Spontaneous endogenous pulsatile release of kisspeptin is temporally coupled with luteinizing hormone in healthy women

Blazej Meczekalski, M.D., Ph.D.,[a] Krzysztof Katulski, M.D., Ph.D.,[a] Agnieszka Podfigurna-Stopa, M.D., Ph.D.,[a] Adam Czyzyk, M.D., Ph.D.,[a] and Alessandro D. Genazzani, M.D., Ph.D.[b]

[a] Department of Gynecological Endocrinology, Poznan University of Medical Sciences, Poznan, Poland; and [b] Department of Obstetrics and Gynecology, Gynecological Endocrinology Center, University of Modena and Reggio Emilia, Modena, Italy

Objective: To evaluate the presence of a spontaneous pulsatile release of kisspeptin and whether it is temporally coupled to LH pulses.
Design: Experimental study.
Setting: Academic medical center.
Patient(s): Thirty young healthy eumenorrheic women aged 20–37 years were included in the study group. All subjects were white women admitted to the Department of Gynecological Endocrinology, Poznan University of Medical Sciences, Poznan, Poland.
Intervention(s): Kisspeptin, FSH, LH, E_2, PRL, and insulin were evaluated in all subjects at baseline.
Main Outcome Measure(s): All women underwent a pulsatility study measuring LH and kisspeptin plasma concentrations to assess the spontaneous episodic secretion of both hormones, sampling every 10 minutes for 2 hours from 9:00 to 11:00 a.m. for a total of 12 blood samples. Detection and specific concordance (SC) algorithms were used to detect pulses and their concordance.
Result(s): A significant endogenous secretory pattern was demonstrated for both LH and kisspeptin over the 2-hour duration of the study (2.4 ± 0.1 peaks/2 h). The computation of the SC index showed for the first time that kisspeptin and LH are cosecreted and temporally coupled at time "0," and their peaks occur at the same point in time.
Conclusion(s): The present study provides evidence supporting the hypothesis that kisspeptin is highly relevant in the regulation and modulation of reproductive functions in humans. (Fertil Steril® 2016;105:1345–50. ©2016 by American Society for Reproductive Medicine.)
Key Words: Kisspeptin, LH, FSH, estradiol, pulses

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/meczakalskib-release-kisspeptin-healthy-women/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Kisspeptin is the main factor controlling gonadotropins secretion. Identified for the first time in 1996 as a suppressor of the malignant melanoma metastasis process in humans (1, 2), it is considered to be a relevant factor for initiating puberty, regulating sex steroid feedback, and controlling fertility in adults. Kiss1 is the gene responsible for encoding kisspeptin.

In 2001 kisspeptin was identified as a ligand for G protein–coupled receptor 54 (GPR54), which was first described in rat brains and later in humans, Kiss-1 mRNA expression has been demonstrated in the placenta and throughout the central nervous system (CNS), including the hypothalamus. Endogenous fragments of kisspeptin 54, 14, and 13 amino acids in length were isolated from the human placenta. The common C-terminal decapeptide shared by these forms was identified as kisspeptin-10. All kisspeptin fragments, including kisspeptin-10, have a similar affinity and efficacy at the previously identified GPR54 receptor (3).

Human kisspeptin neurons are located primarily in two areas: the preoptic area and the hypothalamic arcuate nucleus (ARC), also known as the infundibular nucleus. Kisspeptin modulation of GnRH secretion has been demonstrated to be dependent on sex steroid concentrations. In fact, E_2 and P modulate kisspeptin activity both in the preoptic area and in the ARC receptors. In rodents it has been shown that preoptic kisspeptin neurons

Received July 6, 2015; revised December 24, 2015; accepted January 20, 2016; published online February 6, 2016.
B.M. has nothing to disclose. K.K. has nothing to disclose. A.P.-S. has nothing to disclose. A.C. has nothing to disclose. A.D.G. has nothing to disclose.
Supported by the Polish National Science Centre (grant no. DEC-2011/01/B/NZ7/00616).
Reprint requests: Blazej Meczekalski, M.D., Ph.D., Department of Gynecologic Endocrinology, Poznan University of Medical Sciences, ul. Polna 33, Poznan, Poland (E-mail: blazejmeczekalski@yahoo.com).

