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Kisspeptin-54 Stimulates Gonadotropin Release Most Potently during the Preovulatory Phase of the Menstrual Cycle in Women

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Context: Kisspeptin, the endogenous ligand of the G protein-coupled receptor 54, is a key regulator of the hypothalamo-pituitary-gonadal (HPG) axis. GPR54-null mice exhibit reproductive dysfunction, and exogenous kisspeptin potently stimulates the HPG axis in rodents, primates, and human males. The effects of kisspeptin administration to human females are unknown.

Objective: Our objective was to investigate the effects of kisspeptin on LH release during the menstrual cycle in female volunteers.

Design: Bolus sc kisspeptin-54 was administered to female volunteers, and plasma gonadotropins were measured.

Setting: The study took place at a hospital clinical research facility.

Volunteers: Subjects were healthy female volunteers with regular menstrual cycles.

Intervention: 1) Volunteers received a sc bolus injection of kisspeptin-54 (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 nmol/kg; n = 3–4 per dose) in the follicular phase; and 2) volunteers (n = 8) received a sc bolus injection of either kisspeptin-54 (0.4 nmol/kg) or saline in random order during each phase of the menstrual cycle.

Main Outcome Measures: Plasma gonadotropins were measured.

Results: 1) Kisspeptin-54 caused a dose-dependent increase in mean LH over time at doses from 0.2–6.4 nmol/kg. 2) Kisspeptin-54 increased plasma LH compared with saline injection in all phases of the cycle. The effect of kisspeptin was greatest in the preovulatory phase and least in the follicular phase of the cycle [mean increase in LH over baseline (IU/liter) ± SEM for follicular phase was 0.12 ± 0.17; preovulatory phase, 20.64 ± 2.91 ($P < 0.001$ *vs.* follicular phase); luteal phase, 2.17 ± 0.79 ($P < 0.01$ *vs.* follicular phase)].

Conclusion: Elevation of plasma kisspeptin in human females potently stimulates LH release in the preovulatory phase and provides a novel mechanism for manipulation of the HPG axis in women. (***J Clin Endocrinol Metab* 92: 3958–3966, 2007**)

KISSPEPTIN IS A 54-amino-acid peptide encoded by the *KiSS-1* gene which acts via the G protein-coupled receptor (GPR) GPR54 (1). GPR54 was discovered to be necessary for normal pubertal development when it was demonstrated that mice lacking GPR54 and humans with GPR54-null mutations do not mature sexually and have low circulating gonadotropins and sex hormones (hypogonadotropic hypogonadism) (2–4). Subsequent studies have confirmed that kisspeptin is a potent stimulator of gonadotropin release when administered peripherally or directly into the third ventricle of rats (5–13), mice (14, 15), sheep (15, 16), and monkeys (17–20) and peripherally in human males (21). Kisspeptin appears to operate upstream of GnRH neurons. The GPR54 receptor is expressed in GnRH neurons in the hypothalamus (10, 15, 22), and kisspeptin administration increases the expression of the immediate-early gene product c-Fos in GnRH neurons (6, 10). The effects of kisspeptin on gonadotropin release can be blocked by preadministration of a GnRH receptor antagonist (6, 10, 14, 17–18), and kisspeptin has been shown to release GnRH *in vitro* (8) and *in vivo* (15).

KiSS-1 mRNA is expressed in areas of the hypothalamus implicated in the neuroendocrine regulation of gonadotropin secretion (14, 17) and is regulated by sex steroids (5, 10, 23–26). Kisspeptin is also involved in the regulation of the preovulatory LH surge in female rats (24, 27–29) and sheep (30), and kisspeptin administration stimulates LH secretion in all phases of the estrous cycle in rats (31). In addition, kisspeptin appears to be critical in the onset of puberty (18, 32–34). These data suggest that kisspeptin has an important physiological role in the regulation of the hypothalamo-pituitary-gonadal (HPG) axis and may be a novel target for therapeutic manipulation of the HPG axis.

However, the effects of kisspeptin administration in women are currently unknown. Our studies demonstrate that sc administration of kisspeptin-54 to healthy women results in a stimulation of gonadotropin secretion that is most

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Abbreviations: AVPV, Anteroventral periventricular nucleus; D1, diestrous 1; FEI, free estradiol index; FPLC, fast protein liquid chromatography; GPR, G protein-coupled receptor; HPG, hypothalamo-pituitary-gonadal; icv, intracerebroventricular; IR, immunoreactivity; RFRP, RF amide-related peptide 1.

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potent in the preovulatory phase of the menstrual cycle. These findings identify kisspeptin as a possible novel therapy for the treatment of women with amenorrhea causing infertility.

Subjects and Methods

Kisspeptin-54

Human kisspeptin-54 was synthesized by the Advanced Biotechnology Centre, Imperial College London. It was purified by reverse-phase HPLC, and identity was confirmed by electrospray mass spectroscopy and amino acid analysis as previously described (21). Tests of peptide bioactivity and toxicology were also performed as previously described (21). The *Limulus* amebocyte lysate assay test for pyrogen (Associates of Cape Cod, Liverpool, UK) was negative, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London, UK). Kisspeptin-10, -14, and -54 have similar efficacy *in vitro* (1). However, we used kisspeptin-54 in our study because kisspeptin-54 is more efficacious than shorter kisspeptin fragments *in vivo* (11, 35).

Subjects

Ethical approval for this study was obtained from the Hammersmith and Queen Charlotte's and Chelsea Hospitals Research Ethics Committee (reference number 05/Q0406/142), and subjects gave written informed consent. This study was conducted in accordance with the Declaration of Helsinki. Healthy female volunteers with regular menses were recruited. Mean age ± SEM was 29 ± 1 yr, body mass index was 24.3 ± 1.3 kg/m^2, and length of menstrual cycle observed during the study was 28 ± 0.3 d. Volunteers were taking no medication, including contraceptive medication, and had no allergies or abnormalities on physical examination or electrocardiogram. They had no evidence of abnormal liver, renal or thyroid function. Baseline hemoglobin and glucose were normal, and LH, FSH, estradiol, progesterone, testosterone, SHBG, and prolactin measured in the follicular phase of the menstrual cycle were also normal.

