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Accelerated Episodic LH Release Accompanies Blunted Progesterone Regulation in PCOS-like Female Rhesus Monkeys (*Macaca mulatta*) Exposed to Testosterone During Early-to-Mid Gestation

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Abstract

Background/Aims: Ovarian theca cell hyperandrogenism in women with PCOS is compounded by androgen receptor-mediated impairment of estradiol and progesterone negative feedback regulation of episodic LH release. The resultant LH hypersecretion, likely the product of accelerated episodic release of GnRH from the median eminence of the hypothalamus, hyperstimulates ovarian theca cell steroidogenesis, enabling testosterone (T) and androstenedione excess. Prenatally androgenized female monkeys (PA) exposed to fetal male levels of T during early-to-mid gestation, when adult, demonstrate PCOS-like traits, including high T and LH levels. This study tests the hypothesis that progesterone resistance-associated acceleration in episodic LH release contributes to PA monkey LH excess.

Methods: 4 PA and 3 regularly cycling, healthy control adult female rhesus monkeys of comparable age and body mass index underwent (1) a 10h, frequent intravenous sampling assessment for LH episodic release, immediately followed by (2) IV infusion of exogenous GnRH to quantify continuing pituitary LH responsiveness, and subsequently (3) an SC injection of a progesterone receptor antagonist, mifepristone, to examine LH responses to blockade of progesterone-mediated action.

Results: Compared to controls, the relatively hyperandrogenic PA females exhibited ~100% increase (p=0.037) in LH pulse frequency, positive correlation of LH pulse amplitude (p=0.017) with androstenedione, ~100% greater increase (p=0.034) in acute (0–10 min) LH responses to

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exogenous GnRH, and an absence (p=0.008) of modest LH elevation following acute progesterone receptor blockade suggestive of diminished progesterone negative feedback.

Conclusion: Such dysregulation of LH release in PCOS-like monkeys implicates impaired feedback control of episodic release of hypothalamic GnRH reminiscent of PCOS neuroendocrinopathy.

Keywords

androgens; developmental origins of health and disease; gonadal steroids; gonadotropin-releasing hormone; gonadotropins; hypothalamus; primates; testosterone

Introduction

Polycystic ovary syndrome (PCOS) is the most prevalent endocrinopathy in women's health, contributing not only substantially increased risk of infertility and hirsutism, but also increased risk of type 2 diabetes mellitus, cardiovascular disease and endometrial cancer [1]. Increasing evidence implicates neural androgen receptor (AR) [2,3], dysregulation of hypothalamic GnRH and pituitary LH secretion, as well as enhanced ovarian responsiveness to LH in its pathogenesis [4–7]. PCOS diagnosis derives from a reproductive phenotype, including two out of three of the following criteria: androgen excess, infrequent or absent ovulatory menstrual cycles and polycystic ovary morphology [8]. Any single diagnostic trait can therefore be absent when a diagnosis is made.

High frequency episodic release of GnRH from the hypothalamus typifies many women with PCOS [9,10] and enables both LH hypersecretion contributing to ovarian hyperandrogenism [11] and relative follicle stimulating hormone (FSH) deficiency encouraging ovarian follicular arrest [12,13]. Such gonadotropin imbalance is consistent with recent identification of PCOS risk genes implicated in the regulation of pituitary gonadotropin release [5,14] as well as functional clinical studies demonstrating diminished negative feedback regulation on LH, and probably GnRH release, mediated by estradiol and progesterone [3,15]. Exaggerated episodic release of LH is a prepubertal characteristic of adolescent girls with hyperandrogenism [16–19], including absent nocturnal slowing of LH release episodes [20] and ~ 50% incidence of diminished progesterone-mediated negative feedback on LH [21]. Together, these LH traits are suggestive of pre-PCOS neuroendocrine pathogenesis already established in the immature female hypothalamus.

Not surprisingly, therefore, developmental programming has proved a successful strategy for designing the most comprehensive, experimentally-induced animal models for PCOS, initiated by prenatally androgenized (PA) female rhesus monkeys [22–24]. Progressively increasing evidence of fetal T exposure in daughters of women with PCOS implicates an analogous pathogenic mechanism in women [25–28]. Fetal PA monkeys exhibit LH hypersecretion within weeks of early-to-mid gestation exposure to fetal male testosterone (T) levels [29]. Their LH excess persists into adulthood and is accompanied by ovarian hyperandrogenism, intermittent and absent ovulatory menstrual cycles, as well as polycystic ovaries [30], together with PCOS-typical metabolic sequelae: increased incidence of type 2 diabetes, abdominal adiposity, adipogenic constraint and hyperlipidemia [31,32]. Emulating

LH traits in women with PCOS, elevated LH levels in PA monkeys show resistance to negative feedback suppression from either exogenous estradiol [33] or gonadotropin-hyperstimulated, endogenous ovarian hormone release [34]. In a separate study, adult female rhesus monkeys with naturally occurring hyperandrogenism also exhibit elevated LH levels and accompanying PCOS-like reproductive dysfunction [35,36], together with genital biomarkers indicative of fetal T excess [36]. Numerous studies have since demonstrated fetal T or dihydrotestosterone (DHT) programming of LH hypersecretion as a consequence of elevated episodic release of GnRH/LH in PA adult female sheep [37–39], rats [40,41] and mice [42,43]. Such elevated LH is associated with insensitivity to estradiol- or progesterone-mediated negative feedback [37,43–45], likely mediated within the hypothalamus [46,47]. Such neural origins for LH excess, and diminished LH sensitivity to estradiol/progesterone-mediated negative feedback, however, have yet to be demonstrated in PCOS-like, female nonhuman primates.

