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Hyperactive LH pulse secretion and elevated kisspeptin and neurokinin B gene expression in the arcuate nucleus in a mouse model of PCOS

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Abstract

Polycystic ovary syndrome (PCOS), a common reproductive disorder in women, is characterized by hyperandrogenemia, chronic anovulation, cystic ovarian follicles, and LH hyper-pulsatility, but the pathophysiology is incompletely understood. We recently reported a novel mouse model of PCOS using chronic letrozole (LET; aromatase inhibitor). LET females demonstrate multiple PCOS-like phenotypes, including polycystic ovaries, anovulation, and elevated circulating testosterone and LH, assayed in "one-off" measures. However, due to technical limitations, *in vivo* LH pulsatile secretion, which is elevated in PCOS women, was not previously studied, nor were possible changes in reproductive neurons. Here, we used recent technical advances to examine *in vivo* LH pulse dynamics of freely-moving LET female mice versus control and ovariectomized (OVX) mice. We also determined whether neural gene expression of important reproductive regulators such as kisspeptin, neurokinin B (NKB), and dynorphin, is altered in LET females. Compared to controls, LET females exhibited very rapid, elevated *in vivo* LH pulsatility, with increased pulse frequency, amplitude, and basal levels, similar to PCOS women. LET mice also had markedly elevated *Kiss1, Tac2,* and *Pdyn* expression and increased *Kiss1* neuronal activation in the hypothalamic arcuate nucleus. Notably, the hyperactive LH pulses and increased kisspeptin neuron measures of LET mice were not as elevated as OVX females. Our findings indicate that LET mice, like PCOS women, have markedly elevated LH pulsatility which likely drives increased androgen secretion. Increased hypothalamic kisspeptin and NKB levels may be fundamental contributors to the hyperactive LH pulse secretion in the LET PCOS-like condition, and perhaps, in PCOS women.

Introduction

Polycystic ovary syndrome (PCOS) affects approximately 10% of reproductive-aged women (1-3). PCOS is a complex disorder encompassing multiple phenotypic parameters, with clinical diagnosis typically requiring at least two of the following features: polycystic ovaries, androgen excess (hyperandrogenemia), and chronic anovulation, with the latter two often comprising the predominant features (4). Neuroendocrine hallmarks of PCOS include increased gonadotropin-releasing hormone (GnRH) pulse frequency, increased luteinizing hormone (LH), reduced follicle-stimulating hormone (FSH), and insensitivity to progesterone (P4)-negative feedback (5-7). Along with hyperandrogenemia, disruptions in the neuroendocrine reproductive axis contribute to dysfunctional ovarian maturation and diminished fertility (1-3, 5). In many cases, PCOS is also often associated with metabolic dysfunction, including obesity, increased abdominal adiposity, insulin resistance, glucose intolerance, and a heightened risk of type 2 diabetes (3, 5, 8).

Elucidating the etiology and underlying mechanisms of PCOS has proven challenging, due in part to the heterogeneity of the disease. The development of various animal models to study PCOS has been an important research focus in this regard (9-11). Given the heterogeneity of PCOS phenotypes, different models may be useful in representing different underlying etiologies or classes of PCOS. One common model is prenatal androgenization (PNA), which has been implemented in monkeys, sheep, and rodents. The PNA model elicits many of the reproductive phenotypes associated with PCOS (11-16), including elevated androgens and LH, as well as altered steroid hormone feedback. Although the reproductive effects are quite robust, the PNA models usually lack robust metabolic effects and are often considered models of the "lean PCOS" condition. A more recent mouse model based on elevated exposure to AMH during prenatal life, achieved with injections of AMH given to the mothers during late pregnancy, similarly recapitulates in the female offspring a reproductive phenotype of high LH, hyperandrogenemia, and anovulation, without a strong metabolic phenotype, mimicking the "lean PCOS" condition (17). In contrast to the PNA and AMH models, rodent models of PCOS developed using continuous treatment with letrozole (LET), a nonsteroidal aromatase inhibitor (18), recapitulate the "obese PCOS" (or overweight PCOS) condition. The LET model is based on the finding of lower estrogen/androgen ratios in follicular fluid of PCOS women, suggesting low aromatase activity (19-21), and the fact that genetic variants of the aromatase gene (*CYP19*), which converts androgens to estrogen, are associated with the development of PCOS and hyperandrogenism in women (22-26). Indeed, supportive of this model, ovaries and tissues of PCOS women have decreased aromatase levels (27, 28), and some overweight/obese PCOS women exhibit lower circulating E2 levels (29). The LET model was first reported in female rats, in which continuous LET treatment initiated before puberty (at doses which do not fully deplete E2 levels), develop disrupted estrous cyclicity, increased ovarian weight and cysts, hyperandrogenemia, and anovulation (18, 30). LET rats also exhibit several metabolic features of PCOS,

including increased body weight and insulin resistance (18), suggesting this is a model of the obese or overweight PCOS condition.