Protocol

Establishment of a dose response for the effects of kisspeptin-54 on plasma levels of LH, FSH, and estradiol in the follicular phase of the menstrual cycle. Subjects, but not investigators, were blinded for this component of the study. Volunteers attended the clinical investigation unit between 0800–0900 h. Study days were restricted to the follicular phase of the cycle (defined as d 2–10 inclusive) (36, 37) to control for changes in the HPG axis over the course of the cycle. Individual study days were separated by at least 7 d. Peptide was dissolved in 0.9% saline for bolus injection into the abdominal sc tissue. The doses of kisspeptin used were 0, 0.2, 0.4, 0.8, 2.4, and 6.4 nmol/kg (n = 3–4 subjects at each dose). These doses were selected based on the doses of kisspeptin-54 found to stimulate LH release in our previous iv infusion study in human male volunteers (21). Blood was sampled and blood pressure and heart rate measured at time −30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min after injection. Blood samples were collected into lithium-heparin tubes (LIP, Cambridge, UK) containing 5000 kallikrein inhibitor units of aprotinin (0.2 ml Trasylol; Bayer, Newbury, UK). Samples were immediately centrifuged and plasma separated and stored at −20 C until measurement of LH, FSH, estradiol, SHBG, and kisspeptin at all time points. In addition, plasma progesterone was measured on baseline samples to confirm that progesterone levels were undetectable, consistent with the follicular phase (37).

Investigation of the effects of kisspeptin-54 on stimulation of the HPG axis in different phases of the menstrual cycle. This was a double-blind placebo-controlled study. Volunteers (n = 8) each attended for a total of six study days over a minimum of two menstrual cycles. Two study days were in the follicular, two in the preovulatory and two in the luteal phase of the menstrual cycle. In each phase of the menstrual cycle, volunteers received a sc injection of saline on one day and kisspeptin (0.4 nmol/kg) on the other day. Kisspeptin injection days were randomly alternated with saline injection days over the two menstrual cycles. Individual study days were separated by at least 7 d. The follicular phase studies were performed within d 2–10 of the cycle, the preovulatory phase studies 15–16 d before the start of the next predicted period, and the luteal phase studies 4–10 d before the next predicted period (37). The dose of kisspeptin-54 (0.4 nmol/kg) used in this study caused only a small increase in circulating gonadotropins in the dose-response study above. This dose of kisspeptin-54 was chosen to allow us to determine whether kisspeptin-54 was more effective in stimulating the secretion of gonadotropins in the preovulatory and luteal phases of the menstrual cycle compared with the follicular phase as might be expected from rodent studies (31). The study was otherwise conducted as described above, with the exception that progesterone measurements were made at baseline only in the follicular and preovulatory phases, but on all samples taken during the luteal phase of the cycle.

Analytical methods

LH, FSH, estradiol, testosterone, progesterone, SHBG, and kisspeptin measurement. Measurement of plasma LH, FSH, estradiol, testosterone, and progesterone was performed using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Female testosterone samples underwent ether extraction and reconstitution in bovine calf serum before assay to remove interfering conjugated steroids (38). SHBG was measured using a solid-phase automated enzyme immunoassay (Immulite; Siemans, Llanberis, UK). Reference ranges for females were as follows: LH (follicular), 2–10 IU/liter; LH (midcycle), 20–60 IU/liter; LH (luteal), 4–14 IU/liter; FSH (follicular and luteal), 1.5–8 IU/liter; estradiol (early follicular), less than 300 pmol/liter; estradiol (midcycle), 400-1500 pmol/liter; estradiol (luteal), 200-1000 pmol/liter; testosterone, less than 3 nmol/liter; progesterone, more than 10nmol/liter in the luteal phase indicates ovulation has occurred; and SHBG, 40–80 nmol/liter. Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; testosterone, 4.9%; progesterone, 1.8%; and SHBG, 5.6%. To minimize effects of variation in SHBG on estradiol levels, the free estradiol index (FEI) was calculated as 100 × estradiol/SHBG (39).

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established RIA (21, 40). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF. The assay detected changes of 2 pmol/liter of plasma kisspeptin-IR with a 95% confidence limit. The intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

Characterization of kisspeptin-IR in human plasma. Kisspeptin-IR was characterized in plasma taken from volunteers 90 min after injection of 6.4 nmol/kg kisspeptin-54 using fast protein liquid chromatography (FPLC) as previously described (21, 40). Briefly, plasma (200 μl) was diluted in 800 μl water plus 0.05% trifluoroacetic acid. This was then passed through a 0.2-μm filter (Satorius, Goettingen, Germany). Of the volume collected, 0.6 ml was fractionated by FPLC on a high-resolution reverse-phase (Pep RPC HR 1 ml) C18 column (Pharmacia, Uppsala, Sweden). The column was eluted with a 20–30% gradient of acetonitrile-water containing 0.05% (vol/vol) trifluoroacetic acid for 50 min, and fractions were collected at 1-min intervals. The kisspeptin-IR in all fractions was determined by RIA. The remainder of the sample was used to calculate the percentage recovery. Recovery was calculated as the ratio of kisspeptin-IR (picomoles) recovered from each sample, and kisspeptin-IR loaded on to the FPLC column (picomoles), multiplied by 100 and expressed as a percentage.

Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons across multiple doses of kisspeptin were performed using one-way ANOVA with the Bonferroni *post hoc* correction. Comparisons across both time and dose were performed using repeated-measures two-way ANOVA. For comparisons of the effects of kisspeptin-54 on plasma LH, FSH, and FEI across different phases of the menstrual cycle, LH, FSH, and FEI levels obtained after saline administration were subtracted from the LH, FSH, and FEI levels after administration of kisspeptin-54 in the same phase of the menstrual cycle. Data from each phase were compared