Fertility and Sterility® Vol. 105, No. 5, May 2016 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.01.029

modulate periovulatory positive estrogen feedback to GnRH (4). A limited number of functional studies in humans suggest that both groups (preoptic and ARC) of neurons are involved in this process (5). During the periovulatory period, it is thought that GnRH neurons directly transmit a kisspeptin-controlling signal to the pituitary, understood to be the pulsatile secretion of GnRH, thus stimulating LH and FSH pulsatile release. The negative feedback of E_2 in the follicular phase and of P in the luteal phase on GnRH secretion appears to depend mainly on ARC kisspeptin neurons (5). The role of other kisspeptin neurons identified in some brain regions remains unknown. GnRH neuronal axons lead from the arcuate nucleus to the median eminence, where GnRH is released in a pulsatile manner into portal circulation (5). These findings point to the direct involvement of kisspeptin in GnRH neurosecretion (Supplemental Fig. 1, available online at www.fertstert.org). However, in humans, not all GnRH neurons receive kisspeptin contact (6–9) which may suggest that kisspeptin action/regulation of GnRH secretion involves a more complex mechanism.

Although some studies indicate that kisspeptin directly stimulates the pituitary secretion of LH and FSH based on gene expression and kisspeptin-1 receptor, kisspeptin-induced GnRH secretion appears to be the main physiologic mediator of gonadotropin secretion. Indeed, the role of kisspeptin in the regulation of GnRH secretion was demonstrated in studies using kisspeptin antagonists: Kisspeptin antagonist administration inhibited GnRH production/secretion.

Direct kisspeptin action on GnRH neurons is supported by rodent and primate studies. Humans who develop "inactivating" mutations of the gene encoding kisspeptin and/or its receptor show the occurrence of hypogonadotropic hypogonadism (10). However, in patients in whom "activating" mutations were found, precocious puberty is observed, suggesting that kisspeptin is an important modulator of pubertal maturation and of the pulsatile GnRH secretion (11). Interestingly, in humans, although kisspeptin release stimulates the release of both gonadotropins, the activity for LH is more pronounced.

GnRH secretion models, followed by secretion of LH in different phases of the menstrual cycle, are modulated by a feedback of gonadal steroid hormones. During the follicular phase of the menstrual cycle, GnRH activity and LH secretion are limited by negative feedback effects determined by E_2. The reversal of negative to positive feedback regarding the midcycle LH surge is not yet well understood. Because GnRH neurons do not express estrogen receptors (ERs), the transmission of the ovulation trigger signal, forwarded from the gonads to the hypothalamic GnRH neurons, is likely mediated by a different, distinct neuronal population. Evidence obtained in recent studies indicate that kisspeptin neurons are the putative "missing link," mediating both negative and positive feedback of sex steroids (5).

On such basis, we aimed to evaluate whether a temporal coupling might be measured in plasma concentrations of kisspeptin and LH in a group of eumenorrheic young women, with the use of a simple pulsatility study.

MATERIALS AND METHODS

Study Group

Thirty young healthy women were included in the study group. All of the women were white and admitted to the Department of Gynecological Endocrinology, Poznan University of Medical Sciences, Poznan, Poland.

Inclusion criteria were age 20–35 years (mean 25.6 ± 6.1), regular ovulatory menstrual cycle (28 ± 5 days) during past year, and two recent ovulatory cycles documented with the use of hormonal evaluation (P level in luteal phase >9 ng/mL).

Before her enrollment, each subject gave written informed consent. The protocol was approved by the local Ethics Committee of the Poznan University of Medical Sciences. This study was not registered as a clinical trial, because it was an observational study.

Clinical Evaluation

Each subject was interviewed by a physician, who obtained data on past and current diseases, allergies, family history of endocrinologic, psychiatric, or neurologic illnesses, weight changes, and complete menstrual and medication histories.