We recently developed a mouse model utilizing LET that, similar to rats, strongly recapitulates both the reproductive and metabolic hallmarks of the overweight/obese PCOS condition. Peripubertal female mice implanted with a LET pellet delivering constant, long-term LET develop all the neuroendocrine features of PCOS, including elevated testosterone and LH and decreased FSH (31). The latter findings were supported by increased *Lhb* and decreased *Fshb* mRNA levels in the pituitary (31). As with PCOS women, LET female mice have heavier ovaries, polycystic follicles, and diminished corpora lutea (indicative of decreased ovulation), as well with altered *Fshr* and steroidogenic enzyme gene expression (31). Along with the PCOS-like reproductive alterations, LET mice demonstrated several metabolic impairments, including increased body weight (BW), increased abdominal adiposity and adipose cell size, insulin resistance, and dyslipidemia (31, 32), mimicking similar phenotypes in overweight/obese women with PCOS.

Our original report (31) showed that LET female mice have elevated blood levels of LH, assayed in "one-off" measures from a single blood sample from each animal. At that time, due to long-standing technical and assay limitations in mouse models, *in vivo* LH pulsatile secretion, which is greatly elevated in PCOS women, was not studied in the LET mice. In addition, possible alterations in brain reproductive neural populations known to drive GnRH/LH secretion, such as kisspeptin and neurokinin B neurons, were not examined in the LET models, and thus, mechanistic underpinnings of the elevated LH secretion remain unknown. In the present study, we used recent technical advances (33-35) to examine *in vivo* LH pulse dynamics of awake, freely-moving LET female mice and compared this to control mice and ovariectomized (OVX) mice, the latter of which has robustly elevated LH pulsatility due to complete lack of ovarian hormone negative feedback. Assessment of LH pulse profiles allowed us to determine whether the elevated LH in the single "on-off" measures in the original study reflect increased LH pulse frequency, increased LH pulse amplitude, increased basal LH levels, or a combination of these various pulse parameters, which could be compared to the PCOS pulsatility phenotype. In addition, the present study determined whether gene expression of known reproductive genes, such as *Kiss1*, *Tac2,* and *Pdyn* in the hypothalamic arcuate nucleus, or arcuate kisspeptin neuron activation levels, are enhanced in the brains of LET females, perhaps contributing to their increased LH and androgen secretion. We also determined if hypothalamic gene expression for the inhibitory neuropeptide, RFRP-3, was decreased in LET females, perhaps allowing for less inhibitory input onto the GnRH neural network and therefore increased LH secretion.

90 **Materials and Methods**

Animals and LET Treatment

Female C57BL/6 mice were housed on a 12:12 light-dark cycle, with food and water available *ad libitum*. Mice were housed 2 females/cage. At 4 weeks of age, prior to pubertal completion, female mice were subcutaneously implanted with a LET (50 µg/day) or placebo control (CON) pellet. LET powder was
95 purchased from Sigma and custom 60-day continuous release LET pellets were made by Innovative Research of America (Sarasota, FL). The 50 µg/day dose was similar to that used in previously published LET mouse studies (31, 32, 36, 37). For comparison, an additional control group of C57BL/6 females were OVX at 5 weeks of age to remove all ovarian hormone negative feedback and induce maximally elevated GnRH/LH pulsatility. BW of the mice in all groups was tracked from 4 weeks of age until the end of the experiments at
100 9 weeks of age. All experiments were approved by the UCSD IACUC.

Experiment 1: In Vivo Serial LH Sampling

Beginning at 6 weeks of age (two weeks after LET implantation), LET, control, and OVX littermates underwent daily handling to habituate the mice to the tail-tip bleeding procedure used for collecting serial
105 blood samples. Figure 1 shows the experimental paradigm and timeline. All mice were handled daily for 3 weeks prior to the final day on which the serial bleeding occurred (9 weeks old; 5 weeks after LET implantation). All serial sampling was conducted between 0900 h and 1200 h. On the day of the serial bleeding, the tail tip of each animal was cut and then ~15 min later serial blood samples were collected every 6 min for a total duration of 2.5 hours (n=10-14/group). For each sample, 3 µl of whole blood was pipetted from the tail
110 and mixed with 57 µl of assay buffer, mixed, and then placed on ice until storage at -20C. Animals were awake the entire time and able to freely roam their home cage in between sampling. Serial tail-tip blood samples were assayed for LH levels, as described in the Hormone Assays section. LET mice are acyclic and predominantly arrested in diestrus (DE) (31). In Experiment 1, most LET females were confirmed via vaginal smears to be in DE on the day of LH sampling, with a few mice in estrus (E). All control females were similarly in DE or E.
115 Within each group, there was no difference between the DE and E mice for any LH measure, so group comparisons were made with DE and E mice combined to ensure high statistical power.

