results not only in increased release of pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH)[1], but also increased release of gonadotropin-releasing hormone (GnRH) from the hypothalamus [2]. Similar studies in female New World primates, such as marmoset monkeys, have demonstrated that pituitary chorionic gonadotropin (CG, the New World primate equivalent of LH [3]) emulates the post-OVX elevation of LH found in OWPs[4,5]. An obvious change in hypothalamic GnRH release in OVX marmosets, however, is lacking [6]. Marmosets, nevertheless, exhibit expected increases in pituitary CG release in response to exogenous GnRH administration [4,6-8]and E_2-induced pre-ovulatory CG surges [9,10]. Marmosets also exhibit decreases in pituitary CG release following GnRH antagonist administration and post-ovariectomy E_2 replacement [7,11] typical of those demonstrated for LH in women and a variety of Old World nonhuman primates.

One notable difference in E_2 metabolism in NWPs, is that in pre- and post-OVX female marmosets, E_2 is substantially higher compared with OWPs. The elevated circulating levels of E_2 present in NWPs may reflect a compensatory response [36] to their inherent target organ resistance to steroid hormones[12-16]. E_2 is synthesized from estrone by HSD17B (17beta-hydroxysteroid dehydrogenase) and from testosterone (T)

by CYP19A1 (cytochrome P450-aromatase), as typically found in OWPs. In ovarian antral follicles, thecal cells produce androgens, mostly androstenedione (A4), which is then converted to estrone and E_2 in granulosa cells. Theca and granulosa cells also metabolize A4 into T by HSD17B, but T is usually a relatively minor follicular product except in hyperandrogenic conditions, such as polycystic ovary syndrome, PCOS [17-19]. This two-cell process is stimulated by pituitary gonadotropins LH and FSH. LH, or CG in the marmoset monkey, stimulates the production of androgens in theca cells, whereas FSH enables expression of CYP19A1 in granulosa cells, thus facilitating the regulation of ovarian estrogen production.

PCOS in women is characterized as an infertility disorder marked by anovulation, hyperandrogenism, hypergonadotropism and polycystic ovaries, as well as, in some women, by metabolic dysfunction [20-22]. The dynamics between gonadotropins and PCOS ovarian hormonal pathology is not well understood. FSH has been shown to increase some of the androgenic responses in women [23], but is commonly deficient in PCOS women, necessitating injections of exogenous FSH to stimulate appropriate ovarian folliculogenesis that leads to the selection of a dominant follicle and ovulation [24]. In contrast, LH levels are elevated in ~80% of women with PCOS [25], resulting from increased episodic release of GnRH from the hypothalamus [26]. Such LH hypersecretion is mediated in part by T-induced reduction in the actions of both E_2 [27] and progesterone[28] to engage negative feedback inhibition on LH. These data suggest that in PCOS women, a dysfunction in ovarian steroidogenesis contributes to gonadotropin pathophysiology. The molecular mechanism underlying this dysfunction has yet to be determined in a nonhuman primate model.

In this regard, both enzymes necessary for ovarian E_2 production (CYP19A1, HSD17B) are also expressed in extra-ovarian organ systems and tissues, such as the hypothalamus, of fish, birds and mammals, including primates (HSD17B: [29,30]; CYP19: [27,31,32]). Within the primate brain, intra-hypothalamic locations for CYP19 are reported, with expression residing predominantly in the medial preoptic area (mPOA) and the ventromedial nucleus (VMN)[31]. Intra-hypothalamic expression of both E_2-synthesizing enzymes, together with studies showing production of E_2 from steroid hormone precursors [30] and release of E_2 from the hypothalamus of OVX monkeys [33], suggest that E_2 is produced locally within the hypothalamus, the essential site for E_2-mediated negative feedback control of GnRH and its neuroendocrine regulation of reproductive function.

The use of aromatase inhibition (AI) in diminishing E_2 production in ovary intact female nonhuman primates, such as the marmoset, will enable identification of non-E_2 source(s) of negative feedback regulation of gonadotropin release, and permit a better understanding of the hormonal consequences of hypergonadotropic hyperandrogenism in a female nonhuman primate. Consequently, in this study, we employed a parallel experimental approach in OVX female monkeys to ascertain whether cryptic, non-ovarian sources of E_2 may contribute to female physiological function in primates. We hypothesize that a non-ovarian source of E_2, eradicated by extreme aromatase inhibition, will completely remove negative feedback control of hypothalamic GnRH and pituitary CG, and in ovary intact females, the resultant elevation of gonadotropin CG release will stimulate ovarian androgen release.

3.3 Methods

Animal Use

Animal procedures were carried out according to the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. All procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. The Wisconsin National Primate Research Center (WNPRC) is accredited by the Associated for Assessment and Accreditation of Laboratory Animal Care as a part of the University of Wisconsin-Madison Graduate School.

Sixteen adult (age 2-8 years) female common marmosets with regular ovarian cycles were housed with a gonad-intact male pairmate from at least 8-10 months before study onset until its completion. Females were assigned to ovary intact controls (~200 ul Ensure© vehicle PO, n=3), ovary intact AI (1mg/kg/day letrozole in vehicle PO, n=4), OVX, (vehicle PO, n=3) or OVX+AI (1 mg/kg/day letrozole in vehicle PO, n=6). As the results of this study were robust, a small number of animals were utilized in each group to reduce the animals usage in these experiments. All females were weighed regularly to refine weight-dependent oral dosing. OVX was performed during the follicular phase of the ovarian cycle, as previously described [1]. OVX and treatment onset occurred on the same day and are marked as study day 0. All baseline measures were taken immediately prior to OVX procedure and/or AI/vehicle treatment onset. For OVX surgical procedure, females were anesthetized with ketamine (15 mg/kg, im) and maintained on isoflurane (2%; 0.6 liter/min oxygen). Atropine (0.02 – 0.04 mg/kg i.m.) and buprenorphine (0.01 mg/kg i.m.) for pain management were also administered at the same time as ketamine administration. Each ovary was isolated through a ventral

midline incision and exteriorized for visualization of the fallopian tube and ovarian pedicle. Subsequent histological examination confirmed complete ovary removal. Following surgery, pain management was given per veterinarian discretion and animal behavior and incision was monitored until fully healed.

Letrozole Dosing

Prior to study onset, two ovary intact females (n=2) were treated with the AI letrozole (PO daily, 0.5 mg/kg for 15 days and 1 mg/kg for the remaining 15 days) to determine an appropriate dose to suppress plasma E_2 levels below 3 pg/ml (detection limit of the LC-MS/MS). Blood samples were collected (as described below) every 2-5 days and hormone levels were measured by a validated RIA (CG) and LC-MS/MS (steroid hormones) assay. The latter dose suppressed plasma E_2 levels below assay detection.

Blood Sampling

Females were briefly transported from their home cages and restrained in a marmoset restraint tube for blood sample collection. All blood samples were collected between 6:00-10:00 am. Samples were drawn from the femoral vein using heparinized syringes to minimize blood clotting. Blood samples were centrifuged and plasma was collected and stored at -20° C until hormone assays. Approximately one month prior to study onset, blood samples were collected twice a week from all 16 females to determine stage of ovarian cycle. Samples were analyzed weekly for progesterone, P_4. Approximately 5 days prior to study onset, females were administered IM a 2-day luteolytic regimen of ~1ug injection of prostaglandin F2alpha analogue to standardize study onset (including OVX) during the follicular phase (plasma P_4 <10 ng/mL).

Throughout the duration of treatment, blood samples were collected every 10 days for both RIA and LC-MS/MS assays, described below. As marmosets have low body mass (~350-400g) that constrains maximal blood withdrawals per 30 days, we also collected a single large blood volume at 60 days following treatment onset to provide sufficient sample volume for sufficiently sensitive (< 3pg/ml) LC-MS/MS lower limit of quantitation (LLOQ) for E_2 .

Hormone Assays

Blood samples were analyzed for several hormones. CG levels, (New World primate LH equivalent), were measured by a validated radioimmunoassay (RIA) [5], detection limit 0.661 ng/ml. Intra- and inter-assay coefficients of variation (CoVs) were 17.37% and 8.83%, respectively.

Plasma samples underwent extraction [29] and subsequent analysis on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (LC-MS/MS). The system included two Shimadzu LC20ADXR pumps and a Shimadzu SIL20ACXR autosampler. A sample of 30 µl was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm column (Phenomenex) for separation using a mobile phase: water with 1% formic acid (Solution A) and acetonitrile with 1% formic acid (Solution B), at a flow rate of 200 µl/min. After 3 min, Solution B was increased over the course of 0.1 min to 3% and this was maintained for 3 min, followed by another 0.1 min step-up to 50% Solution B that was maintained for 2.9 min. Subsequent 0.1 min step-ups raised Solution B to 67% for 15 min and then 100% for 10 min. The system was finally returned to initial conditions of 3% Solution B over 0.1 min for the remaining 9.9 min of each sample run. Mass spectrometer results were

generated in positive-ion mode with the following optimized voltages: corona discharge current, 3 V; entrance potential, 10 V. The source temperature was 500°C. The gas settings were as follows: curtain gas, 30 psi; nebulizing gas, 20 psi; collisionally activated dissociation gas, medium. Quantitative results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each compound and internal standard. Each steroid had a MRM used for quantitation and 1 or 2 additional MRMs as qualifiers. The lower limits of quantitation (LLOQ) were 7.0 pg/mL for T, A4, and DHEA; 2.7 pg/mL for E2. Linearity was $r > 0.9990$ and the curve fit was linear with $1/x$ weighting. None of the compounds of interest were detected in blank or double blank samples. Inter-assay coefficient of variation was determined by a pool of marmoset serum. Intra- and Inter- assay CoVs, respectively, were: E_2, 4% and 9%, P4, 4% and 14%, A4, 8% and 17%, T, 11% and 13%, and DHEA, 7% and 11%.

Due to body mass determined constraints on 30-day blood volume withdrawals, LC-MS/MS LLOQ for E_2 was 16 pg/ml for days 0-30 (data reflected in **Fig 1A** and 3 pg/ml for day 60 (data reflected in **Fig 1B).**

Statistical analysis

All results were expressed as mean ± SEM. Parameters not normally distributed were log transformed. Treatment group comparisons were conducted using repeated measures ANOVA followed by Bonferroni multiple comparison post hoc tests using SPSS. Statistical significance was determined as $p < 0.05$.

3.4 Results

Female Characteristics

As illustrated in Table 1, age and body weight were comparable in all female groups at study onset and completion, and were typical for laboratory-housed, adult

female marmosets. Additionally, there was no weight gain in any group throughout the duration of the study.

E_2 and Gonadotropin

AI treatment in both ovary intact and OVX females resulted in diminished ($p<0.001$) E_2, below LC-MS/MS LLOQ (<16 pg/ml) compared with their respective controls throughout the duration of treatment ($p<0.01$) (**Fig 1A).** Additionally, at day 60, and an LLOQ for E_2 by LC-MS/MS of <3 pg/ml, plasma E_2 levels for AI-treated ovary intact ($p<0.001$; 3 ±1 pg/ml; p) and OVX ($p<0.03$; 4 ± 3 pg/mL) females were lower than both ovary intact (250 ±36 pg/ml) and OVX (10±4 pg/ml) females. As expected, plasma levels of E_2 in OVX females were lower ($p<0.001$) than the ovary intact controls (**Figure 1B).** In contrast, circulating levels of CG in ovary intact AI females increased ($p=0.02$) in comparison to ovary intact controls after 30 days on study **(Fig 2).** AUC circulating levels of CG (not shown in figures) between 0-10 and 11-30 days following treatment onset were statistically comparable between the ovary intact female groups, reflecting the relatively tardy increase in CG induced by AI in ovary intact females. Within the ovary intact groups, there was variability in the response to AI treatment, which contributed to the lack of a significant rise in CG. Despite this variability, and lack of statistical differences however, the three highest CG values were in the AI treated group. In the OVX female groups, however, AUC circulating levels of CG in OVX and OVX+AI females both rose from 0-10 days post treatment start, but only the AUC circulating levels of CG in the OVX+AI females continued to rise between 11-30 days (OVX+AI, 172 ± 23; Control, 93 ± 32 ng/mL*20 days; $p<0.04$). After 30 days, CG levels

in OVX+AI females were elevated (p=0.004) above those in all other female groups (**Fig 2**).

Androgen Excess

Plasma androgen levels are illustrated in Figure 3. Increased levels of A_4 (37 ± 26 ng/ml, p<0.03) (**Fig 3a**) and T (1.9 ± 1.1 ng/ml, p<0.05) (**Fig 3b**) were observed in ovary intact AI females after 20 and 30 days, respectively, in comparison to ovary intact controls (A_4: 8.08 ± 1.70, ng/ml at 20 days; T: 0.54 ± 0.20 ng/ml at 30 days). There were, however, no differences in circulating levels of DHEA (**Fig 3c)**. In contrast to ovary intact females, OVX and OVX+AI female marmosets demonstrated similarly low levels of T, A_4, DHEA **(Fig 3a, b, and c, respectively)** 10-30 days after study onset**.** Additionally, in the AI treatment ovary intact females, there was a decreased ratio of DHEA:A_4 (**Fig 4A**; p=0.04**)** and such ratio difference was not detected between the ovary intact groups for A_4: T (**Fig 4B**).

Luteal phase-like levels of circulating P_4

Control females exhibited post-ovulatory luteal phase levels of **P_4** (>10 ng/ml) between 10 and 30 days of treatment **(Fig 5),** most likely reflecting ovulation about 6-8 days following injection with a prostaglandin F2alpha analogue. Ovary intact AI females, interestingly, also exhibited comparable temporal changes in circulating P_4 levels. Despite luteal levels post day 10 in all females and no difference in P_4 levels between the ovary intact groups, the P_4: A_4 ratio is lower in the AI treated group (**Fig 4C**; p<0.001). The P_4:DHEA ratio was not different between groups (**Fig 4D**). P_4 levels in all OVX females were lower (p<0.01) than both ovary intact groups (**Fig 5)** and remained low throughout the study.

3.5 Discussion

In the present study, we demonstrate divergent hormonal phenotypes in adult female marmoset monkeys when contrasting E_2 depletion achieved through aromatase inhibition (AI), OVX or both AI and OVX, combined. Within 30 days of E_2 depletion, AI-treated ovary intact females (E_2: <3 pg/ml) became hypergonadotropic, typical of E_2-depleted OVX female marmosets (E_2: ~10 pg/ml). In contrast, over the same period of time, E_2 depleted, AI-treated OVX females (E_2: <3 pg/ml) became supra-hypergonadotropic beyond the elevated gonadotropin levels observed in either OVX or AI-treated, ovary intact marmosets. OVX is conventionally perceived as maximally removing negative feedback constraint on release of both pituitary gonadotropin and hypothalamic GnRH, mostly due to estrogen depletion, resulting in hypergonadotropism [22]. Our results, however, strongly suggest that in a NHP model, gonadotropin constraining E_2 production is not limited to the ovaries, but also occurs in other locations, such as the pituitary [34] and hypothalamus (neuroE_2) [33]. Such potentially and appropriately situated neuroE_2 opens the possibility for locally acting, brain-produced E_2 contributing towards negative feedback constraint on hypothalamic GnRH release and thus release of pituitary gonadotropin.

Gonadotropic Responses to Aromatase Inhibition in Ovary Intact Females

Such gonadotropic responses to aromatase deficiency have been examined in non-primate species. Ovary-intact rodent models, such as transgenic aromatase knockout mice (ArKO) and AI-treated rats and mice, have provided distinct insight into the separate roles for ovarian and extraovarian E_2 regulation of gonadotropin release. Findings from ArKO mice clearly demonstrate that, in the total body absence of E_2

synthesis, chronic elevations in circulating levels of both gonadotropins occur, leading to hypergonadotropic phenotypes [35,36]. ArKO hypoestrogenic and hypergonadotropic phenotypes are attributed to a lack of E_2 negative feedback on release of both hypothalamus GnRH and pituitary gonadotropin. Pharmacological inhibition of aromatase activity in AI-treated rodents, however, while recapitulating the LH hypersecretion of ArKO mice, fails to elicit FSH hypersecretion [37]. The latter may reflect increased GnRH pulsatility, during which LH secretion is favored over FSH, and thus the LH:FSH ratio is increased [26]. Kauffman and colleagues [37], on the other hand, reported that while hypothalalmic GnRH mRNA expression in AI-treated, ovary intact female mice was similar to controls, pituitary expression of GnRH receptor was increased, suggesting that altered GnRH signaling at the pituitary may explain the differential LH:FSH ratio [26]. Another possible explanation for this differential gonadotropin regulation, not examined in AI-treated rodents, is that ovarian granulosa cell inhibin B provides discrete inhibition on pituitary FSH release [38-41], partly driven by the exaggerated hypergonadotropism of AI. Long-term (>30 days) AI-induced estrogen depletion has not previously been performed in female nonhuman primates, such treatment in adult males generates anticipated elevations in circulating levels of both LH and FSH [42]. In the present study, FSH was not measured due to assay constraints [43].

Aromatase Inhibition in Ovariectomized Females

In the present study, we not only explored the effect of AI manipulation on ovarian-related endocrine responses, but also examined AI manipulation of non-ovarian sources of E_2 on gonadotropin negative feedback. There are many female tissues that express aromatase and are thus capable of synthesizing E_2, including mesenchymal

cells within adipose depots, osteoblast and chondrocytes in bone, vascular endothelium and smooth muscle cells in the cardiovascular system, as well as many brain locations [44,45]. The production of E_2 and its action in extragonadal tissues are markedly distinct from ovarian synthesized E_2. Extragonadal E_2 synthesis exerts local autocrine or paracrine action rather than the conventional endocrine actions of ovarian E_2[46,47]. As gonadotropins are controlled by hypothalamic GnRH, E_2 produced locally in the hypothalamus maybe responsible for regulating gonadotropins in addition to ovarian E_2. In the present study, we thus expected AI treatment of OVX females to produce a supra-hypergonadotropic phenotype beyond that observed in OVX females. We found that both AI and vehicle treated OVX females exhibited a rise in gonadotropins showing that the loss of E_2 in both groups translated to a loss in feedback control of the hypothalamic-pituitary CG output. In the AI treated OVX group, however, gonadotropin continued to rise to supra-hypergonadotropic levels. These data thus provide evidence for a neural (hypothalamic) component of E_2 negative feedback in a female nonhuman primate.

Our findings in ovary intact AI females, however, were not totally as anticipated. AI in ovary intact females only resulted in hypergonadotropism similar to the gonadotropin response observed in OVX alone, and did not yield the expected supra-hypergonadotropism of OVX+AI females. The hypergonadotropic response in the AI-treated, ovary intact females, also was relatively tardy compared with the OVX and the OVX+AI rise in CG. This differential may be explained because AI in additionally E_2 depleted OVX females may effect greater suppression of E_2 synthesis than in ovary intact females that is all below LC-MS/MS detection. Hypergonadotropic

hyperstimulation of ovarian steroidogenesis due to AI-induced may enable recovery of a modicum of aromatase activity and release of E_2 in ovary intact AI females.

Androgen excess and PCOS relevance in ovary intact AI-treated females

Ovary intact AI-treated female monkeys also exhibited increased ovarian steroid hormone levels in addition to hypergonadotropism. Most notably, AI-treated ovary intact females demonstrated hyperandrogenemism, releasing high levels of T and A4. This finding confirms hyperandrogenic phenotypes observed in female ArKO mice, as well as AI-treated female rodents [35-37,48] and recapitulates the hypergonadotropic hyperandrogenism observed in women with PCOS. AI-treatment of female rats enhances expression of *CYP17A1*, a steroidogenic enzyme that is integral to androgen biosynthesis in ovarian theca cells. There is evidence, presented here to suggest that AI treatment in the ovary intact AI-treated female marmosets also display an elevation of CYP17A1. This was demonstrated in the favoring of A4 over P4, despite the cyclical phenotype of P4 observed in the AI-treated females. The hormone ratio data presented here suggest that not only are the ovaries driven towards androgen production, but also that the androgenic steroidogenic pathway is favoring the conversion of DHEA to A4 and T via the enzyme HSD3B2. Unique to adult female primates, including humans, androgen biosynthesis occurs not only in ovarian theca cells, but also in the zona reticularis of the adrenal cortex [49,50]. In females marmoset monkeys, however, increased androgen production from the adrenal cortex following OVX may manifest only after 6 months[50]. Not surprisingly, OVX females in our 30-day study did not exhibit any elevation in androgens. Hyperandrogenism observed in our AI-treated ovary intact

females was therefore likely due to hypergonadotropic hyperstimulation of ovarian androgen biosynthesis.

Other hormonal observations in AI-treated ovary intact females

Ovary intact AI-treated female marmosets in the present study also exhibited ovarian luteinization as circulating levels of P_4 equivalent to those in the luteal phase were achieved 8-10 days after study onset. AI treatment in the present study commenced during the follicular phase of the ovarian cycle. We would have thus expected AI-treated ovary intact females to exhibit follicular phase P_4 values of (<10 ng/ml) because in the complete absence of E_2, it can be expected that ovulation would not occur. This phenomena could be analogous to the premature luteinization seen in women undergoing fertility treatments [51,52], during which gonadotropic hyperstimulation of the ovaries drives increased P_4 release. A notable difference in the treatment used here to women undergoing AI for ovulation induction, however, is the duration of treatment in women is much shorter (5 vs 30+ days for the present study), and the dose employed is approximately 1/40th (0.025 mg/kg) of that administered to marmosets in the present study (1 mg/kg)[33,53]. Thus during ovulation induction protocols employing AI, only a transient, modest suppression of aromatase activity is achieved. Analogous findings were reported for prenatally androgenized, PCOS-like female rhesus monkeys treated with exogenous rh-FSH for in vitro fertilization[54]. High, post-ovulatory luteal phase levels of progesterone, typical of those derived from the ovarian corpus luteum, have also been show to effectively exert negative feedback in OWPs[1,55] in the presence of ineffectual, low levels of E_2. Until the present study, no comparable evidence for such progesterone effects had been demonstrated in NWPs. Such elevated levels of P_4

induced by AI in ovary intact female marmosets may contribute towards the difference observed in pituitary CG output between AI-treated ovary intact and OVX females. In addition to observations in OWPs, the prenatally androgenized mouse models [56] have also shown that elevated P_4 levels are able to blunt a post-OVX rise in gonadotropins.

In summary, we demonstrated that E_2-mediated negative feedback constraint on pituitary gonadotropin release in a female nonhuman primate is not solely provided by ovarian E_2. These data provide evidence for an extra-ovarian, possibly hypothalamic, component of E_2-mediated negative feedback regulation of gonadotropin secretion in a female nonhuman primate. We also provide evidence that in an ovary intact female, elevated levels of P_4 and possibly T may contribute a partial compensatory mechanism for negative feedback on gonadotropin.

3.6 Tables and Figures

	Age (years)	Baseline Weight (kg)	Weight After 30 Days of Treatment (kg)
Ovary Intact Controls	3.5±0.5	378±42	374±42
Ovary Intact+ AI	3.8±0.9	403±41	407±47
OVX	4.0±0.9	380±43	380±48
OVX + AI	3.8±0.6	393±29	399±25

Table 1: Comparable ages and body weights among all female marmoset groups (ovary intact control: n=3, ovary intact +AI: n=4, OVX: n=3, OVX+AI, n=6) throughout the study. Body weight did not change throughout the study for any condition.

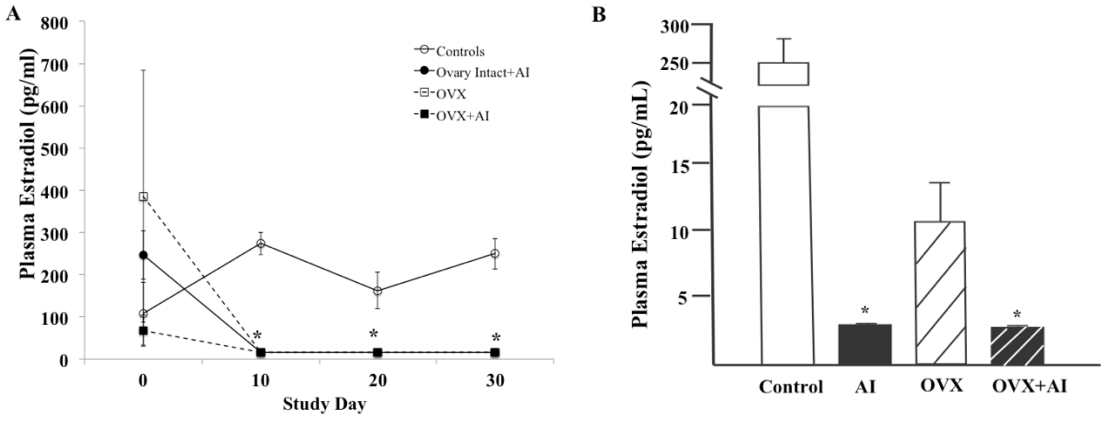

Figure 1: By day 10 following treatment onset, the third generation, non-steroidal aromatase inhibitor, letrozole, diminishes plasma E_2 to undetectable measures (16pg/ml) and remains undetectable throughout the study (*p<0.001) (1A). Additionally, to assess lower values of E_2 a larger sample obtained at 60 days of treatment (1B) shows diminished plasma E_2 levels in AI treated groups (ovary intact and OVX) below assay detection (3 pg/ml by LC-MS/MS) (*p<0.001; **p=0.01). (Treatment groups: ovary intact control: n=3, ovary intact +AI: n=4, OVX: n=3, OVX+AI, n=6)

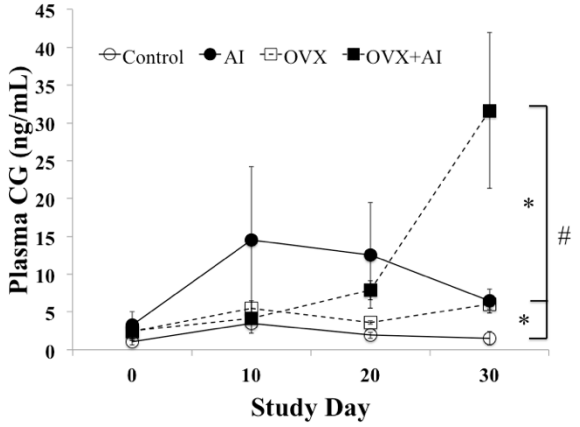

Figure 2: OVX and AI ovary intact females exhibited a hypergonadotropic phenotype above that of control ovary intact females (#p<0.001, * p=0.004, respectively). Following the initial rise in gonadotropin secretion, however, OVX+ AI females exhibited a supra-hypergonadotropic phenotype beyond OVX or AI, alone (*p=0.004). (Treatment groups: ovary intact control: n=3, ovary intact +AI: n=4, OVX: n=3, OVX+AI, n=6)

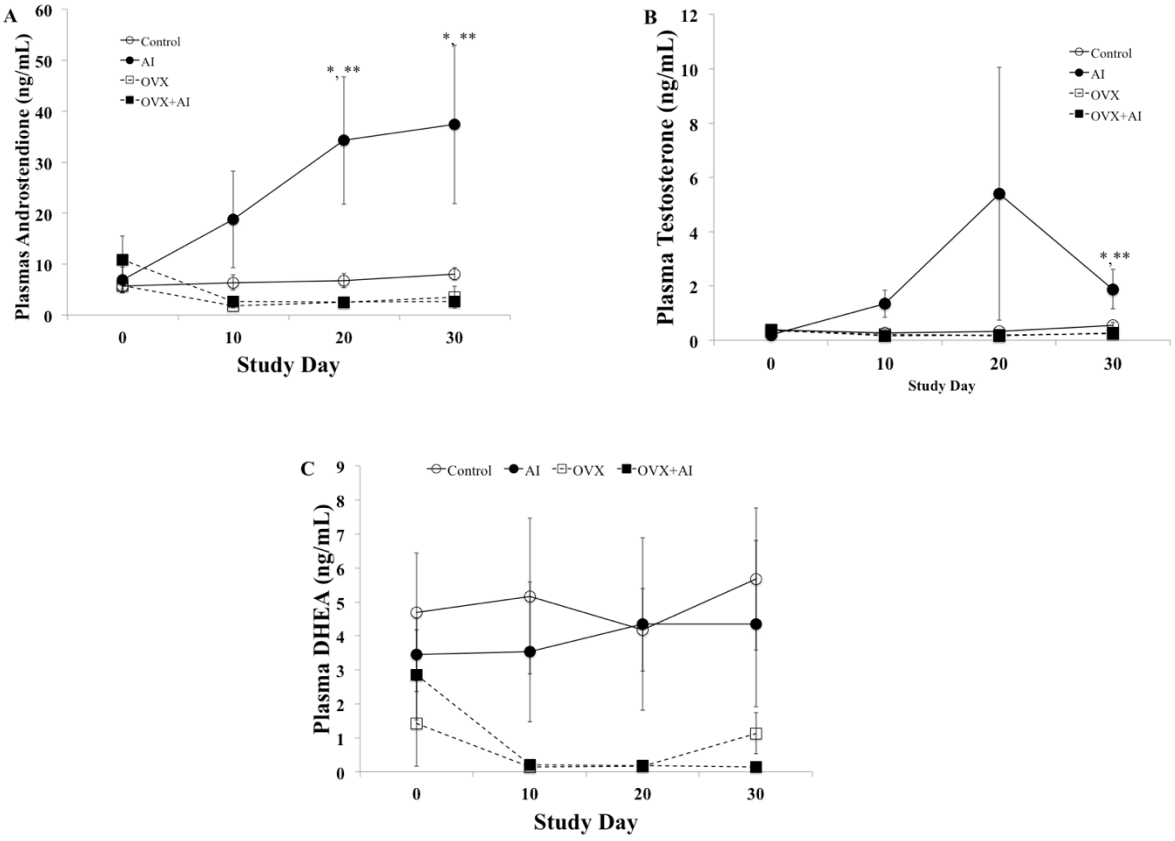

Figure 3: Plasma A_4 (A) and T (B) levels increased in AI-treated females 20 (A_4 only) and 30 days, respectively, following study onset in comparison to ovary intact controls (A_4 **p=0.03,** **p=0.01; T *p=0.05), OVX females (A_4: **p<0.01; T p=0.03) and AI-treated OVX females (A_4: **p<0.01; T *p=0.03). Plasma concentrations of DHEA (C) did not differ between groups. (Treatment groups: ovary intact control: n=3, ovary intact +AI: n=4, OVX: n=3, OVX+AI, n=6)

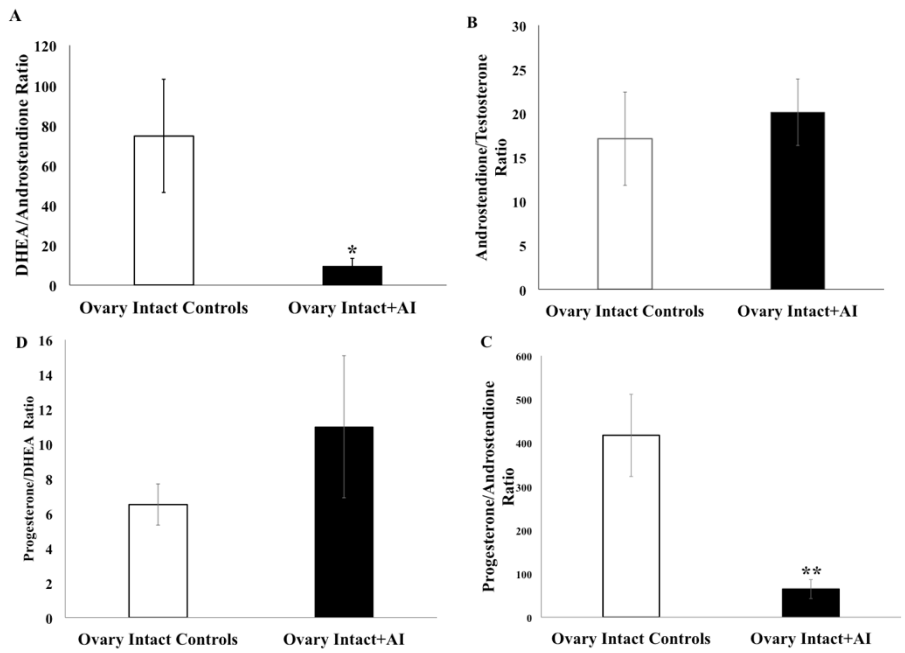

Figure 4: Ovarian hormone ratios show a preference for androgen production, particularly A_4 and T**. (A)** The steroidogenesis in the marmoset ovary of the AI treated females shows an decrease (*p=0.04) in DHEA:A_4 production, identifying an elevation in HSD3B after the conversion of 17OH-P5 to DHEA via CYP17A1. (**B**) There is no difference in the ratio of A_4:T. (**C**) Ovaries in the AI treated females are androgenic as their P_4:A_4 ratios are lower than ovary intact controls (**p<0.001). (**D**) There is no difference in the P_4:DHEA ratio.

