Supplemental Methods

Kisspeptin-54 peptide synthesis and testing

Kisspeptin-54 was synthesised by the Advanced Biotechnology Centre, Imperial College London and purified by reverse-phase high performance liquid chromatography (HPLC). Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide as previously described[19,23]. The peptide was tested for bioactivity and toxicity as previously described[19]. The *Limulus* amebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) was negative for endotoxin, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London). Although kisspeptin-10, -13, -14, and -54 display similar potency *in vitro*, we used kisspeptin-54 due to its higher *in vivo* potency than the other kisspeptin fragments[13,22].

GnRH test Protocol

Subjects were cannulated and given a 100mcg iv bolus injection of GnRH (HRF®, Intrapharm Ltd, Kent, UK) at 0 min. Blood was sampled for measurement of serum LH, FSH and oestradiol at -30, 0, 15, 30, 45, 60, 90, 120 min.

Collection and processing of blood samples

Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA). Samples were allowed to clot prior to centrifugation and separation of serum. Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Beckton Dickson, Franklin Lakes, NJ, USA) containing 5000 kallikrein inhibitor units of aprotinin (0.2ml Trasylol; Bayer, Newbury, UK). Samples were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 4 minutes at 4000rpm, and then separated. Serum and plasma samples were stored immediately at -20^{O}C until analysis.

Other measurements

During each study visit, urine was tested in order to exclude pregnancy (Clearview easy-HCG, Inverness Medical Innovations Inc., Waltham, MA). Blood pressure and heart rate were recorded regularly during all 4h studies and LH pulsatility studies.

Hormone assay reference ranges and performance

Reference ranges for females were as follows: LH (follicular), 2–10 IU/L; LH (midcycle), 20–60 IU/L; LH (luteal), 4–14 IU/L; FSH (follicular and luteal), 1.5–8 IU/L; estradiol (early follicular), <300 pmol/L; estradiol (midcycle), 400-1500 pmol/L; estradiol (luteal), 200-1000 pmol/L. Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; progesterone, 1.8%. Limits of detectability for each assay were as follows: estradiol 70pmol/L; FSH 0.05IU/L; LH 0.07IU/L; progesterone 0.1nmol/L. The estradiol assay had 0.1% cross-reactivity with 12beta-estradiol 3-sulphate, 0.7% cross-reactivity with estrone, and failed to cross-react with 1590 (500ng/ml) nmol/L progesterone or 10microgram/mL aldosterone. *Conversion factors* for hormones from International Units to Mass Units are as follows: Estradiol pmol/L to pg/mL: x*(1/3.67)*; Progesterone nmol/L to ng/mL:x *(1/3.18)*; Luteinizing Hormone IU/L to mIU/mL:x*1*; Follicle Stimulating Hormone IU/L to mIU/mL:x*1*.

Kisspeptin Radioimmunoassay

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 limit of detectability was 2pmol/l, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

Analytical methods

Blood samples were collected as previously described[22]. Serum LH, FSH, estradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Measurement of plasma kisspeptin IR was performed using an established RIA[19, 23].

Supplemental Figure 1. Pituitary responsiveness in healthy women receiving twice-daily injections of saline or kisspeptin-54.

A-B: Serum LH following 100mcg intravenous bolus injection of GnRH at time 0min in women on menstrual day 1 (A; prior to commencing saline or kisspeptin treatment) and menstrual day 15 of the study protocol (B; 24h after stopping saline or kisspeptin treatment). **C:** Summary bar graph comparing mean AUC LH increase following GnRH administration in women before/after saline or kisspeptin-54 treatment. Data are presented as mean±SEM. *, $P<0.05$.

Supplemental Table 1: Baseline characteristics of the 5 healthy female participants at first study visit. All female subjects had regular periods for >24 months, normal reproductive hormone levels, and had not been on contraceptive medication for >12 months, BMI 18.5-24.9. Normal medical history, examination and blood tests were required at screening for study entry. Mean normal cycle length was 28.6±1.1 days.

Participant	Age (years)	Body Mass Index	Normal cycle length (±1 day)
1	24	22.9	31
2	34	24.2	31
3	27	22.0	25
4	36	18.6	28
5	37	22.0	28

Supplemental Table 2: Table showing first menstrual day that corpus luteum (CL) was visualised by ultrasonography in each participant and highest recorded progesterone level during that menstrual cycle (saline or kisspeptin-54 treatment).

	SALINE		KISSPEPTIN-54	
Participant	Day CL observed	Highest Progesterone (nmol/L)	Day CL observed	Highest Progesterone (nmol/L)
1	28	14	28	28
2	21	37	15	28
3	Not seen	47	21	21
4	Not seen	44	18	40
5	21	41	15	32
mean±SEM	23.3±2.3	36.6±13.2	19.4±2.4	29.8±6.9

Supplemental Figure