No subjects were under hormone treatment during the 6 months before the study, and none were receiving hormone therapy at the time of the study. No subjects had chronic diseases requiring continuous therapy, and all subjects denied recent weight loss, eating disorders, or excessive physical activity. No mood or behavior disturbances were reported at the time of the enrollment; psychiatric disorders were excluded with the use of DSM V criteria. No signs of hyperandrogenism (i.e., hirsutism, acne, or balding/alopecia, or a score ≥ 8 on the modified Ferriman-Gallwey scale) were observed at the onset of the study. Patients with current or past infertility and miscarriage diagnoses were excluded. Eleven patients reported at least one pregnancy ending with live term birth, and the other 19 patients were nulliparous with no desire for pregnancy at the time of the evaluation.

The following clinical characteristics were recorded in all subjects: age, age at menarche, history of menstrual cycles, intermenstrual interval length, weight, height, body mass index.

Study Protocol

All subjects underwent a pulsatility study to determine kisspeptin and LH plasma concentrations. The pulsatility study was performed during the middle follicular phase of the menstrual cycle, days 8–10 from the reported onset of last menstrual period. The midfollicular state was confirmed with the use of transvaginal ultrasound, showing the presence of a dominant follicle measuring 10–17 mm, the lack of a luteal body, and serum E_2 concentrations in the range of 100–200 pg/mL. Only patients who ovulated during the studied cycle and whose P concentrations measured >9 ng/mL from day 16–20 of the cycle were included in the study. A heparin-containing intravenous line was placed in the antecubital vein 45–60 minutes before commencing venous sampling, and blood was withdrawn every 10 minutes for 2 hours

(from 09.00 a.m. to 11.10 a.m.) to measure LH and kisspeptin plasma concentrations. Hormone evaluations were performed in the morning to minimize environmental stressors related to daily activity. Patients were asked to fast on the morning of the study.

Additionally, on the morning of the study, a blood sample was drawn to establish baseline hormone parameters of kisspeptin, LH, FSH, E_2, PRL, and insulin.

Blood samples used for kisspeptin assays were collected in EDTA tubes and centrifuged at 1,600*g* for 15 minutes at 4°C. For other hormones, blood was allowed to clot before the serum was centrifuged at 1,500*g* for 5 minutes. The obtained plasma and serum was stored at −70°C until analysis.

Assays

Kisspeptin was measured with the use of ELISA and a Kiss-1 (112–121) Amide/Kisspeptin-10/Metastin (45–54) Amide (Human) EIA Kit 1 (Phoenix Pharmaceuticals). This ELISA kit provides 100% cross-reactivity with longer kisspeptin 1–54 forms, so it detects both forms of active kisspeptin.

Kisspeptin samples from each subject's visits were analyzed together for comparison. The interassay coefficient of variation for all hormonal assays was <9% at the concentrations measured. Intra-assay coefficient of variation for LH was 6.8% with a sensitivity of 0.8 ng/mL.

Serum FSH, LH, PRL, E_2 and insulin concentrations were determined by means of electrochemiluminescence immunoassay (Roche Diagnostics). Concentrations of various serum hormones were measured with the use of a Cobas E601 analyzer (Roche Diagnostics).

Insulin resistance was checked by means of the homeostasis model (HOMA-IR) with the use of the formula fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5, and a HOMA-IR ≥2.5 threshold was interpreted as a measure of insulin resistance (12).

Pulse Detection and Estimation of Degree of Concordance

Pulse detection. Time series of LH and kisspeptin were first evaluated separately to estimate the random measurement error on the duplicates of the time series with the use of the program Predetec.WK1. This is a specific program of the Detect software. Predetec is incorporated into a spreadsheet for Lotus 123 and calculates the mean (X) and variance (s2x) for each set of replicates in the time series. It plots s2x or sx versus X, or log(s2x) versus log(X) and then fits five different models to this relationship, including linear, parabolic, and power function models, selecting the best model in terms of the smallest root-mean-square error for sx. The program is subject to the constraint that the predicted sx can not be negative for the observable range of the data. Finally, the program provides coefficients for the variance model to be used in the Detect program for pulse detection analysis (13).