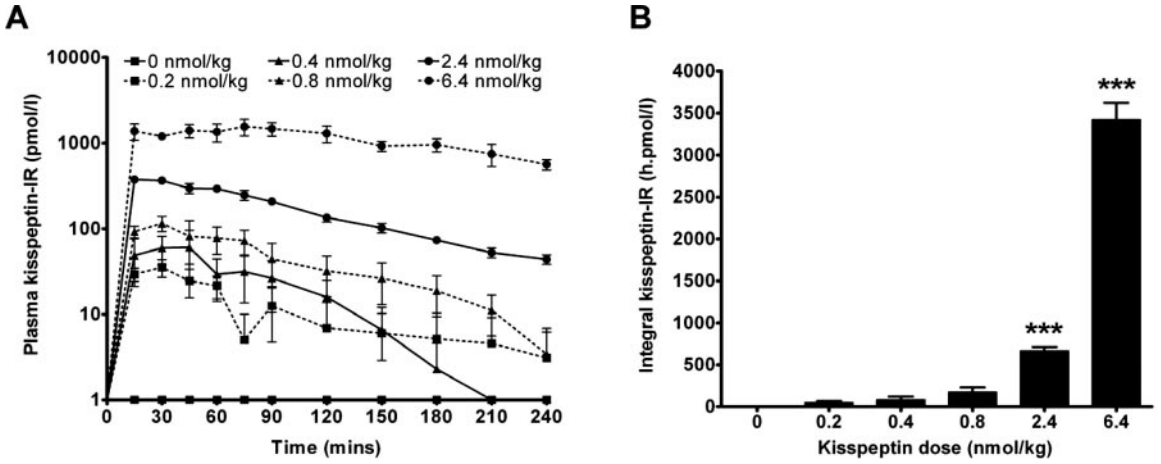

FIG. 1. A, Mean ± SEM plasma kisspeptin-IR after bolus sc injection of 0, 0.2, 0.4, 0.8, 2.4, and 6.4 nmol/kg kisspeptin-54 into healthy human females (n = 3–4 per dose) in the follicular phase of the menstrual cycle. Injections were administered at time 0 min. B, Integrated response (mean area under curve ± SEM) of plasma kisspeptin-IR over the 240-min period after sc injection of increasing doses of kisspeptin-54. ***, $P < 0.001$ *vs.* 0 nmol/kg dose.

using a repeated-measures two-way ANOVA. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

Dose response for the effects of kisspeptin-54 on plasma levels of LH, FSH, and estradiol in the follicular phase of the menstrual cycle

Volunteers reported no nausea or other side effects after injection of kisspeptin-54. There were no significant changes in heart rate or blood pressure during any of the study days compared with baseline measurements taken at time −30 and 0 min (data not shown).

Baseline levels of LH, FSH, and FEI were not significantly different between groups receiving different doses of kisspeptin-54 (data not shown). Baseline progesterone was less than 2 nmol/liter in all volunteers.

Plasma kisspeptin immunoreactivity (kisspeptin-IR) was undetectable (<2 pmol/liter) after injection of saline. Administration of increasing doses of kisspeptin-54 to female volunteers in the follicular phase of the cycle resulted in a dose-dependent rise in plasma kisspeptin-IR (Fig. 1). Exogenous kisspeptin-54 administration caused a dose-dependent increase in plasma LH and FSH (Fig. 2, A and B) but had no significant effect on FEI compared with saline-injected controls (Fig. 2C).

Effects of kisspeptin-54 on stimulation of the HPG axis in different phases of the menstrual cycle

Baseline plasma kisspeptin-IR, LH, FSH, and FEI. Baseline plasma kisspeptin-IR was undetectable (<2 pmol/liter) in all phases of the cycle. Baseline levels of plasma LH, FSH, FEI, and progesterone are shown in Table 1. All eight volunteers were noted to have appropriately raised progesterone levels at baseline in the luteal phase of the cycle (mean ± SEM luteal phase progesterone was 37 ± 3.8 nmol/liter), indicating ovulation had occurred.

Effects of sc kisspeptin-54 on stimulation of the HPG axis in different phases of the menstrual cycle. Bolus sc injection of 0.4 nmol/kg kisspeptin-54 resulted in a rise in plasma kisspeptin-IR. The plasma kisspeptin-IR levels achieved after injection of 0.4 nmol/kg kisspeptin-54 were not significantly different when compared across the three phases studied or compared with the levels achieved at the same dose in the dose-finding study (data not shown).

Injection of 0.4 nmol/kg kisspeptin-54 significantly ele-

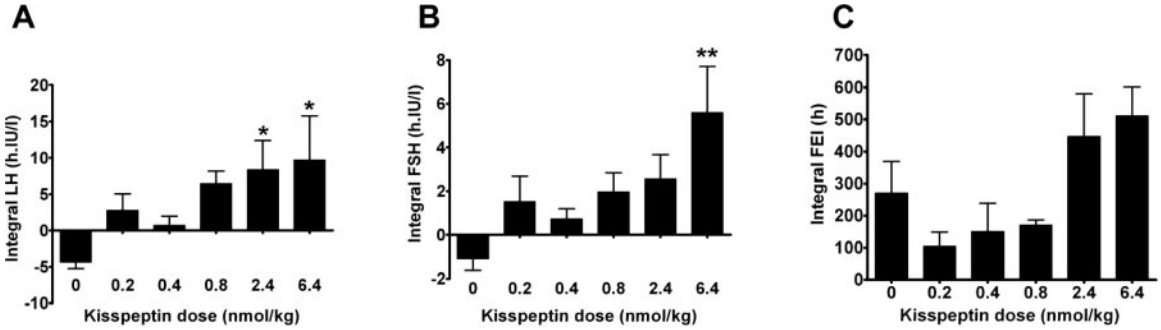

FIG. 2. Graph showing mean area under curve ± SEM increase over baseline of plasma LH (A), FSH (B), and FEI (C) after bolus sc injection of 0, 0.2, 0.4, 0.8, 2.4, and 6.4 nmol/kg kisspeptin-54 into healthy human females (n = 3–4 per dose) in the follicular phase of the menstrual cycle. Injections were administered at time 0 min. *, $P < 0.05$ *vs.* 0 nmol/kg dose; **, $P < 0.01$ *vs.* 0 nmol/kg dose.

TABLE 1. Baseline plasma LH, FSH, FEI, and progesterone in different phases of the menstrual cycle in women with ovulatory periods

	Ovulating volunteers		
	Follicular	Preovulatory	Luteal
LH (IU/liter)	4.18 ± 0.40	14.53 ± 3.07[b]	3.56 ± 0.40
FSH (IU/liter)	4.89 ± 0.18	4.96 ± 0.34	3.12 ± 0.20[a]
FEI	343.1 ± 32.3	1441.4 ± 34.2[b]	701.3 ± 47.7[c]
Progesterone (nmol/liter)	0.56 ± 0.13	0.94 ± 0.21	37.0 ± 3.82[b]

Results are mean ± SEM. Values are provided for the follicular, preovulatory, and luteal phases for volunteers (n = 8) with a luteal phase progesterone of more than 10 nmol/liter, suggesting ovulation. As expected (37), baseline LH levels were significantly elevated in the preovulatory phase compared with both the follicular and luteal phases. Plasma FSH levels in the luteal phase were significantly lower than in both the follicular and preovulatory phases. Baseline FEI was significantly elevated in the preovulatory phase compared with both follicular and luteal phases. And progesterone was significantly elevated in the luteal phase compared with the follicular and preovulatory phases.