Serial blood LH measures were analyzed for endogenous LH pulses. An LH value was determined to be a pulse using a similar criteria as in previous reports for mouse LH pulsatility (38): the value must show a >20% increase from one of the two previous points followed by a decrease by >10% in one of next two
120 subsequent points. In addition, to qualify as a pulse, the pulse amplitude must be ≥0.32 ng/ml, the detection sensitivity of the assay, as in previous reports (39). For identified LH pulses, the following parameters were calculated: 1) pulse frequency (# pulses/60 min); 2) interpulse interval, defined as the time between 2 pulses; 3) pulse amplitude, defined to be the difference between a pulse peak value and a preceding nadir, the lowest

value of the 3 preceding values; 4) pulse peak, defined as the zenith LH value of each identified pulse; 5) basal
125 LH level, defined as the average LH of all the nadirs. In addition to these pulse measures, overall mean LH, the average of all the LH values for an animal for the entire 2.5 h sampling period, was also calculated.

Experiment 2: Analysis of Hypothalamic Gene Expression and Neuronal Activation

LET, control, and OVX littermates (n=6-8/group) generated similarly to Experiment 1 (Fig 1A) were briefly
130 anesthetized with isoflurane and rapidly decapitated (between 11:30-12:30 h) at ~9 weeks of age (5 weeks after LET implantation), similar to the age of pulse bleeding in Experiment 1. All LET and control mice were killed on diestrus (DE), with OVX controls similarly sacrificed the same day. Blood and brains were collected, with the latter immediately frozen on dry ice, and stored at -80°C. Frozen brains were sectioned on a cryostat into 5 sets of 20 uM coronal sections and thaw mounted on Superfrost-plus slides that were stored at -80 C
135 until assaying.

Single-label in situ hybridization (ISH) for ARC *Kiss1* or *Tac2* (NKB) gene expression or for DMN *Rfrp* expression was performed, in each case, on 1 set of brain sections spanning the entire medial-to-caudal hypothalamus which encompassed the entire ARC or DMN region. All ISH assays were performed using published protocols and validated riboprobes (40-43). Briefly, slides with brain sections were fixed in 4%
140 paraformaldehyde, treated with acetic anhydride, rinsed in sodium chloride (SSC), delipidated in chloroform, and dehydrated in graded ethanol. Slides were air dried before the hybridization step, where P33 radiolabeled riboprobe (*Kiss, Tac2, pdyn,* or *Rfrp;* 0.04 pmol/ml) was combined with 1/20 volume yeast tRNA in 0.1 M Tris/0.01M EDTA (pH 8) to produce the probe mix. The probe mix was heat-denatured in boiling water for 3 min, placed on ice for 5 min, and added to pre-warmed hybridization buffer (60% deionized formamide, 5x
145 hybridization salts, 0.1x Denhardt's buffer, 0.2% SDS). 100 μl of this final hybridization mix was added to each slide, which were coverslipped and placed in a humidity chamber at 55C for 16 h. Following hybridization, coverslips were removed and slides were washed with 4x SSC at room temperature. Slides were then placed in RNAse A (10 mg/ml, Roche Biochemicals) in 0.15 M sodium chloride, 10mM Tris, 1mM EDTA (pH 8) for 30 min at 37 C followed by buffer without RNase for another 30 min at 37 C. Slides were then
150 washed in 2X SSC for 30 min and 0.1X SSC at 62 C for 60 min. Slides were then washed for 3 min in 0.1X SSC at room temperature and dehydrated with graded ethanol. After drying, slides were dipped in Kodak NTB emulsion, air dried, and stored at 4 C for 4-9 days (depending on the specific assay) until they were developed and coverslipped.

Double-label ISH for *cfos+Kiss1 mRNAs* was performed on an additional set of ARC brain sections
155 in order to assess neuronal activation of kisspeptin neurons. For the double label assay, slides were treated similar to the single label protocol, with a few modifications. Digoxigenin (DIG)-labeled antisense probe for *Kiss1* was synthesized with T7 RNA polymerase and DIG labeling mix (Roche) and added 1:500 with a *cfos* P33 radiolabeled riboprobe (0.04 pmol/ml) to the probe hybridization mix. The next day, following the 62 C

washes, slides were incubated in 2x SSC with 0.05% Triton X-100 containing 3% normal sheep serum for 75 min at room temp. Slides were then incubated for 16 hr at room temperature with anti-DIG antibody fragments conjugated to alkaline phosphatase (1:500; Roche Biochemical). The next day, slides were washed with Buffer 1 (100mM Tris-HCl, 150 mM Nacl) and then washed in Tris buffer and incubated in a Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. The slides were then rinsed and air dried prior to dipping in emulsion, similar to single label assays.