After calculating the above estimations and using the coefficients of the best model selected, the secretory episodes on each time series were identified with the use of the Detect program as previously described (13), with a *P* value equal to 0.01 (1%) for the nominal false-positive rate.

Estimation of degree of concordance. The presence of any significant concomitance between the secretory events of LH and kisspeptin was assessed by computing the specific concordance (SC) index (13), interposing various lags between each time series couplet under analysis. The time series of each hormone (A and B) was converted into a "quantized" time series, where only the first sample (onset) of each detected peak was taken to represent the occurrence of that peak (13). These quantized data series were then matched, and quantized values for hormone A were compared with the corresponding values for hormone B. The presence or absence of an event (onset) in either or both series was counted and then the SC index was computed. A spectrum of SC values can be constructed by sliding the two series, interposing integral multiples of the sampling interval as the lag. The presence of a positive lag time indicates that an event in A preceded the secretion event in B.

SC spectra were evaluated for each subject and the mean SCs over each group of patients were calculated at each lag time, obtaining a mean SC spectrum. The location of the maximum of the mean SCs for each group of women was also noted.

Monte Carlo simulations were then performed to study the frequency distribution of the SC index under the null hypothesis of random concordance, as previously described (13).

For each of the two pairs of clinical data for each subject, 500 simulated pairs of simulated series were created and frequency distributions of SC obtained. An SC value above the 95% percentile of the frequency distribution generated for that individual or group of subjects resulted in rejection of the null hypothesis at the $P<.05$ confidence level (13).

RESULTS

The hormonal characteristics of the patients studied are summarized in Table 1. In analysis of the time series, all subjects showed the presence of both LH and kisspeptin pulses throughout the 2-hour pulsatility study. Table 2 summarizes the integrated plasma levels and the number of pulses detected for LH and kisspeptin for the subjects. LH and kisspeptin integrated concentrations were computed as the mean of the 12 points of the pulsatility series.

TABLE 1

Hormonal characteristics of the patients included in the study (mean ± SD).

Hormonal characteristic	Value
LH (mIU/mL)	11.20 ± 6.56
FSH (mIU/mL)	5.82 ± 3.67
PRL (ng/mL)	10.52 ± 5.34
E2 (pg/mL)	251.30 ± 156.87
Kisspeptin (ng/mL)	1.42 ± 0.50
T (ng/mL)	0.40 ± 0.24
17OH-P (ng/mL)	0.69 ± 0.54
DHEAS (μmol/l)	5.67 ± 1.23
SHBG (nmol/l)	91,35 ± 45.78
Glucose, fasting (mg/dL)	81.4 ± 8.34
Insulin, fasting (μU/mL)	5.44 ± 2.45

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.

TABLE 2

Correlation between number of pulses of LH and kisspeptin over 2-hour period, and integrated mean concentrations of LH and kisspeptin.

Variable	LH	Kisspeptin	Correlation coefficient
No. of pulses/2 h	2.0 ± 0.6	2.5 ± 0.9	*P*=.0028
Integrated mean concentration	11.20 ± 6.56 mIU/mL	1.42 ± 0.50 ng/mL	*P*=.0032

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.

Kisspeptin and LH concentrations showed a positive correlation (Fig. 1). Indeed, a significant positive correlation was also demonstrated between kisspeptin and LH numbers of pulses/2 h (Table 2) and frequencies (Supplemental Fig. 2, available online at www.ferstert.org) thus confirming that a correlation exists between the occurrence of the secretory events of kisspeptin and those of LH. Mean duration observed for LH and kisspeptin pulses were 39.9 ± 18.2 minutes and 32.9 ± 12.7 minutes, respectively.

After detection of spontaneous episodic kisspeptin secretory events, we evaluated if any temporal coupling was present between kisspeptin and LH pulses. We studied such temporal coupling through the computation of the SC index. This index does not consider the plasma concentrations but the occurrence of the secretory event, as described in the Materials and Methods section.