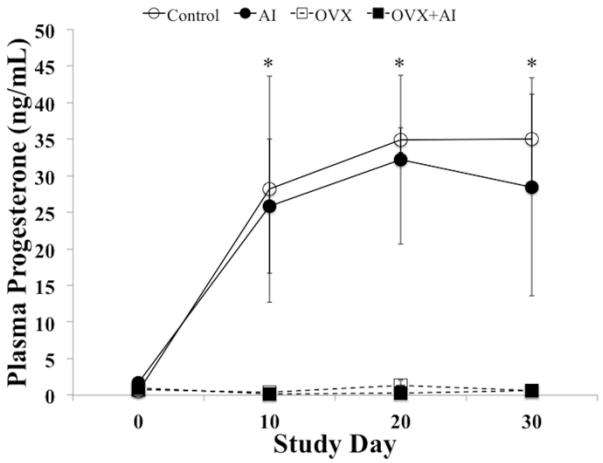

Figure 5: Ovary intact females, both AI and control, exhibited luteinization and/or ovulation, as shown by the rise in P_4 levels above 10 ng/ml. These groups exhibited higher concentrations of P_4 when compared to OVX or OVX+AI conditions ($p<0.01$). (Treatment groups: ovary intact control: n=3, ovary intact +AI: n=4, OVX: n=3, OVX+AI, n=6)

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4. CHAPTER FOUR: Silencing of ERα in the ventromedial nucleus of the hypothalamus disengages female marmoset sexual engagement in the marmoset monkey

Marissa Kraynak, Matthew T. Flowers, David H. Abbott, Jon E. Levine

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4.1 Abstract

Estradiol (E_2) has long been established as a major regulator of female sexual behavior in mammals, including primates. Estrogen receptor alpha (ERα) in the ventromedial nucleus of the hypothalamus (VMN) has been identified as the major molecular gateway facilitating sexually receptive behavior in rodents. In nonhuman primates, however, a molecular gateway has not been identified. We hypothesized that eliminating expression of ERα in the VMN of female marmoset monkeys would result in diminished female receptivity. We ovariectomized female marmosets and assigned them to one of two E_2-replacement conditions: (1) control, scrambled shRNA, and (2) selective ERα protein depletion with ERα gene silencing shRNA. MRI-guided neural infusion surgery enabled delivery of appropriate shRNA. Co-expression of green fluorescent protein (GFP) was used to visualize localization of infected neurons. At least 6 months following treatment onset, female monkeys were observed in six 30-minute behavioral testing sessions with their male partner. Brain tissue was perfused and analyzed by immunohistochemistry to quantify accuracy and effectiveness of gene silencing using NIS-elements computer software. ERα gene silencing shRNA successfully diminished ERα protein expression in the VMN ($p=0.04$). Additionally, in the E_2-replaced, ERα gene silenced females, sexually receptive behaviors were decreased compared with E_2-replaced controls ($p<0.001$). Interestingly, the decreased receptivity was not accompanied by increased sexual rejection. Additionally, E_2-replaced, ERα gene silenced females took notably longer to accept or reject male mounts compared to E_2-replaced controls ($p=0.001$). A lack of responsiveness towards the male partner exemplified by such delayed behavioral response times are not

species typical, suggesting that ERα in the VMN modulates the latency of response to a male partner's sexual advances. Together, these findings suggest that ERα in the VMN may be a component in neural pathways governing female motivation to engage in sexual interaction.

4.2 Introduction

Neuroendocrine signaling of estradiol (E_2) in the hypothalamus regulates many functional aspects of reproduction, including sexual behavior. Ovarian E_2 in both rodents [1,2], as well as nonhuman primate (NHP) models [3,4], has been identified as a major regulator of the expression of female sexual receptivity. In the female rat, greater than 80% specific gene silencing of estrogen receptor alpha (ERα) in the ventromedial nucleus of the hypothalamus (VMN) abolishes sexual behavior [5,6]. The region-specific gene silencing of ERα in the mouse VMN virtually eliminates female receptive behavior comparable to the behavioral extinction also observed during pharmacological blockade of ERα, as well as with genetic knockout models (e.g., ERKO) [7-9], whereas similar manipulations of ERβ do not change female sexual behavior[10].

Female NHPs [3,11-15] have been shown to engage in sexual activity more frequently during periods of peri-ovulatory high E_2. This is despite the absence of estrus during which, in rodents, female sexual behavior expression is closely timed with fertility [16 17] . Additionally, OVX-mediated E_2 depletion of female NHPs, specifically in the marmoset monkey [18] has been shown to decrease the expression of female sexual behavior. In female primates, including women, however, the neural receptor mechanism of E_2 signaling, and its role in regulating female sexual behavior, has not been elucidated.

Similar to female rodents, the hypothalamus, however, has been implicated in the neural region of sexual behavior regulation in NHPs. Chemical and electrolytic lesions of various nuclei within the marmoset hypothalamus have shown that, as found in female rodents, the VMN[19] is the essential hypothalamic region regulating female sexual behavior. Additional lesion studies have also identified accessory roles for other hypothalamic nuclei in regulating female sexual behavior. For example, specific lesioning of the medial pre-optic area (mPOA), results in a loss of proceptive, or sex seeking/soliciting, behavior in female marmoset monkeys. Receptivity, however, is still expressed[20].

The present study is the first to investigate the role for hypothalamic VMN ERα in regulating female sexual receptivity in an NHP. In this study, we used MRI-guided, neural infusions of an adeno-associated virus, AAV8, to deliver a small hairpin RNA (shRNA) either encoding a scrambled virus control, with no known gene targets in this species, or an shRNA specifically designed to associate with ERα mRNA, alone, to prevent translation of ERα protein. We hypothesize that similar to the female rodent literature [5,6], ERα gene silencing in the VMN of female marmoset monkeys will diminish the expression of sexual receptivity.

4.3 Methods

Animals

Eight adult female common marmosets (2-6 years of age) from the Wisconsin National Primate Research Center colony were evenly randomized based on age, body weight into two groups (Table 1): OVX+E2 Replaced+ Scrambled virus shRNA

(Scrambled; n=4) or OVX+E2 Replaced+ ERα Gene Silencing shRNA (ERα gene silenced; n=4). Animals were maintained in these groups for 9-15 months.

All animals lived with a cagemate in 0.60m x 0.91m x 1.83m enclosures and were maintained with 12-h lighting, 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. Animals were fed Mazuri Callitrichid High Fiber Diet #5MI6 (Purina Mills International, St. Louis, MO). The calories in this diet were 20% protein, 6% fat, and 74% carbohydrate. Animals were fed *ad libitum*.

Ovariectomy and estrogen replacement

Following baseline assessments, bilateral OVX was performed in all females at least one month prior to neural surgery. Cloprostenol (Estrumate®, 0.75-1.50 μg intramuscular injection for two successive days approximately 11-60 days after ovulation), an analog of prostaglandin-F2-alpha, was administered prior to OVX to facilitate scheduling of OVX during the follicular phase. All females had maximal E_2-filled silastic capsules implanted subcutaneously following OVX (Figure 1). Silastic capsules were removed and replaced at 3 month intervals post-OVX to maintain consistent E_2 levels.

Neural Infusion Surgery

OVX, adult female marmosets received bilateral stereotaxic injections into the hypothalamic VMN and arcuate nucleus (ARC) of adeno-associated virus 8 (AAV8) expressing shRNA targeting ERα *(ESR1)* for *in vivo* RNAi (ERα gene silenced, n=4) or a control scrambled shRNA (scrambled, n=4), as described below. Within a 22-gauge guide cannula, a sterile 28-gauge cannula attached to a Hamilton syringe controlled by a programmable infusion pump was placed into four areas within the rostral to caudal extents of both left and right VMN and ARC, as modified from[21,22]. Since accurate, within-brain placement of viral vector is required for biological efficacy and specificity, we used anatomical MRI to refine the unique location of each VMN and ARC within each female[23]. With the monkey placed into an MRI-compatible stereotaxic frame, 3-dimensional coordinate locations of both left and right VMN and ARC were obtained from a 26-min, T1 MRI anatomical scan in a next-generation 3T MRI system MR750 (GE Healthcare, Waukesha, WI) performed at least 3 days prior to viral vector infusion (Figure 1A). For viral vector infusion surgery, a solution of viral vector (~2 x 10^{10} packaged genomic particles of AAV8 in 12 μL) mixed with gadolinium-containing Multihance MRI contrast agent (2 mM gadobenate dimeglumine, Bracco Diagnostics Inc.) was infused at 2 μL per minute into eight locations per side: rostral, medial-rostral, medial-caudal and caudal left VMN, and similar locations with the left ARC [21]. The contrast agent was used to confirm accuracy of the infusion site location from a 26-min MRI T1 scan obtained following the first infusion per side (Figure 2). To minimize viral particle dorsal tracking with the infusion cannula, there was a 5-min delay before its removal from the VMN/ARC. The AAV8 virus utilized in this study has been shown to

readily infect neurons[24], and also has been shown in an NHP brain to do so without generating notable inflammation[21].

Steroid Hormone Assay

For steroid hormone analyses, including DHEA, androstenedione, T, estrone and E_2, plasma samples underwent extraction and subsequent analysis on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (LC-MS/MS), as described in Chapter 3[25].

Behavioral Observations

Following treatment onset, pairs were acclimated to the testing cages, as previously described[26]. At least five months after treatment onset, male and female pairmates were placed in single housing for 30 days prior to behavior testing. During the separation period, pairs were out of visual and tactile contact with each other. Following 30 days of single housing, pairs were placed in behavior testing cage for three, 30-minute testing sessions per week for two weeks. During testing, males were placed in a holding box for 5 minutes before being allowed into the main testing cage with the female. Observations (see Marmoset Ethogram in Appendix 1) were video recorded and manually scored by at least two observers. Each test was video recorded and manually scored by at least two observers, and inter- and intra-observer reliability was 80% or greater.

In order to identify and quantify female marmoset sexual receptivity in detail, we employed a novel measurement collected from the video recordings of behavior testing sessions between a variety of animal pairs. In rodent studies of sexual behavior, there is a well-classified reflexive receptive posture, known as lordosis behavior, that readily

enables a male to mount and intromit the female. In the marmoset monkey, prior to this study, no specific receptive posture response to males had been reported for females. Figure 3 depicts quantitative observational findings that define a crucial postural movement by female marmosets to enable intromission during male mounts. Females holding on to vertical substrate (a common location for an a naturally arboreal NHP), move their feet in a posterior, usually downward, movement away from their hands, effectively widening their vertical stance and re-positioning their anogenital region to face more posteriorly than ventrally. Figure 3A shows that intromission occurs only once the female widens her vertical stance to about 5cm ($p<0.001$). Figure 3B illustrates that of mounts quantified, intromission only occurred when a female's feet and hands were at least 5 cm apart. Hand-feet distances were only measured by digital calipers, and only when both hands and feet were clearly visible on the front of the testing cage. The distance measured was from the finger tips holding onto vertical cage mesh, to the tops of the toes also holding on to the vertical cage mesh. This measure of vertical stance taken just prior to mount and compared to that during a male mount, or during intromission when it occurred, provided a clear discriminator between mounts that did and not progress to intromission. Females adjusting their vertical stance once a male mounted to 5cm or more, enabled the mounted male to intromit, xx% of the time.. For the behavioral data in this chapter, a female's receptive posture is defined as this foot movement enabling intromission.

Analysis of Neural Targeting of ERα Gene Silencing

Immunohistochemistry (IHC) procedures for ERα were performed according to Emborg et al[27]. Following upper body perfusion with 4% paraformaldehyde (PFA) (pH

7.6) at necropsy, the brain was removed, post-fixed for an additional 12-16h and cryoprotected in graded (10-30%) sucrose/PBS (pH 7.2) solutions. The brain was then cut frozen (40μm sections) in the coronal plane. Equally spaced sections, spanning the rostral-caudal extent of the hypothalamus from the POA to ARC [28] were immunostained for ERα (mouse monoclonal antibody, 6F11)[29]. DAB-staining was subsequently used to detect primary antibody binding. All eight brains underwent IHC procedure simultaneously.

Brain section images were obtained with a Nikon Microphot FA microscope and digitized using a QImaging Retiga 200R CCD camera and Nikon NIS Elements software. Most images for analysis were obtained using a 4X objective, and those were blended together in NIS Elements to create a whole image of the hypothalamic area. NIS Elements Advanced Research (AR) image analysis software (ver. 5.02) was then used to quantify within MBH subregions the amount of ERα-immunoreactive (ir) labeling, employing measures of cell counts, cell density per unit area (number of cells/mm^2), and labeling intensity (pixel intensity) of individual cells.

Labeled cells were detected using the NIS Elements AR Dark Spot Detection feature. Contrast threshold in the detection algorithm was determined for each case by adjusting cell counts derived from the spot detection algorithm to match cell counts made by two naïve observers on identical images of tissue (n=6 from each of the 8 cases). The counts of the two observers differed by 8.2%, and the NIS-derived cell counts fell within 5% of the average counts of the observers. The NIS Elements AR software calculated mean pixel intensity of each identified cell, and we used this to estimate for intensity of ERα (6F11) staining. The median intensity value of ERα

staining in either the pituitary (when recovered) or the rostral mPOA (section not affected by the shRNA infusions) were calculated to classify cells as dark labeled cells and lightly labeled cells. The median intensity values of the control sections (pituitary or rostral mPOA) were similar across females, and similar between the two regions where these values were taken. The average median intensity (mean ± SEM; pixel intensity of 3776 ± 25) was used as the threshold for cell staining intensity. Utilizing this threshold, cells with a pixel intensity greater than 3776 are 'light' labeled cells, and above 3776 are 'dark' labeled cells.

Density of labeled cells in each of the hypothalamic areas was determined by counting the number of cells in a 1000μ X 500μ ellipse (area of 0.395 mm^2) positioned for all cases in the similar locations in the mPOA, ARC and VMN. We used the average of the three densest estimates as the density measure for each hypothalamic region. One exception is in the most rostral section of the mPOA of a scrambled control female, cj1783: the area with the most ERα expression was lost and thus the estimate for the mPOA in this animal are likely underestimated.

For quantitative analyses, digital images were recorded while the lens was focused on the center of each section. An example of specific immuno-labeling of the hypothalamus is shown in Figure 4. The IHC staining with 6F11 (antibody for ERα; Panel A (control female) and C (ERα gene silenced female) and GFP (rabbit polyclonal antibody) (Panel B) are shown. The smaller square photos on panel A and C denote 40X images to show the difference in staining between a scrambled control female and a ERα gene silenced female. The insert on Figure 4A shows several labeled cells, some that are darker labeled and others that are lighter. The pixel intensity measure using the

NIS elements detects these changes in labeling intensity within animals and across animals. In the insert in Figure 4C, however, there are less cells and they are lightly labeled ERα positive cells, showing an example of gene silencing in the VMN.

Statistical Analysis

Data collected was analyzed utilizing SPSS software. ERα staining intensity, cell density (all intensities included) and behavioral observations were analyzed using independent t-tests to compare means. Plots are expressed as means+/-SEM. Non-parametric Spearman's rho correlations tests were used to detect relationships between the degree of ERα expression in the different regions of the hypothalamus with the observed behavioral phenotypes.

4.4 Results

Targeted Knockdown of ERα in Hypothalamic Nuclei

The degree of gene silencing of ERα varied among females. **Figure 5** shows representative distributions of ERα labeled cells through the hypothalamus in a gene silenced female (cj1554) and a scramble control female (cj1722). In the rostral hypothalamus, ERα cells cluster in the mPOA around the third ventricle. This cluster becomes more dorsal and laterally positioned in the caudal mPOA. Further caudal, the hypothalamus contains a curved distribution of ERα positive cells in the VMN, and a second distribution of cells ventrally in the ARC. ERα positive cells in the ARC extend more caudally than those in VMN. In all females in which the pituitary was recovered and processed (KD n=3, control n=1), it contained a high proportion of darkly labeled cells. Compared to the example of the scramble control female, the hypothalamus of

the ERα gene silenced female contains fewer ERα positive cells, almost all of which are stained lightly.

The between-animal variations in cell density and ERα staining intensity are shown in Figures 6 and 7 as individual plots for all females included in this study. Figure 6 illustrates cell density from rostral to caudal through the MBH, with Figure 6 (A-D) showing the distributions for scrambled control females 1-4. While there is variation between controls, they all exhibit a similar pattern of ERα positively staining cells, with highest density and intensity in the mPOA and lowest in VMN. In Figure 6 (E-H), the cell densities of ERα gene silenced females 1-4 show a similar pattern, but with reduced numbers and staining intensity of ERα positive cells.

The greatest reductions in ERα positively staining cell parameters were found in the VMN of ERα gene silenced, the intended hypothalamic target, but there is also a non-statistically significant trend towards adjacent ERα knockdown in the mPOA and ARC for some animals.

The intensity of the ERα staining in each brain region is shown in Figure 7 for scramble control females 1-4 (Figure 7A-D) and ERα gene silenced females 1-4 (Figure 7E-H). Values plotted to the left (pixel intensity >3776) indicate lighter ERα staining. The curves that appear narrow, (particularly the red lines coding the intensity curves of the VMN for panels E-H) indicate distributions with relatively few darkly-labeled cells (pixel intensity <3776). Control females, with the exception of cj1521, exhibit curves that are wider and to the right, indicating that the ERα staining intensity is darker in a higher proportion of cells. The Scrambled control female, cj1521, had a lower degree of ERα

expression across all three regions of the hypothalamus, both in density of cells (Figure 6D) and in staining intensity (Figure 7D) than the other three Scrambled controls.

The pituitary was recovered in some, but not all of the females. When the pituitary was recovered, the staining intensity is shown in yellow on the intensity plots (Figure 7: D,E,F, and G). The pituitary, as expected, contains many darkly labeled (intensity) ERα positive cells (as seen by the wide, and right shifted curve). Additionally, Table 2 shows descriptive data for the degree of ERα gene silencing achieved in each individual ERα gene silenced female when compared with the mean ERα staining intensity (pixel intensity of 6.1) and mean ERα cell density (555.2 cells/mm^2) of the scramble control group.

Despite between animal variability, intensity of ERα staining was lower in the VMN of ERα gene silenced females (3.7 ± 0.8) compared with the scramble controls (6.1 ± 1.7) (**Figure 8A**, p=0.04). There was no difference in intensity between the groups in the mPOA and the ARC nuclei. Across all brain regions examined, there was no statistical difference between treatment groups for the mean cell density counts (**Figure 8B**). This is perhaps unsurprising given the between-animal variation demonstrated in **Figure 6.**

Behavioral Observations

The responses of male marmoset partners towards their female did not differ between treatment groups (**Figure 9A**). Gene silencing of ERα in the VMN resulted in a lack of female receptive postures (**Figure 9A**; p=0.03) and thus, sexual receptivity in female marmoset monkeys (Figure 9B; p=0.01). These females did not, however, express increased sexual rejection behaviors towards their male partners (**Figure 9B**).

Additionally, ERα gene silenced females displayed a notably latent response to the male after mounting behavior (**Figure 10**).

Behaviors across females in all groups were also correlated with the degree of ERα gene silencing (**Figures 11-13**). In the VMN, receptivity was positively correlated with ERα expression density (**Figure 11A**; r_s=0.95, p<0.001) and ERα staining intensity (**Figure 11B**; r_s=0.786, p=0.02). Percent of rejection was negatively correlated with ERα staining intensity (**Figure 12**; r_s= -0.791, p=0.02). Additionally, ERα staining intensity was also negatively correlated with latency of female responsiveness towards male mounts (**Figure 13**; r_s= -0.738, p=0.04). Analogous correlations between behavior and the staining intensity in other brain regions were also observed (data not shown).

4.5 Discussion

The receptor mechanism governing E_2-dependent expression of female sexual behavior in female primates has not been previously elucidated. The results of this study provide evidence that ERα is a key receptor mechanism for E_2 regulation of female sexual receptivity in NHPs, and likely women.

The present study was modeled after published gene silencing approaches examining ERα's role in discrete nuclei of the hypothalamus in female rats[6]. Compared with the approach in the rat model, we employed MRI-guidance in order to refine individual targeting of the VMN, as NHPs have notable between individual variation in neuroanatomical locations. Based on IHC analysis of ERα expression in marmoset hypothalamic nuclei, ERα expression was significantly diminished in the VMN of females receiving ERα silencing shRNA. In some females, ERα expression in the ARC and mPOA appears to be decreased. This was not consistent, however, across

animals. Behavioral phenotypes were therefore only significantly associated with ERα silencing in the VMN.

This study identifies a clear role for VMN ERα in facilitating the expression of sexual receptivity. The data presented in this chapter further show that the loss of ERα in the VMN, even as little as 21% and 15%, as measured by staining intensity and ERα cell density, respectively, is sufficient to lead to diminished female sexual receptivity. Additionally, a positive correlation between VMN ERα immuno-staining intensity and female marmoset sexual receptivity strongly suggests that expression of female sexual receptivity is dependent upon a major abundance of ERα expression in the female marmoset VMN. These findings coincide with studies in female rodents, however, a notable difference observed involves the degree of ERα gene silencing in the female marmosets. In the analogous studies in female rodents, females are only considered sufficiently ERα silenced when at least 80% of immunoreactive ERα is lost from the VMN, as detected by IHC. The adult female marmoset brain, on the other hand, as identified in the present study, expresses a wide variation of number of immunoreactive ERα cells and staining intensity between individual females. The more ERα expression in the VMN, however, the more sexual receptivity observed by each female. Interestingly, though, there is still a marked decrease in sexual receptivity in all females with at least 15-20% knockdown of ERα in the VMN. Additionally, the more ERα knockdown observed in any individual female, the less sexual receptivity. Therefore showing that even a modicum of ERα expression knockdown in the female marmoset VMN has major behavioral deficit outcomes related to sexual behavior.

Following ovarian E_2 depletion by ovariectomy (OVX), loss of female sexual receptivity is replaced with rejection behavior towards the male partner of the female marmoset (Chapter 2)[18]. When only E_2 action is lost via ERα in the VMN, however, it does not lead to increased rejection behavior. Rejection, nevertheless, negatively correlates with intensity of ERα staining in the VMN. Together, these results suggest that the lack of E_2 signaling through ERα contributes to increased rejection behavior in a quantitative fashion. However, in the absence of an accompanying E_2-depletion-like rejection response, it is possible E_2 action via another VMN estrogen receptor, or via ERα in another hypothalamic nucleus or neural location, provides additional contribution to rejection behavior.

The lack of sexual responsiveness in ERα gene silenced females resembles female responses reported in previous literature examining ERα gene silencing in the VMN of female rodents. In female rodents tested in traditional behavior testing environments (one male and one female rodent in a testing arena) diminished ERα expression in the VMN results in a lack of both proceptive and receptive sexual behaviors[6]. In more socially relevant open, semi-natural behavioral testing environments, ERα in the VMN reduces proceptive sexual behaviors in female rodents, whilst sexually receptive behaviors are still observed[30]. The pairs used in the present study, however, were well-established male-female pairmates, and thus, the social dynamic was species appropriate. The testing arena is also not restricting of movement during behavioral testing sessions in the present study. Thus, the behavioral paradigm in the present study can be paralleled with the semi-natural rodent study[30] depicting that the results presented in this study are socially relevant.

In summary, these data suggest that, similar to female rodents, ERα in the VMN of the female marmoset hypothalamus is the receptor mechanism regulating sexual receptivity and responsiveness towards male partners. Decreased ERα was also negatively correlated with rejection behaviors, suggesting an additional contribution of ERα in a different neural location or of a different estrogen receptor within the VMN. For the first time in a NHP, this study supports a major role for ERα in the VMN in mediating female sexual receptivity and responsiveness.

4.6 Tables and Figures

	Age (years)	Body Weight (g)
Scrambled Controls	3.44 ± 0.55	440.5 ± 24.3
ERα Gene Silenced	3.32 ± 0.31	412.4 ± 32.9

Table 1: Comparable ages and body weights among all female marmoset groups (Scrambled controls, n=4; ERα gene silenced, n=4) at the start of the study.

	PERCENT OF GENE SILENCING ACHIEVED IN VMN	
	ERα Staining Intensity	ERα Cell Density
CJ1554	52%	43%
CJ1755	44%	65%
CJ1730	21%	15%
CJ1684	37%	45%

Table 2: Despite a wide range, ERα gene silenced females all had some degree of ERα gene silencing in the VMN compared with the mean of the control females (Intensity: 6.1; Cell Density: 555.2 cells/mm^2).

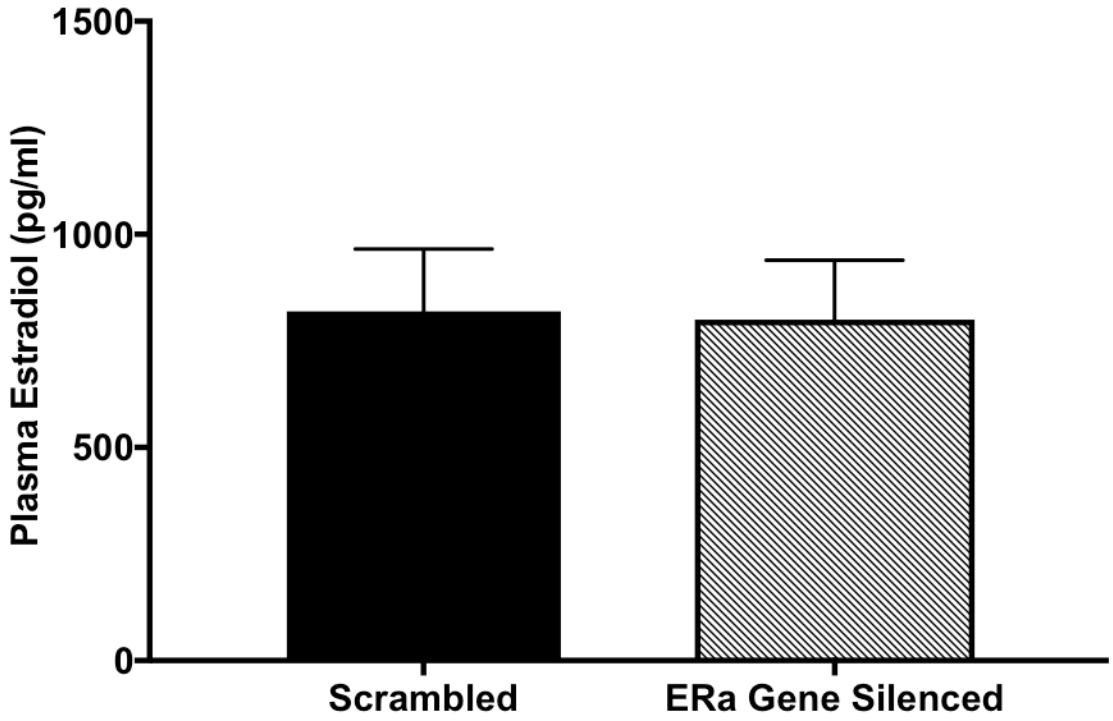

Figure 1: Similar and maximal levels of E_2 are replaced in both groups of females as shown by the plasma estradiol measures. (Scrambled: 918+/-146 pg/ml; ERα Gene Silenced: 800.0+/-140 pg/ml).

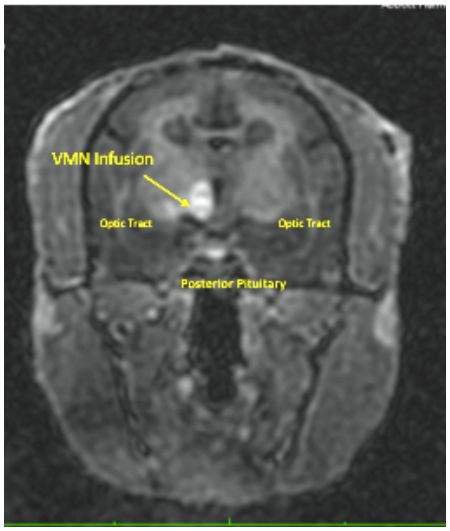

Figure 3: Use of MRI and Multihance, a contrast agent, allows successful targeting of the neural infusion of the viral vector encapsulated shRNA to the VMN of the hypothalamus. MRI images are taken prior to surgery to generate approximate targeting coordinates and following infusions (shown here) to confirm accurate targeting of the VMN.