[a] $P < 0.05$ *vs.* follicular phase.
[b] $P < 0.001$ *vs.* follicular phase.
[c] $P < 0.01$ *vs.* preovulatory phase.

vated plasma LH (Fig. 3, A–C) and FSH (Fig. 4, A–C) in all phases of the cycle after injection of kisspeptin-54, compared with cycle phase-paired saline injection days. A significant effect of 0.4 nmol/kg kisspeptin-54 on FEI was noted only in the luteal phase over the 240-min time period of the study (Fig. 5, A–C).

Comparisons of the effects of kisspeptin-54 on plasma LH, FSH, and FEI across different phases of the menstrual cycle showed that there was a greater increment in plasma LH (Fig. 3D) and FSH (Fig. 4D) after kisspeptin-54 injection in the preovulatory phase of the cycle than in either of the other two phases studied (follicular phase LH *vs.* preovulatory phase LH, $P < 0.001$; preovulatory phase LH *vs.* luteal phase LH, $P < 0.001$; follicular phase FSH *vs.* preovulatory phase FSH, $P < 0.001$; preovulatory phase FSH *vs.* luteal phase FSH, $P < 0.001$). Comparison of incremental changes in FEI (Fig. 5D) across the three cycle phases studied showed they were not significantly different. A significant elevation in luteal phase plasma progesterone was seen after kisspeptin-54 administration (luteal phase saline *vs.* luteal phase 0.4 nmol/kg kisspeptin-54, $P < 0.001$) (Fig. 6).

Characterization of kisspeptin-IR in human plasma

Reverse-phase FPLC was used to further analyze kisspeptin-IR in plasma taken from volunteers 90 min after injection of 6.4 nmol/kg kisspeptin-54. All columns had a recovery greater than 60%. In each plasma sample, the kisspeptin RIA

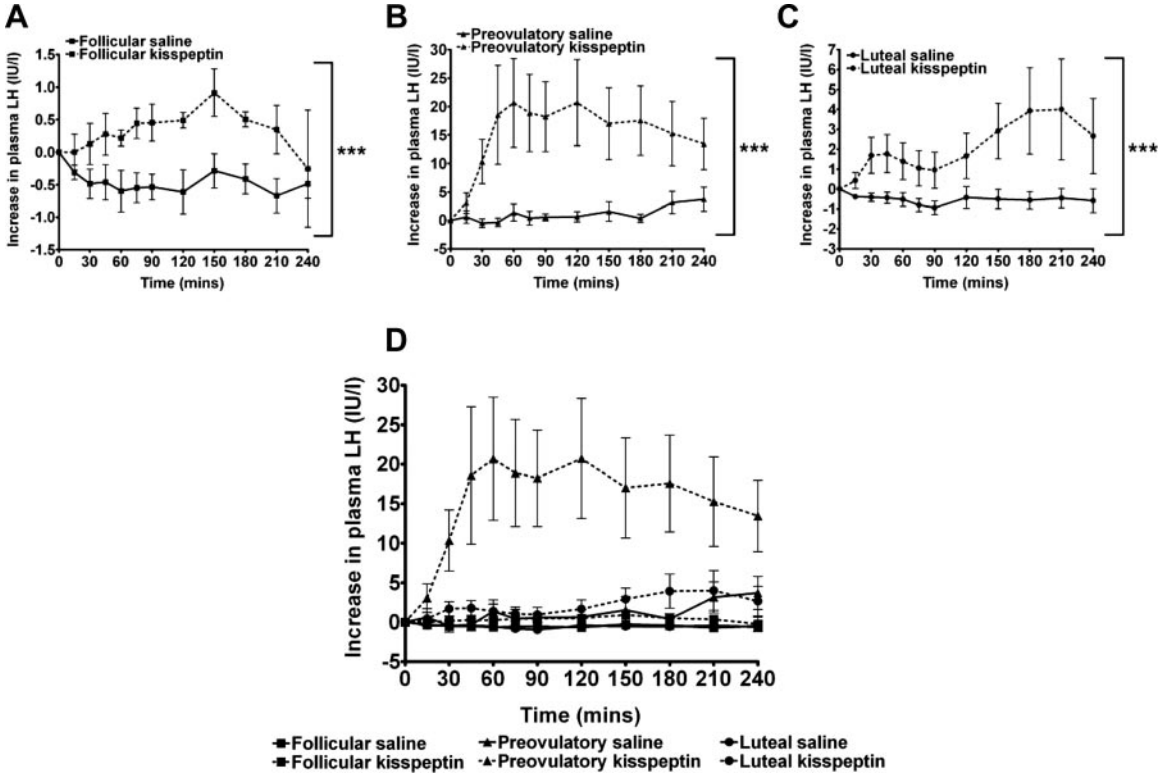

FIG. 3. Mean ± SEM increase over baseline in plasma LH after bolus sc injection of saline or 0.4 nmol/kg kisspeptin-54 into healthy human females (n = 8) in individual phases of the menstrual cycle: A, follicular; B, preovulatory; C, luteal phase. Injections were administered at time 0 min. D, Mean ± SEM increase over baseline in plasma LH in all three phases of the menstrual cycle to allow comparison of the effects of kisspeptin-54 on LH across different phases of the menstrual cycle. ***, $P < 0.001$ kisspeptin *vs.* saline.