It is critical that these PCOS-like neuroendocrine characteristics are established in nonhuman primates since non-primate neuroendocrine regulation of GnRH fundamentally differs from that in primates. For example, unlike non-primates, there is little circadian signal control of preovulatory GnRH surges [48], fetal androgen excess elimination of estradiol-induced ovulatory LH surges [49] and peri-natal androgen programming of GnRH negative feedback [24], while gonadectomized male monkeys with ovarian transplants exhibit cycle ovulatory cycles (50). Moreover, before puberty, there is strong central component of GnRH inhibition of GnRH in primates that consistently involves gamma-amino butyric acid (GABA) as a neural substrate, and has no counterpart in non-primates (51). Evidence for fetal androgen excess programming of PCOS-like GnRH regulation in female rhesus monkeys would therefore, for the first time, implicate a neural developmental window in female primates, including humans, as vulnerabilities for PCOS-like conversion, and would open the door to development of selective pharmaceutical rescue [3,52] with high likelihood of translation.

Consequently, this study was designed to test the hypothesis that gonadotropin dysfunction in PA adult female monkeys is a consequence of increased frequency of GnRH release from the hypothalamus, thus implicating neuroendocrine programming in a PA nonhuman primate model for PCOS at a hypothalamic neuronal (LH pulse frequency) location as well as at a pituitary gonadotrope (LH pulse amplitude) location. Quantitative assessment of episodic LH release in the systemic circulation was used as biomarker for GnRH release, given the reliable association between GnRH and LH episodic release in adult female rhesus monkeys [53,54]. Increased pituitary LH responsiveness to exogenous GnRH was re-confirmed and acute LH responses to a single injection of the progesterone receptor (PR) antagonist, mifepristone, were analyzed for evidence of diminished progesterone negative feedback on LH.

Materials and Methods

Ethics Statement

The Institutional Animal Care and Use Committee of the Graduate School of the University of Wisconsin-Madison approved all procedures used in the study, and the care and housing

of the monkeys was in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and Animal Welfare Act with its subsequent amendments.

Animals

The seven adult female rhesus monkeys (*Macaca mulatta*) used in this study were maintained at the Wisconsin National Primate Research Center at the University of Wisconsin-Madison (WNPRC). The animals were housed in female pairs, under controlled lighting (12 h of light (daytime), 12 h of darkness (nighttime), lights on at 0600 h) and temperature (22˚C), according to standard protocol [55,56]. They were fed Purina monkey chow (product no. 5038, Ralston Purina, St Louis, MO, USA) with occasional supplementation of fresh fruits. This formulation of monkey chow provides 70% of calories as carbohydrate, 13% as fat and 17% as protein. Age and BMI [body weight (kg)/crown-rump length^2 (m^2)] [57] of both female groups were comparable (Table 1). Four of the female monkeys were exposed to gestational T excess by SC injection of their dams with 10 mg testosterone propionate (TP) starting on gestational days 40–44 for 15–35 consecutive days (early-to-mid gestation, PA). Only four of the 14 previously described PA monkeys were studied [31] because they previously exhibited high T levels [58], increased anogenital distance (an anatomical biomarker of early-to-mid gestational T exposure) [59] and intermittent menstrual cycles [58] compared to controls (Table 1), while remaining healthy. The three control female monkeys in this study were not exposed to exogenous T excess *in utero*, were not otherwise manipulated during gestation by other investigators or colony management at WNPRC, and were selected from healthy adult monkeys exhibiting regular menstrual cycles. For some of the female monkeys in this study, somatometric measures, basal levels of ovarian and pituitary hormones, and menstrual cycle duration were previously reported [30,60–63]. Those reported here were obtained specifically for this study. Baseline blood samples providing serum for hormone analyses were obtained from animals accustomed to using a tabletop restraint without anesthesia [61].

All Experiments were performed between September to May, to avoid seasonal oligo- or anovulation [61]. Experiments 1 and 2 were carried out during days 1–5 of the same menstrual cycle or during the same period of anovulation, while Experiment 3 was performed during a different menstrual cycle or anovulatory period.

Experiment 1: Quantitative assessment of episodic LH release.

All monkeys were adapted to the jacket-tether apparatus for several weeks before catheter implantation surgery (see below). This training included three or more separate sessions for each animal. To obtain samples from a remote site without disturbing the research subjects, monkeys were implanted with indwelling catheters in their saphenous vein. The catheter was exteriorized through a tether-jacket system and was connected to a swivel device at the top of each cage through an opaque plastic curtain into an adjacent part of the room, *i.e.* the paired animals were kept in semi-isolation out of visual contact with researchers, but were visible through video camera connection to a monitor from which observers could view their behaviors. To assess between group differences in circulating LH, 0.2-ml blood samples were collected at 10-min intervals for 10 h (0600 – 1600 h). Daytime hours were chosen for examination of LH episodic release to avoid potential nighttime slowing of LH pulse

frequency [64]. After each blood sampling, plasma was separated by centrifugation, and blood cells from the previous sample were re-suspended in sterile saline and re-injected into the monkey. An indwelling catheter made from SILASTIC brand tubing (Dow Corning Corp., Midland, MI) was inserted into the saphenous vein, under isoflurane anesthesia, one day before the start of each experiment, as described previously [65,66]. The catheter was exteriorized through the back, and an extension of the catheter was passed through a cloth jacket with a tether-swivel system, allowing normal movement by the monkey. The catheter extension was connected to a three-way stopcock for blood sampling and injection. The patency of the catheter was maintained by constant infusion of 0.5 ml/h sterile saline containing 5 IU/ml heparin until the initiation of blood sampling after surgery.

LH pulses were detected using the PULSAR algorithm [67]. Pulses of LH were determined using parameters similar to those previously reported [68], with modified cut-off criteria for G1, G2, G3, G4, and G5 of 3.0, 2.5, 1.5, 1.2, and 1.0 sd, respectively. The inter-assay standard variation for LH radioimmunoassay (RIA) was $y = [2.67x + 2.17]/100$, modified from that previously described [68].