ISH slides were analyzed using previously-published techniques with a computer-automated image processing system and custom counting software (GRAINS; Dr. Don Clifton, Univ. of Washington) by a person unaware of the treatment group of each slide (44). For single-label ISH assays, the software counted the number of silver grain clusters representing *Kiss1, Tac2*, *Pdyn*, or *Rfrp c*ells, as well as the number of silver grains in each individual cell cluster (a semi-quantitative measure of *Kiss1, Tac2, Pdyn,* or *Rfrp* mRNA expressed per cell) (45-47). Cells were considered *Kiss1, Tac2, pdyn,* or *Rfrp* positive when the number of silver grains in a cluster exceeded that of background by 3-fold. For double label ISH, DIG-containing cells (ARC *Kiss1*cells) were identified under fluorescence microscopy and the custom grain-counting software then used in dark field to quantify silver grains (representing *cfos* mRNA) overlying each fluorescent cell. Signal-to-background ratios for individual cells were calculated, and a cell considered double-labeled if its ratio was >3, as in previous studies (43, 48, 49).

Hormone Assays

In Experiment 1, serial tail-tip blood samples were measured for LH levels by the University of Virginia Ligand Assay Core using an ultra-sensitive murine LH ELISA. Functional sensitivity was 0.320 ng/mL. Values are reported as ng/ml of whole blood. A subset of mice in each group also had a small retro-orbital blood collection performed at week 3 of the study to confirm that serum T was elevated in LET mice, as previously reported. T was measured by the University of Virginia Ligand Assay Core using a mouse/rat T ELISA assay that has a sensitivity of 10 ng/dl (range 10.0-1600 ng/dL).

In Experiment 2, serum E2 was measured in blood collected at sacrifice to confirm functionality of the pellets. Our prior LET mouse study (31) used an E2 ELISA that was not sensitive enough on the low end of the assay, precluding proper comparison of E2 levels between LET and control females. In the present study, we used a more sensitive tandem liquid chromatography-mass spectrometry (LCMS) assay performed by Prof. Brian Keevil (University Hospital of Manchester, Manchester, UK) with a lower limit of detection of 10 pmol/L (2.7 pg/ml). Given the very large volume of blood serum needed for this assay, serum samples were pooled between animals, with 2 animals of the same treatment per pooled sample (n = 3-4 pooled samples per treatment). E2 values that fell below the limit of assay detection were assigned a value of 2.7 pg/ml to permit comparison to detectable values. In a subset of mice that still had enough serum volume remaining, we also measured serum T levels to confirm elevated T in the LET mice.

195 *Statistical Analysis*

All data were analyzed in GraphPad Prism 6 (La Jolla, CA). Mean data were analyzed as a one-way ANOVA (group), followed by post-hoc Bonferroni's multiple comparison test. All data are expressed as mean ± standard error of the mean (SEM). A value of $p<0.05$ was considered to be statistically significant.

200

Results

Confirmation of the LET hyperandrogenemia and overweight phenotype

To confirm the previously-reported PCOS-like hyperandrogenemia, in Experiment 1 serum T levels were measured in LET and control mice at 7 weeks of age (3 weeks after LET implantation). Circulating T was
205 significantly upregulated in LET females, as expected ($p< 0.05$; Figure 1). Matching the known metabolic phenotype in this model, BWs were also greater in LET than control mice at both 3 and 5 weeks post-LET treatment $p<0.05$; (Figure 1B). In Experiment 2, at sacrifice (5 weeks post-LET implantation), serum E2 levels were measured to be significantly higher in control than LET females, as predicted ($p<0.05$, Fig 1C). Serum E2 levels of LET and OVX females could not be directly compared, as both were below the limit of the LCMS
210 assay detection, unlike control mice whose values were in the detectable range (Fig 1C). Serum T levels and BWs were both significantly elevated in LET mice versus controls, as in the Experiment 1 cohort ($p< 0.05$; Figure 1C).

Experiment 1

215 *Endogenous LH pulses are more frequent and higher amplitude in LET mice*

PCOS women have an abnormally rapid, high amplitude LH pulse profile, but the parameters of *in vivo* LH pulses in LET PCOS models have not been previously determined. To determine if LET mice also exhibit hyperactive LH pulses as in the PCOS condition, blood LH was measured every 6 min for 2.5 h. Figure 2 shows representative LH values during the blood collection period for 3 control females and 3 LET females.
220 Control females exhibited overall low levels of LH with occasional small amplitude pulses. In stark contrast, LET females demonstrated a much more rapid pulsatile LH secretion pattern, along with a higher basal level and higher amplitude of each pulse. Mean analysis of individual pulse parameters indicated significant alterations in virtually all pulse parameters (Fig. 3). Specifically, the number of detectable pulses (pulse frequency) was significantly increased by 2-fold in LET mice versus controls ($p<0.01$; Fig 3A) while interpulse
225 interval (IPI) was correspondingly decreased by ~50% in LET mice ($p<0.01$; Fig. 3B). There were also large increases in mean basal levels (4-fold), mean pulse amplitude (2.5-fold), and mean pulse peak levels (i.e., the zenith of a pulse; 3.5-fold) in LET mice ($p<0.01$ for each parameter compared to control females; Fig 3C-E).