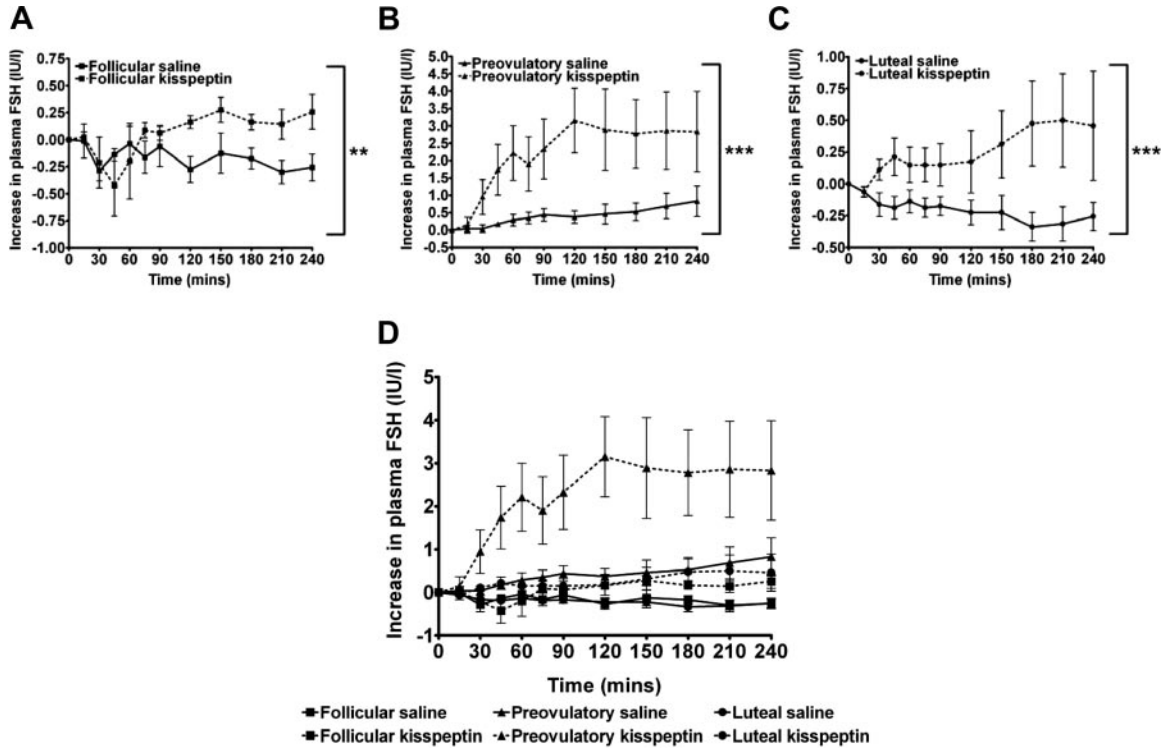

FIG. 4. Mean ± SEM increase over baseline in plasma FSH after bolus sc injection of saline or 0.4 nmol/kg kisspeptin-54 into healthy human females (n = 8) in individual phases of the menstrual cycle: A, follicular; B, preovulatory; C, luteal phase. Injections were administered at time 0 min. D. Mean ± SEM increase over baseline in plasma FSH in all three phases of the menstrual cycle to allow comparison of the effects of kisspeptin-54 on FSH across different phases of the menstrual cycle. **, $P < 0.01$; ***, $P < 0.001$ kisspeptin *vs.* saline.

detected a single immunoreactive peak corresponding to synthetic human kisspeptin-54. A representative profile is shown in Fig. 7.

Discussion

Intravenous administration of kisspeptin potently stimulates gonadotropin release in human males (21), suggesting that it could be used therapeutically to manipulate the HPG axis in reproductive disorders. However, the effects of kisspeptin administration in women are unknown. In addition, iv administration is not an ideal route for therapeutic administration. We have shown that kisspeptin-54 administered sc is absorbed into the circulation and is most effective in stimulating secretion of gonadotropins in the preovulatory phase of the menstrual cycle in healthy women.

Our results show that increasing doses of sc kisspeptin-54 in the follicular phase significantly increased plasma LH and FSH. This is consistent with studies in animals in which sc administration of kisspeptin caused an increase in LH and FSH in female rats (24). Plasma kisspeptin-IR levels increased dose dependently and peaked approximately 15 min after sc administration of kisspeptin-54. This was then followed by a decline in plasma kisspeptin-IR.

It is possible that sc-administered kisspeptin-54 could be degraded to smaller kisspeptin fragments. Forms of kisspeptin 13, 14, and 54 amino acids long have been isolated from human placenta (1). All of these peptides are agonists at the GPR54 receptor. The C-terminal decapeptide common to all the kisspeptins, kisspeptin-10, is the minimum sequence necessary for receptor activation (1, 41, 42), and cultured first-trimester human trophoblasts secrete kisspeptin-10 *in vitro* (43). Our chromatographic analysis of plasma from female volunteers sc injected with kisspeptin-54 suggests that kisspeptin-54 is not significantly degraded to smaller active kisspeptin fragments *in vivo*. However, it is possible there are other degradation products present that are not detected by our kisspeptin assay.

No nausea was observed at any of the doses of kisspeptin tested. Recently, it has been shown that kisspeptin can cause vasoconstriction in *ex vivo* preparations of human arteries (44). However, we found no effects on blood pressure, pulse pressure, or heart rate at any of the doses of kisspeptin tested.

Endogenous kisspeptin is thought to play a role in regulating the preovulatory LH surge in animals. Blockade of local kisspeptin action in the preoptic area with a specific monoclonal antibody to rat kisspeptin completely abolished the proestrous LH surge and inhibited estrous cyclicity (27). Hypothalamic *KiSS-1* mRNA expression in female mice is regulated by estradiol. In the arcuate nucleus, *KiSS-1* mRNA expression increased after ovariectomy and decreased with estradiol treatment. Conversely, in the anteroventral periventricular nucleus (AVPV), *KiSS-1* expression was reduced after ovariectomy and increased with estradiol treatment (24). This suggests that *KiSS-1* neurons in the arcuate nucleus may play a role in the negative-feedback regulation of GnRH secretion, whereas *KiSS-1* neurons in the AVPV may participate in the positive-feedback regulation of GnRH secretion. In addition, it has recently been shown that *KiSS-1*