SC computation facilitated identification of a significant degree of concordance between kisspeptin and LH pulses at time "0." The program computation did not recognize any other moments of concordance between the two hormones when expanding the time series from −100 to +100 minutes, thus reinforcing the finding that kisspeptin and LH are cosecreted; the two hormones have concomitant occurrences of their secretory events. The SC index computed for kisspeptin and LH secretory pulses is shown in Figure 2. The SC index above the threshold of significance ($P<.05$) is at time lag "0," and the $P<.01$ is missed for minimal values.

DISCUSSION

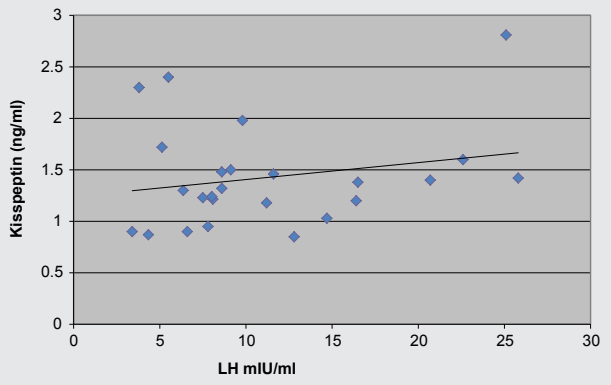
The kisspeptin system has been extensively studied in both nonhuman and human models (14–16) which show kisspeptin's pivotal role in the reproductive system as well as in central regulation of puberty and hypothalamic GnRH secretion (17).

Performing a pulsatility study over a span of 2 hours in a group of young healthy eumenorrheic women, our study captured groundbreaking evidence suggesting that kisspeptin has a spontaneous pulsatile secretory pattern. Additionally, we were able to demonstrate that kisspeptin's pulsatile release is temporally coupled with LH secretory pulses without any lag time: The secretory peaks of the two hormones occur at the same time, i.e., at time "0." The statistically based computerized algorithms used (7, 8) add credibility to the results of this study as in many other studies that used the same analytic methods (6,18–21).

The fact that kisspeptin pulsatile secretion is coupled with LH secretion supports the hypothesis that kisspeptin is deeply involved in the regulation of LH secretion through the kisspeptin-induced GnRH release. This fact appropriately aligns with all previous data showing that peripheral administration of kisspeptin induces LH secretion (14).

A number of studies have shown that intravenous or subcutaneous administration of kisspeptin stimulates LH release in healthy women, as well as in women suffering from

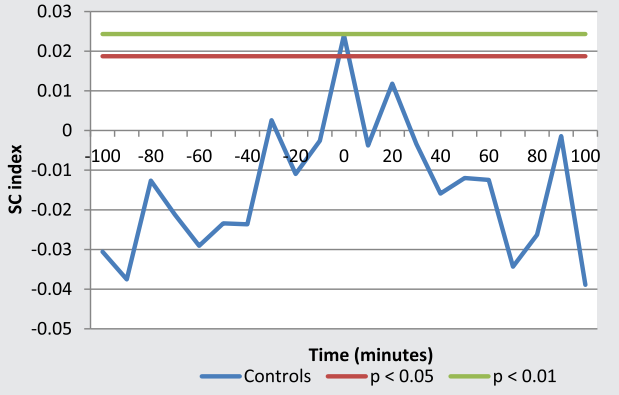
FIGURE 1



Correlation of mean LH and kisspeptin concentrations. Correlation index $R = 0.0017$; $P=.0032$.

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.

FIGURE 2



Specific concordance (SC) index computed after analysis of LH and kisspeptin time series. The SC index was statistically significant ($P<.05$) at time "0," thus demonstrating that the two hormones are cosecreted at the same time. No significant SC index was present at any lag of time (delay or anticipation).

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.

hypothalamic amenorrhea. In a series of papers, Jaysena et al. (22, 23) reported that bolus administration of kisspeptin in the follicular or preovulatory phase is related to a significant (50%–300%) increase in LH secretion. Similarly, Chan et al. (24) reported a stimulatory effect of kisspeptin on LH release during the luteal phase. A single administration of kisspeptin in healthy women seems to influence higher LH pulse frequency, rather than LH pulse secretory mass (22). The data concerning chronic administration of kisspeptin in healthy women is still limited. Chronic kisspeptin administration causes deep tachyphylaxis in women in functional hypothalamic amenorrhea (23). However, the relationship between pulsatile kisspeptin release and LH pulsatility in healthy women remain partly unknown.