Overall mean LH for the entire sampling period was significantly higher, by 4-fold, in LET versus controls ($p<0.01$; Fig 3F).

Hyperactive LH pulse profile of LET mice is not identical to that of OVX mice

To ascertain how the hyperactive LH pulses of LET mice compare to the OVX condition, in which the GnRH pulse generator is completely free of any ovarian negative feedback, we compared LET mice to OVX mice (Figs 2, 3). While LH pulses were rapid and high amplitude in both cases, pulse frequency was significantly lower, by 17%, in LET than OVX females ($p<0.01$; Fig 3A). Pulse amplitude and pulse peak were also significantly lower in LET mice than OVX mice, by ~40% and ~25%, respectively ($p<0.01$ for each; Fig 3C, D). Overall mean LH levels for the entire sampling period was 17% lower in LET females compared to OVX ($p<0.01$; Fig 3F). However, basal LH levels and interpulse interval were not statistically different between LET and OVX mice (Fig 3B, E). Thus, LET and OVX mice had similar baseline starting points for their LH pulses, but the pulses were slightly more frequent and achieved a higher maximum value (pulse peak) in the OVX condition (see representative profiles in Fig. 2).

Experiment 2

The brains of LET mice have greatly elevated Kiss1, Tac2, and pdyn expression

The mechanisms underlying and promoting the elevated LH pulse secretion in PCOS are not yet identified. We examined whether LET mice have elevated reproductive gene expression in the brain, which may be linked to increased stimulatory drive to GnRH and downstream LH secretion. We specifically studied the genes for kisspeptin, Neurokinin B, and dynorphin (encoded by *Kiss1, Tac2*, and *Pdyn* respectively), as these three neuropeptides are co-expressed together in hypothalamic arcuate (ARC) neurons that have been implicated as a key component of the GnRH pulse generator mechanism (50-52).

Kiss1 expression in the ARC, measured via ISH, was greatly increased in LET mice compared to control littermates ($p<0.05$; Fig 4A). Overall *Kiss1* mRNA levels were >150% higher in LET than control females, with both the number of *Kiss1* cells and levels of *Kiss1* mRNA per cell being significantly elevated ($p<0.05$; Fig 4B). The levels of ARC *Kiss1* expression in LET mice was significantly lower than that of OVX females ($p<0.05$; Fig 4B), correlating with the lower LH pulse profile observed in LET versus OVX females (Figs 1, 2). *Tac2* expression levels in the ARC were similarly upregulated, by ~ 60%, in LET mice versus controls ($p<0.05$; Fig 5A, B), with the number of *Tac2* cells being significantly elevated ($p<0.01$; Fig 5B). Unlike *Kiss1,* the number of identifiable ARC *Tac2* cells was not significantly different between LET and OVX mice, though the level of *Tac2* expression per cell was significantly higher in OVX than LET females ($p<0.05$; Fig 5B).

Current models of GnRH pulse generation posit that NKB stimulates neighboring ARC Kiss1/NKB cells to release kisspeptin, after which the inhibitory neuropeptide dynorphin, released from the same cells, is

thought to terminate this process, thereby ending the "pulse" of kisspeptin secretion. Based on this model, we hypothesized that ARC *Pdyn* expression levels would be elevated in LET mice, as faster LH pulses would

265 require both pulse start signals (NKB) and stop signals (dynorphin) to be elevated in order to generate more frequent pulse events. Indeed, we found that LET mice had markedly higher ARC *Pdyn* expression than control mice (p<0.01), both in terms of identified *Pdyn* cells in the ARC and *pdyn* mRNA levels per cell (Fig 6A, B). As with *Kiss1*, *Pdyn* levels were lower in LET than OVX mice (p<0.01), correlating with lower LH output in LET (Fig 6B).

270 Finally, we also assessed *Rfrp* expression levels in the DMN (Fig 7A), as RFRP-3 has been implicated as an inhibitor of reproductive hormone secretion (53-57). We therefore tested whether LET mice have decreased *Rfrp* levels which may indicate less inhibition on the reproductive axis and, thereby, higher GnRH/LH secretion. Contrary to this hypothesis, we did not detect a significant change in *Rfrp* expression in LET mice versus control mice or OVX mice (Fig 7B).

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ARC Kiss1 neuron activation is elevated in LET female mice

In addition to changes in reproductive neuropeptide gene expression, it is also possible that activation and firing of reproductive neurons is altered in the LET PCOS-like condition. We hypothesized that LH levels in LET mice are elevated, in part, by enhanced ARC *Kiss1/Tac2* neuron activation, which may be reflected by

280 increased *cfos* mRNA induction in these neurons (a common measure of neuronal activation). We used double-label ISH to measure *Kiss1+cfos* mRNA coexpression in LET, control, and OVX mice. We found that LET mice had significantly elevated neuronal activation, by 2-fold, of ARC *Kiss1* neurons compared to control females (p<0.05; Fig 8). Though elevated above controls, the degree of *cfos+Kiss1* coexpression in LET brains did not reach the maximally high values observed in OVX females (Fig 8), matching the lower LH pulse

285 secretion and amplitude of LET versus OVX mice.