Experiment 2: Exogenous GnRH stimulation of LH release

To examine pituitary responsiveness to human GnRH (L-7134, Sigma Chemical Company, St. Louis, MO), plasma samples were collected from all seven monkeys involved in Experiment 1, using identical sampling methods. After 10-h of sampling for episodic LH release assessment (see above), 2 μg/kg GnRH (in 0.5 ml sterile saline) was administered through the indwelling IV catheter at 1600h, and 10-min blood sampling was continued for an additional 2h until 1800h (lights off). The GnRH test was designed to assess pituitary gonadotropin responses to exogenous GnRH [51], but not ovarian responses to GnRH-stimulated gonadotropin release. To minimize the total period of sampling, we did not examine the LH response to vehicle.

Experiment 3: LH response to progesterone receptor (PR) antagonism

Each monkey was injected IM with 5 mg/kg mifepristone (PR antagonist; donated by Roussel-UCLAF, Romainville, France) in 0.2ml sterile sesame oil immediately after a blood sample (0.5 ml) was withdrawn at 0 h (07:00–08:00h), and successive 0.5 ml daytime blood samples were withdrawn hourly from 3–10h following injection. Serum was assayed for bioactive LH (bioLH). The dose of mifepristone was comparable to that used to examine gonadotropin responses to acute PR blockade in women [69], and the timing of blood sampling accommodated both attainment of maximum mifepristone levels in blood (at least 2h post-injection) [70] and brain [71], as well as maintenance of blood levels (half-life of ~25–30 h) [72].

Hormone assays

All gonadotropin and steroid hormone assays were performed in the WNPRC Hormone Assay Services Laboratory, as previously described [55,73,74]. Serum concentrations of immunoactive LH and FSH (Experiments 1 and 2) were determined by in-house RIAs [55], and those for bioactive LH (bioLH; Experiment 3) were determined by mouse Leydig cell bioassay using the rhLH-RP1 reference preparation [29]. All assays were validated for

rhesus monkeys. Detection limits, as well as intra- and inter-assay CoVs, were respectively as follows: LH 0.15 ng/ml, 7% and 13%; FSH 0.08 ng/ml, 3% and 13%; bioLH 0.30 ng/ml, 9% and 18%.

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 previously described [74]. A sample of 10 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 incrementally increased to 100% over the course of the next 17 min. 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, androstenedione, progesterone and DHEA; 2.7 pg/mL for estradiol (E_2) and estrone (E_1). 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. Intra-assay LC-MS/MS coefficients of variation were determined from a pool of human serum: E_2, 4%; E_1, 12%; progesterone, 4%; DHEA, 13%; androstenedione, 8%; T, 11%.

Statistical analyses

All results are expressed as mean ± SEM and log transformed to achieve homogeneity of variance and to increase linearity when appropriate [75] prior to t-test (baseline parameters) or appropriately designed ANOVA with one between subject factor (types of female monkeys) and two within subject factors (treatment and time) with Tukey's posthoc means comparisons. Numbers of LH pulses, and immediate (0–10 min) LH responses to GnRH injection, were analyzed the Mann Whitney U-test. Least mean square regression analyses were used to examine parameters associations. Statistical significance was determined as $p<0.05$.

Results

Baseline characteristics

Adult female PA and control monkeys were in their late reproductive years with comparable average to overweight BMI (Table 1) without obesity (>12kg body weight). While 50% of PA females exhibited intermittent ovulatory cycles, in contrast to none of the controls (Table 1), there were no other menstrual cycle parameter differences between the two female groups. PA females, during the early-to-mid follicular phase of a menstrual cycle or a duration of anovulation, exhibited modest aspects of hyperandrogenism and hypersecretion of LH, with trends towards elevated serum ratios for T to E_2 ($p=0.069$) and serum T levels

(p=0.077), as well as an increased ratio of bioLH to immunoactive LH (p=0.033), compared to controls (Table 1).

When all females were considered together, positive correlations were found between circulating T and DHEA levels (r = 1.04, p=0.001), and between circulating E_2 and progesterone levels (r = 1.06, p=0.008), as well as a positive trend between T and the ratio for bioactive to immunoactive LH (r = 0.81, p=0.088) and a negative trend between circulating E_2 levels and menstrual cycle duration (r=0.74, p=0.057). No other correlations were identified (Table 2).

Experiment 1: Quantitative assessment of episodic LH release

PA females exhibited an approximately 120% increase (p=0.037) in the number of LH pulses/hour (pulse frequency) identified by the PULSAR algorithm compared to controls, with accompanying trends towards decreased inter-pulse intervals (p=0.061) and ~64% increase in pulse duration (p=0.089) (Table 3). There were no other differences in quantified LH parameters. Episodic LH release in each PA and control female monkey are illustrated in Figures 1a and 1b, respectively, showing the extent of individual variation in LH episodic release observed in each female monkey group. Controls exhibited LH pulse frequencies typical for daytime values during the early-to-mid follicular phase of rhesus monkey menstrual cycles.

When all females in Experiment 1 were considered together (Table 4), circulating androstenedione concentrations positively correlated with LH pulse amplitude (r=0.84, p=0.017). No other relationships were identified between endocrine or baseline characteristics (Table 4).

Experiment 2: Exogenous GnRH stimulation of LH release

Once the 10-hour frequently sampled IV assessment of episodic LH release was completed, an IV infusion of 50 ng GnRH increased (p=0.001) circulating LH levels in all females. The immediate increase in LH levels during the first 10 min following GnRH infusion was ~100% greater (p=0.034) in PA compared to control females (Figure 2). The continuing daytime LH elevation in response to GnRH was otherwise comparable in the two female groups. No relationships were identified between LH responses to GnRH and baseline steroid hormone concentrations.