Obviously, we were not able to assay and detect hypothalamic secretory GnRH pulses, because it is released into portal hypothalamopituitary circulation. Therefore, based on current evidence, it is inferred with high probability that kisspeptin-LH pulse coupling is mediated by GnRH secretion. However, according to previous data, although kisspeptin may have a direct stimulatory action on gonadotropes, indirect stimulation through the enhancement of GnRH secretion appears to be the principal physiologic pathway for the kisspeptin stimulation of gonadotropin secretion.

Nevertheless, the data in the present study show for the first time that there is a detectable kisspeptin secretory pattern and that kisspeptin and LH pulses are temporally coupled during the midfollicular/preovulatory phase in healthy women.

It is relevant to state that an assay was used for the most active form of kisspeptin, with ten amino acids (kisspeptin-10), which detects 100% cross-reactivity with the longer kisspeptin 1–54 forms. Chan et al. (24) observed that kisspeptin-10 stimulated peak LH secretion within an hour of administration in healthy men. Some authors speculate that kisspeptin-10 acts differently than kisspeptin-54, stimulating a readily releasable pool of GnRH stored within nerve terminals of the median eminence (25). Kisspeptin-54 might act more proximally in the GnRH neuron to stimulate de novo GnRH synthesis (26). On such basis, more precise studies on the physiologic roles of kisspeptin-10 and kisspeptin 1–54 are needed to differentiate their role in stimulating a fast LH response in the preovulatory phase of the menstrual cycle.

Of note, this study was conducted with a relatively large sample size, and it would be valuable to assess whether such temporal coupling exists between kisspeptin and LH secretory episodes during other phases of the menstrual cycle, such as the early follicular and luteal phases. The results in this study can not be generalized to the entire ovarian cycle, because this study was designed to evaluate kisspeptin spontaneous pulsatile release and its coupling with LH during the late follicular phase.

Another relevant point to include is that this study did not evaluate FSH pulsatile secretion. Though FSH is cosecreted with LH during the follicular phase of the menstrual cycle, FSH has a longer half-life than LH, owing to a different metabolic clearance mechanism. Such differences do not permit an optimal determination of FSH pulse frequency on short-term pulsatility studies such as this one (8).

In conclusion, this study demonstrated the presence of spontaneous kisspeptin pulsatile release in eumenorrheic women and that its episodic release is temporally coupled with simultaneous LH secretory episodes. The highly relevant results of this study affect future studies on specific diseases involving abnormal kisspeptin secretion and/or abnormal kisspeptin-induced GnRH release, thus affecting pituitary secretion of gonadotropins in various subtypes of primary amenorrhea, hypothalamic amenorrhea, or anovulation in polycystic ovary syndrome patients.