Discussion

PCOS is a complex disorder encompassing multiple phenotypic parameters, including neuroendocrine and

290 ovarian impairments, and a high prevalence of metabolic perturbation (7, 8, 58). We recently reported a new LET mouse model that recapitulates many of the reproductive and metabolic components of the human overweight PCOS phenotype. LET female mice exhibit significantly elevated circulating androgens, a hallmark of women with PCOS (5, 59-61). In PCOS, hyperandrogenemia is driven by upstream increases in LH pulse secretion. We previously determined that LET mice exhibit increased pituitary *Lhb* mRNA

295 expression and elevated "one-off" measures of circulating LH levels, but endogenous LH pulse patterns were not previously studied due to long-standing technical challenges. Regardless, the increased LH:FSH ratio

originally reported for LET mice was suggestive of enhanced upstream pulsatile GnRH secretion, a neuroendocrine hallmark of PCOS (62). However, until now, there have been no studies in LET animals investigating the presence of persistently rapid GnRH/LH pulsatility. Moreover, the underlying neural
300 alterations underlying the rapid GnRH/LH pulses of PCOS women are completely unknown, and the present study is the first to assess possible brain changes in the LET PCOS-like mouse model (summarized in Fig 9).

In the present study, we analyzed serial blood LH levels of LET mice in samples collected every 6 min for 2 h. In clear contrast to control females, which had low LH levels with infrequent, low-amplitude pulse events (as previously reported (33, 63)), LET females demonstrated robustly elevated, high frequency LH
305 pulses that included significant increases in pulse frequency, pulse amplitude, pulse peak levels, and basal levels. These increased LH pulse parameters, including frequency and amplitude, match similar increases reported for PCOS women (64-68). Thus, the LET model nicely recapitulates the endogenous hyperactive LH pulsatility that is a key neuroendocrine feature of PCOS. Elevated LH pulses have also been reported for other mouse models of PCOS, in particular the PNA model and AMH models (17, 69, 70). Interestingly, in those
310 studies, LH pulses were more frequent in the PCOS-like condition, but basal levels and pulse amplitude were not increased relative to controls (17, 69, 70). Thus, only pulse frequency seems to be altered in the PNA and AMH models, whereas pulse frequency, amplitude, and basal levels are modified in the LET model. It is also of interest that the elevated levels of LH in PNA mice are of lower concentration than the LH levels of LET mice. It is not clear if these differences are simply due to different hormone assay parameters or other technical
315 differences, or if they represent real intrinsic differences in the pulse generator output in the LET versus PNA/AMH models. If the latter, this could provide valuable insights into what may be different etiologies or underlying mechanisms causing the altered LH pulses in each model, and this may relate to different mechanisms occurring across the heterogeneous spectrum of the PCOS disorder (i.e., not all PCOS women are likely to have the same underlying cause for their elevated LH and T levels). Future measurement of *in vivo*
320 LH pulses simultaneously in the different models within the same study will be useful to address this issue.

PCOS women have hyperactive LH pulses but it is not fully understood why this is the case. Because LET females also have hyperactive LH pulses, we studied their brains to gain insight into possible underlying causes of such altered LH secretion. We previously reported no difference in *GnRH* mRNA levels in LET females (31), suggesting possible neural changes may be upstream of GnRH neurons. We therefore
325 hypothesized that arcuate kisspeptin neurons, which have been implicated as essential factors in the GnRH pulse generation mechanism, would show signs of increased activity in LET brains. Indeed, we found that both *Kiss1* and *Tac2* gene expression in the ARC were strongly upregulated in the LET condition, suggestive of higher capacity for these ARC cells to make kisspeptin and NKB. Since both kisspeptin and NKB are stimulatory to GnRH pulse output, higher levels of these factors may contribute to elevated basal LH levels as
330 well as increased LH amplitude and/or pulse frequency. We also measured the neuronal activation levels of kisspeptin cells in the ARC, using *cfos* mRNA induction as a proxy for neuronal activation. We found that

ARC kisspeptin cells have much greater neuronal activation in LET mice versus control mice, correlating with the faster, higher amplitude LH pulses in the former group. Collectively, these findings suggest that ARC kisspeptin/NKB neurons are "turned on" to a greater level in the LET-PCOS like condition, thereby driving
335 enhanced LH pulses (Fig 9). The enhanced kisspeptin/NKB activation of GnRH secretion in LET mice suggested here may also act in concert with previously observed elevations in pituitary GnRH receptor (31) to promote exaggerated LH synthesis leading to heightened basal LH or pulse peak levels.