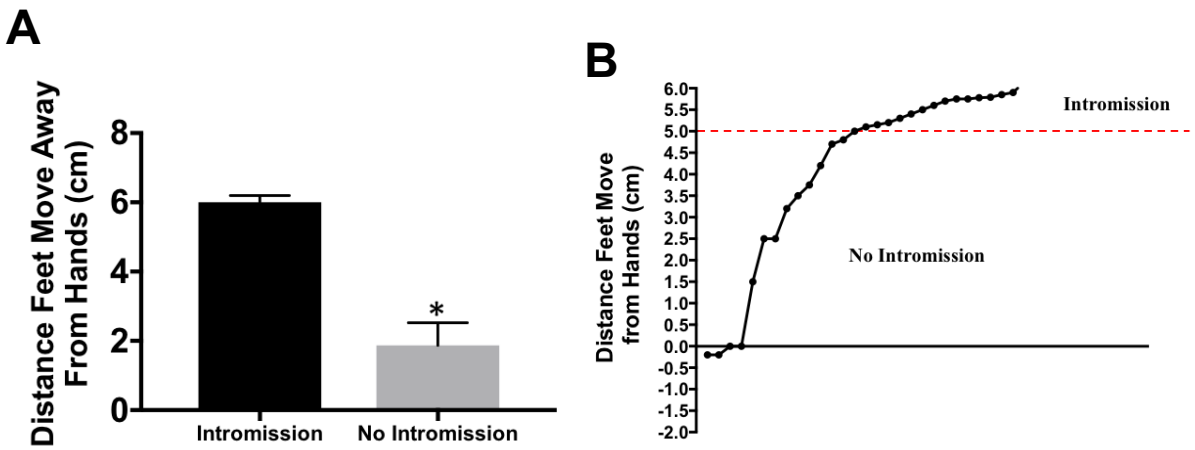

Figure 2: (A) In order for intromission to occur following a male mount attempt, the female must actively move her feet away from her hands. This movement, defined as a receptive posture, is necessary for receptivity of a female marmoset monkey to a male mount. (**B**) Additionally, 5.0 cm distance between feet and hands is the minimum distance for an intromission event to occur.

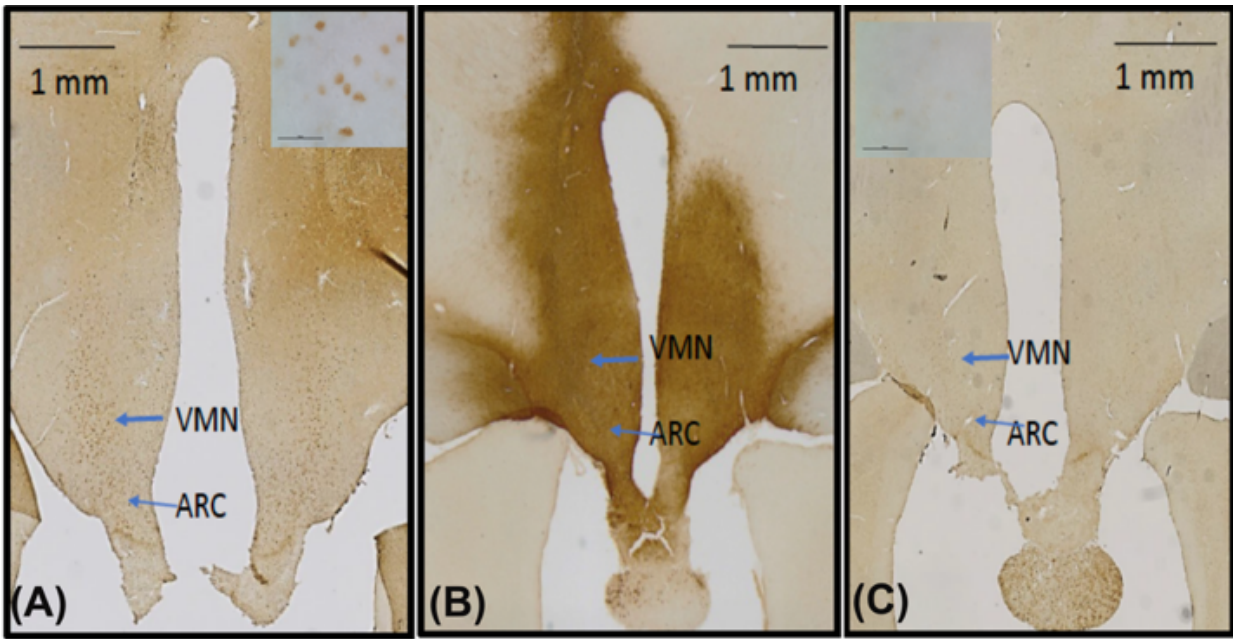

Figure 4: ERα gene silencing shRNA effectively ablates ERα in the VMN of female marmoset monkeys. Left panel (**A**) demonstrates robust ERα immunostaining (a/b: 6F11) in the VMN and ARC of a control female. 40x image of 6F11 staining in the top right corner; the middle panel (**B**) demonstrates expression of GFP (a/b: MAB3580) in an animal receiving the ERα gene silencing viral vector, demonstrating infection of cells throughout the VMN and ARC, while the right panel (**C**) shows absence of ERα immunoreactivity in the VMN and ARC, with robust staining in the pituitary gland, in an adjacent section obtained from the same animal as in panel B. Upper left corner shows a 40X images of this ERα silenced section stained with 6F11.

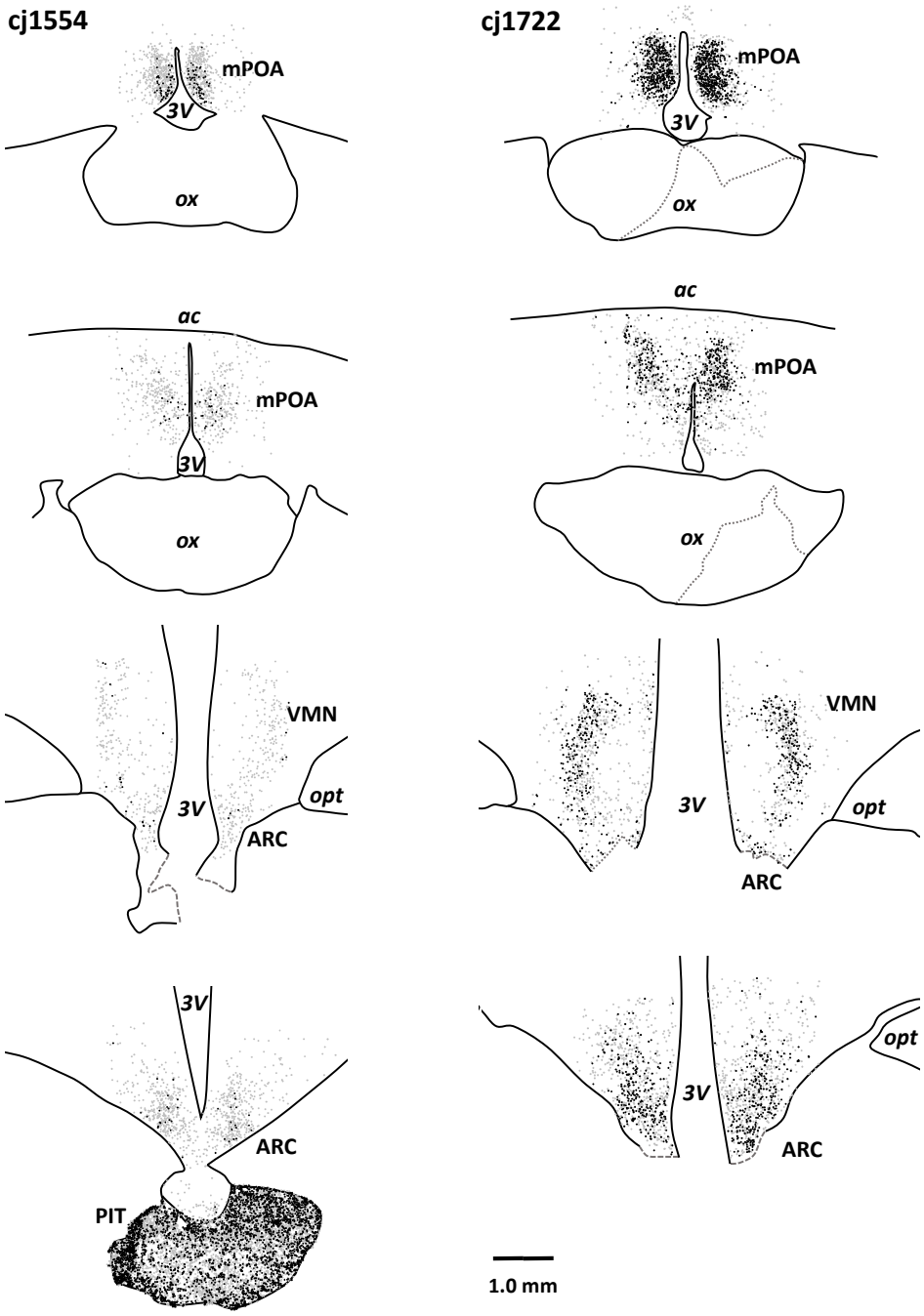

Figure 5: Distribution of ERα labeled cells in the hypothalamus. Representative sections are shown from the rostral (top) to caudal (bottom) hypothalamus for an ERα

gene silenced female (cj1554) and a Scrambled female (cj1722). Black dots indicate the location of darkly stained cells (corresponding to the staining intensities of cells in the darker half of the distributions in the PIT, when available, and Scrambled cells in the rostral mPOA). Dotted lines indicate tears in the tissue. Abbreviations: ox: optic chiasm; ac: anterior commissure; opt: optic tract; 3V: third ventricle

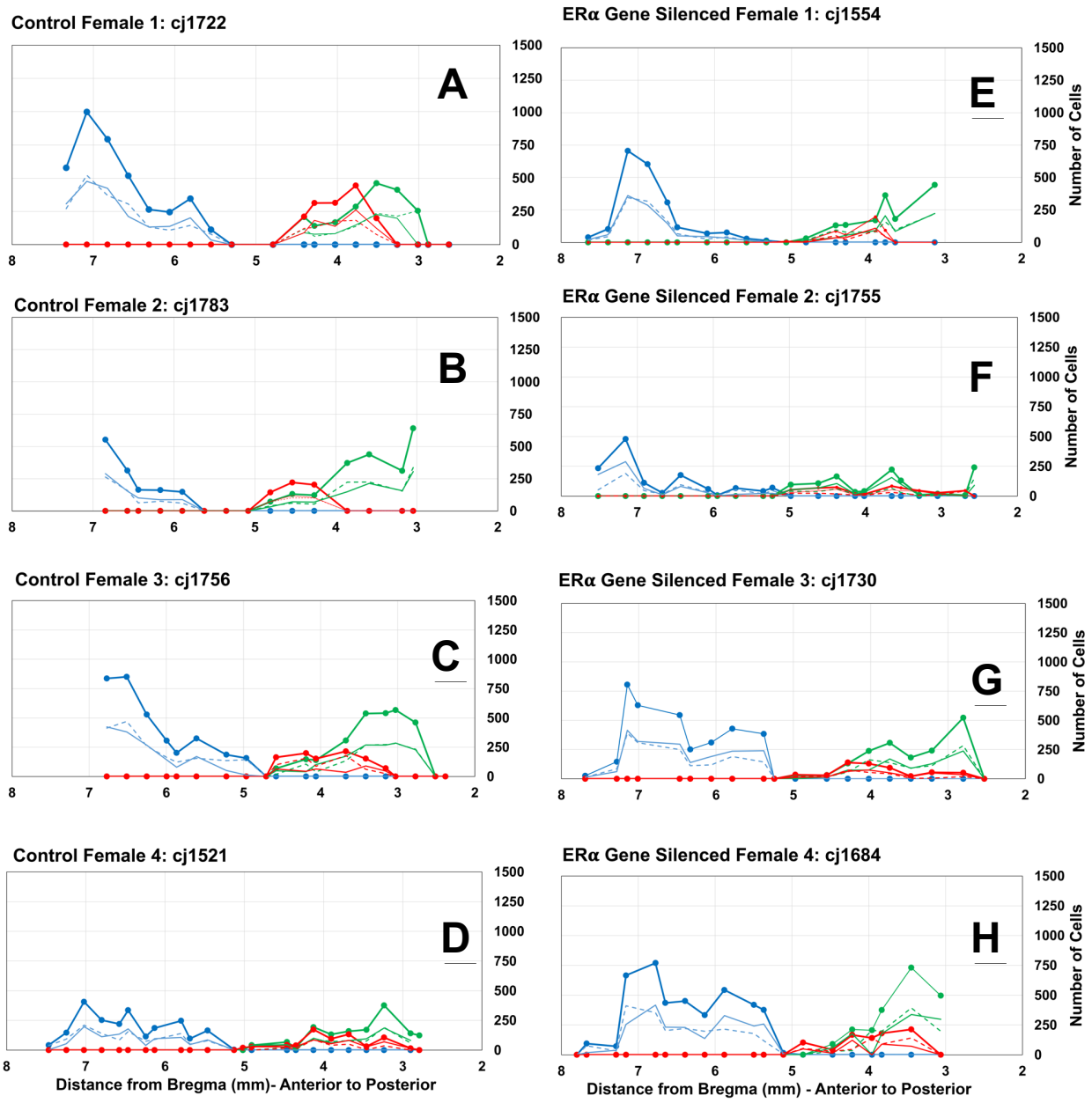

Figure 6: ERα positive cells were count in sections from anterior of the optic chiasm through the posterior sections of the ARC nucleus and the mammillary bodies. Blue line denote counts in the mPOA, red lines=VMN, and green lines=ARC. Marked lines with appropriate colored circle markers are the summation of the right and left side of each nuclei, thin, unmarked lines represent the left (solid) and the right (dotted) cell counts at

each location. Each plot represents one female (Scrambled on left, **A-D**, ERα Gene Silenced on right, **E-H**).

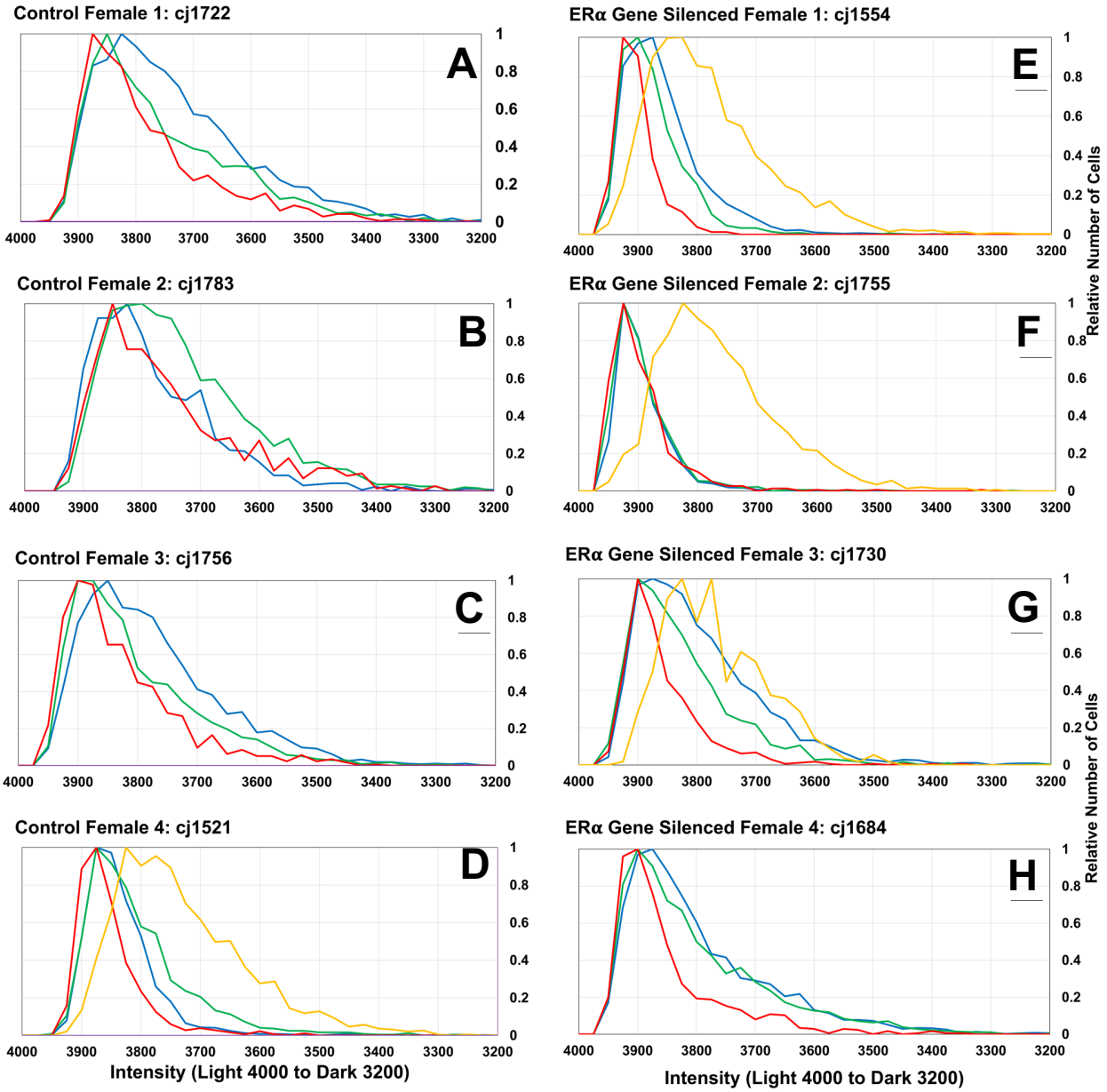

Figure 7: The distribution of ERα staining intensity is shown above. The values are normalized to the peak values where the most number of ERα positive cells reside. The more narrow a plot is, the less number of positive cells there are. The distributions that are shifted to the right and wider depict more cells that are darker stained. Each plot

represents one female (Scrambled on left, **A-D**, ERα Gene Silenced on right, **E-H**). The right panel shows the most narrow distribution being the VMN (red) for all 4 animals, and variations of distributions for the other hypothalamic regions. In females with the pituitary intact show that the pituitary, unaffected by the neural infusions, have many ERα positive cells and they are darkly labeled. Blue=mPOA, red lines=VMN, and green lines=ARC and yellow=pituitary.

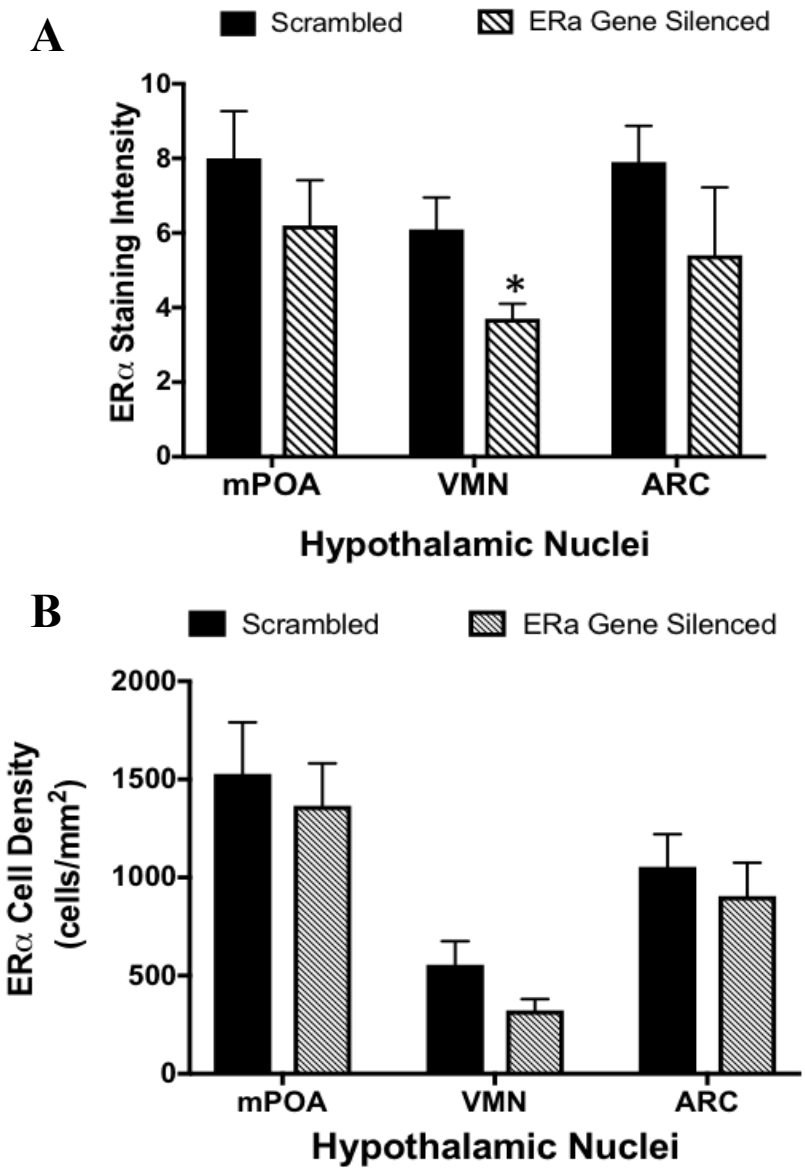

Figure 8: (**A**) ERα staining intensity in the VMN, but not the mPOA and ARC nuclei of the hypothalamus, is markedly less in the ERα gene silenced females (*p=0.04) compared with Scrambled controls. (**B**) The number of cells per unit area, or the ERα

cell density is not statistically different between the groups in any of the hypothalamic nuclei analyzed.

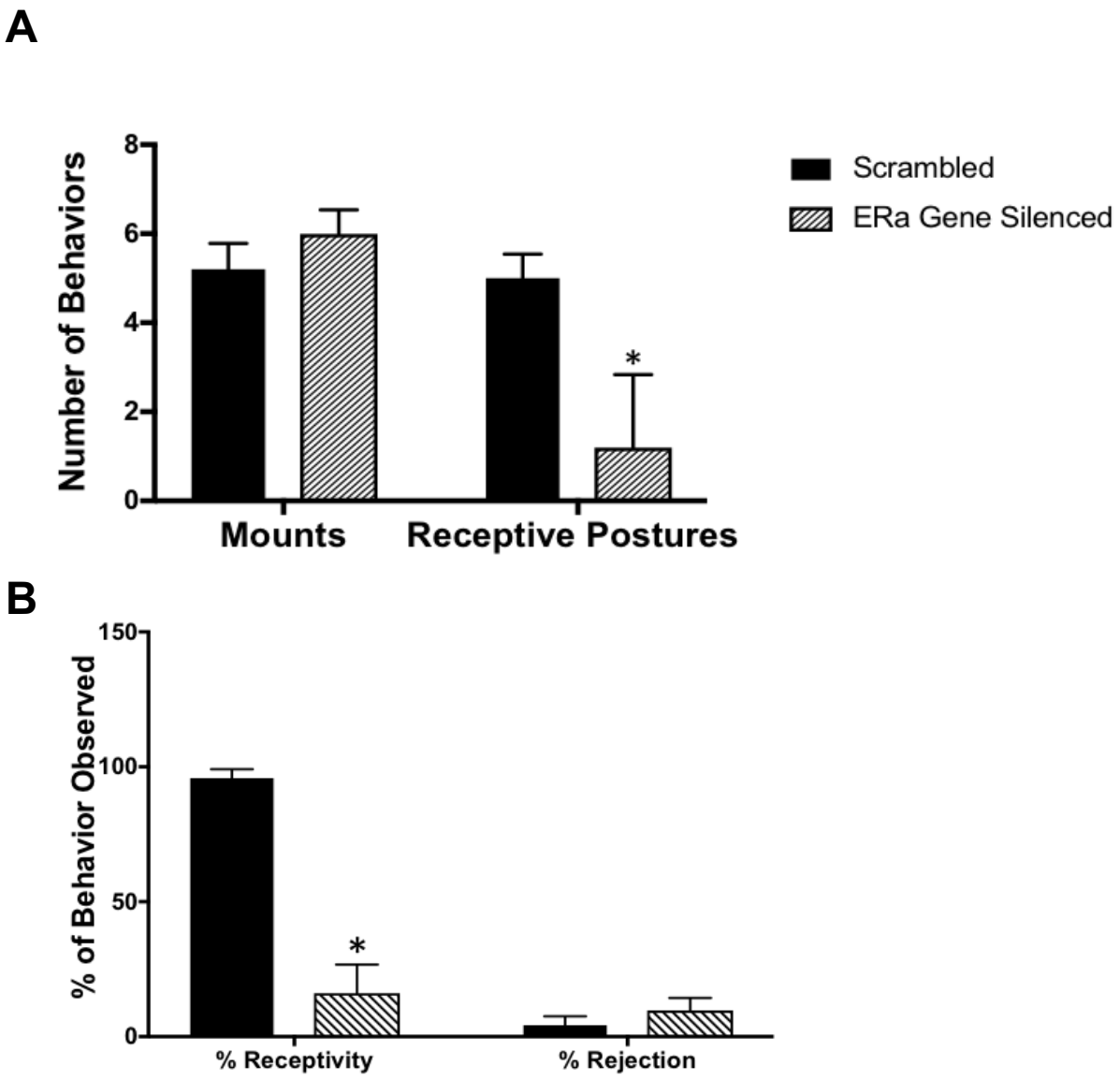

Figure 9: (**A**) Male partners showed no difference in mounting behavior towards females in either treatment group. The number of receptive postures in response to the male mounting, however, was decreased in the ERα gene silenced females (*p=0.001). (**B**) Thus, percent of test where sexual receptivity was observed was diminished in the ERα gene silenced group (*p=0.01). Despite a strong lack of receptivity in the ERα

gene silenced females, they did not exhibit more rejection behaviors than the scrambled controls.

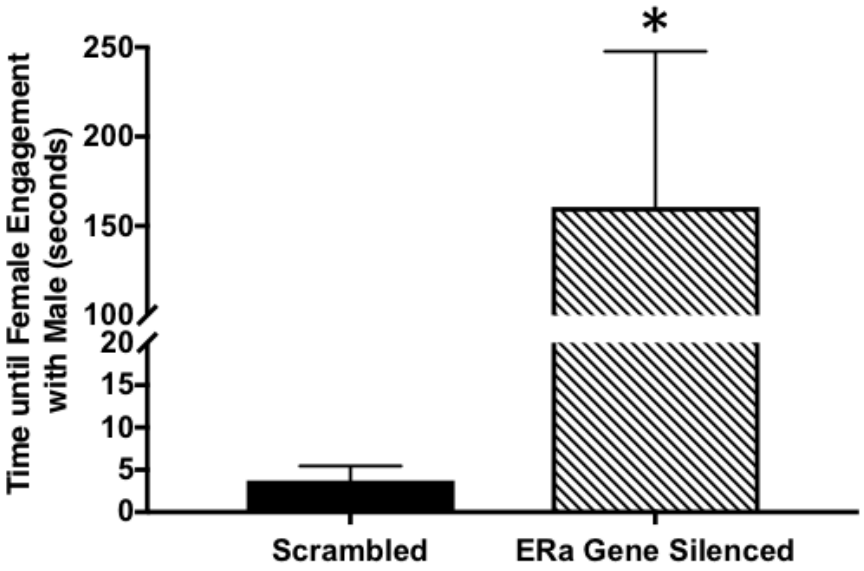

Figure 10: ERα gene silenced females took longer to sexually engage, either receptively or in rejection of, with their male partners (*p=0.001).

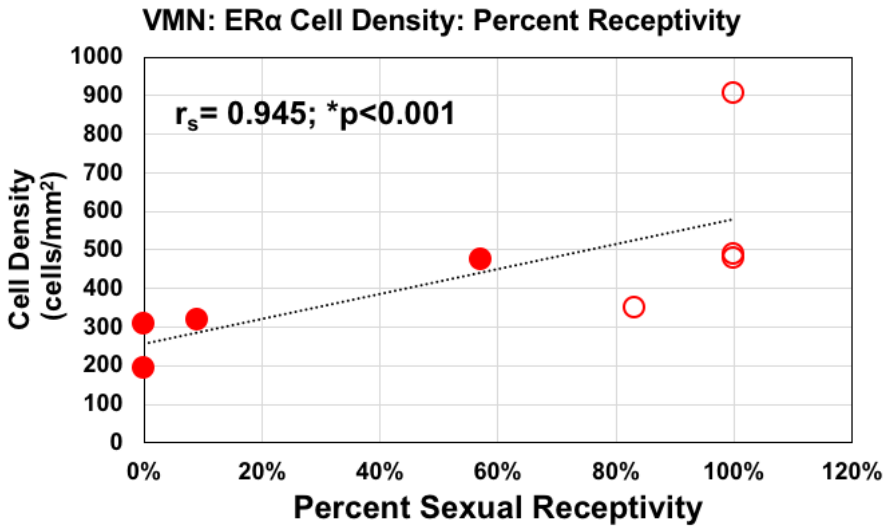

Figure 11: In the VMN, ERα cell density was positively correlated with the percent of receptivity observed (*p=0.001).

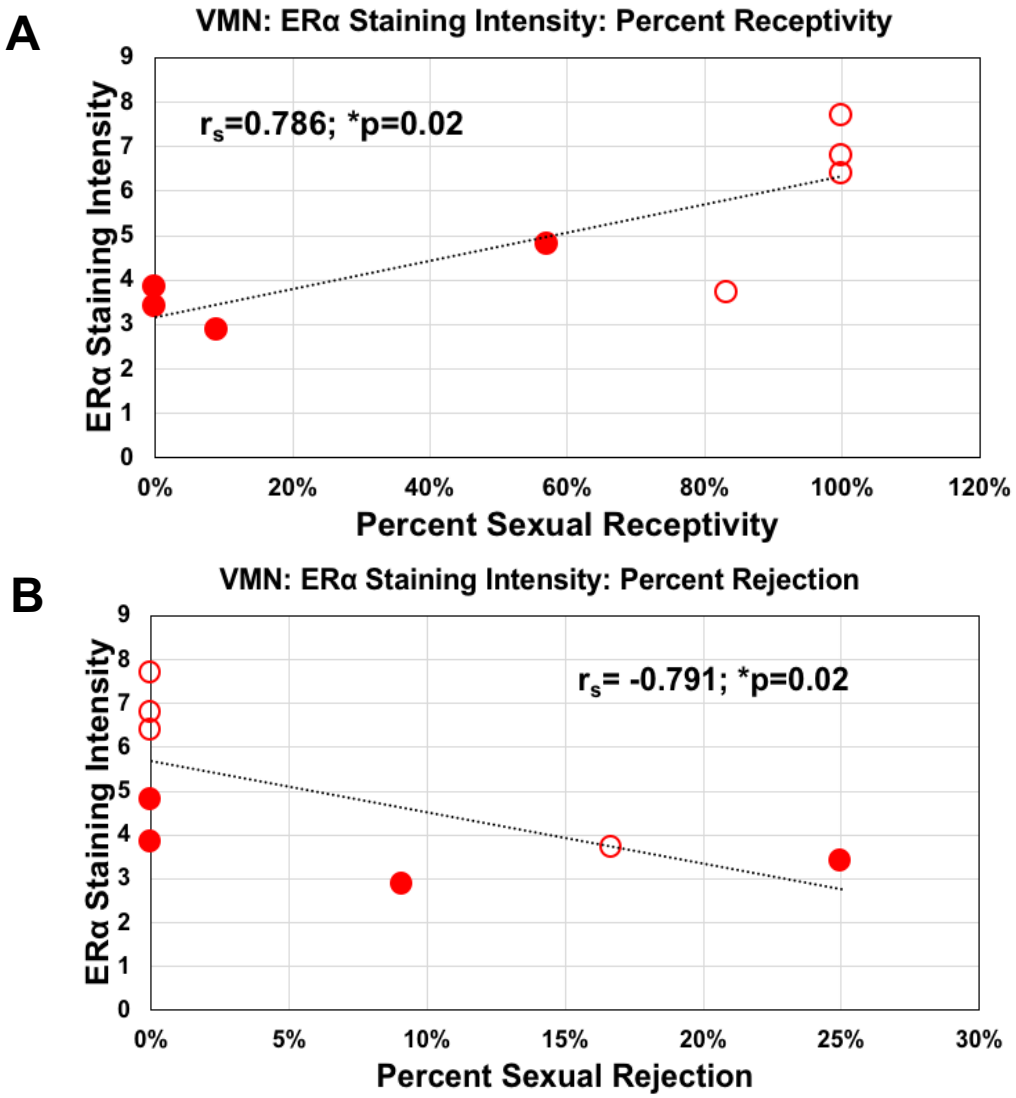

Figure 12: In the VMN, ERα staining intensity is positively correlated with sexual receptivity (**A**; *p=0.02) and negatively correlated with sexual rejection behaviors (**B**; *p=0.02).