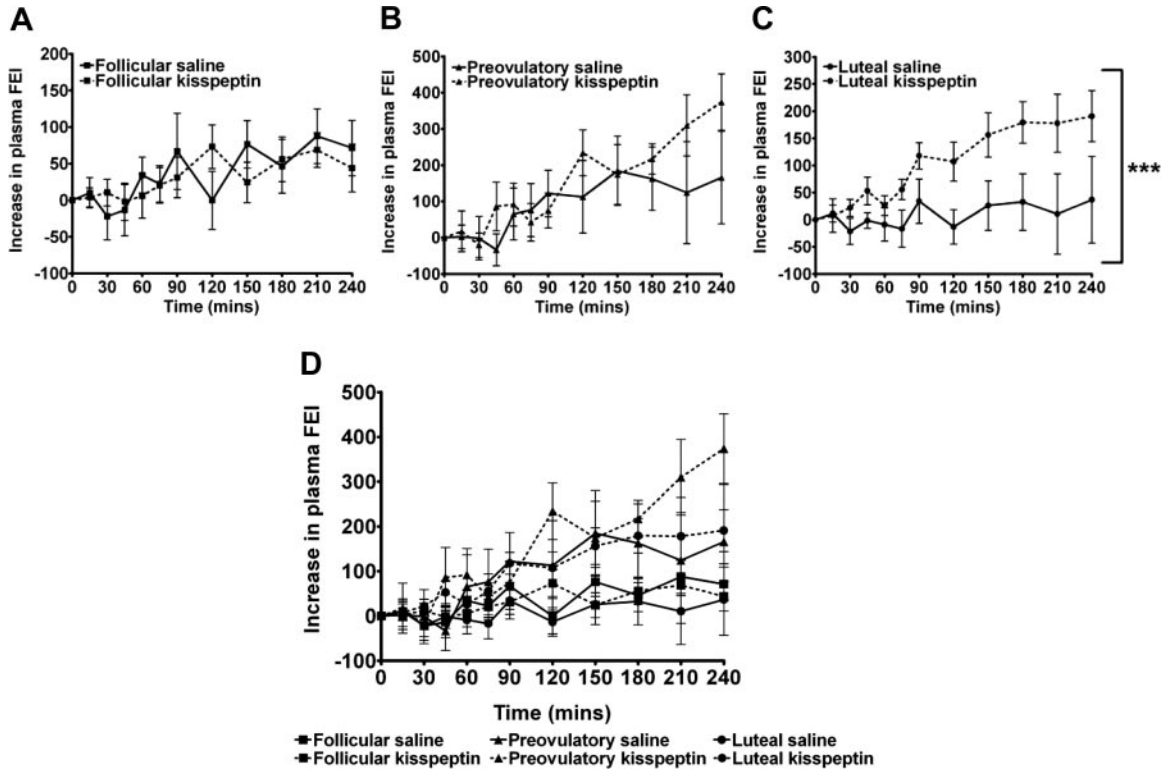

FIG. 5. Mean ± SEM increase over baseline in plasma FEI after bolus sc injection of saline or 0.4 nmol/kg kisspeptin-54 into healthy human females (n = 8) in individual phases of the menstrual cycle: A, follicular; B, preovulatory; C, luteal phase. Injections were administered at time 0 min. D, Mean ± SEM increase over baseline in plasma FEI in all three phases of the menstrual cycle to allow comparison of the effects of kisspeptin-54 on FEI across different phases of the menstrual cycle. ***, $P < 0.001$ kisspeptin *vs.* saline.

mRNA in the AVPV changes during the estrous cycle, with the highest levels occurring during proestrus (29). These data suggest that endogenous kisspeptin may play a critical role in regulating the GnRH/LH surge and ovulation.

To investigate the effects of exogenously administered kisspeptin-54 in different phases of the menstrual cycle, we chose a dose of sc kisspeptin-54 at the lower end of the dose-response curve (0.4 nmol/kg), which resulted in only a small increase in LH and FSH release in the follicular phase. This allowed us to determine whether kisspeptin-54 was more effective in stimulating the secretion of gonadotropins in the preovulatory and luteal phases of the menstrual cycle compared with the follicular phase. We have shown that sc administration of kisspeptin-54 to women in the follicular, preovulatory, and luteal phases of their menstrual cycles significantly increased plasma LH and FSH. The effect of kisspeptin-54 on LH and FSH in the preovulatory phase is approximately 5-fold greater than in the follicular and luteal phases.

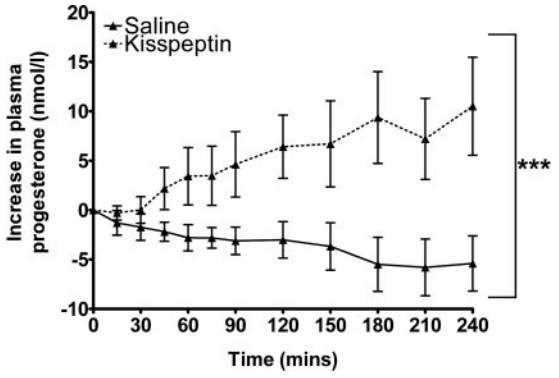

FIG. 6. Graph showing mean ± SEM increase over baseline in plasma progesterone after bolus sc injection of saline or 0.4 nmol/kg kisspeptin-54 into healthy human females (n = 8) in the luteal phase of the menstrual cycle. Injections were administered at time 0 min. ***, $P < 0.001$ kisspeptin *vs.* saline.

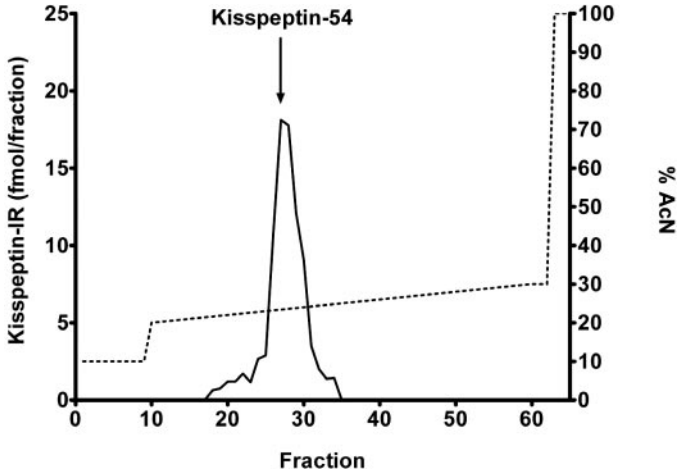

FIG. 7. Representative elution profile of kisspeptin-IR in plasma taken from volunteers 90 min after sc injection of 6.4 nmol/kg kisspeptin-54 and fractionated by reverse-phase FPLC. *Dotted line* represents percent acetonitrile (AcN). Elution position of synthetic human kisspeptin-54 is indicated by an *arrow*.

Kisspeptin-54 was approximately 7-fold more efficacious on LH release compared with FSH release. This is consistent with animal data that suggest that kisspeptin has a more potent effect on LH release than FSH release (9). However, because LH and FSH levels had not returned to baseline at the end of the time course of sampling, it cannot be excluded that kisspeptin evokes a more prolonged FSH response. The time course of LH response after sc kisspeptin-54 administration in our human study was longer than that observed after iv infusion of kisspeptin-10 in primates (19, 20). This difference in effect is likely to be due to the sc route of administration providing a more prolonged stimulus to gonadotropin release. In addition, kisspeptin-54 is more efficacious than shorter kisspeptin fragments *in vivo* (11, 35).