We studied gene expression for dynorphin in the ARC, as this neuropeptide is proposed to play a key role in the pulse generator process, acting to terminate kisspeptin/NKB neuron firing during pulses (51, 71).
340 That is, for every pulse of kisspeptin release initiated by NKB, dynorphin signaling is thought to then terminate kisspeptin neuronal firing, acting as the "brake" to NKB's "accelerator". In this model, faster GnRH/LH pulses may require coordinated elevations in both NKB and dynorphin, as the latter would need to "keep up" with greater, more frequent NKB activity. This prediction was supported here by findings of elevated *Pdyn* levels in the ARC of LET females, relative to control females. In contrast, there was no difference in *Rfrp* levels in
345 the DMN, suggesting that increased LH secretion in the LET condition is not due to diminished inhibitory RFRP-3 input onto the GnRH neural network but rather due to changes intrinsic to the GnRH pulse generator.

In contrast to our present focus on kisspeptin neurons, in the PNA and AMH models of PCOS, the focus has been on ARC GABA neurons, which show increased synaptic and stimulatory input onto GnRH neurons (11, 17, 69, 72). This enhanced GABA-ergic drive to GnRH neurons has been proposed to underlie
350 increased pulsatile LH secretion in those mouse models. Future studies can assess whether GABA modulations also exist in the LET model, though it is interesting to reiterate that the reported LH pulse profiles of the two models do not appear similar in pulse peak or basal levels, and it is therefore possible that the different models have different underlying neural changes that impinge on GnRH output, as might occur in different cases of PCOS.

355 In the present study, we compared LH pulse parameters and brain gene expression of LET mice with those of OVX littermates. In the OVX condition, all ovarian steroid feedback is removed, allowing the GnRH pulse generator to be maximally active, resulting in very rapid, high amplitude LH pulses. We found that, despite being elevated and rapid, the LH pulses of LET females were not as elevated or rapid as in OVX females, with LET LH values being 18-40% lower than OVX (depending on the specific parameter, see Fig
360 3). Thus, the LH pulses of LET females are markedly hyperactive compared to normal controls, but they are not "maximal". Similarly, although they were elevated versus controls, *Kiss1, pdyn,* and *cfos* coexpression levels in LET females were all significantly lower, by 20-35%, than in OVX females. Collectively, these data indicate that the LET model is not just mimicking OVX, as the two conditions do not equate in multiple LH pulse or brain measures. This is further supported by findings that 1) E2 is reduced but not completely absent
365 in LET female rats (in which E2 values are above lower limits of assay detection (18, 30)), 2) certain E2-sensitive measures like *Pgr* mRNA levels are not radically diminished in LET mice relative to controls (31),

and 3) FSH levels and *Fshβ* mRNA are both decreased in LET females but markedly increased in OVX females (31). Unfortunately, in mice, endogenous E2 levels are very low, and most assays are not sensitive enough to accurately detect low endogenous non-proestrus levels of E2. A previously-used E2 ELISA did not detect a
370 difference between LET and control mice, though we did detect a significant difference using a more sensitive LCMS method. However, even that method was not sensitive enough to differentiate E2 values between LET and OVX. Without an even more sensitive assay, we cannot conclude at present that those two groups' E2 differ or not. However, previous evidence summarized above suggests that E2 is likely reduced but not completely absent in LET mice, and the present findings demonstrate that something is mechanistically
375 different in the pulse generation process to result in faster and higher amplitude pulses, as well as higher *Kiss1, Pdyn,* and *cfos* levels, in OVX versus LET females. The mechanistic underpinnings of those differences is unknown but will be an important area of future investigation.

Our findings suggest that ARC kisspeptin/NKB drive to GnRH neurons is a key facet of enhanced LH pulse secretion in the LET model, and perhaps PCOS as well. In support of this, a recent clinical study tested
380 whether reducing NKB action would lower endogenous LH pulses in PCOS women (73). Excitingly, that study determined that pharmacological antagonism of NK3R, the receptor for NKB, did in fact result in reducing LH pulsatility in PCOS subjects (73). This suggested that NKB action, which is known to stimulate kisspeptin neurons and GnRH neurons, may be exaggerated in some PCOS women, but no direct measure of brain NKB or kisspeptin levels have been reported for PCOS women owing to obvious technical limitations of brain
385 analyses in humans. However, our current findings of enhanced NKB levels, along with increase activation of ARC kisspeptin neurons (which contain NKB), supports the possibility that NKB (and/or kisspeptin) are good candidates for being significantly altered in some PCOS women to drive their hyperactive LH secretion.