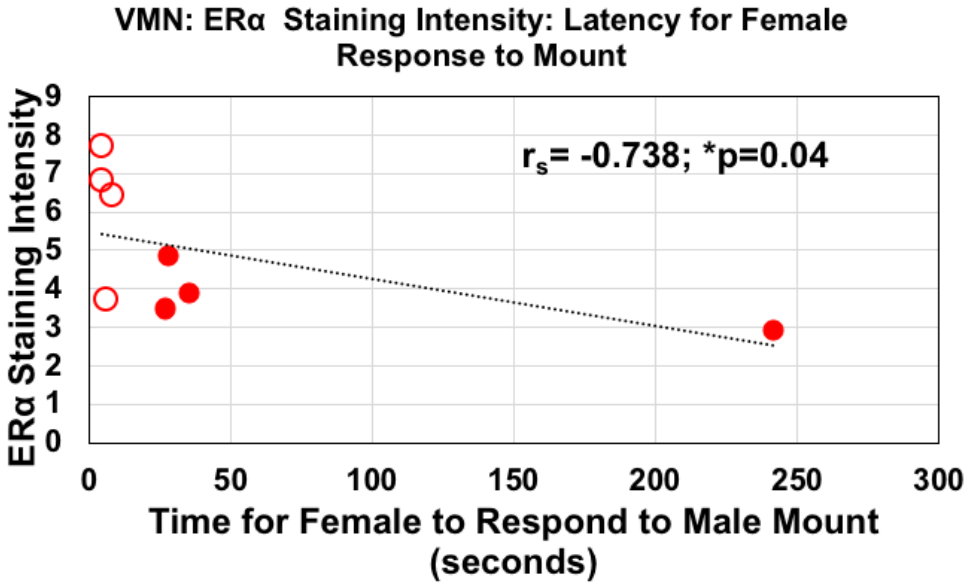

Figure 13: In the VMN, ERα staining intensity is negatively correlated with the amount of time it takes the female to respond to the male mounting behavior (*p=0.04). This correlation is still significant even without the data point to the far right.

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5. CHAPTER FIVE: Aromatase inhibition reveals an extra-ovarian contribution to the regulation of female sexual receptivity in marmoset monkey

Kraynak M, Kuehlmann AL, Kapoor, A, Flowers MT, Levine JE, and Abbott DH

[This manuscript is being prepared for submission to Journal of Clinical Investigation (JCI). Contribution to this manuscript: Designed/carried out experiments, analyzed data, and prepared the manuscript for this thesis and subsequent publication]

5.1 Abstract

Ovarian estradiol, E_2, supports the expression of female mammalian sexual behavior. In rodents, ovariectomy (OVX) abolishes female sexual behavior. In nonhuman primates, however, OVX decreases, but does not abolish female sexual behavior, serving as an example of behavioral emancipation from ovarian E_2. We hypothesize that extra-ovarian E_2 provides key physiological support for female sexual behavior in primates. We employed the use of an aromatase inhibitor, letrozole, to completely eliminate E_2 biosynthesis in a nonhuman primate model. Ten adult female marmosets were OVX and assigned to receive: subcutaneous E_2 containing silastic capsules (E_2; n=4), daily oral treatments of either vehicle (VEH, 1ml/kg, n=3) or letrozole (LET, 1 mg/kg, n=3) for the entire study. Five months following treatment onset, females were separated from their male partners and singly-housed for 30 days before commencing 30 minute testing 3x/week for two weeks while singly housed. Intra- and inter-rater reliability was >80%. E_2-treated females displayed more sexual acceptance and receptive head turns ($p=0.01$) than VEH or LET females. LET females exhibited the least receptive behaviors ($p=0.002$), while escalating rejections towards males, including aggressive hitting not observed in VEH or E_2-treated females. LC-MS/MS quantification of hypothalamic steroid hormones revealed a diminished E_2 level in the hypothalamus of the LET treated females only. Androgens, however, increased in both OVX and LET treated females, diminishing the hypothalamic E_2:T ratio in the LET treated females, thus suggesting a possible role for testosterone in the regulation of sexual rejection behaviors. Steroid hormones and sexual behavior expression were additionally correlated with the mRNA expression ratios of key serotonergic receptors in the hypothalamus that may be

possible targets for treating sexual dysfunction. Our findings provide the first evidence for extra-ovarian E_2 support of female sexual receptivity in a female primate. These findings also suggest the necessity of both ovarian and extra-ovarian E_2 in facilitating full expression of receptivity in the female marmoset, and identified androgens in the hypothalamus as a potential mechanism for driving sexual rejection. Such primate emancipation from complete dependence on ovarian E_2 may enable opportunistic female sexual engagement in complex social environments.

5.2 Introduction

Personally distressing sexual dysfunction, or female sexual interest and arousal disorder (FSIAD) as described in the Diagnostic and Statistical Manual of Mental Disorders[1] occurs in about 14% of women. The diagnostic criteria for FSIAD include having 3 out of 6 of the following symptoms for at least 6 months: 1) an absence or decrease of interest in sexual interest; 2) difficulty in initiating sex with a partner; 3) absence or decrease in responsiveness to partner; 4) decrease in sexual excitement or pleasure; 5) decrease in responsiveness to sexual cues; 6) decreased sensation during sexual activity[1]. Notably, some have criticized the 6-month criterion for the symptom duration and suggest that personally distressing sexual dysfunction should be treated after only 3 months of persistent symptoms [2]. Despite the number of women experiencing sexual health issues, there is only one FDA approved pharmaceutical treatment for FSIAD. The lack of understanding of specific biological mechanisms regulating sex drive/libido in women largely contributes to the lack of effective treatment options.

Ovarian E_2 has been established as a major contributor to the regulation of female sexual behavior in many mammalian species including rodents (rats, mice, guinea pigs) and nonhuman primate (NHP) species[3-10]. In rodents, the expression of sexual receptivity is entirely dependent on, and is strictly timed with, an ovulatory surge of E_2 from the ovary and a subsequent rise of progesterone[11-13]. Furthermore, surgical OVX abolishes female sexual receptivity, an effect that can be rescued by E_2 replacement[14,15]. The receptor mechanism governing sexual receptivity in female rodents has also been elucidated. Ovarian E_2 mediates sexual receptivity largely through estrogen receptor alpha (ERα)[16,17] in the ventromedial nucleus of the hypothalamus (VMN)[18]. Other components of female sexual behavior, such as proceptivity and sexual motivation, are also mediated through ERα, but more so through the population of ERα expressing neurons in the medial pre-optic area (mPOA). Furthermore, female sexual behavior expression has been linked to E_2-dependent neurochemical changes in reward system pathways, such as the dopaminergic and serotonergic systems, as reviewed in[19].

In NHP species such as the Great Apes[4-6], rhesus monkey[20-23], and the model used in this study, the marmoset monkey[24], females exhibit E_2-dependent increases in sexual receptivity. These behaviors, however, are noticeably not limited to mid-cycle rises in E_2. Additionally, in female marmoset monkeys, OVX diminishes sexual behaviors, but does not abolish sexual receptivity. The behavioral patterns observed in NHP species, compared with female rodents, and especially in marmoset monkeys, are more similar to behavioral patterns observed in women. Thus, female marmosets

provide an NHP model to examine the hormonal regulation of sexual behavior that can be relevant to sexual function in women.

Women and females in certain NHP species, such as rhesus monkeys[25], exhibit a naturally occurring menopause or a decline in ovarian function. Thus the female NHP lifespan extends beyond the reproductive years. Despite the sharp decline of circulating E_2, however, not all menopausal women become disinterested in sex, or develop FSIAD. In fact, there is conflicting evidence regarding the prevalence and degree of decline in libido in women post-menopause reviewed in[26]. Thus, there is an ovarian-independent, unidentified mechanism capable of supporting female sexual behavior in women and NHPs. As we have previously shown in female marmoset monkey that extra-ovarian E_2 functionally maintains negative feedback on gonadotropin secretion in the absence of the ovaries[27], it is plausible that female marmoset extra-ovarian E_2 can also support sexual receptivity.

Aromatase, a cytochrome P450 enzyme, encoded by the *CYP19A1* gene, converts testosterone (T) to E_2, as well as androstenedione (A4) to estrone (E_1). The major source of E_2 in the female body is the ovaries. E_2, however, is also produced in various other tissues where aromatase is expressed, such as the liver, breast tissue skin, pituitary gland, and in various brain regions[28-30]. In particular, neural production of E_2 has been identified in birds[31,32], rats[33-35], and in monkeys[36-38]. Brain aromatase is expressed at its highest levels in the medial basal hypothalamus (MBH), preoptic area (POA) and amygdala. A role for brain aromatase in regulating GnRH release in female macaques was recently demonstrated to show that 1) E_2 is produced and released at detectable levels within the OVX monkey pituitary stalk-median eminence (SME), 2) E_2

synthesis and release depends upon aromatase activity in the SME, and 3) hypothalamic E_2 can rapidly stimulate GnRH release in the SME [37-39]. These studies, in addition to our previous study in the female marmoset[27], provide evidence that E_2 is produced in the female marmoset brain and thus can consequently have physiological action in NHPs.

In the present study, we employed the use of the aromatase inhibitor, letrozole©, to diminish E_2 production in OVX female marmosets to enable investigation into extra-ovarian E_2 source(s) regulating of female sexual behavior in a NHP. We hypothesize that an extra-ovarian source of E_2, likely from the brain, will be greatly diminished or extinguished by body-wide aromatase inhibition and subsequently will abolish the expression of female receptivity in female marmoset monkeys compared with both E_2 replaced females and those experiencing the loss of ovarian E_2 alone. Additionally, we also examine if E_2-dependent changes in female behavior correlate to changes in hypothalamic mRNA expression of behaviorally-related serotonin and dopamine receptors as a means to gain some insight into the possible mechanism of extra-ovarian E_2 regulation of female sex behavior in the marmoset monkey.

5.3 Methods

Animals

Eleven adult female common marmosets (2-6 years of age) from the Wisconsin National Primate Research Center colony were OVX and randomly assigned to one of three treatment groups (Table 1): E2 replaced (E2; n=5), ovarian E_2 depleted, (OVX; n=3), or total E_2 depletion, OVX plus daily letrozole administration (LET; n=3). Treatment groups were balanced by age and body weight prior to the onset of the study

(**Table 1**). E_2 replacement was achieved through subcutaneous E_2-filled capsules that maintained a sufficient systemic level of E_2 (853 $\pm$ 65 pg/ml) to suppress gonadotropin secretion. To maintain constant E_2 levels, capsules were replaced every 3 months throughout the study. OVX females received a daily oral dose of 1ml/kg ensure© as a vehicle control. LET females were given a daily oral dose of 1 mg/kg of letrozole dissolved in 1ml/kg nsure©. This effective dose was previously determined in[27].

All marmosets lived with a well-established cagemate in 0.60m x 0.91m x 1.83m enclosures and were maintained with 12-hour lighting (0600h-1800h), 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. Animals were fed ad-libitum with Mazuri Callitrichid High Fiber Diet #5MI6 (Purina Mills International, St. Louis, MO). The calories in this diet were 20% protein, 6% fat, and 74% carbohydrate.

Ovariectomy and estrogen replacement

Following baseline assessments, bilateral OVX was performed. Cloprostenol (Estrumate®, 0.75-1.50 μg intramuscular injection for two successive days approximately 11-60 days after ovulation), an analog of prostaglandin-F2-alpha, was administered prior to OVX to facilitate scheduling of OVX during the follicular phase. Treatment onset was the day of the OVX procedure.

Hormone Assay

For steroid hormone analyses, both plasma and hypothalamic tissue samples (previously published methods for steroid hormone extractions[40]) underwent extraction. Hypothalamic samples were composed of hemi-hypothalami sectioned into 50-75mg aliquots that were combined after the extraction process. Each combined hypothalamic sample was subsequently analyzed on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (LC-MS/MS) as previously described[27].

Behavioral Observations

Following treatment onset, pairs were acclimated to the testing cages, as previously described[41]. 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. Following this single housing period, pairs were reunited for behavioral testing. The pairs were tested for three 30-minute testing sessions per week for two weeks. Each test was video recorded. At least two observers behaviorally scored each test and inter- and intra-observer reliability was 80% or greater.

Gene Expression Analysis

Hypothalamic tissues were dissected and frozen at necropsy. 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 PCR 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 E2 group. Primer sequences were designed using NCBI Primer-Blast[42] and are listed in Supplemental **Table 2**.

Statistical Analysis

Data collected was analyzed utilizing SPSS software (IBM, Armonk, NY). Hormone measures, behavioral observations and gene expression data were analyzed with a one-way ANOVA test, followed by Bonferroni multiple comparison post hoc tests. The steroid hormone data was log transformed prior to the ANOVA. Nonparametric spearman's correlation tests were utilized to detect relationships between gene expression, hormone levels, and behavior. Spearman's rho coefficients are expressed as r_s. Statistical significance was determined at $p \leq 0.05$.

5.4 Results

Hormone Measures (Peripheral and Hypothalamic)

Hypothalamic E_2 levels (Figure 1A) in LET females were significantly lower than the OVX ($p=0.04$) and E2 treated females ($p=0.03$). Plasma E_2 levels (Figure 1B) were diminished in OVX and LET females ($p<0.001$) compared to E2 replaced controls. In this experiment, OVX and LET plasma E_2 levels were not statistically different. Other published experiments in this thesis (Chapter 3)[27], however, show that LET diminishes E_2 below OVX alone.

Hypothalamic androgens are shown in Figure 2. Hypothalamic androstenedione (A4) (Figure 2B) was elevated in both OVX ($p=0.05$) and LET ($p=0.05$) females compared with E2 replaced controls. Despite what appears to be a trend towards elevated T (Figure 2A) in the hypothalamus, there was also no statistical difference between any groups, nor was there any treatment effect on hypothalamic DHEA (**2C**).

Circulating plasma androgen levels show no treatment effect on T (Figure 3A) or A4 (3B). Plasma DHEA, however, (3C) was elevated in OVX (p=0.02) and LET (p=0.01) treated females compared to the E2 replaced controls. Both hypothalamic and plasma P4 were also analyzed in this study, however, there were no treatment effects in either P4 measure.

Selected ratios of hypothalamic steroid hormones were also calculated and analyzed between the groups. The ratio of hypothalamic A4:DHEA was higher (Figure 4A) in LET (p=0.005) compared to E2 replaced females and there was a trend (p=0.06) for higher hypothalamic A4:DHEA ratio in OVX compared to E2 replaced females. There were no treatment effects for the ratio of hypothalamic T:A4 (Figure 4B). Finally, the ratio of hypothalamic E_2:T was elevated (p=0.004) (Figure 4C) only in LET females compared with E2 replaced controls. Given the steroid hormone ratios, the hypothesized shift in favored steroidogenic pathways in each treatment groups is illustrated in Figure 5. Each diagram represents the likely steroid hormone synthesis pathway in one treatment group (Figure 5A: E2; 5B: OVX; C: LET). In addition to the steroid hormone levels in the hypothalamus, mRNA expression analysis (Figure 6) revealed no difference in expression of *CYP19* (aromatase) or the androgen synthesizing enzyme *CYP17A1*. There was, however, an overall treatment effect on the mRNA expression of *CYTB5B* (cytochrome b5, type B) (ANOVA: p=0.03), however, post hoc testing revealed only a trend (p=0.06) of LET females expressing more hypothalamic *CYTB5B* than E2 replaced controls.

Behavioral Analysis

The behavioral analysis showed evidence that both ovarian and extra-ovarian E_2 have essential roles in the regulation of female sexual behavior. Figure 7A illustrates that diminishing ovarian E_2 decreased sexual receptivity and increased rejection behavior compared with the E_2 treated females (p=0.03). The depletion of extra-ovarian E_2 via aromatase inhibition diminished female sexual receptivity and increased female sexual rejection beyond that of OVX alone (p=0.003). Additionally, Figure 7B depicts that extra-ovarian E_2 is not sufficient to support intimate sexual receptivity beyond females accepting mounts by the male, as indicated by the stark decrease in receptive head turns in OVX females (p=0.003). Despite the expression of receptivity in OVX females, full receptivity is not exhibited by these females. LET females did not exhibit initial receptive behaviors, thus no copulation led to sexually intimate behavior accompanying mounts. Notably, however, diminishing extra-ovarian E_2 leads to not only increased rejection, but the rejection is more aggressive as indicated by an increase in hitting behavior towards the male partners (p=0.004). The sexual aggressiveness exhibited by LET females is a novel progression of behavior compared to existing literature examining female sexual behavior in NHPs.

Relationships Between Hormones, Behavior and Hypothalamic Gene Expression

No treatment effects were detectable for any of the behaviorally related target genes, despite a trend (ANOVA: p=0.07, *HTR5A:HTR2C).* There were, however, a variety of correlational relationships detected between gene expression, steroid hormones, and behavior.

Relationships between hypothalamic and plasma levels of steroid hormones, behavior, and hypothalamic gene expression were also analyzed. Table 4 shows the

Spearman's correlation coefficients (r_s) and associated significance level for each relationship identified. Overall, there was a positive relationship between female plasma E_2, but not hypothalamic E_2, with the expression of female sexual receptivity ($r_s = 0.97$; $p<0.001$) and an inversely negative relationship with female sexual rejection ($r_s = 0.96$; $p<0.001$). The expression of both receptivity and rejection were also found to be negatively correlated with the ratio of serotonin receptor 5A (*HTR5A*) to serotonin receptor 2C (*HTR2C*) (Receptivity: r_s= (-) 0.91, Rejection: p=0.002; r_s= (+) 0.88, p=0.001). The ratios of gene expression for *HTR5A:HTR2C* (r_s= (-) 0.91, p=0.001) and *HTR5A:HTR2A* (r_s= (-) 0.83, p=0.006) were both negatively correlated with plasma E_2 levels. While hypothalamic E_2 was not correlated directly with sexual receptivity, it was positively correlated with hypothalamic mRNA expression of *HTR2C* ($r_s = 0.60$; p=0.05) and with the dopamine receptor ratio *DRD4:DRD1* ($r_s = 0.68$; p=0.02), and negatively correlated with the ratio of *HTR1A:HTR2C* ($r_s = 0.67$; p=0.05).

Female hypothalamic androgens were also correlated with their behavior and gene expression. Hypothalamic androgens were negatively correlated with female sexual receptivity and positively correlated with female sexual rejection behaviors: T ($r_s = 0.75$; p=0.008), A4 ($r_s = 0.79$; p=0.004), and DHEA ($r_s = 0.63$; p=0.04). In the periphery, only female plasma DHEA levels were negatively correlated with female sexual behavior (Receptivity: r_s = (-) 0.83; p=0.006; Rejection: r_s = (+) 0.81; p=0.01). Hypothalamic androgens, and plasma T and A4 levels, were not associated with any changes in serotonergic or dopaminergic receptor mRNA expression in the hypothalamus. Plasma DHEA levels, however, were positively correlated with the

mRNA expression ratio of *HTR5A:HTR2A* (r_s = 0.74; p<0.02) and *HTR1A:HTR2C* (r_s = 0.73; p=0.03).

5.5 Discussion

In the present study, we demonstrated that E_2, from both ovarian and extra-ovarian sources, is crucial for any expression of female sexual receptivity in a female NHP, the female marmoset monkey, and further that both ovarian and extra-ovarian E_2 are necessary for the full expression of female sexual receptivity. Importantly, we have also identified that E_2 is not the only steroid hormone in the brain that may be contributing to the regulation of female sexual behavior expression. This study identifies a potential antagonistic role for hypothalamic androgens, inhibiting female sexual receptivity. These results thus support the hypothesis that, at least in female marmoset monkeys, extra-ovarian E_2, likely produced in the hypothalamus, is the major regulator of the expression of sexual receptivity.

E_2 and Sexual Receptivity

Although ovarian E_2 has been shown to elevate the expression of sexual receptivity in female NHPs[4,24,43], the inability to completely deplete female receptivity has been an enigma in the understanding of female sexual function in NHPs and women. Such persistent receptivity differs from its strictly regulated expression in female rodents, in which mid-cycle ovarian E_2 is necessary for the reflexive expression of the receptive posture, lordosis [14,15]. Ovarian E_2 depleted female monkeys in this study still express receptivity towards at least 35% of male mounting behaviors. It is only when the production of extra-ovarian, possibly hypothalamic, E_2 is removed in addition to OVX that female marmosets are truly, and consistently, rendered

unreceptive to male sexual advances. While E_2 is necessary for female marmoset receptivity, however, the present data also suggest that in concurrence with the loss of ovarian E_2, androgens in the hypothalamus may also play a critical role in the inhibition of female sexual receptivity and the expression of sexually rejecting behaviors.

Androgens and Sexual Receptivity

In the present study, female hypothalamic A4 as well as plasma DHEA levels, are elevated in LET females. In addition, all of these female androgen measures negatively correlate with female sexual receptivity and positively correlate with female sexual rejection behaviors. This evokes the subsequent question of what role do hypothalamic androgens play in the regulating female sexual behavior. The varying effects of androgens on female sexual receptivity have been studied in female rodents, as well as in NHPs. In female rodents and rhesus monkeys, T and A4 have been shown to increase or enhance female sexual behavior[22,44]. This effect, in female rodents, has been attributed to the aromatization of T to E_2 through analogous studies showing that the non-aromatizable androgen, 5a-dihydrotestosterone (DHT) antagonizes E_2-induced sexual receptivity [44-48]. Potential mechanisms for androgen antagonism of E_2 mediated female receptivity have been elucidated in female rodent studies. The effects of DHT on female receptivity in the female rat, can be blocked by the androgen receptor (AR) antagonist, flutamide[49]. Additionally, androgens in the hypothalamus have been shown to antagonize E_2 activity by downregulating estrogen receptor expression and activity[50].

Interestingly, however, in previous marmoset monkey studies, the expression of sexual receptivity and/or rejection behavior in T-treated OVX females[9] were similar to OVX females, and not increased/enhanced as observed in both the rhesus monkey and

the female rodents. E_2 has been repeatedly shown in other publications[9,24] as well as in this study, to robustly increase female sexual receptivity, thus if T were being aromatized to E_2 in the hypothalamus, it would be expected that female sexual receptivity would have increased. Given that OVX females in this study were not hypoestrogenic in the hypothalamus compared to E_2 replaced females, it can be postulated that E_2 in the hypothalamus, likely locally produced, may be sufficient to antagonize any androgen-facilitated rejection behavior, at least in female marmoset monkeys. Even in the absence of elevated T, the hypothalamic ratio of E_2:T was diminished in only OVX + LET females compared to both OVX and E_2 replaced females. It is thus plausible that E_2 and T in the hypothalamus, have an antagonistic relationship regulating female sexual behavior. This hypothesis is, in part, supported by studies in the rodent showing that increased E_2 can reduce the receptivity-inhibiting actions of DHT [51]. Thus, the significantly elevated E_2:T ratio in LET females could be one possible reason and mechanistic explanation for the increased and robust display of rejection behavior by LET female marmosets.

Ovarian E_2 Depletion and Hypothalamic Steroid Hormones

The finding that hypothalamic E_2 in OVX females was comparable to that of females with mid-cycle circulating E_2 is evidence that in a female NHP, E_2 is synthesized locally in the hypothalamus independently of gonadal status. The elevated A4 detected in the hypothalamus as a consequence of ovarian E_2 depletion is another novel finding of this study. Unlike the hypothalamic E_2 results, however, the rise in hypothalamic androgen is not necessarily due to local production in the hypothalamus, alone. One probable contribution to increased hypothalamic A4 includes circulating

levels of DHEA. Ovarian E_2 depletion in the female marmoset has been shown to induce the protein expression of cytochrome b5 in a newly differentiated zona reticularis (ZR) of the adrenal cortex. This then leads to the increased activity of 17,20 lyase portion of CYP17A1 in the adrenal cortex, resulting in increased production of adrenal DHEA[52]. This phenomenon is very likely the direct cause of increased circulating DHEA in LET female marmosets in the present study. It is possible that the increased circulating DHEA is reaching the hypothalamus and consequently utilized as a substrate to synthesize A4. Adrenalectomy and OVX combined in female marmosets, however, do not result in the robust lack of sexual receptivity observed in the present study, thus while the elevated adrenal androgens may result in female hypothalamic hyperandrogenism, the hypothalamic E_2 in OVX marmosets is likely produced de novo from androgen substrate locally in the brain[53]. An abundance of DHEA made available to the brain from the adrenal cortex may account for the biosynthetic pathway shift from DHEA to A4 to T (and then E_2 in OVX; Figure 5B) observed in the OVX and LET females groups alike, rather than through androstenediol to T to E_2 (Figure 5A). It is also possible that, like in the OVX marmoset adrenal cortex, cytochrome b5 is upregulated in the hypothalamus as well with the removal of ovarian hormones. The final consideration for the source of the hyperandrogenism observed in the hypothalamus after OVX is that DHEA is produced *de novo* from 17-hydroxypregnenolone. The data from these experiments, however, only permit speculation.

Previous literature in rodents and avian species have shown that sex steroids, both E_2 and androgens, also contribute to the regulation of the aromatase enzyme protein[54-57] and mRNA[58]{Shen 1995}[59] expression and activity[60-64] in the hypothalamus.

In the present study, however, mRNA expression of aromatase was not altered by either diminished circulating E_2 or diminished hypothalamic E_2. Thus, further studies are necessary to identify if hypothalamically-produced sex steroids alter hypothalamic aromatase activity.

Steroid Hormones, Serotonin, and Behavior

The analysis of behaviorally-relevant gene expression in the female hypothalamus analysis reveals possible estrogenic targets that may be integral to understanding of neural mechanisms of female sexual receptivity specific to NHPs, and possibly humans. Serotonin receptors (5HTRs), in particular, are identified as potential transcriptional targets of steroid hormones in this study. Serotonin has been mainly implicated as an inhibitor of sexual receptivity. Previous literature in both female rodents[65] and in marmoset monkeys[41] have pharmacologically shown that $5HTR_{1A}$ drives inhibition of female sexual receptivity. Contrastingly, $5HTR_{2A/C}$ are serotonergic receptors that have facilitative effects on female sexual receptivity[66]. The 5HT receptor ratio expression changes in the female marmoset hypothalamus in this study contributing to speculation that elevated androgen and E_2 to androgen ratios in the hypothalamus are supporting female sexual rejection. The ratio of *HTR5A:HTR2A/C* is positively correlated with the hypothalamic levels of A4 and the ratio of E_2:T, two measures that are increased in LET females which exhibited increased female rejection behavior, including aggressive rejection. In addition, plasma E_2 is negatively correlated with this ratio and is shown to increase female sexual receptivity. Hypothalamic E_2, however, is not correlated with the occurrence of female marmoset receptivity, but is positively related to specific gene expression patterns, such as the expression of *HT2C,*

permissive of female sexual receptivity. Thus, the finding that full expression of female marmoset sexual receptivity requires both hypothalamic and ovarian E_2 may be because both are necessary to fully engage female receptivity inducing serotonin receptor dynamics in the female marmoset hypothalamus.

The present study also suggest a species difference in inhibitory serotonergic pathways. In female rodents[67] and rhesus monkeys[68], E_2 has been shown to downregulate $5HTR_{1A}$ specifically in the hypothalamus. This receptor historically has been understood to be the major inhibitory receptor pathway for female sexual behavior. In female marmosets, while the $5HTR_{1A}$ agonist, 8-OH-DPAT has been shown to also increase female sexual rejection, the results in this study suggest that in addition to $5HTR_{1A}$, $5HTR_{5A}$ may be another major receptor-mediated inhibitory 5HT mechanism, at least in the female marmoset. These findings suggest that there may be marmoset or primate specific hypothalamic 5HT receptor subtypes that affect female sexual behavior, given the understanding that a wide variety of 5HT receptors are expressed and act in a complex, dynamic ways in many brain regions. In generating these hypothalamic mRNA expression data, this study has enabled consideration of such new hypotheses for hypothalamic-specific E_2 mechanisms governing female sexual behavior in a NHP model.

The lack of effective treatment options for women with FSIAD is likely directly related the lack of primate and human specific understanding of the biology of female sexuality. Prior to this study, there had been a lack of studies examining another role for E_2 in mediating female sexual function in primate. The present study suggests that E_2 is absolutely key to the regulation of female sexual receptivity in NHPs. The implications of

these findings open up a wealth of opportunities to explore mechanistic approaches to treating sexual dysfunction in women.

Currently, the only FDA approved treatment for FSIAD in pre-menopausal women, is flibanserin, is a serotonin receptor 1A agonist and serotonin receptor 2A antagonist[69]. This pharmaceutical has been shown in previous marmoset studies[41] to increase relationship quality between male-female marmoset pairs and in pre-menopausal women[41] as well as increasing sexually satisfying events in women. The present study is the first suggesting transcriptional regulation of 5HT receptor dynamics in the hypothalamus comparing across models of both ovarian and extra-ovarian E_2. The correlation between E_2-dependent female sexual behavior phenotypes and specific 5HT receptor ratios of mRNA expression imply that $5HTR_{5A}$ and $5HTR_{2c}$ may be important neural transcriptional targets of both E_2 and androgens, and should be considered as treatment targets for women with FSIAD. Studies examining $5HTR_{5A}$ in any mammalian species are lacking. It is known, however, that this receptor is inhibitory towards anxiety-like behaviors[70] and gene variants in humans have been linked to aberrant sexual expression in individuals with schizophrenia[71,72]. It is worthwhile to note that in female rodents, within the 5HT 5 class of 5HT receptors, $5HTR_{5B}$ is the receptor subtype identified in more behavioral regulation than $5HTR_{5A}$ (even despite little research within this class, likely due to the lack of effective and selective pharmaceutical options). Also notably, the gene for $5HTR_{5B}$ is absent from the human, but not the mouse, genome[73]. This may suggest one possible species difference in serotonin signaling between rodents and primates, including humans.