The effects of intracerebroventricular (icv) injection of kisspeptin to cyclic female rats on LH and FSH secretion during each of the four major stages of the estrous cycle [diestrous 1 (D1), D2, proestrous, and estrous] have recently been reported (31). The D1 and D2 phase of the rat cycle is equivalent to the follicular phase of the human menstrual cycle, during which there is follicular development. The proestrous phase of the rat cycle is equivalent to the preovulatory phase of the human menstrual cycle. Ovulation occurs in the estrous phase of the rat cycle, and unlike in humans, there is no luteal phase. The icv injection of kisspeptin significantly increased LH levels in all four phases of the estrous cycle in female rats. The highest LH levels were achieved after kisspeptin administration during proestrus, but the highest fold increase in LH over corresponding control values was seen in the estrous phase. Kisspeptin increased circulating FSH levels compared with vehicle-injected controls in D1 and D2 but had no effect on FSH levels at proestrus and estrus (31). In contrast, in our study, we found that sc administration of kisspeptin-54 to healthy females in the preovulatory phase resulted in highest LH and FSH levels and highest fold increase in LH and FSH over corresponding control values compared with the follicular and luteal phases of the menstrual cycle. The differences in the effects of kisspeptin on gonadotropin release in female rats compared with human female volunteers may be due to a number of factors. There are differences in the estrous cycle in rats and menstrual cycle in females as detailed above. The route of kisspeptin administration may also have an effect. The rodent studies administered kisspeptin icv, whereas our human study used sc administration.

Peripheral administration of kisspeptin is thought to mediate its effects predominantly via the release of GnRH (6, 8, 10, 14, 15, 17, 18). Therefore, one might expect peripheral administration of GnRH to have similar effects on LH and FSH release in the follicular, preovulatory, and luteal phases in human female volunteers as observed with kisspeptin-54 in this study. Consistent with this, iv administration of GnRH has been shown to have the greatest effect on LH release during the preovulatory phase, followed by the luteal phase, with smallest effects in the follicular phase (45–50). After GnRH administration, the response of plasma FSH levels was found to parallel the change in the levels of LH, but these changes were less pronounced (45–47, 49, 50). Thus, the effects of GnRH administration to healthy female volunteers are similar to the effects of kisspeptin-54 on LH and FSH secretion in our study and are consistent with the peripheral effects of kisspeptin-54 administration being mediated via GnRH release. However, an additional direct effect of kisspeptin on the pituitary gland cannot be ruled out.

Interestingly, we found that kisspeptin-54 stimulates progesterone release in the luteal phase of healthy female volunteers with ovulatory menstrual cycles. Administration of GnRH to human female volunteers has been shown to increase progesterone levels in the luteal phase of the menstrual cycle (51) via an increase in LH release (52). Therefore, the kisspeptin-54-stimulated progesterone release observed in our study in the luteal phase of healthy female volunteers may be mediated via the secretion of GnRH and subsequently LH. However, it has recently been shown that there is strong intensity of kisspeptin and GPR54-IR in the corpus luteum, particularly in the steroidogenic luteal cells (53). It is therefore possible that the increase in progesterone after peripheral kisspeptin-54 administration in our study represents a direct effect of kisspeptin on the corpus luteum.

Our data show that sc administration of kisspeptin-54 can stimulate gonadotropin release in healthy female volunteers. It has been suggested that peripheral administration of kisspeptin may result in a more physiological release of gonadotropins compared with GnRH by inducing the secretion of the endogenous releasable pool of gonadotropins (11). Kisspeptin-54 may therefore provide a novel mechanism for manipulation of the HPG axis for the treatment of women with amenorrhea causing infertility.

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References

1. **Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M** 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 276:34631–34636
2. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976
3. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
4. **Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL** 2003 The KiSS-1 receptor GPR54 is essential

for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357–1363
5. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145:4565–4574
6. **Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T** 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem Biophys Res Commun 320:383–388
7. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146:156–163
8. **Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR** 2004 Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 16:850–858
9. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. Endocrinology 146:1689–1697
10. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA** 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264–272
11. **Tovar S, Vazquez MJ, Navarro VM, Fernandez-Fernandez R, Castellano JM, Vigo E, Roa J, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2006 Effects of single or repeated intravenous administration of kisspeptin upon dynamic LH secretion in conscious male rats. Endocrinology 147:2696–2704
12. **Patterson M, Murphy KG, Thompson EL, Patel S, Ghatei MA, Bloom SR** 2006 Administration of kisspeptin-54 into discrete regions of the hypothalamus potently increases plasma luteinising hormone and testosterone in male adult rats. J Neuroendocrinol 18:349–354
13. **Castellano JM, Navarro VM, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2006 Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes 55:2602–2610
14. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077
15. **Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA** 2005 Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci USA 102:1761–1766
16. **Arreguin-Arevalo JA, Lents CA, Farmerie TA, Nett TM, Clay CM,** KiSS-1 peptide induces release of LH by a direct effect on the hypothalamus of ovariectomized ewes. Anim Reprod Sci 101:265–275
17. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM** 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 102:2129–2134
18. **Plant TM, Ramaswamy S, Dipietro MJ** 2006 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 147:1007–1013
19. **Seminara SB, Dipietro MJ, Ramaswamy S, Crowley Jr WF, Plant TM** 2006 Continuous human metastin 45–54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male rhesus monkey (*Macaca mulatta*): a finding with therapeutic implications. Endocrinology 147:2122–2126
20. **Ramaswamy S, Seminara SB, Pohl CR, Dipietro MJ, Crowley Jr WF, Plant TM** 2007 Effect of continuous intravenous administration of human metastin 45–54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (*Macaca mulatta*). Endocrinology 148:3364–3370
21. **Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR** 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 90:6609–6615
22. **Pompolo S, Pereira A, Estrada KM, Clarke IJ** 2006 Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology 147:804–810
23. **Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA** 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976–2984
24. **Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA** 2005 Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686–3692
25. **Smith JT, Clay CM, Caraty A, Clarke IJ** 2006 KiSS-1 mRNA expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150–1157
26. **Rometo AM, Krajewski SJ, Voytko ML, Rance NE** 2007 Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab 92:2744–2750
27. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K** 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431–4436
28. **Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M** 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774–1783
29. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K** 2007 Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53:367–378
30. **Estrada KM, Clay CM, Pompolo S, Smith JT, Clarke IJ** 2006 Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinol 18:806–809
31. **Roa J, Vigo E, Castellano JM, Navarro VM, Fernandez-Fernandez R, Casanueva FF, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2006 Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female Rat. Endocrinology 147:2864–2878
32. **Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2004 Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J Physiol 561:379–386
33. **Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE** 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25:11349–11356
34. **Clarkson J, Herbison AE** 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147:5817–5825
35. **Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, Cooke JH, Jethwa PH, Todd JF, Ghatei MA, Bloom SR** 2006 Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. Am J Physiol Endocrinol Metab 291:E1074–E1082
36. **Hashimoto T, Miyai K, Izumi K, Kumahara Y** 1973 Gonadotropin response to synthetic LH-RH in normal subjects: correlation between LH and FSH. J Clin Endocrinol Metab 37:910–916
37. **Neill JD** 2006 Knobil and Neill's physiology of reproduction. St. Louis: Academic Press
38. **de Gomez MS, Frei A, Vitins P, Cekan SZ** 1981 Influence of antisera, purification procedures, and tracers on validity of a testosterone radioimmunoassay. Ann Clin Biochem 18:281–286
39. **Belgorosky A, Escobar ME, Rivarola MA** 1987 Validity of the calculation of non-sex hormone-binding globulin-bound estradiol from total testosterone, total estradiol and sex hormone-binding globulin concentrations in human serum. J Steroid Biochem 28:429–432
40. **Dhillo WS, Savage P, Murphy KG, Chaudhri OB, Patterson M, Nijher GM, Foggo VM, Dancey GS, Mitchell H, Seckl MJ, Ghatei MA, Bloom SR** 2006 Plasma kisspeptin is raised in patients with gestational trophoblastic neoplasia and falls during treatment. Am J Physiol Endocrinol Metab 291:E878–E884
41. **Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M** 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613–617
42. **Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC** 2001 AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem 276:28969–28975
43. **Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W, Knofler M, Andreae F, Wagner O, Quaranta V, Desoye G** 2004 Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci 117:1319–1328
44. **Mead EJ, Maguire JJ, Kuc RE, Davenport AP** 2007 Kisspeptins are novel potent vasoconstrictors in humans, with a discrete localization of their receptor GPR54, to atherosclerosis prone vessels. Endocrinology 148:140–147
45. **Yen SS, Rebar R, Vandenberg G, Ehara Y, Siler T** 1973 Pituitary gonado-