Experiment 3: Acute (3–10h) bioLH responses to progesterone receptor antagonist, mifepristone

Progesterone receptor blockade by IM injection of mifepristone revealed a relatively positive % difference (p=0.008) in daytime bioLH levels compared to baseline (mean of −1 h and 0 h values) in control, but not PA, monkeys (Figure 3). Eighty-three percent of bioLH values exceeded baseline in control females in response to mifepristone, in contrast (p=0.002) to only 25% in PA monkeys. Mifepristone-induced responses in bioLH levels were expressed as % of each individual female's baseline value prior to mifepristone administration since bioLH levels remained elevated (p=0.049) throughout (baseline: 0.93 ± 0.21 ng/ml; 10h: 0.68 ± 0.07 ng/ml) in PA compared to control (baseline: 0.30 ± 0.08 ng/ml; 10h: 0.42 ± 0.14

ng/ml) monkeys. When all females were considered together, % change in LH tended to negatively correlate ($r=-0.70$, $p=0.083$) with baseline T levels. No other relationships were identified.

Discussion

This study provides the first evidence for PCOS-like increases in daytime episodic release of pituitary LH, and thus probably hypothalamic GnRH, during the early follicular phase or anovulatory period in PCOS-like, PA adult female rhesus monkeys. The more rapid LH pulse frequency (~ 1 LH pulse/h) observed in PA monkeys is comparable to frequencies previously reported for women with PCOS (~ 1 LH pulse/h) [76], as well as long-term ovariectomized adult (monkey: ~ 0.9 LH pulses/h; human: ~ 1 LH pulse/h) [77–79] and post-menopausal, ovary intact (monkey and human: ~ 1 LH pulse/h) individuals [68,80,81], all of whom approximate the frequency of episodic GnRH release from isolated human hypothalamic neurons [82] and fetal monkey tissue explants [83]. Healthy women and adult female rhesus monkeys with regular menstrual cycles exhibit slower episodic LH release rates, but during the early follicular phase, women approximate PCOS-like rapid frequency of LH release [84].

Since frequency, and not amplitude, discriminates PCOS-like LH release in PA monkeys from that in controls, neuronal circuitry governing hypothalamic GnRH pulse generation is implicated as providing a neural substrate for PCOS-like pathogenic origins during a specific developmental window. Since not all fetal T programming translates from non-primates to primates, including humans [51,85], it was crucial to establish this specific neuroendocrinopathy in a nonhuman primate model. In female rodents and sheep, for example, fetal T commonly abolishes maturation of ovarian E_2-induced GnRH/LH surges in adulthood, preventing ovulation [51,85]. In contrast, adult female monkeys exposed to naturally occurring [36] or experimentally-induced [33,61] fetal T excess retain the ability to demonstrate ovarian E_2-induced LH (and likely GnRH) surges and ovulatory menstrual cycles. Women with PCOS [86], and with known exposure to fetal T excess [87], also demonstrate E_2-induced LH surges or ovulatory menstrual cycles.

In fetal rhesus monkeys, GnRH neurons initiate their migration from embryonic origins in the nasal epithelium about 32–36 days of gestation and complete their distribution in the hypothalamus by ~ 60 days [88], closely emulating GnRH neuronal embryonic progression in humans at 6–7 weeks of gestation [85], and approximating the gestational ages encompassed by experimentally induced T exposure of PCOS-like monkeys in the present study. Such discrete T exposure programs impaired negative feedback on LH, as manifest by diminished sensitivity to E_2 and ovarian hormone negative feedback [33,51]. Observable aspects of this PCOS-like neuroendocrine defect, such as elevated LH levels, are present in PA female monkeys from late gestation [29]. In addition, persisting relative hyperandrogenism in adult PA monkeys, may further contribute to AR-mediated negative feedback impairment, as reported for women with PCOS [89]. A T-responsive, hypothalamic developmental window may close after mid-gestation, since female monkeys experiencing T exposure after mid-gestation exhibit normal basal LH levels in adulthood [51]. Such a developmental window may re-open at puberty, when onset of exogenous T exposure in

female monkeys accelerates LH pulse frequency [90], but despite continued exposure to T, LH pulse frequency reverts to normal in adulthood [91].

In our aging, but premenopausal, PA monkeys and controls in the present study, neither hyperandrogenism nor LH hypersecretion are reliably exhibited, findings concordant with previous studies of aging, PCOS-like monkeys [55] and in aging, premenopausal women with PCOS [92,93]. Trends towards elevated T levels and T to E_2 ratio, as well as modest elevations in bioLH levels (Expt. 3 throughout) and episodic LH release are, nevertheless, consistent with less prominent PCOS-like reproductive signs and symptoms during aging [52,93,94].

Since GnRH neurons do not express androgen receptors (AR), and AR are implicated in T-driven, PCOS-like gestational programming [2], including increased LH pulse frequency [47], any pathogenic neural location must lie within the transynaptic afferent neuronal network to GnRH neurons [43,95]. In PA female sheep, fetal T exposure reduces synaptic input to GnRH neurons [96], including diminished synaptic connections from hypothalamic AR-expressing kisspeptin/neurokinin B/dynorphin (KNDy) neurons [97] and diminished synaptic inter-connections between hypothalamic KNDy neurons accompanying lower post-synaptic expression for the neurokinin B receptor [97,98]. Since the kisspeptin and neurokinin B network plays a key role in neuronal stimulation for GnRH release in rhesus monkeys [99,100] and, together with dynorphin, is functionally implicated in regulating GnRH release in men [101], fetal T exposure may either enhance kisspeptin and neurokinin B stimulation of episodic GnRH release or impair dynorphin-mediated GnRH inhibition. A KNDy neuronal site for PCOS reproductive pathogenesis is consistent with therapeutic results obtained from neurokinin B NK3-receptor antagonist treatment of women with PCOS. In a recent clinical trial [102], the NK3 receptor antagonist AZD4901 resulted in diminished basal LH, LH pulse frequency, and basal T, and there was an 18% versus 6% incidence of ovulation in the placebo group. Thus, novel therapeutic targeting of PCOS neuroendocrine reproductive pathophysiology has the potential to ameliorate PCOS reproductive and endocrine dysfunction. The comparable efficacy of NK3 receptor antagonist, ESN364, in diminishing the frequency of episodic LH release in ovariectomized ewes, LH levels in castrated male monkeys and pre-ovulatory LH surges in female monkeys, reinforces such therapeutic potential [103].