Implications of Hypothalamic E_2 in the Treatment of Sexual Dysfunction

The findings related to hypothalamic E_2 and gene expression in this study may reveal unique insights into the effectiveness of flibanserin on sexual function in women. In addition to the modulation of serotonergic receptors, flibanserin was also shown to be a weak agonist of dopamine receptor 4 (DR4)[74]. In vitro, a high abundant is necessary for flibanserin to activate DRD4. Data from the present study suggest that there may be more relevance to this pharmacological effect of flibanserin. In the female marmoset monkey, we have identified a positive relationship between hypothalamic E_2 and mRNA expression ratio for dopamine receptors *DRD4:DRD1*. Thus, if hypothalamic E_2 permits the favoring of DR4 expression in women, then it is possible that another, less discussed neural mechanism of action for flibanserin could be through increased dopamine signaling. In the female marmosets in this study, the hypothalamic E_2 levels in both OVX and E2 replaced, but not LET females, varied among individuals (average range of 2.2 ng/ml). If such variation in hypothalamic production of E_2 occurs in women as well, the availability of the DR4 in the brain might enhance the effectiveness of flibanserin. This is one possible explanation that can be derived from this study to provide insight into the inconsistent effectiveness of flibanserin in women.

In conclusion, this study identifies a novel role for both hypothalamic E_2 in the regulation of female sexual receptivity and accompanying androgenic regulation of female sexual rejection in marmoset monkeys. Furthermore, these data also identify key relationships between steroid hormones, behavior, and behaviorally-related genes that suggest possible neural mechanisms for E_2 regulation of female sexual behaviors. The findings from this study have key implications and insights into sexual function in a female NHP, and possibly women.

5.6 Tables and Figures

	AGE (YEARS)	BODY WEIGHT (G)
OVX+ E2	3.24 ± 0.51	402.0 ± 25.9
OVX +VEH	3.5 ± 0.53	394.0 ± 49.8
OVX+LET	3.18 ± 0.34	389.0 ± 10.1

Table 1: Treatment groups were balanced for age and body weight prior to treatment onset.

Gene	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
CYP17A1	ATCATCAATCTGTGGGCGCT	CGCTCAGGCATGAACTTGTC
CYP19	TGGTCTTGTTCGCATGGTCA	TCAACACGTCGATGAAGCCT
CYTB5B	GCGAAGCGCAACTCCTTGAA	CGGGTGACATCGTAGACTCG
TBP	CCATGACTCCTGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA

Table 2: Marmoset specific primers for the behaviorally related genes are listed above.

Tata-binding protein (TBP) was the housekeeping gene used in this study.

	TREATMENT GROUP	HYPOTHALAMIC E_2 MEASURE COMPARED TO THE CONTROL GROUP
CJ1892	OVX	Increased 26%
CJ1896	OVX	Decreased 68%
CJ1848	OVX	Increased 61%
CJ1867	LET	Decreased 93%
CJ1884	LET	Decreased 97%
CJ1946	LET	Decreased 70%

Table 3: The table above shows descriptive information about the hypothalamic E_2 measured in the OVX and LET treated females compared with the E2 replaced controls. The percentage reflects the percent E_2 measured, either increased or decreased compared with the mean control hypothalamic E_2 values (mean=1.05pg/ml). The response of OVX on hypothalamic E_2 was widely variable among the three females included in this study. Two out of three of these females increased hypothalamic E_2 following OVX, while another (cj1896) showed diminished hypothalamic E_2 that resembles the levels seen in the LET treated females, all of which show decreased E_2.

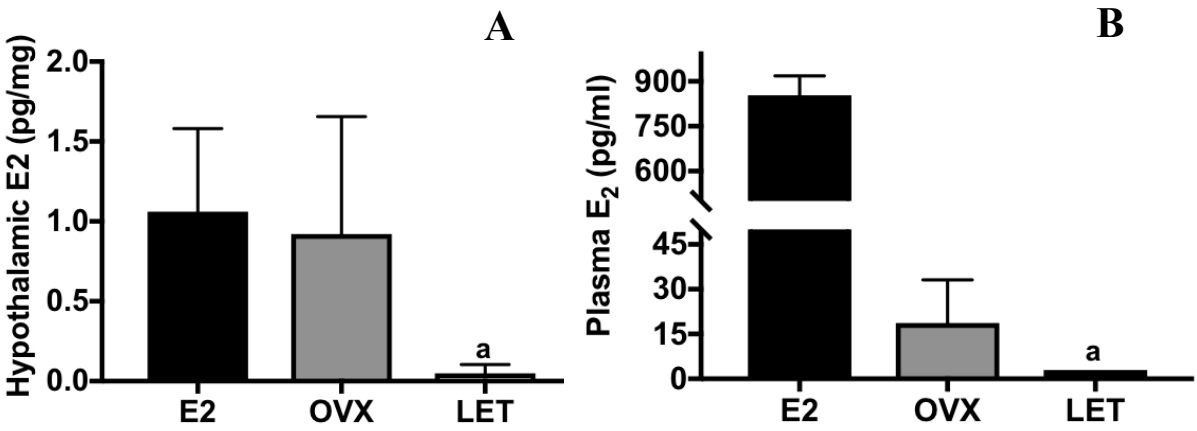

Figure 1: (**A**) Hypothalamic E_2 is decreased in the LET treated females compared only with the E_2 treated controls ($p<0.001$). (**B**) Plasma E_2 however, was diminished in the OVX and LET females ($p<0.001$). (a = difference shown is compared with the E2 replaced control group)

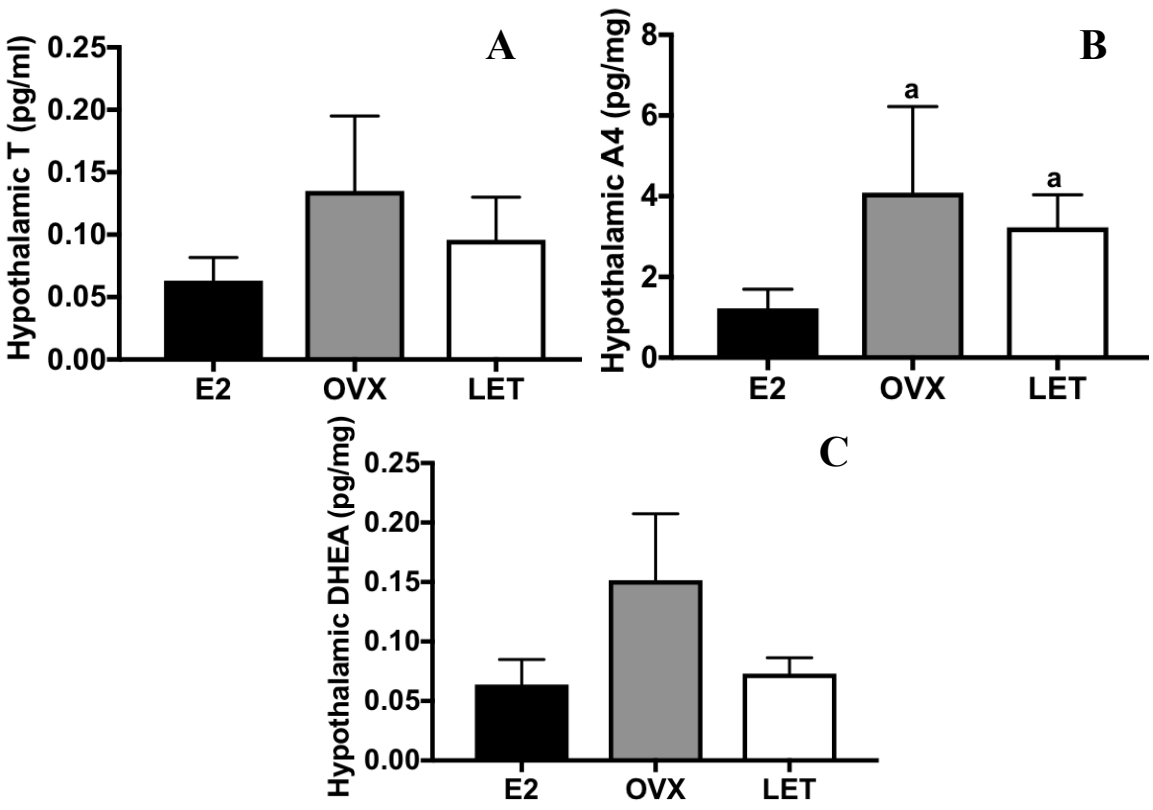

Figure 2: Hypothalamic T (**A**) and A4 (**B**) were increased in the OVX (p=0.002) and LET (p=0.001) females compared with the E2 replaced controls. DHEA (**C**) in the hypothalamus was only increased in the OVX females (p=0.04). (a = difference shown is compared with the E2 replaced control group)

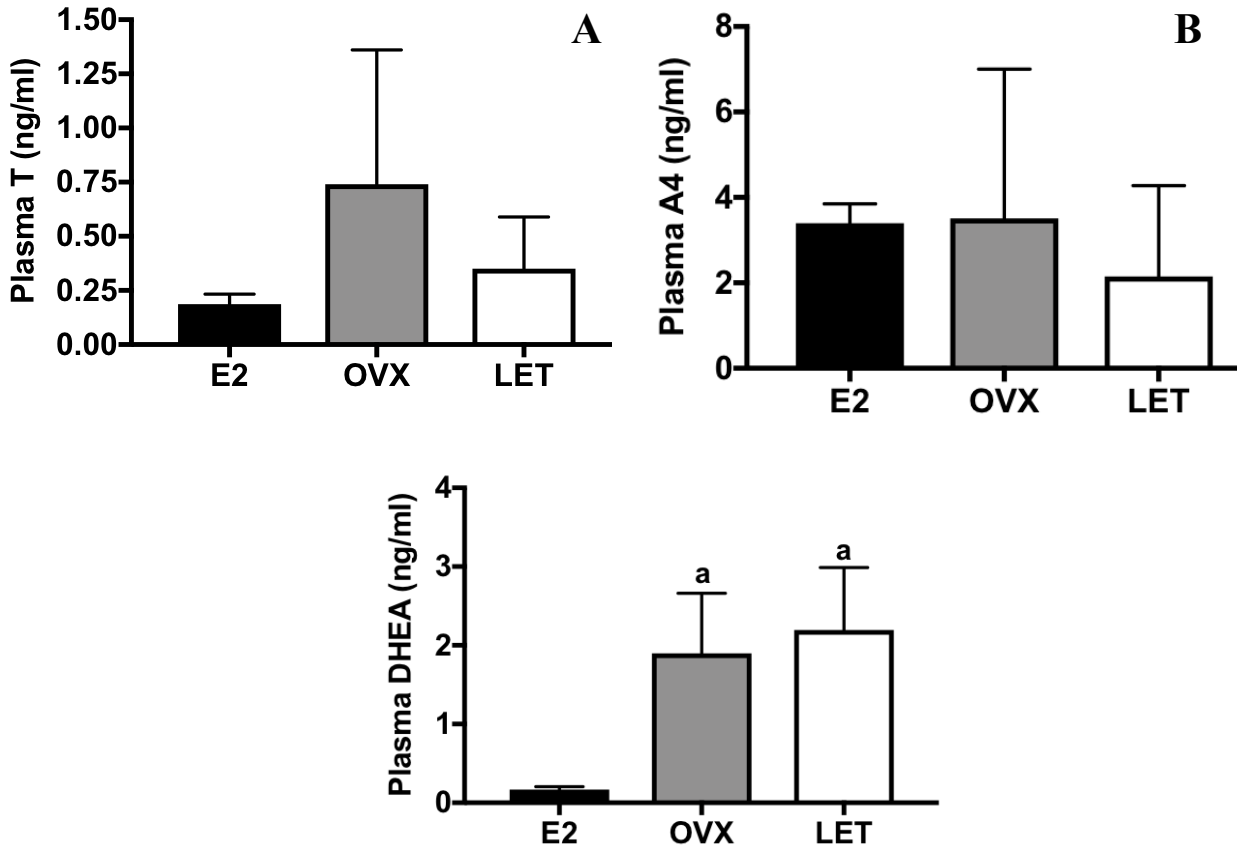

Figure 3: Peripheral T (**A**) and A4 (**B**) is not altered due to OVX or LET. Peripheral DHEA, however, is increased in the both the OVX ($p=0.02$) and LET ($p=0.02$) compared with the E2 treated control. This is likely a result of the release from E_2 suppression of the adrenal gland [52]. (a =difference shown is compared with the E2 replaced control group)

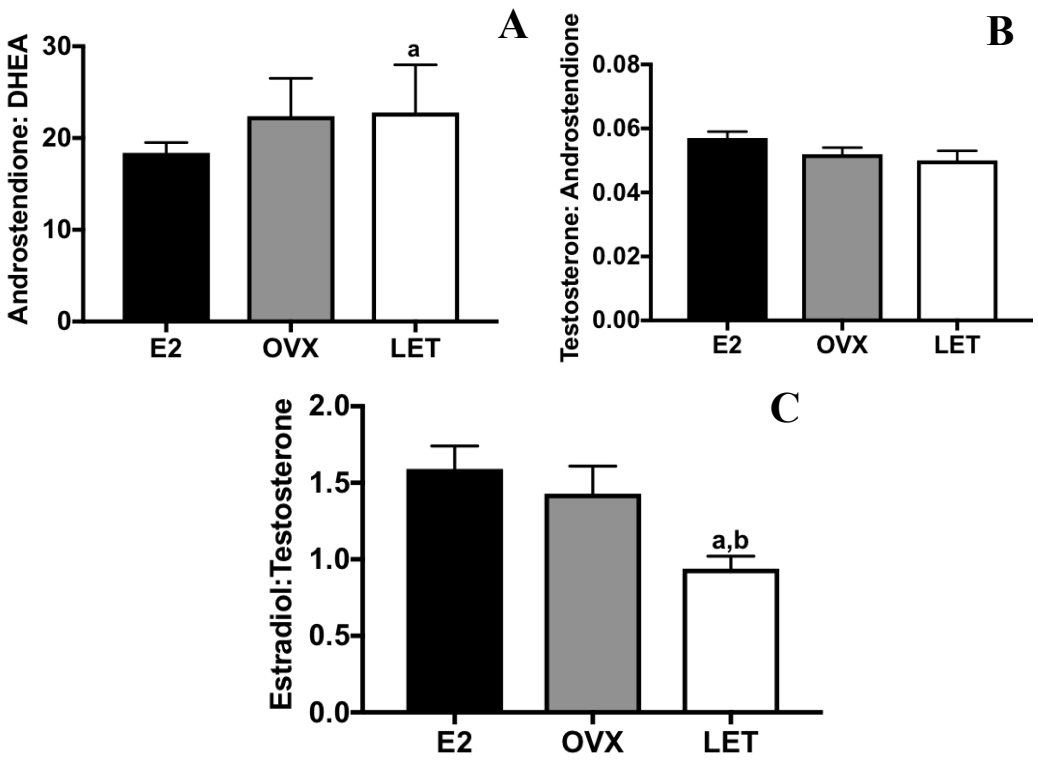

Figure 4: Steroid hormone ratios of hypothalamic androgens show that E_2 replacement favors the typical ∆5 steroidogenesis pathway in the hypothalamus, favoring the throughput to E_2. The LET treated females have a higher A4:DHEA (p=0.001) compared to the E2 replaced controls (**A**). There was also a trend of the OVX A4:DHEA ratio compared with the E2 replaced controls(p=0.056). There was no difference in T:A4 ratio (**B**) between any group. Additionally, the E_2:T ratio (**C**) was elevated in the LET treated group compared to both OVX (p=0.04) the E2 replaced controls (p=0.004). (a =difference shown is compared with the E2 replaced control group; b= difference shown is compared with the OVX group)

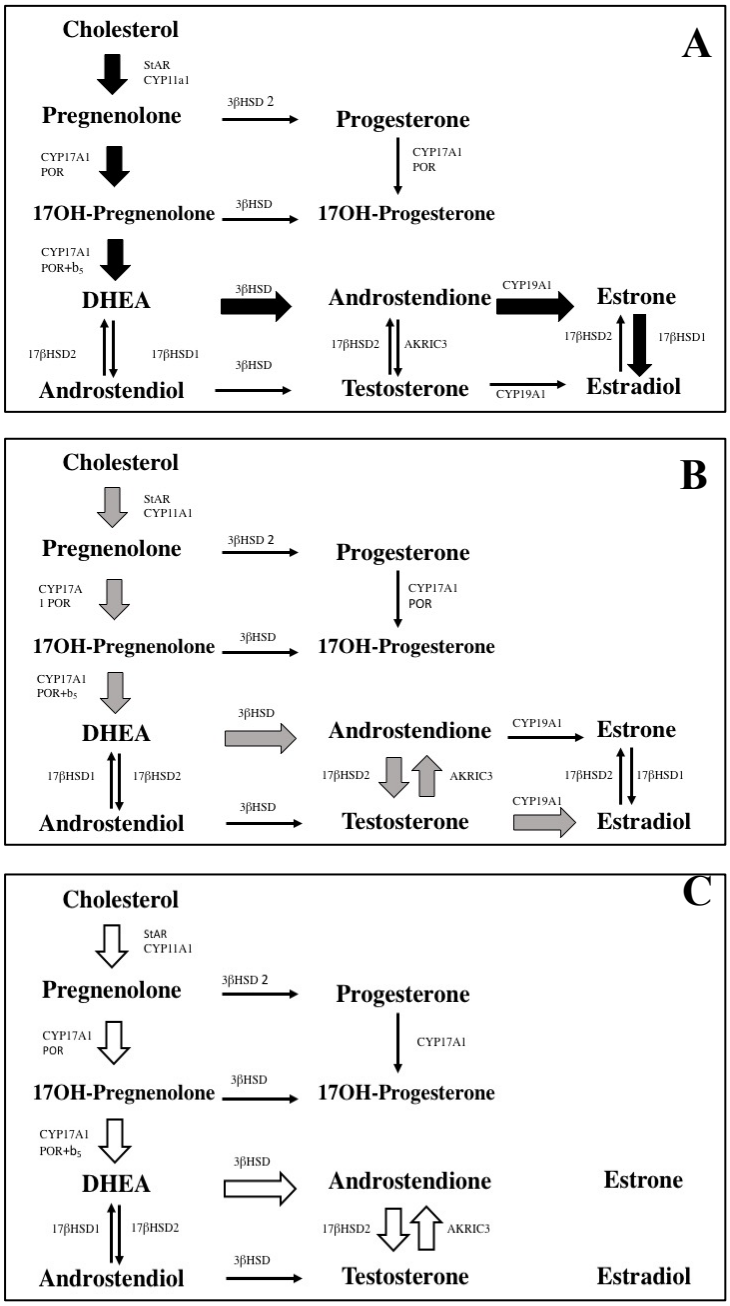

Figure 5: The ratios of hypothalamic steroid hormones identify that ovarian E_2 depletion shifted the major pathways of steroid hormone synthesis. Further, LET treatment mediated aromatase inhibition diminished aromatase activity in the brain blocking the majority of the production of E_1 and E_2 in the hypothalamus. These diagrams show the

proposed pathways for E2 replaced females (**A**), OVX (**B**), and LET (**B**) elucidated by the hypothalamic steroid hormone ratio data.

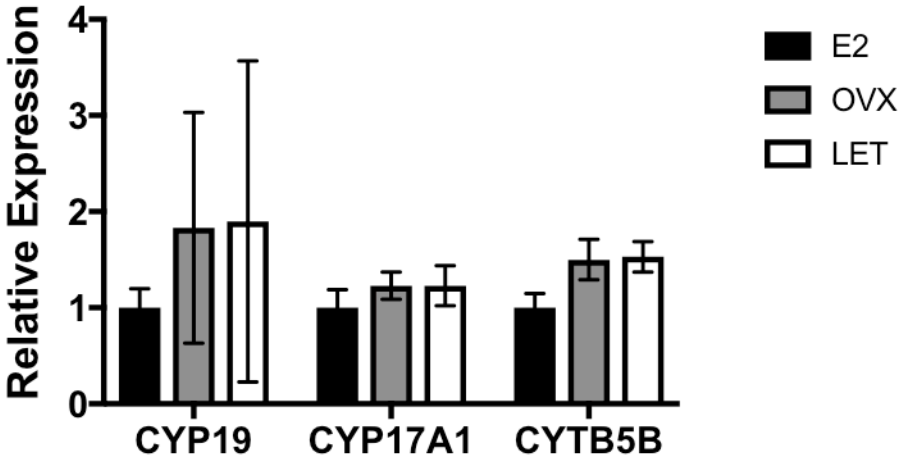

Figure 6: The gene expression for steroidogenic enzymes *CYP19* (aromatase) and *CYP17A1* were not different between the treatment groups. A one-way ANOVA detected a difference in mRNA expression of cytochrome b5 (*CYTB5B*) (ANOVA: p=0.03), however, post hoc testing revealed only a trend (p=0.058) between E2 treated females and LET treated females. All gene targets are normalized to the E2 treated control group.

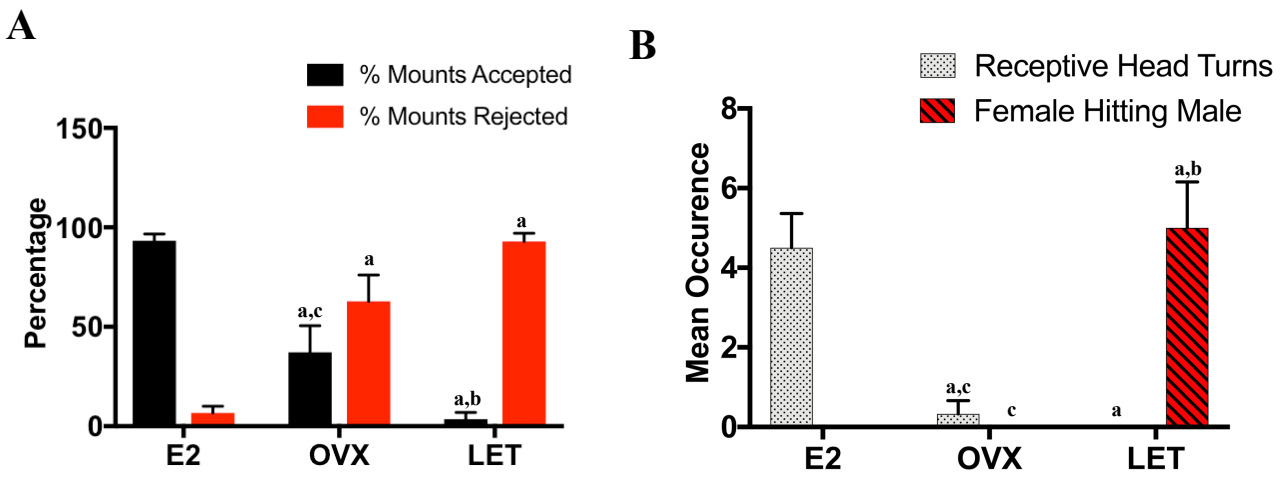

Figure 7: (**A**) Sexual receptivity is diminished in response to OVX (a: p=0.001) and further diminished in the LET treated females (a: p=0.003; b: p=0.04). Sexual rejection is elevated in both OVX and LET treated females (p=0.03). (**B**) Additionally, the number of receptive head turns observed during sexual activity was diminished in the absence of ovarian and extra-ovarian E_2 (p=0.003). LET treated females exhibited not only increased rejection, but there was also a increase in hitting behavior towards the male partner (a,b: p=0.004). (a= compared with the E2 replaced females; b= compared with the OVX females; c= compared with the LET treated females).

Hormone	Sexual Behavior		mRNA Expression
	Receptivity	**Rejection**	
Plasma E_2 (pg/ml)	r_s = (+) 0.97 p<0.001	r_s = (+) 0.96 p<0.001	*HTR5A:HTR2A* r_s = (-) 0.83, p=0.006
			HTR5A:HTR2C r_s = (-) 0.91, p=0.001
Hypothalamic E_2 (pg/mg)	r_s = (+) 0.38 NS	r_s = (-) 0.42 NS	*HTR2C* r_s = (+) 0.60, p=0.05 *HTR1A:HTR2C* r_s = (-) 0.67, p=0.008
			DRD1:DRD4 r_s = (-) 0.68, p=0.02
Hypothalamic T (pg/mg)	r_s = (-) 0.75, p=0.008	r_s = (+) 0.75, p=0.008	No gene expression correlations detected
Hypothalamic A4 (pg/mg)	r_s = (-) 0.79, p=0.004	r_s = (+) 0.79, p=0.004	*HTR5A:HTR2A* r_s = (+) 0.61, p=0.05 *HTR5A:HTR2C* r_s = (+) 0.63, p=0.04
Hypothalamic DHEA (pg/mg)	r_s = (-) 0.63, p=0.04	r_s = (+) 0.63, p=0.04	No gene expression correlations detected
Plasma DHEA (ng/ml)	r_s = (-) 0.83, p=0.006	r_s = (+) 0.81, p=0.01	*HTR5A:HTR2A* r_s = (+) 0.74, p=0.02
			HTR5A:HTR2C r_s = (+) 0.73, p=0.02

Table 4: The plasma and hypothalamic measures of E_2 and androgens exhibited significant relationships with sexual behavior expression as well as the dynamics of hypothalamic mRNA expression of serotonergic receptors. This table represents correlations of the steroid hormone (left column) with the behaviors and mRNA expression data (middle and right column, analyzed separately). Relationships are expressed by r_s, the Spearman's correlation coefficient.(-) represents a negative

relationship, and (+) represents a positive relationship. The level of significance was p=0.05. No other relationships between the steroids and other serotonergic receptors or dopamine receptors were detected, and thus are not shown here.

5.7 References

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6. CHAPTER SIX: Behavioral Sequence Analysis of Female Marmoset Sexual Behavior in the Context of Estrogen Manipulation

Marissa Kraynak and David H Abbott

6.1 Abstract

The context of both animal and human behavior is important for understanding regulating mechanisms. Female sexual expression is no different. One important aspects includes reproductive hormones, such as estradiol (E_2). Behavioral sequence analysis employs statistical methodology that can be used to discern meaningful species-typical patterns of behavior. This chapter utilizes this approach to identify behavioral patterns in the context of each manipulation of E_2 production: OVX + E_2 replaced (E2), removal of ovarian E_2 production (OVX + VEH), and removal of ovarian and extra-ovarian E_2 production (OVX + LET), as well as diminished E_2 action via ERα in the VMN of the hypothalamus. These analyses of behavioral transitions aid in generating a theoretical understanding of how E_2 activates female sexual behavior in the hypothalamus of a NHP, the marmoset monkey.

6.2 Introduction

The expression of any one behavior does not occur alone, but rather is influenced by the context in which it occurs. The influential context of sexual behavior, in particular, varies by species-specific reproductive patterns. Thus, in female mammals, hormonal balance is a crucial influence regulating sexual behavior expression. For example, a large proportion of the in-depth understanding of hormonal regulation of female sexual behavior has emerged from studies in female rodents. Female rodent reproductive behaviors are intimately coordinated with ovulation and fertility, even down to a few hours during nighttime hours, in order to increase the chances of successful reproduction. In NHPs, and certainly in women, sexual behavior expression is not necessarily tied to fertility. Motivation for females engaging in sexual behavior is

therefore not the same for all species. In this regard, it is not surprising that the hormonal regulation of female sexual behavior also differs between species. Consequently, the understanding of hormonal and neural mechanisms governing behavioral patterns in female rodents, while fairly well studied, has little translatability to the effective treatments for women with sexual health issues. Solving the enigma of sexual dysfunction in women requires a better understanding of the context in which different behavioral patterns occur. It is clear that in women, circulating balance of hormones deriving from the ovaries does not fully explain the wide variety of female behavioral patterns. If this were the case, all women would naturally and unanimously cease to be sexually active outside of the peri-ovulatory period and following menopause. The marmoset monkey is a NHP species that naturally displays analogous dynamics of social and sexual behaviors observed in humans. For example, the occurrence of sexual behavior can be controlled by both partners alike. Both partners also have the ability to promote, engage, and interrupt behaviors.

The present study utilized rigorously controlled studies to gain an understanding of the different aspects of E_2 production or E_2 action on female marmoset sexual behavior patterns. More specifically, this study analyzed behavioral transitions to predict female sexual behavior patterns changes in response to E_2 production and action. This analysis combines findings from more than one series of experiments in order to build hypothetical models for E_2-mediated sexual behavior.

6.3 Methods

Animals

Nineteen adult female marmosets and their male partners were observed during behavioral observation sessions, as described in Chapters 4 and 5. The treatment group details can be found in their corresponding chapters. There are five different treatment groups included as followed:

1.) Mid-cycle E_2 Replacement, Control: E_2 (n=5; Chapter 5)

2.) Control for ERα Viral Vector Surgery: Scrambled (n=4; Chapter 4)

3.) Ovarian E_2 Depletion: OVX (n=3; Chapter 5)

4.) Ovarian and Extra-ovarian Depletion: LET (n=3; Chapter 5)

5.) ERα gene silenced (n=4; Chapter 4).

Groups 1 and 2 were both analyzed separately in order to rule out any behavioral changes in ERα gene silenced females that could be due to neural infusion surgery. Behaviorally, however, the scrambled control group was statistically comparable to the E2 replaced control group. Thus, for simplicity, the graphical depictions of the data show these two groups combined as “E2 Replaced” females.

Female marmosets lived with a well-established male cagemate in 0.60m x 0.91m x 1.83m enclosures and were maintained with 12-h lighting, 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. Animals were fed ad-libitum with Mazuri

Callitrichid High Fiber Diet #5MI6 (Purina Mills International, St. Louis, MO). The calories in this diet were 20% protein, 6% fat, and 74% carbohydrate.

Sequence Analysis of Sexual Behavior

Behavioral observations were collected from each male-female pair as described in Chapters 4 and 5. Pairs from Chapter 2 were excluded because a different behavioral paradigm employed. The behaviors utilized in the present analysis, and their abbreviations, can be found in Table 1.

Sequence analysis was used to identify statistically significant sequences of female sexual behaviors observed within each treatment group pair. A behavioral transition in this study, included any behavior that followed within 10 seconds of a previous behavior. For example, when “male mount” is the starting behavior, interacting marmosets commonly transition from there to either “reject mount” or a “receptive posture” by the female, that would be scored as two separate behavioral transitions. Contingency tables and chi-squared test statistics were used in order to analyze the probability of each “two-act” transition occurring within a treatment group. These methods were adapted for this study from those previously described in[1,2].