In summary, we show for the first time that, compared to control females, LET females exhibit very rapid, dramatically elevated *in vivo* LH pulsatility, with increases in pulse frequency, pulse amplitude, and
390 basal levels, similar to LH patterns in PCOS women. LET mice also exhibit highly elevated *Kiss1* and *Tac2* expression, a corresponding increase in *Pdyn* levels, and enhanced *Kiss1* neuronal activation in the hypothalamic ARC nucleus. These findings indicate that LET mice, like PCOS women, have markedly elevated LH pulsatility which likely drives their increased androgen secretion. Moreover, the increased hypothalamic kisspeptin and NKB drive to downstream GnRH neurons may be a fundamental contributor to
395 the hyperactive endogenous LH pulse secretion in the LET PCOS-like condition. Whether similar brain alterations also contribute, fully or in part, to the rapid LH pulses of some PCOS women is a possibility that currently remains unknown and an important future direction for the field.

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Figure Legends

Figure 1. A) Schematic of experimental paradigm for LET induction of PCOS-like phenotype in female mice and time-line for serial bleeding (Experiment 1) and brain collection (Experiment 2). **B)** Mean BWs over the course of Experiment 1 and serum T at week 3 (pnd 49). **C)** Mean BWs over the course of Experiment 2 and serum T and E2 at week 5 (pnd 63). All LET and OVX samples were below the limit of E2 assay sensitivity (2.7 pg/ml). *, $p<0.05$ versus control females.

Figure 2. Representative profiles of *in vivo* LH secretion in 3 LET-treated female mice (middle column), as well as 3 control female (left column) and 3 OVX female (right column) littermates. LH was measured in serial tail-tip bleeds from awake animals every 6 min for 2.5 hours. Identified pulses are indicated by *. LET females had more frequent pulses, along with higher pulse amplitude and elevated basal levels, than control mice.

Figure 3. Mean pulse parameters of LET, control (CON), and OVX mice from 2.5 hours of *in vivo* serial LH sampling. LH pulse frequency (pulses per h) (**A**), inter-pulse interval (IPI; the number of minutes between pulses) (**B**), pulse amplitude (**C**), pulse peak (zenith value of a pulse) (**D**), basal LH level (**E**), and mean LH across the entire sampling period (**F**) were all significantly different in LET vs CON females. Different letters above bars indicate significantly different ($p<0.05$) from each other.

Figure 4. Elevated *Kiss1* levels in the arcuate nucleus of LET mice. **A**) Representative microscope image of *Kiss1* mRNA expression in the ARC nucleus, as determined by radiolabeled *in situ* hybridization. **B**) Mean *Kiss1* cell number and *Kiss1* mRNA per cell in the arcuate nucleus. Different letters above bars indicate significantly different ($p<0.05$) from each other.

Figure 5. Increased *Tac2* expression levels in the arcuate nucleus of LET mice. **A**) Representative microscope image of *Tac2* mRNA expression in the ARC nucleus, as determined by radiolabeled *in situ* hybridization. **B**) Mean *Tac2* cell number and *Tac2* mRNA per cell in the arcuate nucleus. Different letters above bars indicate significantly different from each other.

Figure 6. Increased dynorphin gene expression in the arcuate nucleus of LET mice. **A**) Representative microscope image of *Pdyn* mRNA expression in the ARC nucleus, as determined by radiolabeled *in situ* hybridization. **B**) Mean *Pdyn* cell number and *Pdyn* mRNA per cell in the arcuate nucleus. Different letters above bars indicate significantly different from each other.

Figure 7. Unaltered *Rfrp* gene expression in the DMN of LET mice. **A**) Representative microscope image of *Rfrp* mRNA expression in the DMN, as determined by radiolabeled *in situ* hybridization. **B**) Mean *Rfrp* cell number and *Rfrp* mRNA per cell in the DMN. There were no significant differences in *Rfrp* measures between any groups.

Figure 8. Increased *Kiss1* neuronal activation levels in the arcuate nucleus of LET mice. **A**) Representative microscope image of *Kiss1* (red fluorescence) + *cfos* (silver grain clusters) mRNA co-expression in the ARC nucleus, as determined by double-label *in situ* hybridization. Yellow arrowheads denote some example co-labeled *Kiss1* cells; blue arrowheads denote example *Kiss1* cells lacking significant *cfos* co-labeling. **B**) Mean

Kiss1+cfos co-expression in the ARC of each group, counted by investigator blind to treatment. Different letters above bars indicate significantly different from each other.

Figure 9. Cartoon summary of neuroendocrine changes in the LET PCOS-like model. LET females exhibit
655 greater numbers of detectable *Kiss1/Tac2/Pdyn* cells and higher *Kiss1, Tac2*, and *Pdyn* mRNA levels per cell in the ARC nucleus (represented by larger cell size and darker green color), as well as increased ARC *Kiss1* neuron activation (represented by the yellow star bursts), relative to normal control females. LET females correspondingly demonstrate faster, higher amplitude LH pulse patterns compared to normal females, providing greater gonadotropin drive to the ovary. Whether or not similar brain alterations are present in some
660 PCOS women is a possibility but currently unknown.