Fetal T exposure has also been shown to alter hypothalamic pre-synaptic glutamate and gamma-amino butyric acid (GABA) neuronal inputs to GnRH in both PA female mice [104] and sheep [96]. Increased GABA synaptic connections [43] may enable increased $GABA_A$ receptor-mediated drive to GnRH neurons in PA mice [42] likely mediated through depolarization of GnRH neurons [105,106] increasing GnRH release frequency. Such an additional putative neuronal site for PCOS reproductive pathogenesis would complement contributions from KNDy neurons, and would be consistent with a higher incidence of PCOS in GABA-enabled psychiatric disorders, such as epilepsy and bipolar disorder [107]. PCOS incidence is further exaggerated by valproate, an anti-convulsant and GABAergic therapeutic, especially among young (<26 years) women [108]. Perhaps most interestingly, hypergonadotropic ovarian hyperandrogensim was recently induced in a mouse model for PCOS by the systemic administration of antimullerian hormone (AMH), likely resulting in

increased hypothalamic GABAergic synaptic connections to GnRH neurons driving hyperactive GnRH release [109]. While it is not known whether PA monkeys have altered hypothalamic pre-synaptic glutamate and gamma-amino butyric acid (GABA) neuronal inputs to GnRH, or whether they release GnRH in response to AMH, as demonstrated in female mice [110], they do not exhibit elevated AMH levels in their late reproductive years [55], making ovarian AMH an unlikely contributor to the present nonhuman primate findings.

A single injection of the selective progesterone receptor antagonist mifepristone, on the other hand, modestly differentiated LH responses in the two female monkey groups, suggestive of blunted, PCOS-like diminished progesterone negative feedback on daytime LH levels [111] in PA monkeys. Interestingly, there was a trend for circulating levels of T to negatively correlate with % LH response to mifepristone in all female monkeys combined, potentially implicating prevailing hyperandrogenism in disrupting progesterone-regulated negative feedback on GnRH/LH release. While LH responses to progesterone receptor blockade have not been reported for women with PCOS, administering luteal phase levels of exogenous progesterone to PCOS women fails to diminish their rapid LH release frequencies in contrast to the same hormone's clear efficacy in suppressing LH release episodes in healthy women with regular menstrual cycles [4]. In addition, mifepristone is inconsistent in elevating LH levels when administered to healthy women during the early follicular phase [112–114] and fails to reverse exogenous E_2 and progesterone-induced slowing of GnRH pulse frequency in healthy women when it replaces 10 days of prior exogenous progesterone therapy [69]. Such unexpectedly mixed effects of mifepristone may stem from its suppression of Ca^{++}-mediated membrane depolarization [115] crucial for exocytosis of GnRH and LH, and from its antagonism at the glucocorticoid receptor [116], potentially inducing compensatory elevation in hypothalamic CRH, and potentially, GnRH suppression [117].

Endogenous E_2 levels in our study subjects are relatively low for adult female rhesus monkeys [34,118], possibly reflecting our employment of a more specific and sensitive LC-MS/MS assay method that generates considerably lower circulating values than immunoassays [29,75], in addition to previously reported and continuing impairments in ovarian folliculogenesis and luteal sufficiency in PA subjects [58,118]. Comparable ovarian defects also manifest in women with PCOS [119]. While such characteristics might be considered as signs of approaching menopause (~25–27 years of age in rhesus monkeys) [120,121], monkeys in both study groups demonstrate comparable LH and FSH levels to those previously reported at younger ages (~21–23 years of age) [55] and typically found in premenopausal adult female rhesus monkeys undergoing regular or intermittent ovulatory menstrual cycles [121]. Additionally, no monkeys in this study were amenorrheic.

With regard to strengths of this study, we employed the most comprehensive animal model for PCOS, PA female rhesus monkeys exposed to gestational T excess during early-to-mid gestation, and used previously validated quantitative assessment of LH episodic release in adult female rhesus monkeys, together with a highly specific and sensitive LC-MS/MS assay method. Limitations include small numbers of monkeys per treatment group, in compliance with contemporary refinement and reduction practices for laboratory nonhuman primates;

the use of systemic circulation concentrations of LH as a secondary measure for hypothalamic GnRH release; and the use of adult female rhesus macaques in their late reproductive, pre-menopausal years, thus PA monkeys in this study, typical of comparable aged women with PCOS [93], exhibited less pronounced hyperandrogenism and hypergonadotropism, and combined ovulatory with oligo-ovulatory females, as occurs when PCOS is diagnosed using Rotterdam 2003 criteria [8].

In sum, these results suggest a neural pathogenic site for PCOS-like reproductive endocrinopathy in a nonhuman primate model, distinguished from its non-primate counterparts by singular disruption of negative, but not positive, feedback regulation. This opens the door to novel and site-specific, central nervous system therapeutic targeting of PCOS neuroendocrine reproductive pathophysiology with the potential to ameliorate infertility, hyperandrogenemia and their sequelae.