Frequencies of initial behaviors and transitions were tabulated from all testing sessions and used to determine the expected frequency occurrence of each behavioral transition in adult marmoset pairs (Table 2). The expected frequency was derived from 52.5 hours of behavioral observations over all 19 male-female pairs. Only testing sessions with usable video recordings were utilized to collect these data. That definition isolated five behavioral transition sequences analyzed in this study. Chi-squared statistics were generated for each group in the context of the number of observational

hours and compared to the expected frequency of the transition generated from the entire cohort of 19 pairs. These statistics are listed in Table 3. The higher the chi-squared value, the more likely the transition was not due to chance and was a transition in a behavioral sequence. A chi-squared distribution table was utilized in order to determine whether the transition was statistically likely to occur (significance level $p<0.05$). Each plot and table represents just one treatment group, and arrows are only present between two behaviors if there is a significant likelihood for that transition to occur.

Sweet Water Testing

Sweet water preference test, was adapted from behavioral testing described in[3]. Several treatment groups of animals were combined to form three groups:

1) E2 Replaced (n=7): comprising n=3 from ovariectomized (OVX)+E_2 scrambled controls and E2 replaced (n=4) females in Chapter 4 and Chapter 5,

2) E2 Depleted (n=6): comprising OVX (n=3) and letrozole treated (LET) (n=3), both from Chapter 5,

3) ERα gene silenced females (n=3) from Chapter 4.

There were no statistical differences, or apparent different trends between the groups that were combined to form the E2 Replaced and E2 Depleted groups. This behavioral testing occurred while females were singly housed ~5 months post treatment onset. To determine if E_2 modulates the incentive for female marmosets to engage with a rewarding stimulus, females were offered two water bottles at the front of their home cages for 30 minutes per test. One water bottle was filled with 100ml of regular water and the other was 100ml of a 2mM solution of sucralose (a non-calorific sweetener).

Home cage water supply was detached during the testing period. Following 30 minute testing, the remaining volume left in the water bottle was recorded and the amount of water and sweet water consumed were calculated. There were three, 30-min testing sessions per female. These results were analyzed in two ways utilizing SPSS software (IBM, Armonk, NY). The mean consumption of water and sweet water were analyzed with a one-way ANOVA test, followed by Bonferroni multiple comparison post hoc tests. Additionally, a Repeated Measures-ANOVA was conducted to detect any change in consumption over the three testing sessions.

6.4 Results

Behavioral Sequence Analysis

Sexual receptivity is diminished by several E_2 manipulations (Figure 1; one -way ANOVA $p<0.0001$). The two control groups, E2 and scramble did not differ and both exhibited high expression of receptivity. Thus, any differences observed in the ERα gene silenced group were not due to non-specific effects of MRI-guided neural surgery. For simplicity, throughout the rest of this chapter, these two groups (E2 and Scrambled controls) will be discussed together as a single E2 Replaced control group. Loss of ovarian E_2 diminishes sexual receptivity compared to controls ($p<0.0001$). Loss of extra-ovarian E_2 in addition to OVX, diminishes sexual receptivity beyond OVX alone ($p=0.04$), and markedly increases rejection and aggression.

Sequence analysis revealed different aspects of marmoset sexual behavior patterns that were altered by the different manipulations of E_2 production and E_2 action. **Table 2** is a frequency distribution for each group and analyzed transition. In this table, the bracketed number is the expected frequency within the entire cohort of pairs used in

this dataset. The unbracketed numbers depict the observed frequency of that treatment group for that transition. **Table 3** then shows the x^2 statistics for each of those transitions and the chi-squared distribution table derived p-values. The higher the chi-squared value, the more likely that transition was not due to chance and is a probable transition in a behavioral sequence. Figure 2 is a series of transitional diagrams for each treatment group. Arrows represent a significant probability that transitions will occur ($p<0.05$). Chi-squared statistics and p-values for each transition are shown in Table 3. Figure 2A shows a marmoset-typical sexual behavior pattern between male and female pairmates in the context of mid-cycle, maximal levels of E_2 replacement. In this figure, the most likely sequence following male initiation of a mount is: mount to receptive posture transition, M -> RP ($p<0.001$) followed by Receptive Posture -> Intromission ($p<0.0001$) followed by Intromission -> Receptive Head Turn ($p<0.0001$). Figure 2B subsequently shows that if there is a loss of ovarian E_2 it is only more likely that the female will reject the male partner's mount ($p<0.005$). Loss of both extra-ovarian and ovarian E_2, however, as shown in Figure 2C not only increases the likelihood of sexual rejection, but also changes the most likely subsequent sexual behavior in response to the male mount to Mount ->Rejection ($p<0.0001$) followed by an aggressive sequence of behavior, Rejection -> Hitting ($p<0.0001$). Finally, in Figure 2D, loss of hypothalamic ERα results in the absence of any predictable sequence of behavior in response to male mounting.

Non-Reproductive Goal-Oriented Behavior

In regards to a non-reproductive goal-oriented behavior, Figure 3A illustrates E2 Replaced females compared to E2 depleted females. E2 replaced females

demonstrated increased consumption of sweet water ($p=0.03$), but not of regular, unflavored water, offered at the same time. ERα gene silenced females did statistically differ from either E2 Replaced or E2 Depleted females. It appears, however, that there was an intermediate phenotype associated with the loss of ERα. Additionally, Figure 3B reveals that E2 Replaced females compared to E2 Depleted females progressively increased the amount of sugar water consumed over the three consecutive testing sessions (RM-ANOVA: $p=0.04$). ERα gene silenced females, in contrast, failed to increment sugar water consumption.

6.5 Discussion

Female Sexual Behavior

Sexual function in female NHPs and in women is mediated by both peripheral and neural production and action of steroid hormones. These data provide an broad overview of the subtle changes mediated by various manipulations of E_2 production and action. Together, these data reveal that in female marmoset monkeys, the balance of circulating and hypothalamic E_2 are essential for understanding female sexual behavior. Peri-ovulatory levels of E_2 support a complete sequence of sexually receptive behavior by female marmoset monkeys that includes intimate, female-initiated interactions with the mounted male during copulation. Each manipulation of E_2 production and action modulates E_2-dependent, sequence-typical behavior pattern in different ways. In any single M->RP transition, or display of female sexual receptivity, either or both ovarian or extra-ovarian E_2 are necessary. Interestingly, however, the loss of E_2 action in the VMN via ERα results in the lack of all receptivity. The lack of any receptive sexual behavior sequence identified in the ERα gene silenced females shows that female marmoset

receptivity is dependent on expression of ERα. The prominent difference identified in this analysis, however, shows that ERα gene silenced females did not exhibit a sexual rejection behavioral sequence.

Sexual rejection specific behavioral sequences, in contrast to receptivity, are seemingly driven by an absence of E_2. Ovarian E_2 depletion (OVX), alone, diminishes but does not abolish receptivity. It does, however, change behavioral sequences exhibited by female marmosets during sexual interactions with their male partners. OVX females are more likely to respond to male mounting behavior with sexual rejection. This sequence, however, ends after rejection of the male mount. Ovarian (OVX) and extra-ovarian E_2 depletion (OVX + LET), interestingly, exhibit an escalated enhancement of sexual rejection, demonstrating that not only are OVX + LET females more likely to reject sexual advancements, but they are also aggressive and violent in rejecting their male partners. From the data shown in Chapter 5, hypothalamic testosterone, rather than E_2, may be a major neural driver of female marmoset sexual rejection behavioral sequences. Further, the more severely the balance of E_2:testosterone ratio in the female hypothalamus favors testosterone, the more robust and violent her sexual rejection. The lack of a similar sexual rejection by ERα gene silenced females, however, suggests that E_2 action via ERα in the VMN is not the only neural mechanism regulating the expression of female rejection behavior. Circulating plasma E_2 levels of the ERα gene silenced females were comparable to the E2 Replaced females. Thus, given that hypothalamic A4 increases in the OVX + LET females, but not the E2 replaced females, it is reasonable to speculate that hypothalamic androgen concentrations, including testosterone, are low in ERα gene

silenced females. Further evidence that E_2 may not be the facilitator of sexual rejection behavior lies in the lack of any predictable sequence of sexual behavior in ERα gene silenced females.

The relationships that have been identified between E_2 and serotonergic receptor (HTR) gene expression changes in the hypothalamus (results in Chapter 5) also provide insight into possible contributors to sequence behavior. While there is a wide breadth of studies examining HTR expression in response to steroid hormones, the mechanisms for these changes are not clear. In female rodents[4] and rhesus monkeys [5], E_2 has been shown to downregulate hypothalamic $5HTR_{1A}$, the historically identified serotonergic mediator of sexual inhibition. ERα antagonists, however, have not been able to induce similar same changes[6]. Additionally, no studies have classified 5HTR expression and density in the brain of estrogen receptor alpha knock out (ERαKO) mice. ERα in HT producing neurons in the dorsal raphe nucleus, however, modulates the gene expression of the serotonin transporter gene (SERT), subsequently slowing the clearance of HT in the synapse[7]. ERα also increases the binding sites for SERT in the hypothalamus[8] which suggest that one possible mechanistic role for ERα in the serotonergic regulation of behavior may be in the dynamics of serotonin availability in the synapse. Notably, however, there is a lack of literature on ERα specific regulation of HTRs in the hypothalamus, and thus ERα's role in the 5HTR relationships revealed in the hypothalamus in response to the loss of ovarian and extra-ovarian E_2 depletion. In regards to the possible role for androgens in this mechanism, even less is understood. Unlike estrogen receptors that are highly co-expressed within HT neurons, androgen receptors, in the female rodent brain, are less expressed in HT neurons[9]. Thus, in lieu

of more evidence in a NHP model, androgen signaling via androgen receptor, may not be connected with the serotonergic regulation of female sexual behaviors.

Sweet Water- Non-sexual, Rewarding Behavior

In addition to the sexual behavior sequence analysis, the sweet water testing employed in this study examines another type of goal-oriented behavior in the context of E_2 production and action. In this testing, females that exhibited mid-cycle levels of E_2 indulged in more of the rewarding, incentivized sweet water consumption than E2 depleted females. This was in the absence in the increase of regular, unflavored water offered at the same time. The E_2 depleted females, in contrast, did not increase their intake of either unflavored water or sweet water throughout the testing sessions. Taken together, these data suggest that E_2 may enhance the salience and incentive properties of rewarding stimuli. Speculatively, this is an effect that may also be related to female sexual behavior. In response to the sweetened water, however, the ERα gene silenced females did not resemble either E2 replaced or E2 depleted females, but rather showed an intermediate phenotype. One reason may be that the ERα gene silencing was targeted to the VMN. The major brain regions that have been implicated are nucleus accumbens derived dopaminergic neurons innervating the mPOA[10,11]. While ERα gene silencing was found to be varied between females, the difference in ERα expression in the mPOA was non-significant. Previous literature in female rats has shown that ERα in the mPOA regulates both sexual motivation[12] as well as other goal-oriented behaviors such as a female rat retrieving her pups [13]. In NHPs, it is also possible, given the lack of understanding regarding ERα specific modulations to reward system pathways, that ERα in the hypothalamus mediates E_2-faciliated sexual receptivity and sexual

motivation, however, the role of hypothalamic ERα in other types of goal-oriented behavior is still to be determined.

In conclusion, Figure 4 shows a theoretical sequence diagram for the hormonal regulators of each female marmoset sexual behavior transition. This theoretical diagram postulates, given the data, that one contributor to the regulation of sexual receptivity and subsequent rejection is the steroid hormone environment in the hypothalamus. The behavioral pattern of sexual receptivity in female marmoset monkeys is facilitated by mid-cycle levels of ovarian E_2, as this is the only hormonal environment in the present study in which sexual receptivity sequences are likely in the female marmoset monkey. In the VMN of the hypothalamus, ERα is the receptor for E_2-mediated sexual receptivity in the female marmoset. Sexual rejection, however, relates to both E2 depletion and diminished action via ERα in the VMN, AND increase in hypothalamic androgens, with rejection increasing as hypothalamic steroid hormone dynamics favor androgens. Prior studies have implicated the antagonist role for androgens and E_2-induced receptivity, in the female rodents, however, prior to the present study, the diminished E_2:T ratio, specifically in the hypothalamus, has not been elucidated. Further studies manipulating testosterone and androgen action in the hypothalamus are needed in order to further understand the contribution of hypothalamic T to female sexual rejection.

6.6 Tables and Figures

Behavior	Abbreviation
Male Mount	M
Receptive Posture	RP
Mount Rejection	RJ
Receptive Head Turn	RHT
Intromission	I
Hitting	H

Table 1: Behaviors and their scoring abbreviations that were analyzed in the sequence analysis are listed in the table above.

		Subsequent Behavior					
		Treatment Group	**RP**	**RJ**	**RHT**	**I**	**H**
Initial Behavior	**M**	**E2**	1.77 (0.78)	0.31 (0.48)	-	-	-
		OVX	0.67 (0.78)	1.22 (0.48)	-	-	-
		LET	0 (0.78)	1.56 (0.48)	-	-	-
		Scrambled	2.27 (0.78)	0.09 (0.48)	-	-	-
		ERα Gene Silenced	0.57 (0.78)	0.19 (0.48)	-	-	-
	RP	**E2**	-	-	1.69 (0.52)	1.77 (0.57)	-
		OVX	-	-	0.11 (0.52)	0.67 (0.57)	-
		LET	-	-	0 (0.52)	0 (0.57)	-
		Scrambled	-	-	2.09 (0.52)	2.27 (0.57)	-
		ERα Gene Silenced	-	-	0.38 (0.52)	0.57 (0.57)	-
	RJ	**E2**	-	-	-	-	0 (0.2)
		OVX	-	-	-	-	0 (0.2)
		LET	-	-	-	-	1.67 (0.2)
		Scrambled	-	-	-	-	0 (0.2)
		ERα Gene Silenced	-	-	-	-	0 (0.2)

Table 2: The frequency distribution table above shows the frequencies of the subsequent behaviors following the initial behaviors as the unbracketed numbers in each cell. The bracketed numbers in each cell represents the calculated expected frequency of the transition for the entire cohort of male-female pairs and their interactions.

A. Behavioral Transition	Treatment Group: E2
M->RP	x^2 : 12.55 ; p=0.02
M->RJ	x^2 : 0.06; ns
RP->RHT	x^2 : 30.01; p<0.0001
RP->I	x^2 : 25.23; p<0.0001
Rj->H	N/A

B. Behavioral Transition	Treatment Group: Scrambled
M->RP	x^2 : 28.57; p<0.001
M->RJ	x^2 : 3.2; ns
RP->RHT	x^2 : 59.08; p<0.0001
RP->I	x^2 : 50.86; p<0.0001
Rj->H	N/A

C. Behavioral Transition	Treatment Group: OVX
M->RP	x^2 : 0.16; ns
M->RJ	x^2 : 11.48; p=0.005
RP->RHT	x^2 : 0.41; ns
RP->I	x^2 : 0.16; ns
Rj->H	N/A

D. Behavioral Transition	Treatment Group: LET
M->RP	N/A
M->RJ	x^2 : 24.10; p<0.0001
RP->RHT	N/A
RP->I	N/A
Rj->H	x^2 : 107.5; p<0.0001

E. Behavioral Transition	Treatment Group: ERα Gene Silenced
M->RP	x^2 : 0.56; ns
M->RJ	x^2 : 1.75; ns
RP->RHT	x^2 : 0.05; ns
RP->I	x^2 : 0.00004; ns
Rj->H	N/A

Table 3: Each table above (**A-E**) shows the x^2 statistic for all transitions analyzed. Each individual table contains the statistics for one treatment groups. These are represented in the transition charts in **Figure 2**.

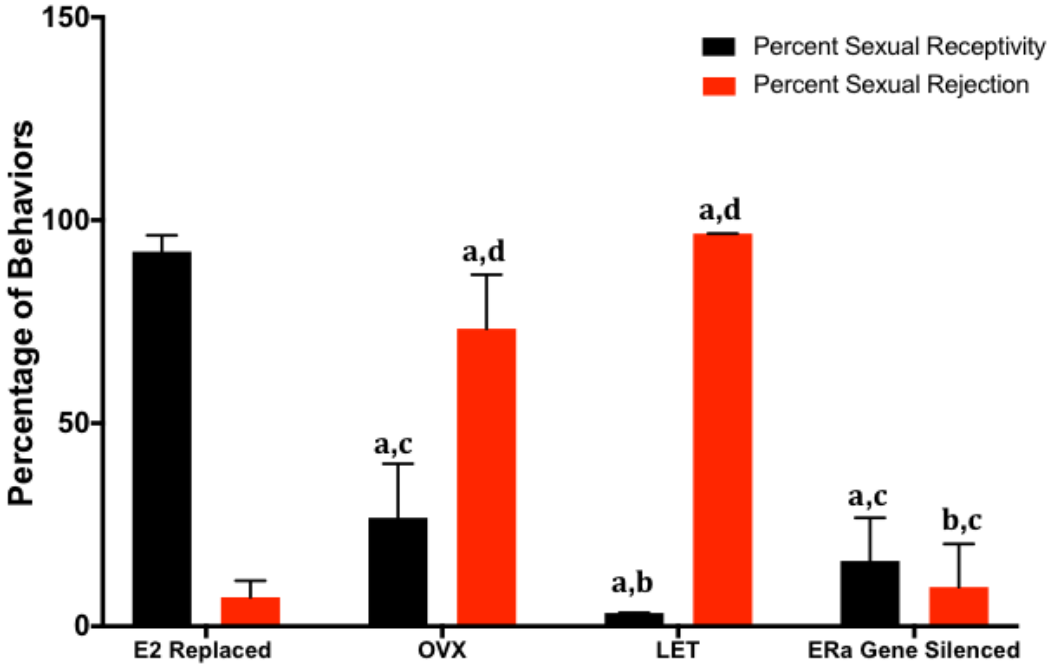

Figure 1: Estradiol, E_2, facilitates increased expression of sexual receptivity. The loss of ovarian E_2 (OVX; p=0.01) and the loss of hypothalamic ERα expression (ERa Gene Silenced; p<0.001) decreases sexual receptivity compared with E_2 replacement. Depletion of extra-ovarian on top of OVX diminishes sexual receptivity beyond both OVX and ERα gene silencing (LET; p<0.001). The expression of sexual rejection, however, only increases due to the loss of E_2 (OVX (p<0.001)and LET (p<0.001) groups) but not in response to the loss of E_2 action. (a= different compared to E2 replaced; b=different compared with OVX; c=different compared with LET; d=different compared to ERα Gene Silenced)

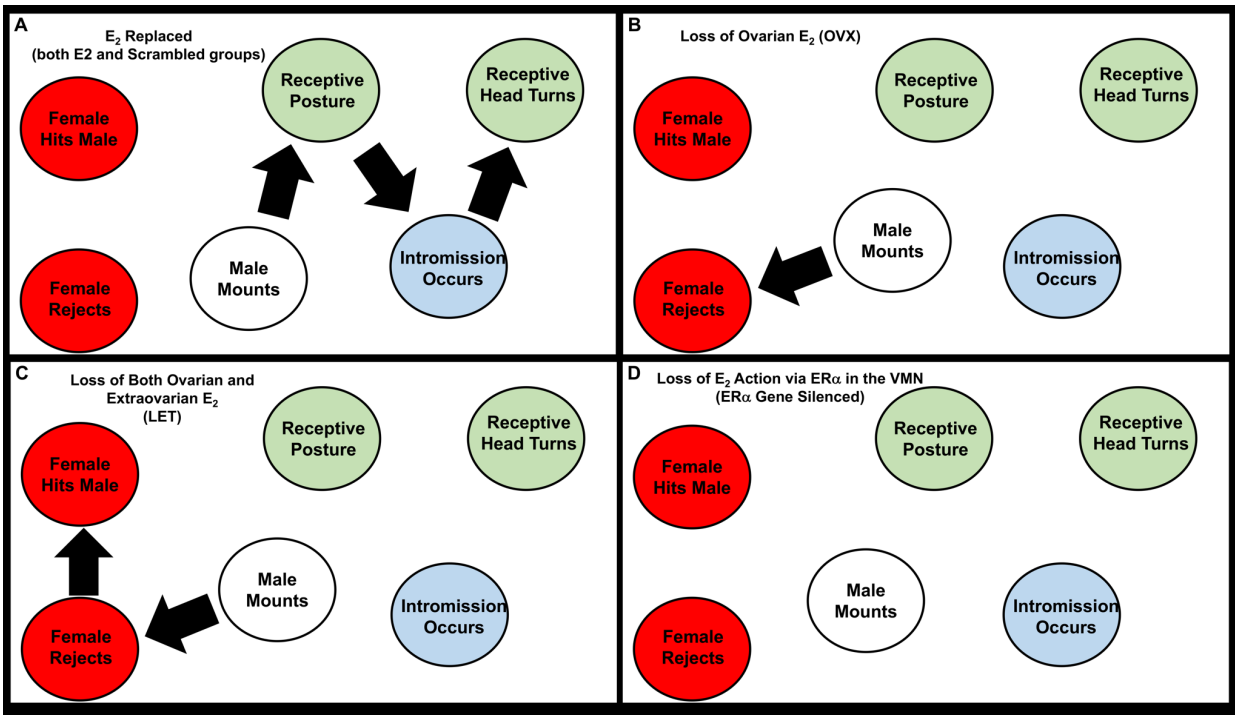

Figure 2: (**A**) The sexual behavior pattern most likely to occur with estradiol, E_2, replacement. (**B**) Loss of ovarian E_2 switches the likelihood of receptivity to sexual rejection. (**C**) Total E_2 loss results in not only a high probability of sexual rejection, but also the likelihood that sexual rejection will also lead to aggressive hitting behavior. (**D**) Loss of E_2 action via ERα in the VMN of the hypothalamus, however, disengages any sexual responses of the female towards the male pairmate. Each black arrow represents a statistically significant ($p<0.05$) transition between the behaviors they connect.

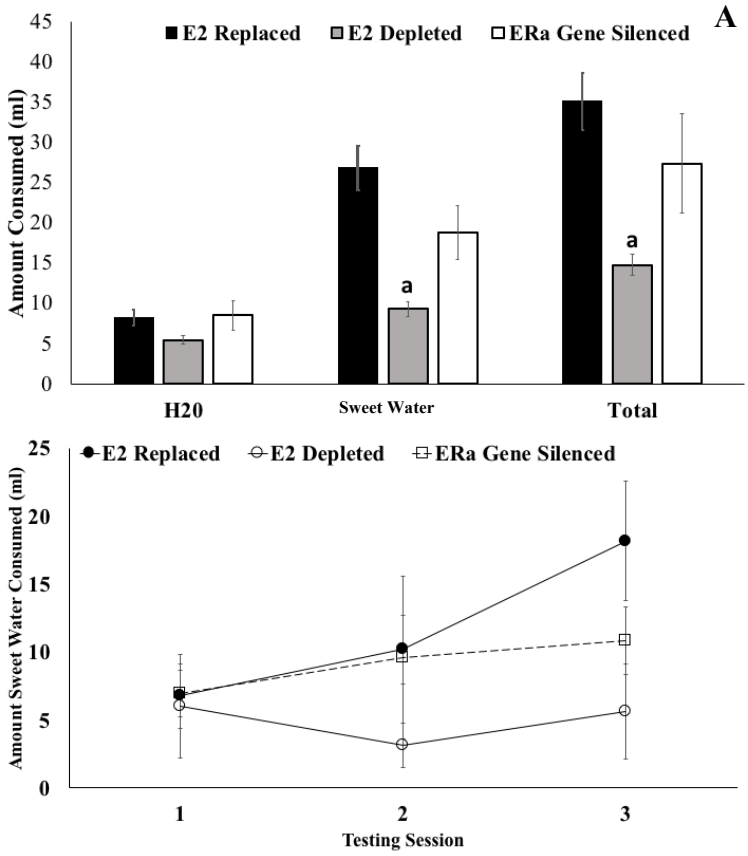

Figure 3: (**A**) The consumption of sweet water (p=0.03) was decreased in the E2 Depleted group compared to the E2 replaced controls. There was a subsequent increase in total consumption as well (p=0.04) but, there was no treatment effect on water consumption. (**B**) Additionally, there was a treatment x time interaction (p=0.04) detecting that the sweet water consumption in E2 Replaced females increased with each subsequent testing session. This effect is not observed in E2 depleted groups. In both measures, the ERα gene silenced females did not consume either the sweet water or the regular water more than either E2 replaced or E2 depleted females.

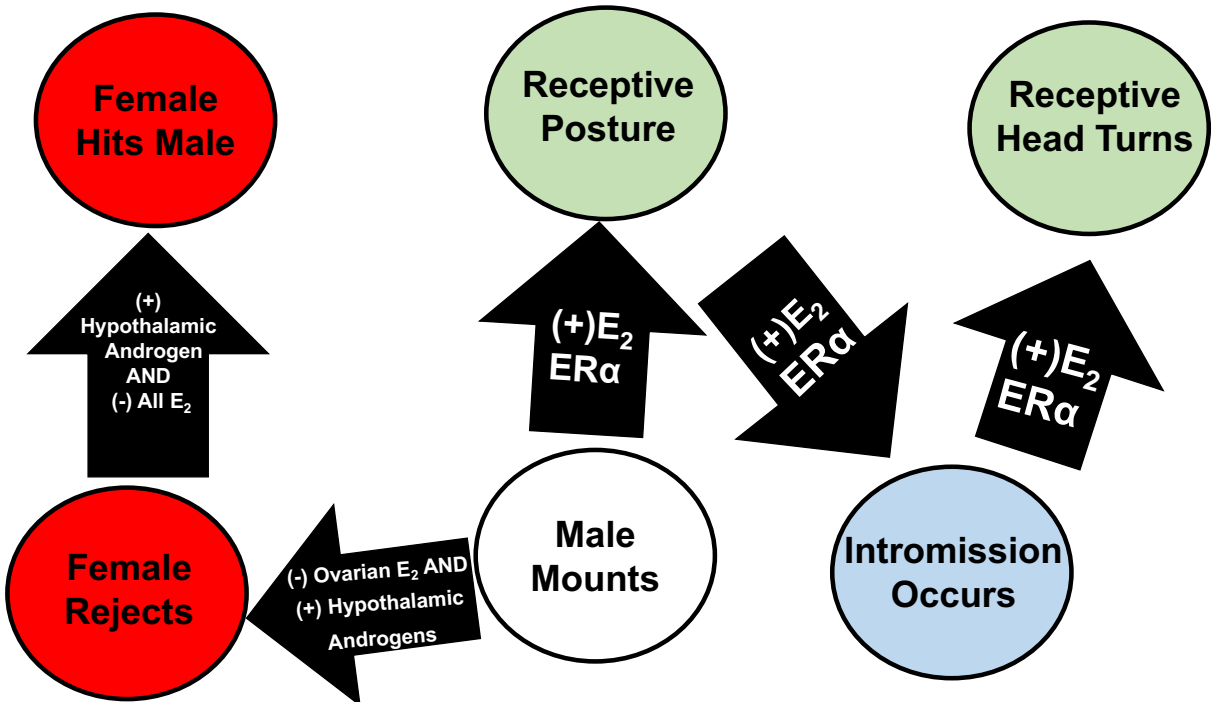

Figure 4: Theoretical diagram of the estrogenic regulation of each sexual behavior transition.

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7. CHAPTER SEVEN: Future Directions

All contributions made by Marissa Kraynak.

Sexual function is an integral, yet often ignored aspect of women's health and well-being. The lack of effective treatment options for personally distressing sexual dysfunction in women, Female Sexual Interest and Arousal Disorder (FSIAD)[1], is concerning. This is largely due to a lack of understanding of the neuroendocrine regulation of female sexual responsiveness, as well as sexual motivation and desire, specific to female NHPs and women. Previous to the research presented in this thesis, neural mechanistic understanding of E_2-mediated female sexual behavior was based upon animals models in which the sole purpose for sexual behavior was procreation[2]. In female rodents, sexual expression is precisely and reliably timed with fertility. In women and NHPs, however, sexual expression is not limited to periods of fertility. In fact in many higher primates, including humans, sexual interactions have evolved to include functional roles in social relationships and partner bonding[3]. This is one highly plausible explanation for emancipation of female primate sexual expression from procreation.

7.1 Neural Mechanism Permitting Female Sexual Receptivity Independent of Fertility

The studies in this thesis identify major neural mechanisms of E_2-mediated sexual behaviors in female marmoset monkeys. Prior to these studies, and for the first time in any female NHP, we have established that hypothalamic E_2 can support female sexual receptivity in the absence of ovarian E_2. Additionally, the loss of at least 15-20% of ERα-mediated E_2 action in the VMN results in diminished sexual responsiveness towards the male partner. Taken together, these data suggest that (1) the major mechanism for E_2-mediated sexual receptivity is via ERα in VMN of the hypothalamus, and (2) extra-ovarian E_2 alone is sufficient to maintain neural support of female sexual responsiveness and receptivity in marmoset monkeys. These conclusions suggest that in a female NHP, a major driver of female sexual receptivity is neuro- or

hypothalamic E_2. These findings provide insight that hypothalamic E_2 may also play an integral role in sexual function in women.

7.2 Neural Mechanism Permitting Female Sexual Rejection

The studies in this thesis also provide preliminary evidence for a separate mechanism permitting sexual rejection in female marmoset monkeys. Inhibition of female sexual receptivity in the female marmoset is not mechanistically driven by ERα in the hypothalamus in that not all ERα gene silenced females in these studies showed an increase in rejection behavior, as typically observed with the loss of ovarian and/or extraovarian E_2 production. The results from this thesis (Chapter 5) suggest that hypothalamic androgens may play a more prominent role in the neural mechanism driving sexual rejection. It was previously demonstrated[4] that one consequence of long-term OVX in female marmoset monkeys is the initiation of adrenal androgen production. Prior to this study, this phenomena was not studied in any other steroidogenic organs. Ppreliminary results shown in Chapter 5, however, suggest that the brain, specifically the hypothalamus, may also become hyperandrogenic after long-term OVX. Consequently, it is only with increased androgens in the brain that female marmosets exhibit an increase in rejection behaviors and rejection becomes more robust and aggressive when the E_2:T ratio decreases. Thus, a further hypothesis from these studies is that part of the E_2 mechanism facilitating sexual receptivity is the inhibition of androgen-facilitated sexual rejection, since a diminished E_2:T ratio in the hypothalamus leads to increased displays of rejection behavior. In order to further the understanding for an androgenic mechanism permitting sexual rejection, however, future studies are needed to identify sources of androgen (such as adrenal cortex, adipose depots, areas of the brain), and the mechanism of androgen action in the brain, through parallel designed studies to those presented in this thesis. It would also be interesting for future studies to examine whether

periods of low E_2 during the marmoset ovarian cycle are associated with any changes in the E_2:T ratio in the hypothalamus facilitating rejection behavior in an ovary intact female.