trophin responsiveness to synthetic LRF in subjects with normal and abnormal hypothalamic-pituitary-gonadal axis. J Reprod Fertil Suppl 20:137–161
46. **Aono T, Minagawa J, Kinugasa T, Tanizawa O, Kurachi K** 1973 Response of pituitary LH and FSH to synthetic LH-releasing hormone in normal subjects and patients with Sheehan's syndrome. Am J Obstet Gynecol 117:1046–1052
47. **Shaw RW, Butt WR, London DR, Marshall JC** 1974 Variation in response to synthetic luteinizing hormone-releasing hormone (LH-RH) at different phases of the same menstrual cycle in normal women. J Obstet Gynaecol Br Commonw 81:632–639
48. **Nakano R, Kotsuji F, Tojo S** 1975 Pituitary responsiveness to synthetic luteinizing hormone releasing hormone (LH-RH) during the menstrual cycle and in female hypogonadism. Br J Obstet Gynaecol 82:805–811
49. **de Kretser DM, Burger HG, Dumpys R** 1978 Patterns of serum LH and FSH in response to 4-hour infusions of luteinizing hormone releasing hormone in normal women during menstrual cycle, on oral contraceptives, and in postmenopausal state. J Clin Endocrinol Metab 46:227–235
50. **Jewelewicz R, Dyrenfurth I, Ferin M, Bogumil J, Vande Wiele RL** 1977 Gonadotropin, estrogen and progesterone response to long term gonadotropin-releasing hormone infusion at various stages of the menstrual cycle. J Clin Endocrinol Metab 45:662–667
51. **Loucopoulos A, Ferin M** 1987 The treatment of luteal phase defects with pulsatile infusion of gonadotropin-releasing hormone. Fertil Steril 48:933–936
52. **McLachlan RI, Cohen NL, Vale WW, Rivier JE, Burger HG, Bremner WJ, Soules MR** 1989 The importance of luteinizing hormone in the control of inhibin and progesterone secretion by the human corpus luteum. J Clin Endocrinol Metab 68:1078–1085
53. **Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere M** 2006 Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? Endocrinology 147:4852–4862

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Erratum

In the article "Health Status, Mood and Cognition in Experimentally-Induced Subclinical Hypothyroidism" by M. Samuels, K. Schuff, N. Carlson, P. Carello, and J. Janowksy (*The Journal of Clinical Endocrinology & Metabolism* **92:**2545–2551), the authors report an error in the free T_3 concentrations in Table 1. The corrected values and related correlations are described below and shown in the revised table. *The authors regret the error.*

TABLE 1. Slightly low free T_3 levels were seen in 5 subjects at baseline, 2 at the end of the euthyroid arm, and 10 at the end of the subclinical hypothyroid arm. Differences in the SF-36 mental component summary (MCS), as well as the bodily pain (BP), mental health (MH), role physical (RP), social functioning (SF), and vitality (VT) subscales, were related to changes in free T_3 levels between the two study arms ($P = 0.005$-0.09). The difference in immediate paragraph recall was related to changes in free T_3 levels between the two study arms ($P = 0.04$).

TABLE 1. Clinical parameters and thyroid function tests at the end of each arm of the study (euthyroid and subclinical hypothyroid)

Measure	Baseline (mean ± SEM)	Euthyroid (mean ± SEM)	Subclinical hypothyroid (mean ± SEM)	*P* value, paired *t* test
TSH (mU/liter)	3.53 ± 1.12	2.19 ± 0.35	17.37 ± 3.04	<0.0001
Free T_4 (ng/dl)	1.32 ± 0.065	1.43 ± 0.03	0.99 ± 0.04	<0.0001
Free T_3 (pg/dl)	256.2 ± 11.0	259.6 ± 8.4	210.5 ± 11.0	<0.0001
Weight (kg)	77.37 ± 2.64	78.0 ± 3.0	78.4 ± 3.1	0.41
BMI (kg/m^2)	28.13 ± 1.16	28.4 ± 1.3	28.5 ± 1.3	0.43
Pulse (beats/min)	75.53 ± 2.84	74.9 ± 3.0	74.2 ± 3.1	0.77
Systolic blood pressure (mm Hg)	127.89 ± 3.38	126.7 ± 5.1	126.1 ± 3.6	0.89
Diastolic blood pressure (mm Hg)	74.74 ± 2.65	76.6 ± 2.5	75.5 ± 2.9	0.75
Billewicz scale	2.95 ± 0.57	1.74 ± 0.33	2.58 ± 0.62	0.08

P values refer to differences between the end of the euthyroid arm and the subclinical hypothyroid arm of the study.