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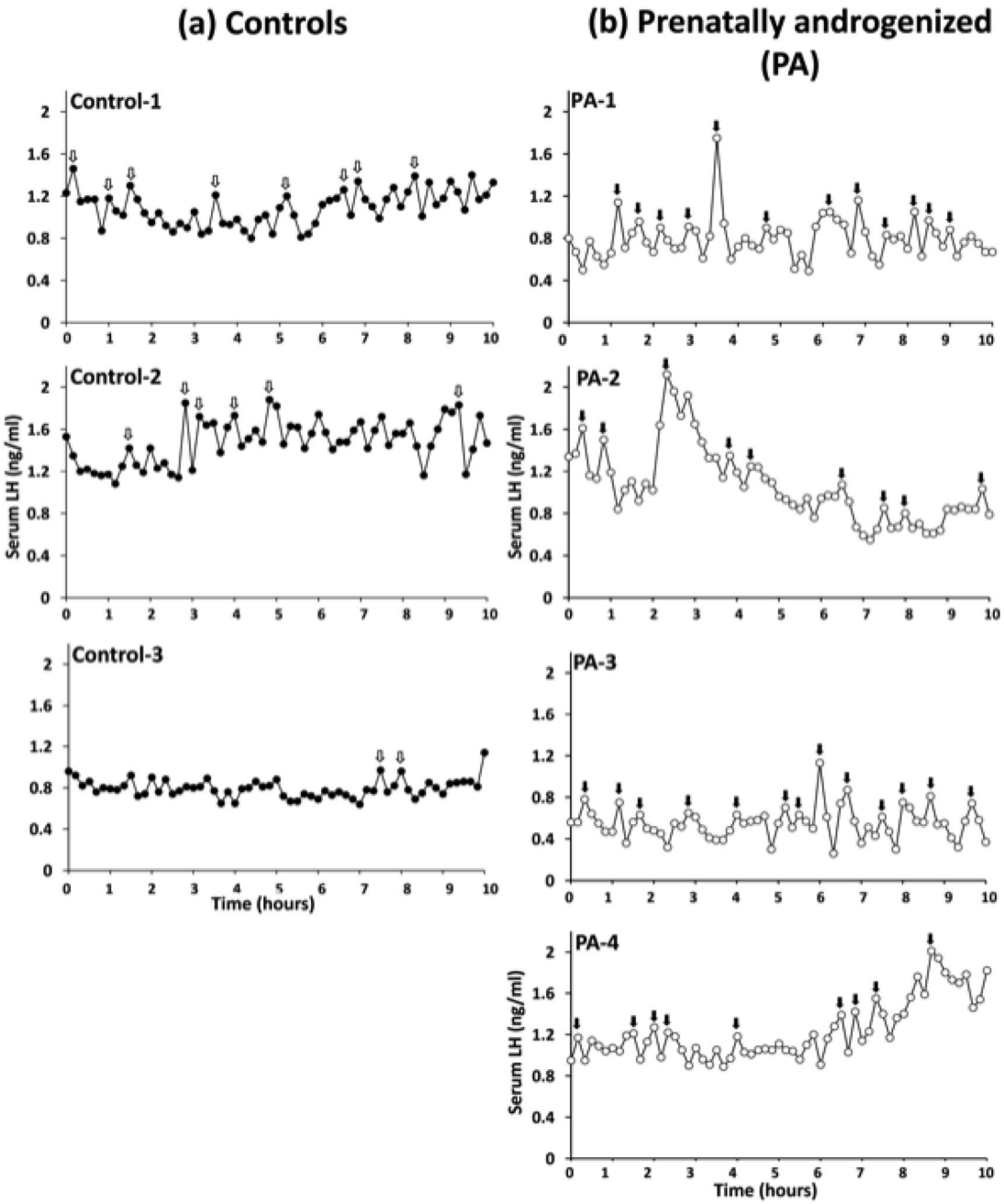

Figure 1.

Serum immunoactive LH levels in (a) three adult control (closed symbols), and (b) four adult PCOS-like PA (open symbols), female rhesus monkeys during the early follicular phase of the menstrual cycle or period of anovulation, and obtained from 10-min interval blood sampling from an indwelling IV cannula between 0800h and 1600h (daytime). Cannulae were housed within a jacket backpack that attached to a reinforced tether and swivel permitting remote sampling. PULSAR algorithm identified peak episodes of LH release are indicated by arrows: a) Control-1, 8 pulses/10h; Control-2, 6 pulses/10h;

Control-3, 2 pulses/10h, and (b) PA-1, 12 pulses/10h; PA-2, 9 pulses/10h: PA-3, 13 pulses/10h; PA-4, 9 pulses/10h.