7.3 Steroid Hormone Regulation of Proceptive Sexual Behavior

Interestingly, throughout all of the studies in this thesis, proceptive behaviors were not observed in the various manipulations of E_2 production and action in this study. Thus the conclusions regarding E_2-mediated sexual behaviors can only be applied to sexually receptive behaviors. Interestingly, previous studies in marmoset monkeys have observed increased proceptivity during the periovulatory period of the ovarian cycle (high E_2)[5], as well as in E_2 replaced female marmoset monkeys[6]. There may be two possible explanations for these differential findings. One major difference between the current study and those previous involves the testing environment. Male-female pairmates in past studies have been singly housed throughout the duration of behavioral testing. In the present study, pairmates were housed together for at least two months prior to study onset and only separated for 90 minutes prior to behavior testing (Chapter 2) or for an entire 30 days prior to testing onset (Chapters 4-6). There have been many studies in mammalian species[7,8] that have shown the importance of socially-relevant testing arena and environments in understanding sexual behavior regulation. It is possible that proceptive behaviors are not observed in the paradigm employed throughout the present studies because the male-female pairs are familiar with one another, thus leading to a lack of sexual solicitation. There is one study in female Goeldi's Monkey, a NWP species related to marmosets, that examined the role of mate familiarity in female sexual proceptivity. The study found that during the peri-ovulatory period, E_2 consistently increased female sexual proceptivity, however, the different male partners were only made 'familiar' to the female for one week, whereas the current study establishes male-female familiarity for at least two months[9]. Mate

familiarity effects on sexual proceptivity in female marmoset monkey, however, has not been studied. Another explanation, or possibly a synergistic explanation for the lack of proceptivity could be that proceptivity is not regulated by E_2 in familiar, well-established male-female marmoset pairmates. In the present studies, E_2 replacement or (ovarian/extraovarian) E_2-depletion was constant throughout the study.

Progesterone (P4), however, was not part of any of the current treatment regimens and subsequently was not different between the different female groups. In female rodents, P4 has been shown to have both facilitative effects on sexual proceptivity within 10-12 hours after ovulation[10-12] and inhibitory effects on sexual receptivity and proceptivity following estrus[13,14]. In female primates, in contrast, a facilitative effect of P4 has never been elucidated. Luteal phase levels of P4 (>10ng/ml) robustly diminish receptive and proceptive behaviors in ovary intact-cycling female marmosets[5] and in OVX females that received luteal phase levels of P4[6]. It is also known that P4 action is enabled through mid-cycle levels of E_2 inducing increased expression of P4 receptors in the hypothalamus[11,15]. It would be interesting to examine whether central P4 production and/or action can permit sexual solicitation behavior in female marmoset monkeys. It would also be interesting if both the social housing context combined with the lack of P4 action in the various treatment groups in the present study synergistically contributed to the lack of sexual solicitation. Further studies manipulating these social and hormonal environments are necessary to understand the regulation of sexual proceptivity in female marmoset monkey.

7.4 Applications to Understanding and Treating Sexual Dysfunction in Women

The major findings regarding the mechanism regulating sexual behavior in female marmoset monkeys in this study provide insights into potential novel therapeutic targets for treating women with sexual dysfunction. In menopausal women, for example, it is not known

whether or not hypothalamic steroid hormone production increases following the demise of the ovary as a major producer of circulating E_2 . If menopause results in an increase in hypothalamic E_2 production, then neural E_2 may be responsible for the maintenance of female sexual function for women later in life. It is also unknown whether women who undergo surgical menopause (hysterectomy, oophorectomy) also have a change in central steroid hormone production. Both groups of women lack ovarian E_2 and are populations affected by FSIAD[16]. Given the wide range of hypothalamic E_2 levels between individual females within both E_2 replaced and OVX female marmoset groups, such individual variance of E_2, should it occur in the hypothalami of women, could potentially explain a the wide range of variance in female sexuality.

These phenomena are not exclusively applicable to menopausal women, alone. There may be a supporting role specifically for hypothalamic E_2 in regulating sexual function prior to menopause in women. In female primates, including women, the maintenance of sexual receptivity/sexual function outside of the peri-ovulatory period in respective reproductive cycles, could also be due to hypothalamic E_2 action, likely acting through ERα. Thus, altered E_2 production and/or action in the hypothalamus, could potentially be a key understanding in the pathology of female sexual dysfunction in women.

Targeting E_2 as a therapeutic, however, has a serious limitation in that E_2 is a highly effective, proliferation-inducing hormone and poses the risk of cancer. Therefore, safer and more effective therapeutic options would be to pharmacologically manipulated to enable only E_2-modulated neurochemical systems . This study, in these regards, has also implicated serotonin and dopamine receptor targets of circulating and/or hypothalamic E_2. Hypothalamic androgens, while not specifically manipulated in the present studies, may also play a role in regulating neurochemical signaling related to female sexual behavior.

The relationships between steroid hormones, behavior, and gene expression have generated hypothesized neural mechanisms mediating female sexual receptivity. One of the more consistent and unique findings is the link between serotonin receptor dynamics involving $5HTR_{5A}$ and $5HTR_{2A/2C}$. Both androgens and plasma E_2 are correlated with the ratio of these two receptors. It can be hypothesized from the data in this study that $5HTR_{5A}$ might be an effective target for a therapeutic to relieve female sexual dysfunction that has not been implicated by rodent studies. Yet, the increase in $5HTR_{5A}$ in relation to the decrease in $5HTR_{2A/2C}$ appears to be a potential serotonergic regulator of female sexual inhibition, or rejection. An androgenic role in serotonergic function has also not been studied in the hypothalamus in any species. Thus, there are many mechanistic avenues in the brain for examining the specific mechanisms permitted by hypothalamic E_2 and/or androgens and how these mechanisms can be pharmacologically manipulated in order to treat sexual dysfunction in women.

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8. APPENDIX

1. Sexual Behavior Ethogram

SEXUAL BEHAVIORS				
Behavior	**Type**	**Code**	**Subject**	**Definition**
Erection	Event	E	Male	Male's penis is fully erect
Proceptive Tongue Flick	Event	0	Female	Rapid protrusion and retraction of the tongue
Proceptive Stare	Event	1	Female	Females stares fixedly at the male and flattens her ear tuffs
Mount Attempt	Event	2	Male	Male displays mounting behavior, but fails to reach final mounting position.
Mount	Event	3	Male	Male mounts the back of the female, gripping with hands and feet.
Accept Mount	Event	4	Female	Female lets male get in mounting position, normally goes into frozen posture
Reject Mount	Event	5	Female	Female refuses male's mount or mount attempt by moving away, biting/pushing male off and/or using verbal threats. This behavior occurs before intromission.
Early Termination of Mount	Event	7	Female	Female dismounts from male following a successful mount but before ejaculation.
Intromission	Event	8	Male	Male mounts the back of the female, back legs are on substrate, and he shows several bouts of thrusting (penis bob). Tail coiling may occur. Female generally reacts with a receptive head turn.
Receptive Head Turn	Event	9	Female	Female turns around and nuzzles or bites the male once he has mounted.
Ejaculation	Event	10	Male	Ejaculation terminates a successive mount. Can be scored by either visible ejaculate or the male licking his penis.
Receptive Tongue Flick	Event	11	Female	Female tongue flicks during copulation. Protrusion and retraction of the tongue.

Hitting	State	H	Female	Female physically hits, swats at, or slaps male.

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Ovarian Estradiol Supports Sexual Behavior but not Energy Homeostasis in Female Marmoset Monkeys

Marissa Kraynak[1,2,*], Ricki J. Colman[1,3*], Matthew T. Flowers[1,4], David H. Abbott[1,5], Jon E. Levine[1,4]

Wisconsin National Primate Research Center[1]; Endocrinology and Reproductive Physiology Training Program[2]; Department of Cell and Regenerative Biology[3]; Department of Neuroscience[4;] Department of Obstetrics and Gynecology[5]
University of Wisconsin-Madison, Madison, WI

* These authors contributed equally to the completion of this study

Abstract

Objective: In adult female rodents, ovarian estradiol (E_2) regulates body weight, adiposity, energy balance, physical activity, glucose-insulin homeodynamics, and lipid metabolism, while protecting against diet-induced obesity. The same E_2 actions are presumed to occur in primates, but confirmatory studies have been lacking.

Methods: We investigated the consequences of ovariectomy (OVX) and E_2 replacement in female marmoset monkeys on major metabolic and morphometric endpoints. Sexual behavior and uterine diameters were assessed as positive controls for E_2 treatment efficacy. Metabolic parameters were measured 1 mo prior to OVX, and 3 and 6 mo thereafter. During OVX, animals received empty or E_2-containing silastic s.c.

implants. To test the interaction between E_2 and diet, both treatment groups were assigned to either a higher fat diet (HFD) or a low-fat diet (LFD).

Results: As anticipated, OVX animals exhibited diminished frequency ($p=0.04$) of sexually receptive behavior and increased rejection behavior ($p=0.04$) towards their male partners compared to E_2-treated OVX females. OVX also decreased ($p=0.01$) uterine diameter. There were no treatment effects on total caloric intake. There were no significant effects of OVX, E_2 treatment, or diet on body weight, body composition, energy expenditure, physical activity, fasting glucose, or glucose tolerance. Regardless of E_2 treatment, serum triglycerides were higher ($p=0.05$) in HFD than LFD females. Postmortem qPCR analysis of hypothalamic tissues revealed higher mRNA expression ($p<0.001$) for *PGR* in E_2-treated monkeys versus OVX controls regardless of diet, but no differences between groups in other selected metabolic genes. In contrast, regardless of E_2 treatment, there was a decreased mRNA expression of PGC1α (*PPARGC1A*), *HTR1A* and *HTR5A* in HFD compared to LFD females.

Conclusions: Our findings, overall, document a greatly diminished role for ovarian E_2 in the metabolic physiology of a female primate, and encourage consideration that primates, including humans, evolved metabolic control systems regulated by extra-ovarian E_2 or are generally less subject to E_2 regulation.

Introduction

In many rodent biomedical research models, ovarian estradiol (E_2) regulates body weight and composition, energy balance, and insulin-glucose homeodynamics. E_2 regulated control mechanisms are particularly pronounced in rodents, since ovariectomy (OVX)-mediated E_2 depletion is reliably accompanied by increased body weight and

visceral adiposity, reduced physical activity and energy expenditure(1), as well as diminished glucose tolerance and insulin sensitivity. All these effects are prevented or reversed by physiological E_2 replacement (2, 3). Furthermore, while intact female mice are resistant to high-fat diet-induced obesity and its associated sequelae, in contrast to male mice, OVX-mediated E_2 depletion abolishes this protection(4, 5). Virtually all of these E_2 actions are mediated by estrogen receptor alpha (ERα; *ESR1*), as mice bearing null mutations of the *ESR1* gene exhibit body weight, adiposity and energy metabolism phenotypes that largely mimic those observed in long-term OVX mice(6-8).

Substantial progress has been made towards clarifying molecular and cellular mechanisms engaging ovarian E_2 regulation of energy homeostasis in rodents, particularly those actions that are transduced by hypothalamic neurons expressing ERα (9, 10). Female-specific stimulatory effects of E_2 on energy expenditure, for example, have been shown to be transduced in ERα expressing neurons of the ventromedial nucleus (VMN) of the hypothalamus(11) by non-classical ERα signaling (10) coupled to activation of PI3-kinase (12). E_2 has also been shown to regulate gene expression associated with regulation of food intake and energy expenditure in the hypothalamus, largely through ERα activation (9, 13). It has yet to be substantiated, however, whether the same mechanisms exist among non-rodent biomedical research models, and in humans. In addition to the role of ovarian E_2, high-fat diets in female rodents, as well as the marmoset monkey, have had effects on neuromodulators of satiety, including 5-hydroxytryptamine (5HT; serotonin) receptors. In female rodents, pharmacological activation of $5HT_{1A}$ and $5HT_{2c}$ receptors induce hyperphagia; however, only the $5HT_{2c}$ receptor is consistently accompanied by an obese phenotype (14). In the female

marmoset monkeys used in this study, it has been previously published that the higher fat diet (HFD) attenuates mRNA expression of serotonin production related genes in the presence of E_2 (15). In regards to 5HT action, hypothalamic mRNA expression of serotonin receptors in the hypothalamus in response to either OVX or HFD in female primates, including women, has not yet been identified prior to this study.

Metabolic functions of ovarian E_2 in women, in contrast to rodents, have been difficult to define, partly due to logistical and ethical constraints in designing definitive experiments with rigorous control. Numerous studies have attempted to dissociate the effects of normal aging versus declining E_2 levels on adiposity, energy balance and cardiometabolic health in menopausal women (16-18). 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 (19-21). While these changes parallel those observed in OVX rodents (22), a causal relationship between declining ovarian E_2 in menopause and altered body composition and energy balance has been difficult to confirm. Most randomized controlled studies have demonstrated that both oral and transdermal E_2 therapy in postmenopausal women is associated with a reduction in central adiposity and increased lean body mass (23, 24), as well as reduced insulin resistance and fasting glucose, new-onset diabetes, blood lipids, blood pressure, and adhesion molecules and procoagulant factors (25). Of the few studies of energy expenditure after menopausal hormone replacement therapy (HRT) comprising a variety of estrogenic formulations, some demonstrate increases in fat oxidation and energy expenditure (26, 27), while others reveal acute decreases in fat mass, lipid

oxidation and energy expenditure (28). There are similarly conflicting data on the effects of HRT on insulin sensitivity, with some suggesting beneficial effects (25) while others find no consistent improvement (29-31). Differences in ages of the subjects, as well as in amount, composition, timing, duration and route of administration of HRT, and difficulties in controlling for diet, life-style and environmental factors, likely contribute to inconsistencies among clinical studies.

Female nonhuman primates possess reproductive and metabolic control systems that most closely parallel those in women, including ~28-day ovulatory cycles that feature E_2 surges during the peri-ovulatory phase followed by a definitive luteal phase. Nonhuman primates have therefore served as important biomedical research models with which to study the actions of E_2 on sexual behavior and the control of ovarian cyclicity. Comprehensive studies of the roles played by ovarian E_2 on energy balance and body composition in nonhuman primates, by contrast, are scarce. OVX-mediated E_2 depletion has small effects on female body weight, with no change in body mass index (BMI) in female rhesus macaques (32), and no effect on female body weight in cynomolgus macaques (33, 34). While a putative selective estrogen receptor modifier (SERM) promotes weight loss in OVX rhesus monkeys (35), E_2 replacement therapy has no effect on body weight in OVX cynomolgus macaques (34, 36). The effects of OVX and E_2 replacement on energy expenditure, physical activity, body composition, glucoregulation and lipid metabolism have yet to be comprehensively assessed in any female nonhuman primate to determine if the metabolic actions of ovarian E_2 in adult female primates resemble those identified in rodents.

To investigate the metabolic functions of ovarian E_2 in a female nonhuman primate, we selected the common marmoset monkey (*Callithrix jacchus)*, a higher primate modestly susceptible to diet-induced obesity (37, 38). Additionally, marmoset monkeys do not exhibit seasonal breeding, in contrast to many Old World primates (OWPs) (39) utilized in biomedical research, including rhesus macaques, that can confound issues related to shifts in reproductive demands on energy homeostasis (40, 41). We explored whether OVX-mediated E_2 depletion imparts a greater susceptibility to diet-induced obesity as observed in female mice (4, 5). Apart from practical reasons to perform this study in marmosets (i.e., ease of handling, compressed life cycle), we chose to study this New World primates (NWPs) so as to permit analysis of female energy expenditure and physical activity levels in male-female pairs that maintain a wide range of species-specific social and sexual interactions. Marmosets also exhibit predictable sexual behavior responses to OVX and E_2 treatments (42) that provided valid quantitation in the present studies confirming efficacy of E_2 regimens in eliciting anticipated biological responses. There is one notable difference, however, between circulating E_2 levels in NWPs compared to female OWPs, including women. In pre- and post-OVX female NWPs, such as female marmosets, circulating levels of E_2 are substantially higher compared to E_2 levels in female OWPs(43). The elevated circulating levels of E_2 in NWPs appear to reflect compensatory responses to comprehensive target organ resistance to steroid hormone action (44, 45).

We report here that ovarian E_2 sustains female marmoset sexual behavior that is otherwise diminished at 6 mo following OVX, as anticipated from previous studies (42). Neither OVX alone nor E_2 replacement, however, produce significant changes in any of

the metabolic and morphometric parameters quantified in the same females, regardless of low- or higher fat diet, suggesting major differences in metabolic regulation by ovarian E_2 in at least one primate species versus its rodent counterparts.

Methods

Animals

Sixteen adult female common marmosets (2-6 years of age) from the Wisconsin National Primate Research Center colony were evenly randomized based on age, body weight and fasting triglyceride values into 4 groups (Supplementary Table 1): E_2 replaced + low-fat control diet, LFD, (E2+LFD), E_2 replaced + higher fat diet, HFD, (E2+HFD), E_2 depleted + LFD (OVX+LFD), E_2 depleted +HFD (OVX+HFD). Following onset of experiment, one female was excluded from the study (from E2+LFD group) due to diet noncompliance. Animals were maintained in these groups for 6 months after which they went to necropsy to permit tissue collection for gene expression analysis.

All females lived with a male cagemate in 0.60m x 0.91m x 1.83m enclosures and were maintained with 12-hour (h) lighting, 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.

Diets and food intake

Prior to study onset, animals were fed Mazuri Callitrichid High Fiber Diet #5MI6 (Purina Mills International, St. Louis, MO) comprising approximately 53% carbohydrate,

20% protein, 6% fat and 10% fiber by weight, with a metabolizable energy of 3.3 kcal/g (approximately 61%, 23% and 16% kcal from carbohydrate, protein and fat, respectively). All animals were then switched to either a low-fat diet (LFD) or higher fat diet (HFD) 1 month prior to baseline assessments and OVX to allow for dietary acclimation. Both the LFD and HFD were semi-purified customized diets designed by Teklad Custom Research Diets (Madison, WI), and comprised lactalbumin, dextrin, sucrose, soybean oil and cellulose with additional vitamins and minerals, as described in (15). The HFD was supplemented with anhydrous milk fat and additional sucrose, at the expense of a lower dextrin content, to increase the sucrose and fat content of the HFD by approximately 2-fold, relative to the LFD. The LFD (TD.110278) comprised 64.3% carbohydrate, 14% protein, 5.6% fat and 5.0% fiber by weight, with a caloric density of 3.6 kcal/g (70.7%, 15.4% and 13.9% kcal from carbohydrate, protein and fat, respectively). The HFD (TD.110277) consisted of 56.5% carbohydrate, 15.4% protein, 12.7% fat and 5.0% fiber by weight, with a caloric density of 4.0 kcal/g (56.2%, 15.3% and 28.4% kcal from carbohydrate, protein and fat, respectively). Females were separated from their male cagemates for ~1 hour in the morning and ~1 hour in the afternoon for feeding to allow for accurate assessment of individual animals' food intake. Food was only available to females during these 1-hour times. All food (base diet plus enrichment) eaten was quantified daily throughout the study.

Ovariectomy and estrogen replacement

Following baseline assessments, bilateral OVX was performed on all females to provide gonadal hormone deficiency. Cloprostenol (Estrumate®, 0.75-1.50 μg intramuscular injection for two successive days approximately 11-60 days after ovulation),

an analog of prostaglandin-F2-alpha, was administered prior to OVX to facilitate scheduling of OVX during the follicular phase. At the time of OVX, either empty or E_2-filled (to maintain consistent peri-ovulatory E_2 levels, E_2 replaced) silastic capsules were implanted subcutaneously. Silastic capsules were removed and replaced at 3 months post-OVX to maintain consistent E_2 levels. At this time, empty capsules in the OVX groups were replaced as well to maintain consistent conditions among the groups.

As an indicator of functional E_2 depletion, uterine dimensions were obtained monthly by transabdominal ultrasonography. Using the scanner's calibrated, digitized calipers, uterine trans-fundus length (transverse uterine diameter) and dorso-ventral uterine diameter were measured from transverse views, and fundus-cervix length was measured from sagittal views.

Hormone Assay

For steroid hormone analyses, plasma samples underwent extraction and subsequent analysis on a QTRAP 5500 quadruple linear ion trap mass spectrometer (AB Sciex) equipped with an atmospheric pressure chemical ionization source (LC-MS/MS) (43). The system included two Shimadzu LC20ADXR pumps and a Shimadzu SIL20ACXR autosampler. A sample of 30 µl was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm column (Phenomenex) for separation using a mobile phase: water with 1% formic acid (Solution A) and acetonitrile with 1% formic acid (Solution B), at a flow rate of 200 µl/min. After 3 min, Solution B was increased over the course of 0.1 min to 3% and this was maintained for 3 min, followed by another 0.1 min step-up to 50% Solution B that was maintained for 2.9 min. Subsequent 0.1 min step-ups raised Solution B to 67% for 15 min and then 100% for 10 min. The system was finally

returned to initial conditions of 3% Solution B over 0.1 min for the remaining 9.9 min of each sample run. Mass spectrometer results were generated in positive-ion mode with the following optimized voltages: corona discharge current, 3 V; entrance potential, 10 V. The source temperature was 500°C. The gas settings were as follows: curtain gas, 30 psi; nebulizing gas, 20 psi; collisionally activated dissociation gas, medium. Quantitative results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each compound and internal standard. Each steroid had a MRM used for quantitation and 1 or 2 additional MRMs as qualifiers. The lower limits of quantitation (LLOQ) was 2.7 pg/mL for E_2. Linearity was $r > 0.9990$ and the curve fit was linear with $1/x$ weighting. None of the compounds of interest were detected in blank or double blank samples. Inter-assay coefficient of variation was determined by a pool of marmoset serum. Intra- and Inter- assay CoVs, respectively, were 4% and 9%.

Behavioral Observations

Following treatment onset, pairs were acclimated to the testing cages, as previously described (46). Behavior testing took place five months post treatment onset. There were two 15-minute tests per week for two weeks. Well-established (>6 months) male-female pairmates were used. Pairs were deprived of visual and olfactory contact with one another for 90 minutes immediately before behavioral observations were taken. Following the 90-minute separation period, males were placed in a holding box for 5 minutes before being allowed into the main testing cage with the female. Observations were recorded digitally using JWatcher and via manual scoring of behaviors for 15 minutes. Each test was recorded and inter- and intra-observer reliability was 80% or greater.

Body composition

Animals were weighed weekly. Body dimensions were assessed monthly in awake, manually restrained animals. Measurements included abdomen, chest, arm and leg circumference (by tape measure), and crown-rump length (by osteometric board). Body mass index (BMI, body weight [kg]/height[m^2]) was calculated from crown rump length. At baseline, 3 and 6 months post-OVX, total body composition was further assessed by dual-energy x-ray absorptiometry (DXA, iDXA, GE/Lunar Corp., Madison, WI) on sedated animals.

Locomotor activity

Monthly a small accelerometer (Actiwatch Mini, CamNtech Ltd., Cambridge UK) was added to each animal's standard collar. Activity and intensity of movement were recorded over an ~ two-week period after which the accelerometers were removed. The accelerometer sampled activity counts every 30 seconds and these data were averaged for every hour, day (during lights on), night (during lights off), morning (0600-1200 h), afternoon (1200-1800 h), and 24h.

Energy expenditure by $D_2{}^{18}0$ (doubly labeled water [DLW])

Total energy expenditure (TEE) was determined at baseline and 3 and 6 months post-OVX by the doubly labeled water technique ($D_2{}^{18}O$). On day 1 of this procedure, immediately following a pre-dose urine collection, sterile deuterium oxide and oxygen-18 labeled water ($D_2{}^{18}0$) mixed with normal saline was administered via intraperitoneal injection. The respective non-radioactive isotope doses were ~0.16 and 0.24 g/kg body weight. On days 2 through 4, additional urine samples were collected. For urine collection, each marmoset was manually captured and released into a small urine-collection

chamber (15 x 20 x 15 cm), constructed of stainless steel mesh and Plexiglass, which was positioned inside the animal's home cage. The marmoset remained in the chamber until a sample was produced (~10 minutes). Urine was stored at -20ºC in cryogenically stable tubes until analysis by isotope ratio mass spectrometry as previously described (47) and CO_2 production was calculated according to the equation of (48). Data is represented as TEE/free fat mass (FFM) for each animal at each time point recorded.

Glucoregulation

Glucoregulation was assessed at baseline and 3 and 6 months post-OVX in overnight fasted, awake animals by oral glucose tolerance test. 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 following sucrose administration and assessed for glucose. Glucose was measured by glucometer (Accu-Check Aviva, Roche Diagnostics, Indianapolis, IN). The trapezoidal rule was used to calculate area under the curve (AUC).

Lipid Metabolism Measure

A standard lipid panel (UW-Meriter hospital clinics, Madison, WI) was used to measure circulating lipids (cholesterol, triglycerides, HDL and LDL) in fasted serum samples collected at baseline during oral glucose tolerance testing.

Gene Expression Analysis

Medial basal hypothalamus tissues were dissected and frozen at necropsy. 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 PCR 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 E_2+LFD group. Primer sequences were designed using NCBI Primer-Blast (49) and are listed in Supplemental Table 2.

Statistical Analysis

Data collected were analyzed utilizing SPSS software. Repeated measures ANOVA were used to analyze data collection that repeated throughout the duration of the study. Behavioral observations and gene expression data were analyzed with a two-way ANOVA. Statistical significance was determined at $p<0.05$.

Results

Reproductive Physiology

OVX effectively diminished E_2 levels, regardless of diet condition. Females that received E_2 replacement sustained peri-ovulatory phase levels of E_2 over the course of the study, as shown in Figure 1A ($p=0.003$). The achieved level of circulating E_2 in the E_2 replaced females maintained pre-OVX uterine diameter, a key indicator of effective ovarian E_2 replacement, whereas OVX resulted in a diminished uterine diameter over the 6-month experiment ($p=0.01$, Figure 1B). Additionally, E_2 replacement physiologically supported the expression of female sexual behaviors in E_2 replaced females. Figure 2 shows that by 5 mo post OVX, depletion of E_2 leads to an expected decrease (~50%) in sexually receptive behavior (A; $p=0.04$) and a complementary increase in species-characteristic sexual rejection behavior towards the male partner's mounting behavior (B; $p=0.04$).

Metabolic Physiology

Marmosets are known to prefer a high-sugar diet (50). Therefore, we designed a low-fat diet (LFD) and higher fat diet (HFD) that are both high in sugar (sucrose, dextrin), with the HFD containing approximately 2-fold more sucrose and fat, relative to the LFD diet. Prior to OVX, all animals were assigned to either the LFD or HFD. Contrary to reproductive responses to OVX, metabolic functions were minimally affected by E_2 depletion or diet in female marmosets over 6 months. Similar body weights (Figure 3A; p=0.97) and BMI (Figure 3B; p=0.58) were observed across all treatment groups throughout the study. Total body and regional fat mass, measured by DXA were also similar among all females over the 6 months (Supplemental Table 3, total body fat, p=0.82; chest, p=0.69; abdomen, p=0.87; legs, p=0.68). Additionally, measures of energy intake and expenditure were similar across all female groups (Figure 4 A-C). These measures included caloric intake (A, p=0.95), locomotor activity (B, p=0.81) and doubly-labeled water estimates of energy expenditure (C, TEE/FFM, p=0.88). Fasted serum lipids, including cholesterol measures (cholesterol: p=0.16; HDL: p=0.66; LDL: p=0.71; Chol:HDL Ratio, p=0.76) (Table 1) were similarly comparable across all female groups. In contrast to all other metabolic measures, circulating triglyceride levels did produce an interaction between time and diet (p=0.04). As the study progressed, females fed the HFD exhibited elevated triglyceride levels compared with females receiving the LFD. Fasting blood glucose levels and AUC glucose from GTTs were not different between the treatment groups and did not change (fasting glucose, p=0.054; AUC glucose, p=0.91) throughout the study (Table 1).

Hypothalamic Gene Expression

Gene expression analysis, via qPCR, of the medial-basal hypothalamus showed that E_2 depletion resulted in a decrease in the expression of *PGR*, which encodes progesterone receptor (Figure 5A, p=0.01). *PGR* expression exhibited no effect of diet and no interaction between E_2 treatment and diet condition. Other selected, E_2-regulated, neuroendocrine and behaviorally-related genes did not exhibit any E_2 dependent changes in hypothalamic mRNA expression (Supplemental Table 4). There was a decrease in mRNA expression for *PPARGC1A*, which encodes PGC1α (Figure 5B, p=0.03), and the serotonin receptors *HTR1A* (Figure 5C, p=0.01) and *HTR5A* (Figure 5D, p=0.01) in females fed the HFD compared to the LFD. Although there was no effect of E_2 treatment and no interaction between E_2 treatment and diet condition for expression of *PPARGC1A, HTR1A* or *HTR5A*, the effect size of diet treatment appeared attenuated with E_2 treatment relative to OVX for these genes. Other metabolically-related genes, however, exhibited no expression differences between any treatment groups (Supplemental Table 4).

Discussion

In women, the natural decline in ovarian E_2 and fertility during the menopausal transition is associated with detrimental metabolic changes, including increased body weight, central adiposity and impaired glucoregulation (16). In developing countries, obesity and diabetes are prevalent among women (17) due to many factors, including increasingly sedentary lifestyles and diet changes involving higher fat and sugar content. When metabolic detriments overlap with menopause, they place women at high risk for type 2 diabetes mellitus, T2DM, compared with healthy, pre-menopausal women (51). There is, however, a lack of evidence identifying ovarian E_2 as a causal agent of

metabolic dysregulation in postmenopausal women, and whether changes in physical activity, metabolic rate and food intake occur because of a decline in E_2 signaling.

In the present study, we used a nonhuman primate model, the female marmoset monkey, to understand the effects of both OVX-mediated E_2 depletion and a diet higher in fat and sucrose on various aspects of metabolic function. Marmosets demonstrate modest susceptibility to diet-induced obesity from either a high-sugar or high-fat diet (37, 38). Ovary intact female mice are protected against diet-induced obesity and metabolic disturbances relative to OVX mice (4, 5). We therefore hypothesized that in OVX female marmosets, E_2 depletion will permit the HFD, which has increased sucrose and fat content relative to the LFD, to promote increased body weight and adiposity, together with decreased glucose tolerance. The results, however, do not implicate ovarian E_2 as a major contributor to female primate metabolic health. We found a lack of change in energy balance in female marmosets due to either OVX and/or HFD. There were no differences in feeding behavior, or in locomotor activity and energy expenditure, in response to either OVX or diet. Unsurprisingly, given the lack of change in energy balance, these female marmosets did not exhibit increases in body weight, BMI or subsequent changes in adiposity and glucose metabolism, regardless of E_2 or diet condition. These results do not implicate ovarian E_2 as a major contributor to female primate metabolic health.