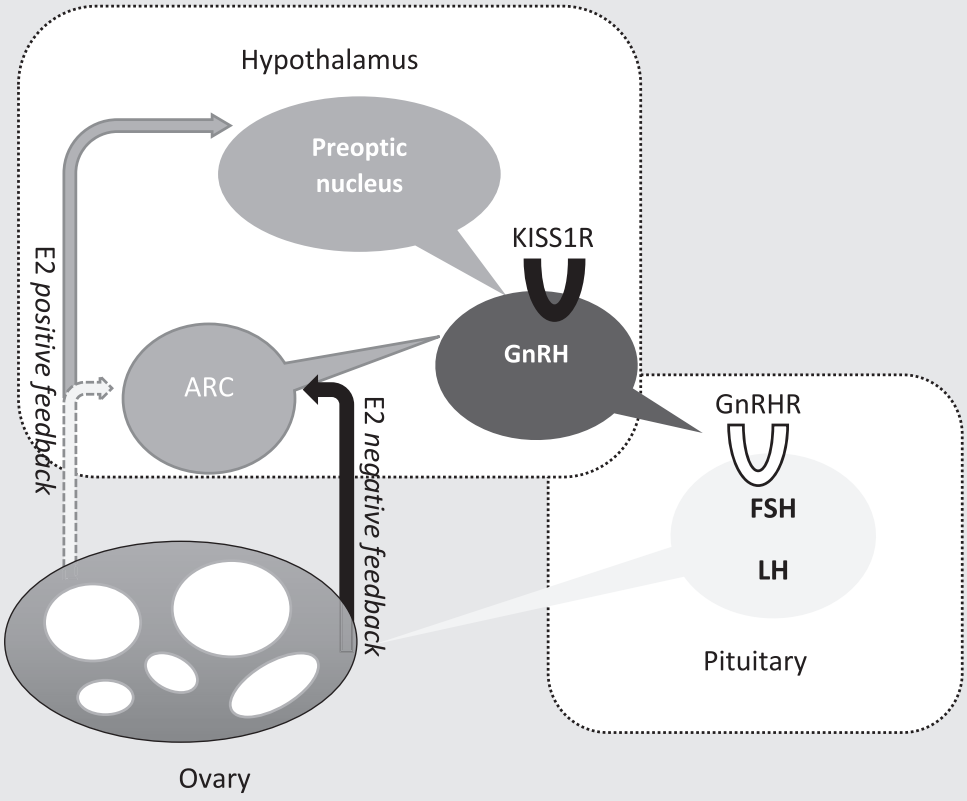
Acknowledgments: The authors thank Kurtis S. Kaminishi, M.D., M.B.A., for language editing.

REFERENCES

1. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ. Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin in vivo. Endocrinology 2008;4:1951–9.
2. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, et al. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of Kiss-1 mRNA in the male rat. Neuroendocrinology 2004;4:264–72.
3. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, le Poul E, et al. The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 2001;276:34631–6.
4. Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). Brain Res Rev 2008;57:277–87.
5. Hrabovszky E. Neuroanatomy of the human hypothalamic kisspeptin system. Neuroendocrinology 2014;99:33–48.
6. Genazzani AD, Luisi M, Malavasi B, Strucchi C, Luisi S, Casarosa E, et al. Pulsatile secretory characteristics of allopregnanolone, a neuroactive steroid, during the menstrual cycle and in amenorrheic subjects. Eur J Endocrinol 2002;146:347–56.
7. Genazzani AD, Petraglia F, Benatti R, Montanini V, Algeri I, Volpe A, et al. Luteinizing hormone (LH) secretory burst duration is independent from LH, prolactin, or gonadal steroid plasma levels in amenorrheic women. J Clin Endocrinol Metab 1991;72:1220–5.
8. Genazzani AD, Petraglia F, Volpogni C, d'Ambrogio G, Facchinetti F, Genazzani AR. FSH secretory pattern and degree of concordance with LH in amenorrheic, fertile and postmenopausal women. Am J Physiol 1993;264:E776–81.
9. Meczekalski B, Genazzani AR, Genazzani AD, Warenik-Szymankiewicz A, Luisi M. Clinical evaluation of patients with weight-loss related amenorrhea: neuropeptide Y and luteinizing hormone pulsatility. Gynecol Endocrinol 2006;22:239–43.
10. Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, et al. Inactivating Kiss1 mutation and hypogonadotropic hypogonadism. N Engl J Med 2012;366:629–35.
11. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, et al. A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med 2008;358:709–15.
12. Henderson M, Rabasa-Lhoret R, Bastard JP, Chiasson JL, Baillargeon JP, Hanley JA, et al. Measuring insulin sensitivity in youth: How do the different indices compare with the gold-standard method? Diabetes Metab 2011;37:72–8.
13. Guardabasso V, Genazzani AD, Veldhuis JD, Rodbard D. Objective assessment of concordance of secretory events in two endocrine time series. Acta Endocrinol 1991;124:208–18.
14. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, et al. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 2004;145:4073–7.
15. Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via