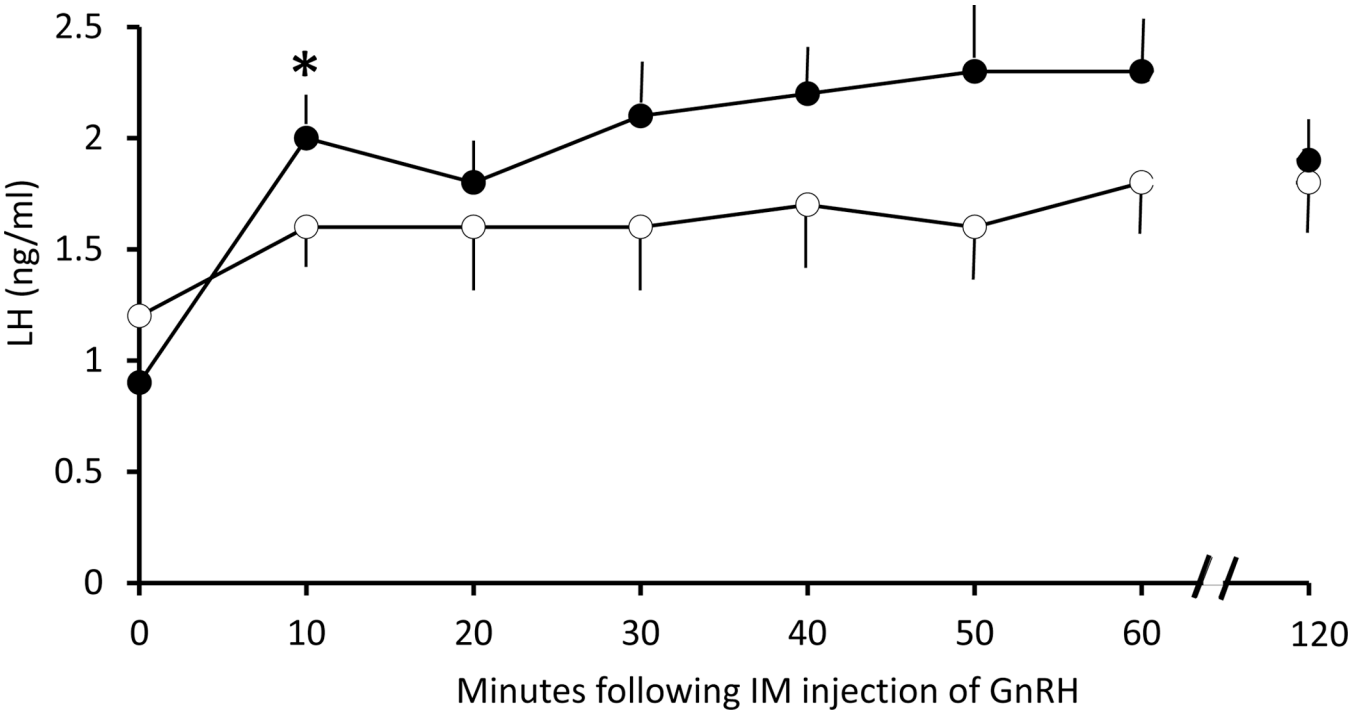

Figure 2.

Serum immunoactive LH responses (mean ± SEM) to IV infusion of 2μg/kg GnRH in adult female control (open symbols) and PA (closed symbols) monkeys immediately following the 10-h assessment of episodic LH release, as described in Figure 1. Only values from 0–60 min and 120 min following GnRH infusion are shown. * p=0.034 vs. 0 min, PA monkeys, alone.

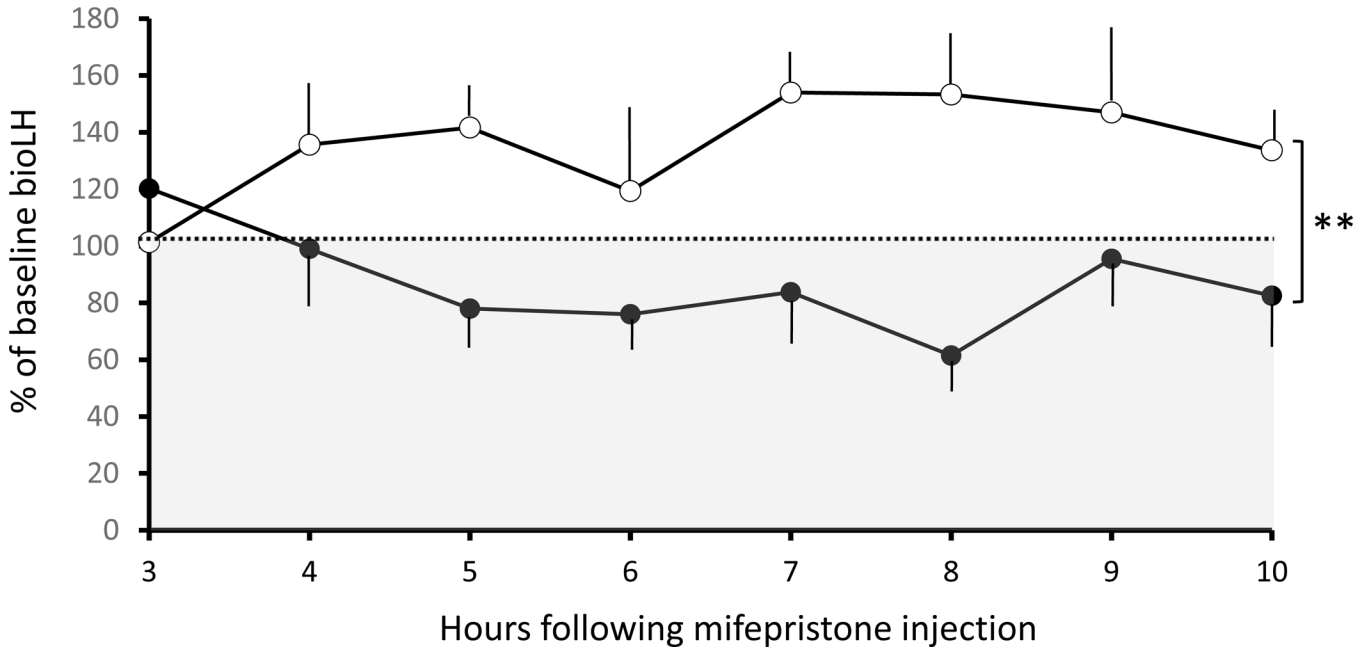

Figure 3.

% change in serum bioLH levels (mean ± SEM) from baseline (mean of −1h and 0h values = 100% and dashed line) in response to IM injection of 5 mg/kg mifepristone in adult female control (open symbols) and PA (closed symbols) monkeys between 0600h and 1800h (daytime). Shaded grey area indicates LH values below baseline. ** p=0.008, control vs. PA monkeys, overall.

Table 1.

Baseline characteristics of adult, ovary intact control and PCOS-like EPA female rhesus monkeys during the early follicular phase of a menstrual cycle or anovulatory period.