In female rodents (52), OVX can transiently increase food intake and decrease metabolic energy expenditure and locomotor activity. In rhesus macaques one study has documented a 3% increase in body weight at 6 wk post-OVX, an effect that was exacerbated by a typical HFD containing approximately 35% of calories from fat (32).

No changes in body weight were reported in cynomolgus macaques maintained on a normal fat diet (~11% calories from fat) after long-term OVX (33, 34). Body weight changes in female cynomolgus macaques have yet to be studied, however, when long-term OVX is combined with HFD.

The HFD fed to female marmoset monkeys in the present study was approximately 2-fold higher in fat than the LFD (28.4% vs. 13.9% calories from fat) and contained twice as much sucrose compared to the LFD. Although the HFD (4.0 kcal/g) contained a higher caloric density than the LFD (3.6 kcal/g), the caloric intake between the LFD and HFD groups was similar. Furthermore, the HFD did not induce any phenotypes consistent with metabolic dysfunction in either OVX or E_2-treated females. Given the lack of E_2- and diet-related changes in body composition and energy homeostasis, it was unsurprising that gene expression of many of the known hypothalamic genes regulating metabolism (SupplementaryTable 4) were not different between any of the female groups. Despite the lack of metabolic phenotype, however, mRNA expression of *PPARGC1A* was downregulated in female marmosets receiving HFD*. PPARGC1A* encodes PGC1α, a co-activator primarily implicated in regulating thermogenesis and mitochondrial biogenesis in hepatocytes, adipocytes and skeletal muscle tissue (53). Prior to this study, the regulation of hypothalamic *PPARGC1A* mRNA expression had been examined only in obese mice fed 'Western style diets'. In male, but not female mice, hypothalamic PGC1α was shown to be a regulator of hypothalamic ERα and its mRNA expression was diminished in response to a 'Western diet'(54). In contrast, in the present nonhuman primate study, HFD induced a decrease in hypothalamic *PPARGC1A* expression in female marmosets, independent of E_2

condition, and without an accompanying decrease in the expression of *ESR1*, which encodes ERα (Supplemental Table 4). Together, these findings suggest that, unlike female rodents, E_2 does not protect female nonhuman primates from altered lipid metabolism induced by HFD. The absence of a subsequent decrease in *ESR1* expression in female marmosets in response to diet induced downregulation of *PPARGC1A* expression further points to an ovarian E_2 independent mechanism of metabolic regulation in this study.

Interestingly, HFD decreased hypothalamic gene expression for 5-HT receptors 1A and 5A (*HTR1A*, *HTR5A*). Pharmacological blockade of HTR1A in female rodents has been implicated in increasing food consumption (55). In the present study, however, there were no changes in food consumption observed in monkeys fed HFD, despite the decrease in $5HT_{1A}$ receptor expression. Notably, in the current study there was no difference in hypothalamic mRNA expression of *HTR2C,* which encodes $5HT_{2c}$ receptor. However, a dramatic increase in food intake behavior is observed in mice with a genetic deletion of $5HT_{2c}$ receptor as well as pharmacological agonist treated female mice (14). *HTR5A* gene expression, nevertheless, was not previously studied in animal models with regard to feeding behavior and appetite. In the current marmoset study, *HTR5A* expression is downregulated by HFD. In the marmoset monkey, gene expression relevant to 5HT production is diminished in the dorsal raphe nucleus in response to HFD (15). In the hypothalamus, given the data presented here, it appears that 5HT action may also be diminished via decreased receptor expression, independent of ovarian E_2.

The diet-related decrease in gene expression for *HTR1A* and *HTR5A* in this study may indicate an underlying dysregulation of satiety, possibly in response to altered metabolic function in the brain, even in the absence of obvious food intake changes and obesity. It is worthwhile to note, however, that the limitations of our experimental food intake measures may have confounded our ability to accurately measure calorie intake. We measured food intake only during 1-hour feeding periods, potentially altering diet or E_2 effects on food intake that may be better detected through free feeding methodology with separating females from their male cagemates. Together with our earlier report (15), this study is the first to identify diet-induced changes of *PPARGC1a*, as well as 5HT production and action, in a female primate model. These findings suggest that in female marmoset monkeys, HFD induces neural molecular changes in the hypothalamus and dorsal raphe (15) prior to any increase in body and fat mass and energy homeostasis.

Despite a lack of E_2 specific regulation of body weight, body composition and glucose and energy homeostasis, E_2 replacement in this study maintained other female-typical biological functions in female marmosets, including receptive sexual behavior (42). In the present study, the pairmates used were well-established pairs that lived together continuously prior to testing and throughout the testing periods. This paradigm is very applicable to relationship paradigms in humans. E_2 replacement in the present study was also shown to induce an increase in hypothalamic mRNA expression of *PGR* that encodes for the progesterone receptor. This has not been examined previously in marmoset monkeys, but is another well-established function of E_2 in both rodents(56) and other nonhuman primates (57). Additionally, E_2 replacement was able to maintain

the diameter of the uterus found in ovary intact female marmosets, in contrast to the diminished diameter found in OVX females. Taken together, these results provide evidence of sufficient replacement of the ovarian contribution to circulating E_2 levels in our female marmoset subjects.

It is worthwhile to note that NWPs, such as marmoset monkeys, have species-specific differences in steroid hormone metabolism compared with OWPs, including humans (39). There are naturally occurring states of hypoestrogenism, such as anovulatory, reproductively subordinate states (46, 58) in female marmoset monkeys. This is one possible explanation for the lack of metabolic response to OVX observed in this study. Subordinate females with very low E_2 do not exhibit changes in body weight (46) or even bone density (Colman, personal communication). Reproductive subordination in the female marmoset is a naturally-occurring phenomenon that suggests an evolutionary advantage of this species to maintaining adult female body weight and bone density in a low estrogenic state (39). This is markedly different from other mammalian species, including other primates, in which detriments to bone metabolism and possibly energy homeostasis occurs in the presence of low or insufficient circulating levels of E_2, normally contributed by the ovaries (59). NWPs, or at least marmosets, may have a heightened sensitivity to E_2 (46). In the OVX state, female marmosets exhibit low, but detectable E_2 levels (43), and thus it is possible that female marmosets have evolved to regulate metabolic homeostasis with relatively little circulating E_2. On the other hand, female marmosets may have developed compensatory increases in bioavailable E_2 in a variety of organs systems, in order to maintain metabolic homeostasis and skeletal integrity during natural occurring

hypoestrogenism observed in socially subordinate females (60). Another possible explanation for the lack of metabolic response to OVX is that, in primates, including women, it is possible for extra-ovarian E_2 produced in tissues such as adipose (61) and the hypothalamus (62), to sufficiently regulate metabolic function. It has been shown that extra-ovarian E_2 can exert negative feedback regulation of gonadotropin release in female marmoset monkeys (43), which suggests that extra-ovarian E_2 in females in this study may also contribute to maintaining body weight and adiposity. Evolutionarily, an emancipation from ovarian E_2 regulation of metabolism would be relevant and advantageous because it allows for homeostatic regulation of metabolism during the post-reproductive years, a phase of life not uncommon in higher primates.

It is also notable that this effect may not be limited to the marmoset monkey, other nonhuman primates, and/or women. In rodent models, obesity associated with ablation of ERα (10, 11) appears to exceed that induced by OVX alone (1) (63). This may suggest that even in the rodent, extra-ovarian E_2 may protect against obesity. Nevertheless, it is clear from this study that in female marmoset monkeys, ovarian E_2 is not necessary to maintain homeostatic regulation of metabolism even when fed a HFD.

While this study suggests that female marmoset monkeys are resistant to metabolic changes due to HFD, and also to OVX, it is not without its limitations. Although the HFD contained approximately 2-fold more fat than the LFD (28.4% vs. 13.9% calories from fat), increased saturated fat content due to the addition of anhydrous milk fat, 2-fold more sucrose (30% vs. 15% by weight) and an increased caloric density (4.0 kcal/g vs. 3.6 kcal/g), this formulation of HFD insufficient to induce changes in body composition and adiposity. Most western-style high-fat diets used to

induce obesity and/or metabolic derangements contain 35% to 60% kcal from fat (64). It is possible that a longer duration of time on HFD, or an increased fat content of the HFD, would elicit increases in body weight and adiposity, and subsequently induce altered glucoregulation in these female nonhuman primates. The marmoset LFD and HFD diets used in this study are both semi-purified diets, as opposed to standard commercial marmoset diets that are often hybrid diets comprising a mix of natural and purified ingredients. Although the marmosets in this study readily consumed both the LFD and HFD diets, it is possible that the dietary composition of the semi-purified diets may need to be optimized to improve palatability and to promote an increased caloric intake sufficient for positive energy balance. It has been previously shown that feeding marmosets a high-glucose diet (Mazuri 5MI5) elicited earlier and more sustained increases in both fat mass and hyperglycemia relative to feeding a high-fat diet (38). Marmosets may therefore prefer diets high in simple sugars (especially sucrose and glucose), as opposed to increased fat content (50). Our findings, nevertheless, demonstrate an absence of ovarian E_2 influence on homeostatic regulation of female metabolism in a nonhuman primate.

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Table and Figure Legends

	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Cholesterol:HDL	Triglycerides (mg/d	Glucose (mg/dl)	Glucose AUC (mg/dl
Baseline							
E2+LFD	138±1	55±5	59±6	2.6±0.3	121±20	92±9	20983±4969
E2+HFD	157±12	67±14	62±5	2.5±0.3	139±13	86±8	17227±1112
OVX+LFD	170±15	64±11	74±6	2.8±0.3	161±22	77±6	20820±3081
OVX+HFD	142±14	59±6	55±14	2.4±0.3	137±31	94±13	20155±2566
Month 3							
E2+LFD	114±2	45±2	51±2	2.5±0.1	90±6	101±17	23404±8695
E2+HFD	166±6	72±12	69±7	2.5±-0.3	126±2	83±5	20001±3384
OVX+LFD	132±15	50±6	52±10	2.7±0.2	146±16	79±6	21383±2048
OVX+HFD	145±18	74±17	40±4	2.1±0.2	157±21	111±21	21248±1872
Month 6							
E2+LFD	155±14	65±8	70±4	2.4±0.1	99±15	112±6	25931±8856
E2+HFD	170±11	74±6	71±10	2.3±0.1	123±9	97±8	21053±2981
OVX+LFD	154±16	66±7	66±8	2.4±0.2	111±22	88±6	23012±2746
OVX+HFD	179±22	82±15	57±19	2.3±0.1	200±58	118±15	23307±3060

Table 1: Fasted measurements of serum cholesterol (total, LDL and HDL), and triglycerides were taken across the 6-month study. The only effect observed occurred at Month 3: higher triglycerides in HFD females ($p=0.05$), independent of E_2 treatment. This effect was no longer significant at 6 months; however, there was a trend towards HFD elevation of triglycerides. There were no cholesterol differences between treatment groups. There were also no changes in fasted blood glucose and glucoregulatory function between groups throughout the study. All groups showed similar responses in glucose tolerance test ($p=0.91$)

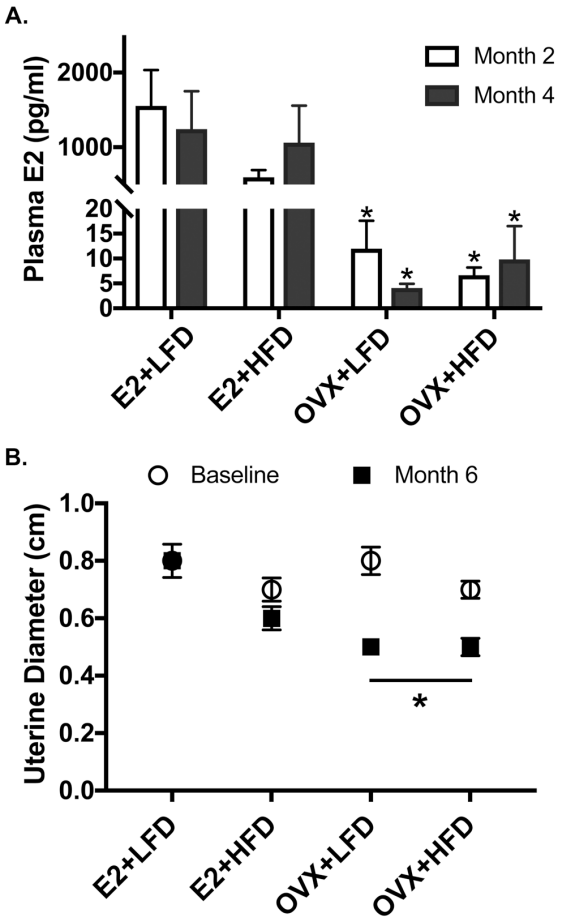

Figure 1A-B: (A) Estradiol, E_2, was diminished in both LFD and HFD OVX groups. Peri-ovulatory levels of E_2 (1.0 $\pm$ 0.3 ng/ml at 2 months; 1.1$\pm$0.3 ng/ml at 4 months) were obtained with subcutaneous capsules throughout the study. (**B**) After 6 months of treatment onset, uterine diameter was diminished in OVX females ($p<0.01$). E_2 replacement maintained pre-OVX uterine diameter.

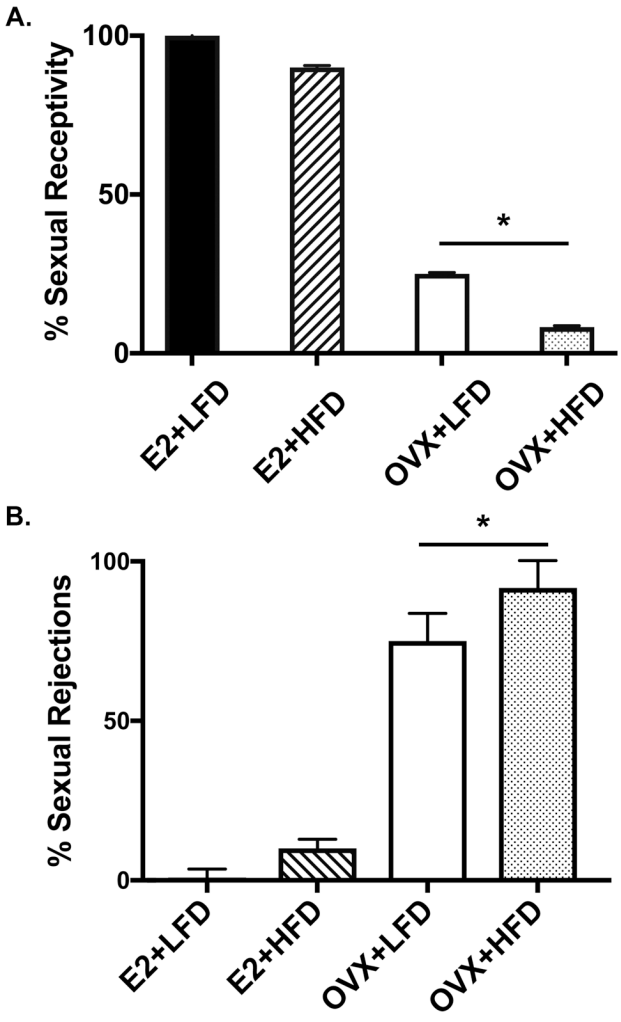

Figure 2A-B: (**A**) After 5 months of treatment onset, OVX, regardless of diet condition, induced typically diminished expression of sexually receptive behaviors (p=0.04) compared to E_2 treated females. (**B**) OVX females, complimentary to the receptivity decline, exhibited an increase in rejection behavior towards their male partners (p=0.04).

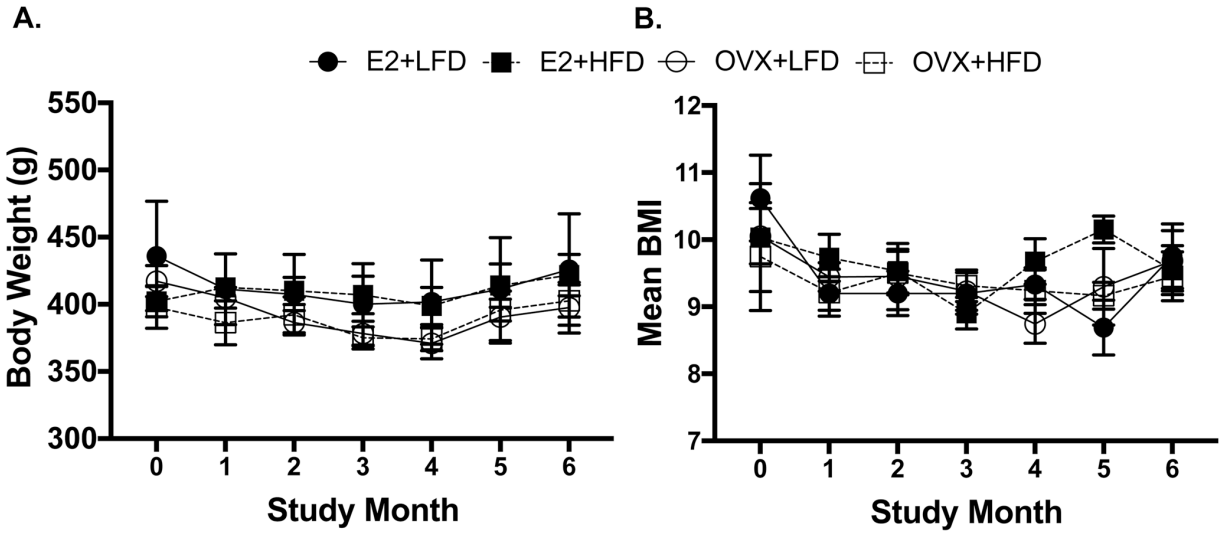

Figure 3A-B: Across all study groups, body weights and BMI measures remained similar throughout the study duration (**A**: p=0.97; **B**: p=0.58, respectively).

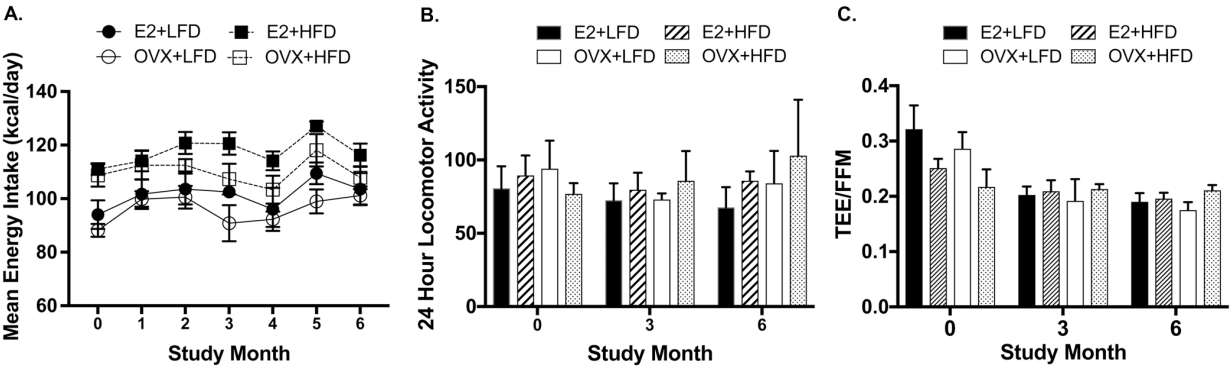

Figure 4A-C: Energy intake, assessed by food intake (**A**, p=0.95), and energy expenditure, estimated through activity collars (**B**, p=0.81) and by doubly labeled water expressed as total energy expenditure/free fat mass (TEE/FFM)(**C**, p=0.88), did not differ between female groups.

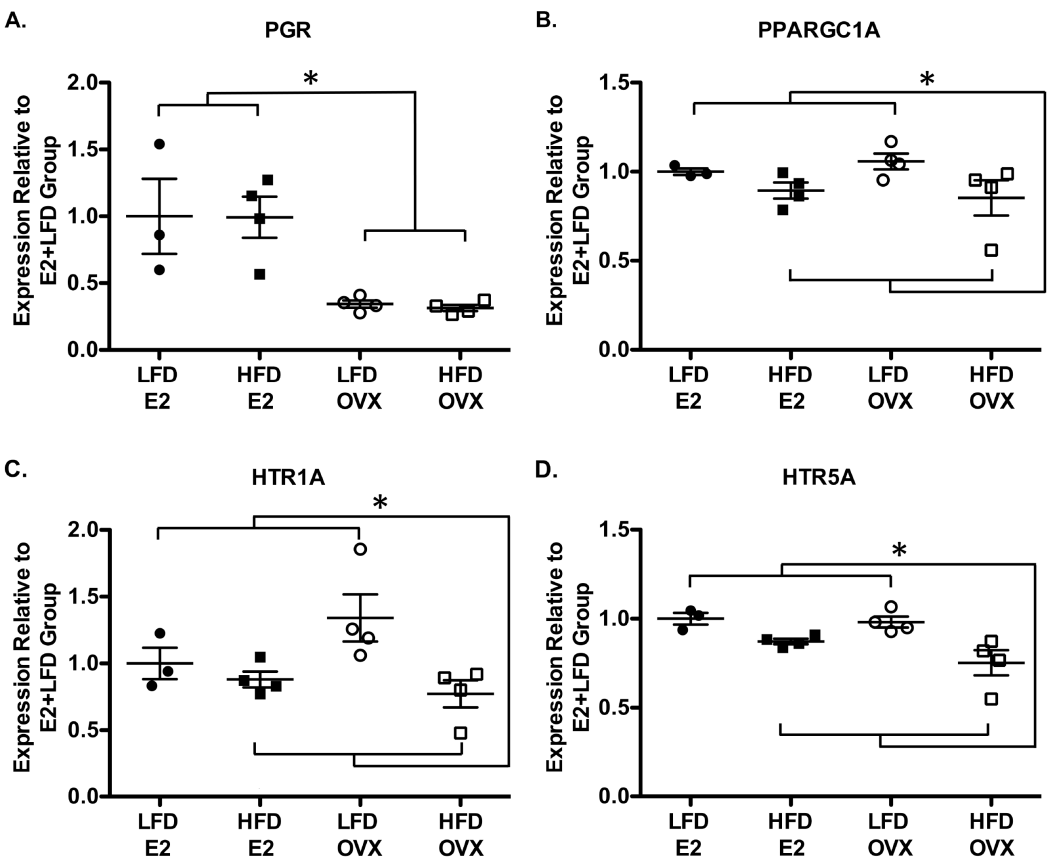

Figure 5 A-D: qPCR-determined mRNA expression of progesterone receptor (PGR) **(A)** was diminished in OVX compared to E_2 replaced females ($p<0.01$), but there was no effect of diet and no interaction between E_2 treatment and diet condition. There were, however, decreases in PGC1α(*PPARGC1A)* (**B**, *p=0.03), *HTR1A* (**C**, *p=0.03) and *HTR5A* (**D**, *p=0.01) mRNA expression in females fed the HFD compared to the LFD, despite an absence of obvious changes in body weight and body composition. There was no effect of E_2 treatment and no interaction between E_2 treatment and diet condition.

Treatment Group	Age (yrs)	Body Weight (g)	Fasting Triglycerides (mg/dl)
E2+LFD	3.47 ±0.27	437.67 ±38.86	120.67 ±19.6
E2+HFD	3.59 ±0.52	404.00 ±12.32	138.75 ±12.7
OVX+LFD	3.45 ±0.57	384.25 ±14.10	161.25 ±21.5
OVX+HFD	3.43 ±0.28	386.00 ±14.67	136.5 ±31.1

Supplemental Table 1: Females in all groups were of similar ages and body weights prior to study onset.

Gene	Forward Primer (5' --> 3')	Reverse Primer (5' --> 3')
AGRP	AACGCCTTCTGCTACTGTCG	TGGTCCCATCCTTTATTCGCC
AVP	AGGGAACACCTGCGGACATA	ATCTTGGTGCACTCAGGTGG
CARTPT	CGAGCCCTGGACATCTACTC	GGGGACTTGGCCATACTTCTT
DRD1	CAGACTTTGCCCTGTGACGA	ACATCGCAGCCCCATTGTTA
DRD2	GCCTCCTTCCTTGACCTTCC	GGCCTTGAAGGGTGTGAACT
DRD4	TTGGCTGGGCTACGTCAACA	CGGCGTTGAAGACCGTGTA
ESR1	TCCGCAAATGCTACGAAGTG	CTCCCTCCTCTTCGGTCTTTTC
GNRH1	CCTGAAAGGAGCTCTGGAAAGT	TCCTTCTGGCCCAGTGGATT
HNRNPAB	TTTGTCGGGGGTCTGAATCC	CCTCAATCTCCCCAAAATCGC
HTR1A	TTAGCAAGGACCACGGCTAC	ATGCGCCCATAGAGAACCAG
HTR1B	TGGGTCTCCTGTGTACGTGA	CCTAGCGGCCATGAGTTTCT
HTR2A	TCAACTCCAGAACGAAGGCA	ATCGTCGGCGAGTAAGCAAC
HTR2B	CAAGCCACCTCAACGCCTAA	CAGAGCCTTGTCCTTCCGAG
HTR2C	CCAAGCAACGCCATCCTTC	TTAGGTGCACAAGGAACGAA
HTR5A	CGTGGTGCTCTTCGTGTACT	TCGGATACGGGTGAGACACT
KISS1	AGGCAAGCCTCAAGGCACTC	GCCATGTCTGATGACTCCCC
NPY	ATCTAGCCCGGAGACACTGA	TGAAGGGTCTTCAAGCCGAG
OXT	CTCGATGTGCGCAAGTGCC	TCCACGCAGCAGATATTCGG
OXTR	ATGCGCCTAAGGAAGCCTCA	GTGACCCGTGAAGAGCATGT
PGR	CCCTGCATGTCGCCTTAGAA	ATTTGGAATGCCCACTGGCT
POMC	GTGTCAGGATCTCACCACCG	ATTGCCAGGGAACACTGGAG
PPARGC1A	AAGTCCCACACACAATCGCA	TGGGGTCATTTGGTGACTCG
TBP	CCATGACTCCTGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA

Supplemental Table 2: Primers used for qPCR gene expression analysis.

	Total Body Fat (g)	Chest Fat (g)	Abdominal Fat (g)	Leg Fat (g)
Baseline				
E2+LFD	207±35	62±17	51±17	46±7
E2+HFD	199±12	51±5	43±4	54±1
OVX+LFD	199±11	59±3	44±4	47±1
OVX+HFD	199±12	54±4	40±5	50±1
Month 3				
E2+LFD	168±23	45±7	33±5	45±3
E2+HFD	137±13	40±5	27±3	36±2
OVX+LFD	157±10	41±2	31±1	4±1
OVX+HFD	141±8	39±3	27±1	36±1
Month 6				
E2+LFD	136±23	43±10	30±7	30±1
E2+HFD	115±13	37±6	23±3	28±1
OVX+LFD	112±14	37±7	22±3	24±1
OVX+HFD	118±15	41±6	24±4	26±1

Supplemental Table 3: DXA scans were utilized to determine body composition. There were no differences in adiposity between female groups.

Gene Symbol	Gene Name	E2+LFD	E2+HFD	OVX+LFD	OVX+HFD
Metabolism					
AGRP	Agouti-related Protein	1.00 ±0.76	0.94 ±0.40	1.84 ±0.82	0.73 ±0.26
CARTPT	Cocaine- and -amphetamine-regulated Transcript	1.00 ±0.08	1.21 ±0.12	1.61 ±0.34	1.15 ±0.21
NPY	Neuropeptide Y	1.00 ±0.26	1.31 ±0.29	1.70 ±0.51	0.83 ±0.15
PPARGC1A	**Peroxisome Proliferative Activated Receptor, Gamma, Coactivator 1 Alpha**	**1.00 ±0.02**	**0.89 ±0.04**	**1.06 ±0.04**	**0.85 ±0.10**
POMC	Proopiomelanocortin	1.00 ±0.65	0.37 ±0.20	0.47 ±0.23	1.20 ±1.04
Neuroendocrine					
AVP	Vasopressin	1.00 ±0.14	0.96 ±0.13	1.56 ±0.33	1.06 ±0.11
ESR1	Estrogen Receptor Alpha	1.00 ±0.25	0.73 ±0.14	0.79 ±0.07	1.27 ±0.72
HNRNPab	Heterogeneous Nuclear Ribonucleoprotein A/B	1.00 ±0.01	0.99 ±0.05	0.99 ±0.09	0.92 ±0.08
KISS1	Kisspeptin	1.00 ±0.55	0.77 ±0.22	1.88 ±0.62	1.27 ±0.33
GNRH1	Gonadotropin-releasing Hormone	1.00 ±0.15	1.17 ±0.10	0.89 ±0.09	1.00 ±0.09
PGR	**Progesterone Receptor**	**1.00 ±0.28**	**0.99 ±0.15**	**0.34 ±0.03**	**0.31 ±0.02**
Behaviorally Related					
DRD1	Dopamine Receptor D1	1.00 ±0.13	0.89 ±0.04	1.41 ±0.22	0.87 ±0.13
DRD2	Dopamine Receptor D2	1.00 ±0.25	0.83 ±0.11	0.76 ±0.06	1.03 ±0.18
DRD4	Dopamine Receptor D4	1.00 ±0.03	0.62 ±0.21	1.17 ±0.23	0.76 ±0.13
HTR1A	**Serotonin Receptor 1A**	**1.00 ±0.12**	**0.88 ±0.06**	**1.34 ±0.18**	**0.77 ±0.10**
HTR1B	Serotonin Receptor 1B	1.00 ±0.06	0.81 ±0.11	0.79 ±0.03	0.75 ±0.10
HT2RA	Serotonin Receptor 2A	1.00 ±0.12	0.84 ±0.09	0.91 ±0.03	0.80 ±0.15
HT2RB	Serotonin Receptor 2B	1.00 ±0.14	0.99 ±0.12	0.94 ±0.05	0.90 ±0.05
HT2RC	Serotonin Receptor 2C	1.00 ±0.04	0.65 ±0.12	0.67 ±0.05	0.62 ±0.13
HTR5A	**Serotonin Receptor 5A**	**1.00 ±0.03**	**0.87 ±0.02**	**0.98 ±0.03**	**0.75 ±0..07**
OXT	Oxytocin	1.00 ±0.13	1.00 ±0.15	1.23 ±0.26	0.99 ±0.08
OXTR	Oxytocin Receptor	1.00 ±0.08	0.83 ±0.08	0.79 ±0.15	1.10 ±0.40

Supplemental Table 4: qPCR-determined mRNA expression of selected metabolic, reproductive, and behaviorally related genes are shown here. Significant effects are shown in the text and included here. mRNA of additional selected genes were not difference among groups.