G protein-coupled receptor 54. Proc Natl Acad Sci U S A 2005;102:1761–6.
16. Plant TM, Ramaswamy S, Dipietro MJ. Repetitive activation of hypothalamic G protein–coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (*Macaca mulatta*) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology 2006;147:1007–13.
17. Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, et al. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 2005;90:6609–15.
18. Genazzani AD, Guardabasso V, Petraglia F, Genazzani AR. Specific concordance index defines the physiological lag between LH and progesterone in women during the midluteal phase of the menstrual cycle. Gynecol Endocrinol 1991;5:175–84.
19. Genazzani AD, Petraglia F, Gastaldi M, Volpogni C, Gamba O, Massolo F, et al. Evidence of additional control mechanism regulating LH and FSH episodic secretion in prepubertal children and in postmenopausal women. Hum Reprod 1994;9:1807–12.
20. Rochira V, Zirilli L, Genazzani AD, Balestrieri A, Aranda C, Fabre B, et al. Hypothalamic-pituitary-gonadal axis in two men with aromatase deficiency: evidence that circulating estrogens are required at the hypothalamic level for the integrity of gonadotropin negative feedback. Eur J Endocrinol 2006;155:513–22.
21. Genazzani AD, Lanzoni C, Ricchieri F, Santagni S, Rattighieri E, Chierchia E, et al. Acetyl-L-carnitine (ALC) administration positively affects reproductive axis in hypogonadotropic women with functional hypothalamic amenorrhea. J Endocrinol Invest 2011;34:287–91.
22. Jayasena CN, Comninos AN, Veldhuis JD, Misra S, Abbara A, Izzi-Engbeaya C, et al. A single injection of kisspeptin-54 temporarily increases luteinizing hormone pulsatility in healthy women. Clin Endocrinol (Oxf) 2013;79:558–63.
23. Jayasena CN, Nijher GM, Chaudhri OB. Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotropin secretion in women with hypothalamic amenorrhea, but chronic administration causes tachyphylaxis. J Clin Endocrinol Metab 2009;94:4315–23.
24. Chan YM, Butler JP, Pinnell NE. Kisspeptin resets the hypothalamic GnRH clock in men. J Clin Endocrinol Metab 2011;96:E908–15.
25. d'Anglemont de Tassigny X, Fagg LA, Carlton MB, Colledge WH. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 2008;149:3926–32.
26. d'Anglemont de Tassigny X, Ackroyd KJ, Chatzidaki EE, Colledge WH. Kisspeptin signaling is required for peripheral but not central stimulation of gonadotropin-releasing hormone neurons by NMDA. J Neurosci 2010;30:8581–90.

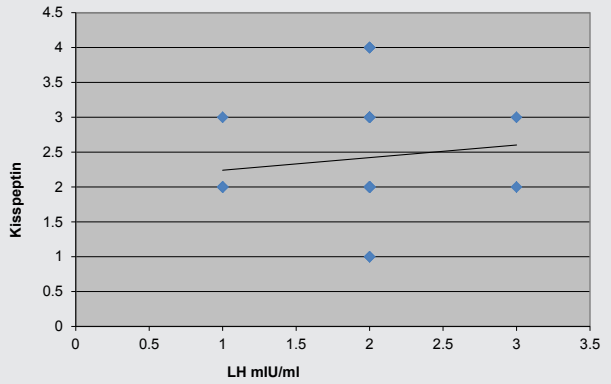
SUPPLEMENTAL FIGURE 1



The schematic representation of kisspeptin neurons' role in regulation of the menstrual cycle. Kisspeptin neurons are located in two hypothalamic nuclei: preoptic and arcuate (ARC). Both nuclei modulate GnRH secretion and indirectly drive the FSH and LH pituitary secretion. Downstream ovarian E_2 secretion controls the kisspeptin release. Estrogens can up-regulate kisspeptin expression in the preoptic nucleus (*grey arrow*) and probably the ARC nucleus (*light grey*). The negative E_2 feedback is mediated mainly by the ARC nucleus (*black arrow*). KISS1R = kisspeptin-1 receptor; GnRHR = GnRH receptor.

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 2



The correlation of the number of LH and kisspeptin pulses in the study group over 2 hours. Correlation index $R = 0.1666$; $P = .0028$.

Meczekalski. Kisspeptin release in healthy women. Fertil Steril 2016.