Parameter (mean ± SEM)	Control (n=3)	PCOS-like EPA (n=4)	p-value
Age, size, menstrual cycle			
Age (years)	24.0 ± 0.6	24.8 ± 0.5	0.350
Body weight (kg)	9.1 ± 0.4	8.8 ± 1.1	0.750
BMI (kg/m^2)	42.3 ± 2.4	38.4 ± 4.0	0.440
Menstrual cycle (days)	30.6 ± 3.0	47.0 ± 11.6	0.296
Intermittent menstrual cycles	0% (0/3)	50% (2/4)	0.429
Hormones			
Testosterone (ng/mL)	0.12 ± 0.03	0.19 ± 0.02	0.077
Androstenedione (ng/mL)	0.40 ± 0 11	0.40 ± 0.10	0.980
DHEA (ng/mL)	0.11 ± 0.02	0.17± 0.02	0.107
Total androgens (nmol/L)	2.20 ± 0.39	2.67 ± 0.34	0.388
Estradiol (pg/mL)	14.40 ± 4.55	6.40 ± 3.94	0.241
Estrone (pg/mL)	3.20 ± 0.40	3.51 ± 0.34	0.613
Progesterone (ng/mL)	0.15 ± 0.07	0.07 ± 0.06	0.282
LH (ng/mL) [immunoactive]	1.12 ± 0.20	0.91 ± 0.15	0.226
bioLH (ng/mL) [bioactive]	0.21 ± 0.01	0.23 ± 0.02	0.387
FSH (ng/mL)	2.68 ± 0.56	2.73 ± 0.32	0.944
Hormone ratios			
Testosterone : Estradiol	12.54 ± 8.45	36.84 ± 7.32	0.069
Androstenedione : Testosterone	3.70 ± 1.00	2.40 ± 0.86	0.355
Estrone:Estradiol	0.34 ± 0.20	0.74 ± 0.16	0.177
Androstenedione:Estrone	114 ± 32	115 ± 28	0.832
LH : FSH	0.52 ± 0.13	0.28 ± 0.02	0.152
bioLH : LH [immunoactive]	**0.17 ± 0.01**	**0.32 ± 0.06**	**0.033**

Table 2.

Correlations between baseline (early-to-mid follicular phase or anovulatory period) circulating concentrations of ovarian hormones and gonadotropins, as well as menstrual cycle duration for both adult female monkey groups combined (n=7).

Correlations[a]	Slope	r-value	p-value
Baseline Testosterone vs:			
Androstenedione	−0.28	0.24	0.609
Estradiol	−0.19	0.08	0.867
Estrone	0.27	0.52	0.232
Progesterone	−0.44	0.15	0.751
DHEA	**1.04**	**0.99**	**0.001**
LH [immunoactive]	−0.45	0.44	0.317
bioLH	0.23	0.53	0.280
Ratio of bioLH: LH [immunoactive]	0.81	0.75	0.088
Menstrual cycle duration	−15.69	0.12	0.798
Baseline Androstenedione vs.:			
Estradiol	−0.04	0.02	0.969
Estrone	−0.05	0.11	0.814
Progesterone	0.17	0.07	0.883
DHEA	−0.13	0.16	0.737
LH [immunoactive]	0.49	0.57	0.183
bioLH	−0.05	0.10	0.859
Ratio of bioLH: LH [immunoactive]	−0.58	0.46	0.360
Menstrual cycle duration	8.99	0.08	0.861
Baseline Estradiol vs.:			
Estrone	0.11	0.52	0.234
Progesterone	**1.06**	**0.88**	**0.008**
DHEA	−0.03	0.07	0.876
LH [immunoactive]	0.11	0.26	0.571
bioLH	0.07	0.37	0.473
Ratio of bioLH: LH [immunoactive]	−0.16	0.34	0.510
Menstrual cycle duration	−39.17	0.74	0.057
Baseline Progesterone vs.:			
DHEA	−0.06	0.16	0.736
LH [immunoactive]	0.19	0.57	0.181
bioLH	0.06	0.42	0.412
Ratio of bioLH: LH [immunoactive]	−0.20	0.54	0.270
Menstrual cycle duration	−24.32	0.55	0.198

[a]Hormonal parameters are all log transformed.

Table 3.

Characteristics of LH episodic release during a 10-hour frequently sampled intravenous assessment, together with associated parameters in adult, ovary intact control and PCOS-like EPA female rhesus monkeys during the early follicular phase of a menstrual cycle or anovulatory period.

Parameter[a] (mean ± SEM)	Control (n=3)	PCOS-like EPA (n=4)	p-value
LH episodic release			
# of LH pulses/h	**0.5 ± 0.2**	**1.1 ± 0.1**	**0.037**
Inter-pulse interval (min)	110 ± 24	59 ± 8	0.061
LH pulse amplitude (ng/mL)	0.33 ± 0.10	0.36 ± 0.10	0.526
LH pulse duration (min)	18.9 ± 4.5	31.0 ± 3.6	0.089
10-hour LH mean (ng/mL)	1.12 ± 0.20	0.92 ± 0.14	0.447

[a]All parameters were log transformed except # of LH pulses.

Table 4.

Correlations with LH episodic release parameters during a 10-hour frequently sampled intravenous assessment in adult, ovary intact female rhesus monkeys, control and PCOS-like EPA groups combined (n=7) during the early follicular phase or anovulatory period.

Correlations	Slope	r-value	p-value
Baseline testosterone:			
No. of LH pulses/h	6.54	0.26	0.579
Inter-pulse interval	−51.57	0.20	0.673
LH pulse amplitude	−0.10	0.25	0.583
LH pulse duration	15.65	0.24	0.603
Baseline androstenedione:			
No. of LH pulses/h	−2.65	0.12	0.790
Inter-pulse interval	36.76	0.17	0.719
LH pulse amplitude	**0.28**	**0.84**	**0.017**
LH pulse duration	22.61	0.42	0.353
Baseline estradiol:			
No. of LH pulses/h	−5.87	0.57	0.181
Inter-pulse interval	70.66	0.67	0.102
LH pulse amplitude	−0.06	0.36	0.425
LH pulse duration	−16.97	0.65	0.116
Baseline progesterone:			
No. of LH pulses/h	−4.50	0.52	0.227
Inter-pulse interval	47.14	0.53	0.217
LH pulse amplitude	−0.02	0.18	0.700
LH pulse duration	−11.14	0.51	0.242